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DEVNERTOX

Toxic threats to the developing nervous system:
in vivo and *in vitro* studies on the effects of mixture of neurotoxic substances potentially contaminating food

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DEV NER TOX

Toxic threats to the developing nervous system: *in vivo* and *in vitro* studies on the effects of mixture of neurotoxic substances potentially contaminating food

Participants:

- **Karolinska Institutet**, Stockholm, Sweden; Prof. Sandra Ceccatelli (coordinator) and Prof. Jan-Åke Gustafsson.
- **University of Pavia**, Italy; Prof. Luigi Manzo.
- **University of Parma**, Italy; Prof. Antonio Mutti and Prof. Lucio Costa.
- **Fundación C.V. Centro de Investigacion Principe Felipe**, Valencia, Spain; Dr. Vicente Felipo.
- **Catholic University of Louvain**, Brussels, Belgium; Prof. Alfred Bernard.
- **Uppsala University**, Uppsala, Sweden; Prof. Per Eriksson.
- **Nofer Institute**, Lodz, Poland; Prof. Slawomir Gralewicz.
- **Trinity College**, Dublin, Ireland; Prof. Roger Anwyl.

General introduction

Food, health and the environment

The presence of contaminating substances in food is an issue that has received a lot of attention. Because of the sensitivity of the developing central nervous system to toxic insults there is a particular concern about potential neurotoxic effects of substances that fetuses and children are exposed to, *in utero*, via breast milk or food (Grandjean and Landrigan 2006).

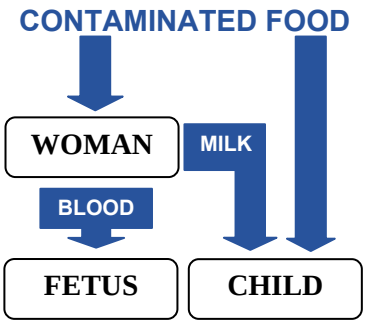

Figure 1. Routes for developmental exposure to food contaminants in humans.

Methylmercury (MeHg) and polychlorinated biphenyls (PCBs) are persistent pollutants that enter the lowest stages of the food chain and then steadily become more concentrated at each stage. Although the developmental neurotoxic effects of these substances are well established, their mechanisms of action are not fully understood. In addition, few studies have investigated the effects of simultaneous

exposures, which is the real-life situation for individuals consuming polluted fish, the main source of human exposure to MeHg and PCBs.

The DEVNERTOX project

DEVNERTOX is the acronym for the Specific Targeted Research Project entitled "Toxic threats to the **dev**eloping **ner**vous system: *in vivo* and *in vitro* studies on the effects of mixture of neuro**tox**ic substances potentially contaminating food", which involves, ten European academic research groups, with expertise ranging from behavioural sciences to molecular biology. The project has focused on generating experimental models to improve developmental neurotoxicity testing and risk assessment for neurotoxic food contaminants. Three compounds have been studied alone and in combination, MeHg and two PCBs with different chemical properties; the non-dioxin-like di-*ortho*-substituted PCB 153 and the coplanar dioxin-like PCB 126.

The major goals of DEVNERTOX were to:

1. Develop standardised testing protocols based on the use of several *in vitro* experimental models.
2. Identify specific biochemical, molecular and functional endpoints based on the mechanism of action of MeHg and PCBs, both alone and in combination.
3. Evaluate the neurotoxic effects of MeHg and PCBs during development, including long-term consequences and gender-related aspects.
4. Define quantitative measures of observed effects for risk-assessment purposes, and to derive guidelines for exposure limits.

Project execution and dissemination of knowledge

As outlined in Fig. 2, the first part of the project was dedicated to the establishment of *in vitro* and *in vivo* experimental models and methodologies. The *in vitro*-systems were used to identify toxic mechanisms of MeHg and PCBs on relevant neural cells, especially focusing on effects on neurotransmission, differentiation and cell death. In the *in vivo* studies, neurophysiological outcomes were investigated in experimental animals exposed during development with emphasis on effects on behaviour and different neurotransmitter systems. Age- and gender-related aspects were considered and the results from *in vivo* and *in vitro* studies were evaluated with regard to one another, as well as to previous studies in humans and different experimental systems.

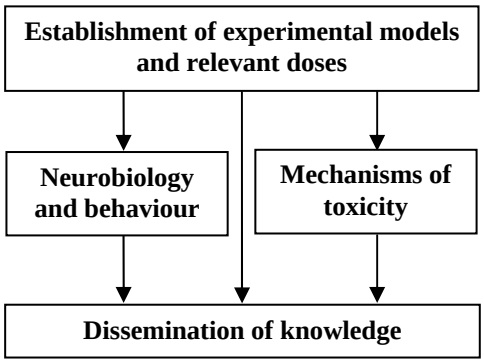

Figure 2. Outline of the workplan for the DEVNERTOX project.

The results and conclusions from the DEVNERTOX project are summarised below. The data are or will be published in international peer reviewed scientific journals, and presented at several scientific congresses and workshops, including the *International Conference on Food Contaminants and Neurodevelopmental Disorders*, in Valencia, Spain, December 3-5, 2006, which was organized by the DEVNERTOX consortium (see: http://www.fundacioncac.es/eng/fundacion/actividades/actividadesficha.jsp?idActividad=64). For the general public two brochures have been created and more information can be found at the DEVNERTOX website: http://www.imm.ki.se/DEVNERTOX/

Work performed and end results

Implementation of the *in vitro*-models

One of the main objectives of the DEVNERTOX project was to set up and evaluate *in vitro*-models to study neurodevelopmental effects of food contaminants. Nine different cell culture systems, listed in Table 1, have been implemented and studied with regard to a number of endpoints including cell viability, differentiation and effects on neurotransmission. In addition, the actual intracellular target doses have been assessed in the most relevant models.

Table 1. *In vitro*-models studied in the DEVNERTOX project

Cell lines	*Primary cultures*
C17.2 (mouse neural progenitor cells)	cNSC (rat embryonic neural stem cells)
HT22 (mouse hippocampal cells)	aNSC (mouse adult neural stem cells)
AtT20 (mouse pituitary cells)	CGC (rat cerebellar granule cells)
PC12 (rat pheocromocytoma cells)	
SK-N-MC (human neuroblastoma cells)	
D384 (human astrocytoma cells)	

Dose-dependent effects on cell viability were assessed in all models at various time points. Figure 3 shows in summary at what doses (in the cell medium) significant effects on biological functions and cell viability were observed in the different *in vitro* models.

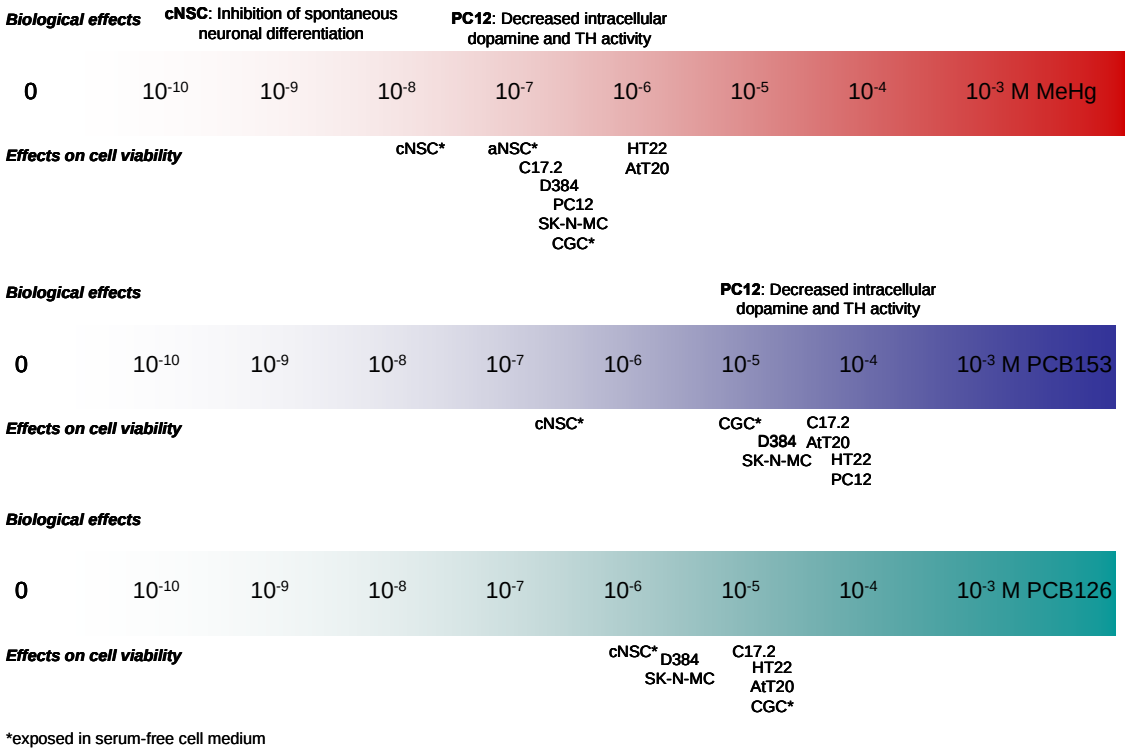

Figure 3. Sensitivity to toxic stimuli in different *in vitro*-models after 24h exposures. Concentrations in the cell medium corresponding to the lowest observed adverse effect levels (LOAELs). See Table 1 for cell model abbreviations.

Studies on cell death and survival in neuronal, neuroendocrine and glial cells

Among the parameters studied in the DEVNERTOX *in vitro* models were the effects of exposure to MeHg and/or PCBs on cell viability and characterization of the mechanisms of cell death. Cell death in the nervous system can occur either by apoptosis, which is an active process requiring energy, or by necrosis, a passive form of cell death. The intensity and duration of an insult can determine the type of cell demise. Apoptosis is characterized by morphological changes, such as cell shrinkage, condensation of chromatin, formation of apoptotic bodies, externalization of phosphatidylserine at the plasma membrane level that promote the engulfment of apoptotic cells by phagocytes.

Biochemically, apoptosis is characterized by a cascade of degradative events, which can be identified by specific patterns of proteolytic cleavage and DNA fragmentation. A number of genes have been shown to modulate the sensitivity of cells to apoptosis. Modulation of the expression of these genes can shift the balance of cells towards death or survival. An important part of apoptosis is the proteolytic cleavage of a number of key proteins. Calpain and caspases are the predominant proteases that can perform this function (Gorman et al. 2000).

The exact mechanisms of toxicity of most food contaminants and their specific cellular targets are for the most part unknown. Even less is known about possible

interactions between different toxic agents. One way forward in elucidating the effects of combined exposure is by mathematical models. As part of the DEVNERTOX project a mathematical approach has been developed for *in vitro* co-exposure studies that could be used to investigate the effects of concomitant exposure to various types of neurotoxicants (Vettori et al. 2006).

When it comes to studies of interaction between compounds, the term "additivity" is used when several (two or more) compounds act without any interaction among them and the total effect does not differ from what can be expected from the dose-effect relations of the individual agents. The terms "antagonism" and "synergism" are used when there is an interaction among toxic compounds, i.e. when the total effect is lower (antagonism) or higher (synergism) than expected (Groten et al., 2001). In molecular terms, the toxicity of a compound depends among other things on its affinity to target sites at cellular level and it might be decreased or increased by the presence of other toxic substances that biologically modify cellular conformation and expression, sometimes affecting the cellular defence system and detoxification capability.

HT22 (mouse hippocampal cells)
Hippocampal neurons are highly sensitive to most neurotoxicants, and therefore effects on cell viability were assessed in the HT22 hippocampal cell line (Elinder et al. 2005). Mainly necrotic, and to a lesser extent apoptotic cell death, was induced after exposure to MeHg and/or the PCBs. Analysis of data from co-exposures to lower doses suggested competitive interactions between MeHg and both PCBs. Similar to the other *in vitro* models studied, co-exposure to doses that were not cytotoxic by themselves did not induce significant amounts of cell death when combined.

In brief, treatment of HT22 cells with all three toxicants appears to cause activation of calpains and cathepsins, possibly by disruption of intracellular Ca^{2+}-signaling and further alteration of mitochondrial function. There was no activation of caspases and pre-treatment with antioxidants could partially protect from MeHg- but not PCB-induced cell death.

AtT20 (mouse pituitary cells)
The pituitary constitutes a known target organ for PCB-toxicity and cell viability was studied in the AtT20 pituitary cell line after exposure to MeHg and/or the selected PCBs (for details see Johansson et al., 2006). Similar to the HT22 cells, and to a previous study of dioxin-treated AtT20 cells (Huang et al. 2005), mostly necrosis was detected after exposure. Simultaneous exposure to moderately toxic doses of PCBs and MeHg resulted in additive or slightly synergistic effects on the induction of cell death (indicated by the Bliss independence criterion followed by *t*-tests).

Our studies show that both coplanar (PCB 126) and non-coplanar (PCB 153) PCBs induce cell death in AtT20 pituitary cells via activation of calpains and lysosomal proteases, possibly through disruption of mitochondrial function and intracellular calcium signaling (Johansson et al. 2006). Results from pre-treatment with protease-inhibitors are shown in Fig. 3. No activation of caspases was detected and all tested toxicants induced primarily necrotic cell death. Oxidative stress does not seem to play a role in PCB toxicity in this *in vitro* model. In contrast, production of ROS was detected after MeHg exposure and, in accordance with the HT22 data, pre-treatment with an antioxidant (trolox) could partially protect from MeHg- but not PCB-induced cell death.

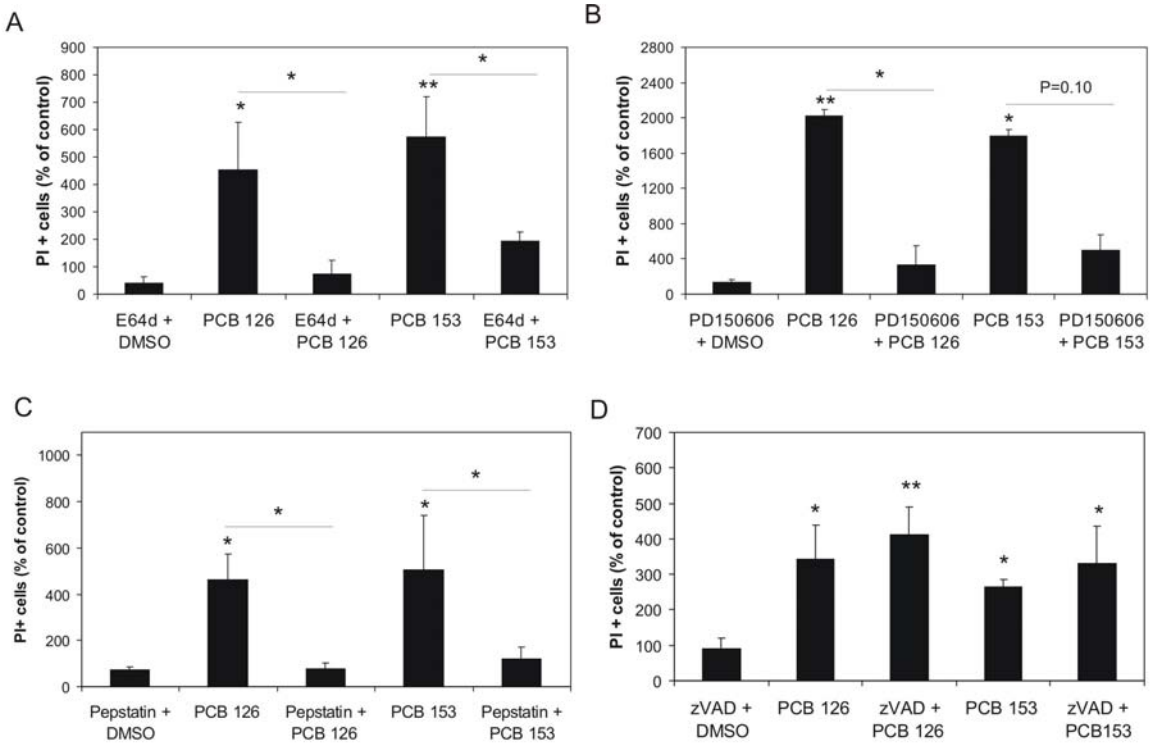

Figure 4. Protective effects of protease inhibitors in PCB-exposed AtT20 cells indicating involvement of calpains and cathepsins but not caspases. Necrosis (detected as propidium iodide (PI) permeability) after 24 h exposure to 50 μM PCB 126 and 75 μM PCB 153, with or without **(A)** cystein protease inhibitor E64d (25 μM, 30 min pre-treatment), **(B)** calpain specific inhibitor PD150606 (100 μM, 1 h), **(C)** cathepsin D inhibitor pepstatin (100 μM, 1 h), or **(D)** pan-caspase inhibitor zVAD-fmk (20 μM, 1 h). Mean % of control of triplicates ±SEM, representative of three independent experiments (two for the z-VAD-fmk analysis). ANOVA followed by Fisher's PLSD: *p<0.05, **p<0.01.

PC12 (rat pheocromocytoma cells)

The PC12 cell line was selected as a model to study the dopaminergic system. Viability in PC12 cells was evaluated after 24h exposure to MeHg or PCB 153 (for details see Vettori et al., 2006). In the analysis of synchronous co-exposure to MeHg and PCB 153, a 3D graph (Fig. 5) was generated according to the Loewe additivity model (Berenbaum., 1985; Greco et al. 1995) using the pure curve equations (of cell viability after single exposures). A comparison was then made between theoretical non-interaction points and the experimentally observed viability results after combined exposure to different concentrations. Briefly, for combinations of some concentrations (MeHg 5e-7 M and PCB153 1e-4 and 2e-4 M ; MeHg 1e-6 M and PCB153 5e-5 M), a statistically significant antagonist effect was observed. A similar effect was observed for co-exposure to a low dose of MeHg (1e-7 M) and to doses of PCB153 higher than the EC_{50} for cytotoxicity (4e-4 M).

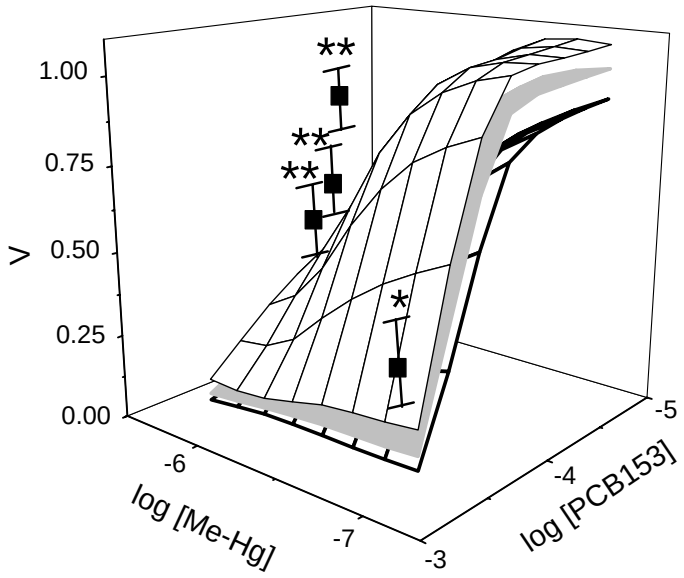

Figure 5. Comparison between some viability experimental data points and the zero interaction surface for PC12 cells exposed to MeHg and PCB 153. * $p<0.05$; ** $p<0.01$. For details see Vettori et al. 2006.

In order to test if the same competitive effect could also be found for other end-points, lipid peroxidation (indicative of oxidative stress) was studied after co-exposure to MeHg and PCB 153, by measuring thio-barbituric acid reactive substances (TBARS). The levels of TBARS were dose-dependently increased in single dose experiments, for both compounds tested (data not shown). Figure 6 summarizes the results after single and combined exposure, using combinations of concentrations derived from the cell viability results. Lipid peroxidation was lower in co-exposed samples, as compared to single compound exposure, confirming the competitive effect of MeHg and PCB 153 in PC12 cells at these doses.

Co-exposure to MeHg and PCB 153 was also studied with asynchronous exposure. When cells were exposed first to PCB 153 and then MeHg the effects were in general additive. The competitive effect was significant only when PC12 cells were first exposed to MeHg, indicating that whereas MeHg can modulate PCB 153 toxicity, the opposite seems not to be true.

Target cellular doses were measured after single and combined exposures. Even if the data has to be validated with additional experiments, combined exposure seemed to cause accumulation of Hg and PCB 153 inside the cells, as compared to single exposures. Comparison with other cell lines exposed to the same MeHg concentrations in the cell medium revealed that PC12 cells contained slightly lower Hg levels than C17.2 neural progenitor cells and slightly higher Hg levels than SK-N-MC neuroblastoma cells. For PCB 153 PC12 and C17.2 cells had similar intracellular concentrations while the SK-N-MC cells contained significantly more.

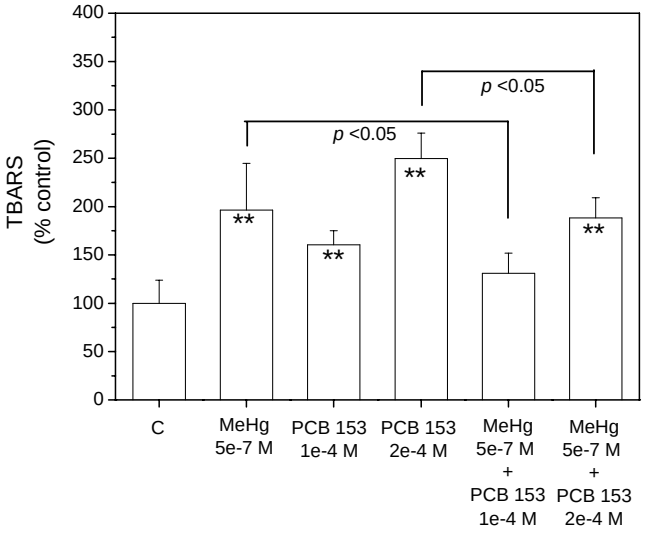

Figure 6. Antagonistic effects of combined exposure to certain doses of MeHg and PCB 153 on lipid peroxidation in PC12 cells. One-way ANOVA followed by Tuckey *post-hoc* tests, ** $p<0.01$.

SK-N-MC (human neuroblastoma cells)
Viability in SK-N-MC cells (Goldini et al. 2003; Vettori et al. 2005) was also evaluated and no differences were seen between single and co-exposed cells at the MeHg and PCB 153 doses tested. As mention, the intracellular concentration of PCB 153 was higher in SK-N-MC than in PC12 and C17.2 cells exposed to the same dose. In line with the PC12 results, combined exposure seemed to cause accumulation of Hg and PCB 153 inside the SK-N-MC cells, as compared to single exposures. Additional target dose measures are ongoing for SK-N-MC as well as several other *in vitro* models, to increase the accuracy and test the generality of the data.

Cerebellar granule cells (CGC)
Viability studies were performed in primary cultures of glutamatergic cerebellar neurons (Miñana et al. 1998; Castoldi et al., 2000) exposed *in vitro* to MeHg and/or the PCBs under serum-free conditions for 24h. Viability was assessed by the MTT assay as well as by means of cell double staining with calcein-AM to label viable cells and propidium iodide to label cells with damaged plasma membrane. Both methods yielded similar results.

Concentrations of MeHg which *per se* did not significantly alter cell viability, or reduced it slightly, produced a more marked cytotoxic effect when combined with non-cytotoxic concentrations of PCB 153. Concentrations of MeHg higher than 1 μM and PCB concentrations over 20 μM were too toxic by themselves to be susceptible of exacerbation by addition of PCB 153 or MeHg, respectively. No interactive effects on cytotoxicity were observed between PCB 126 and MeHg at the doses tested.

In addition to these experiments, long-term exposures (10-14 days) of CGCs to MeHg or PCB were performed in the presence of serum. Unlike the results from the 24h serum-free exposures, the CGCs were more sensitive to PCB 126 than PCB 153 under these conditions (see Fig. 13). Co-exposure to MeHg and PCB 153 caused increased cell death compared to MeHg exposure alone at PCB 153 concentrations $\geq$30 μM.

D384 (human astrocytoma cells)

Astrocytes have been implicated in both defensive and facilitating capacities for many toxic injuries, and astrocytic cultures have proven to be a valid tool for evaluating neurotoxicity. Effects on viability in D384 cells (Gorman et al. 2000) was assessed after 24h exposure to MeHg or PCB. Similar to the SK-N-MC cells, no differences were seen between single and co-exposed D384 cells at the MeHg and PCB 153 doses tested.

Studies on differentiation, cell death and survival in neural stem cells

Neural stem cells (NSCs) have the ability both to self-renew and to differentiate into various neural cell types. They play an essential role in the development and maturation of the nervous system and constitute a sensitive and highly relevant potential target for toxic effects on neurodevelopment. Depending on the dose, apoptotic and/or necrotic cell death could be detected in NSCs after exposure to the toxicants. For studies of the involvement of specific cell death pathways, doses inducing apoptosis but not necrosis were used, while sub-lethal doses were found to affect differentiation. In total three NSC *in vitro*-models have been studied in DEVNERTOX:

C17.2, a neural progenitor cell line originally derived from the developing mouse cerebellum. These cells have the ability to differentiate into neurons, astrocytes or oligodendrocytes, both *in vitro* and *in vivo* (Snyder et al. 1992;1997).

cNSCs are primary rat embryonic cortical neural stem cells (Johe et al. 1996; Hermanson et al. 2002) that, depending on the culture conditions, can differentiate into neural cell types as well as smooth muscle cells. These cells demonstrated the highest sensitivity to toxicant exposure during 24 hours (Fig. 3) and were used to investigate effects on differentiation.

aNSCs are mouse adult neural stem cells (Sleeper et al. 2002) and cell viability studies indicated that these cells are less sensitive to MeHg than the embryonic cNSCs but more susceptible than the C17.2 cells (Fig. 3).

To test whether MeHg may affect the spontaneous neuronal differentiation of neural stem cells, we exposed the cNSC to non-cytotoxic concentrations that are relevant for human developmental exposure (Tamm et al. 2006). We observed that 2.5 or 5 nM MeHg significantly impaired the neuronal differentiating potential, as visualized by a clear decrease in Tuj1 positive neuronal cells as compared to the control (Fig. 7). The intracellular Hg levels in cNSC exposed to 5 nM MeHg were only slightly higher than the detection limit for the method and in the same range as what has been measured in the few studies of Hg brain levels in normal human infants (see Fig. 17, Choi et al. 1978; Lapham et al. 1995)

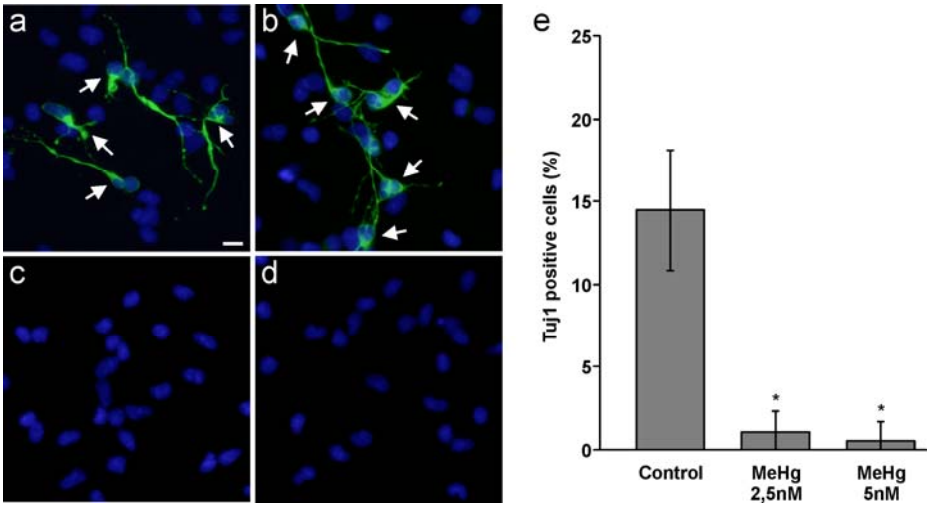

Figure 7. Non-cytotoxic levels of MeHg cause an inhibition of neuronal differentiation in cNSCs. Cells were fixed and immunocytochemically stained with the early neuronal marker Tuj1 (arrows) and analysed under fluorescent microscope (scale bar = 10 μm). Compared to the untreated control cells **(a, b)**, single doses of MeHg (2.5 and 5 nM) **(c, d)** decreases neuronal differentiation of cNSCs. Semiquantitative analysis shows that the decrease in cNSC neuronal differentiation is significant **(e)**. Mean values ± SD (n=3), ANOVA followed by Fisher's PLSD test: *p<0.05.

Our studies on cell death in C17.2 and cNSC showed that particularly the cNSC are highly sensitive to the toxic effects of MeHg and PCBs. The intracellular Hg and PCB 153 levels in C17.2 cells were similar to those measured in PC12 cells exposed to the same doses. Viability studies of combined exposures to apoptosis-inducing doses of MeHg and either PCB suggested antagonistic effects in C17.2 and cNSC.

After MeHg-exposure, NSCs undergo apoptosis via activation of two parallel pathways involving caspases and calpains (Fig. 8). Bax activation and cytochrome *c* release from the mitochondria occurred in both NSC models (for details see Tamm et al. 2006). For the PCBs, the effects were different: Although condensed nuclei were detected in both cNSC and C17.2, PCB-exposure did not cause apoptosis characteristic effects on the mitochondria or activation of caspase or calpain proteases. Instead we detected an effect on the lysosomes with release of cathepsins into the cytosol.

Cells could, at least partly, be rescued from toxicant induced cell death by pre-treatment with protease inhibitors. For example, similar to the HT22 and AtT20 results described above, the cathepsin D inhibitor pepstatin and protected against the PCBs. In accordance with the caspase activity results (Fig. 3a), the pan-caspase inhibitor z-VAD-fmk was only protective against MeHg in NSCs. This protection was increased by adding a calpain inhibitor.

Pre-treatment with β-estradiol partially protected the C17.2 cells from MeHg-induced apoptosis. In addition, β-estradiol also reduced MeHg-induced cleavage of the cytoskeletal α-fodrin into proteolytic fragments characteristic for caspases (120 kD) and calpains (150 kD).

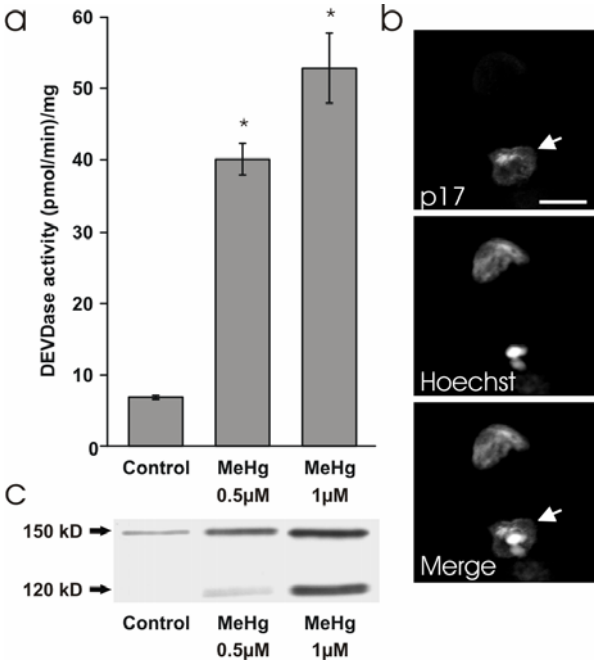

Figure 8. (a) MeHg induces caspase-3-like activity in neural stem cells. Controls and MeHg-exposed C17.2 cells were harvested, and caspase-3-like activity was assessed by DEVD-AMC cleavage. Mean values ± SD (n=3), ANOVA followed by Fisher's PLSD test: *p<0.05. **(b)** cNSCs exposed to 0.05 µM MeHg for 24 h show activated caspase-3 (=p17) and apoptotic-like nuclear condensation detected by Hoechst 33342 staining. Scale bar = 10 µm. **(c)** Western blot analysis of C17.2 cells exposed to 0.5-1 µM MeHg for 24 h showing increased specific breakdown of α-fodrin by calpains (150 kD fragment) and caspases (120 kD fragment).

Implementation of the *in vivo*-models

Experimental animal models were set up to study the neurodevelopmental effects of MeHg, PCB 153 and PCB 126. Rat and mouse models, with the latter also including knock-out animals, were used to assess neurotransmission, behavioural and electrophysiological endpoints. In addition, serum sex hormone measurements and determination of the actual target doses (*i.e.* the levels of the toxicants in blood and brain tissue) have been performed.

The standard diets as well as the tap water given to experimental animals were analysed to test the background exposure to Hg and PCBs. In the three diets tested, Hg levels were comparable and averaged about 10-15 ng/g chow. The water Hg content was below the detection limit of the method (<0.3 µg/l). The total intake of Hg through food and water consumption accounted for 1/2300 of the daily MeHg rat dose of 0.5 mg/kg b.w./day, and can therefore be considered negligible. PCBs were undetectable in food and water.

The exposure conditions are described in the *Materials and Methods* -section. Briefly, to study maternally mediated exposure during gestation and lactation all rats and one group of mice were exposed from gestational day (GD) 7 to postnatal day (PND) 21 and a second group of mice were exposed from GD7 to PND7. This type of exposure was used to mimic the situation in humans subjected to food contaminants during development (Fig. 1). In addition, a neonatal mouse model with single dose exposure at PND10 was used to study effects on the 'brain growth spurt'. This is a defined critical period of brain development which in neonatal rodents spans over the first 3-4

weeks of life, but in humans begins during the third trimester of pregnancy and continues throughout the first two years of life. There was no evidence of overt toxicity caused by exposures to the toxicants in the dams or the pups.

Tissue levels of toxicants in exposed animals

The concentrations and distributions of Hg and the PCBs in the brain and blood of rat offspring and dams were determined at different time-points of postnatal development: PND1, PND21 (weaning), PND36 (puberty) and PND90 (adulthood). In general, the chemical agents displayed a time-dependent reduction in their intra-tissue levels, although with different elimination kinetics.

In rat offspring, brain Hg concentrations rapidly decreased from birth to PND21 despite the continuous exposure to MeHg through lactation. By PND90 brain Hg levels of MeHg-exposed rats had reached control levels. The elimination curve for Hg ($t_{1/2}$ = 2.8 days) is presented in Figure 9. About 86.5% of the total Hg was eliminated in the first kinetic phase, whereas the lower fraction (13.5%) was eliminated with slower kinetics. Hg brain levels were also measured in MeHg-exposed mice, but at fewer time points (Fig. 17).

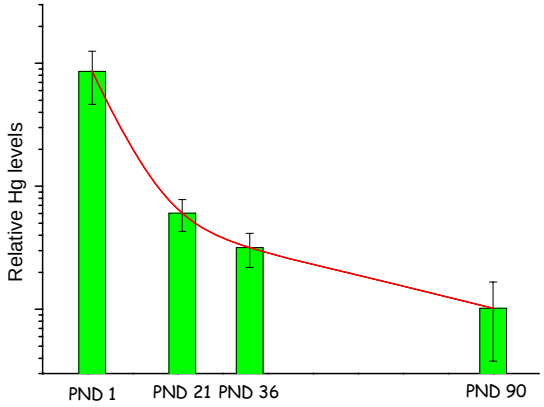

Figure 9. Time-course changes in total Hg concentrations in the brain of pups developmentally exposed (GD7-PND21) to MeHg through maternal oral intake of 0.5 mg/kg/day.

The PCB 153 concentrations decreased slowly in brain during the pre-weaning period (only 15%), due to the continuous exposure of pups *via* contaminated milk. After exposure cessation, PCB 153 levels rapidly dropped to reach about 45% of the PND21 levels at puberty (PND36). At PND90, rats perinatally exposed to 5 mg, but not 1 mg PCB 153/kg/day, still displayed slightly higher PCB153 levels than control animals. The $t_{1/2}$ for PCB 153 elimination was 30.6 days (Figure 10).

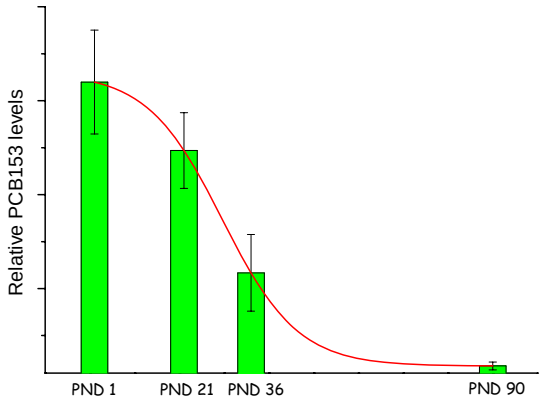

Figure 10. Time-course changes in PCB 153 concentrations in the brain of pups developmentally exposed (GD7-PND21) through a maternal oral intake of 5 mg/kg/day.

Concerning PCB 126, the exposed rats displayed brain levels significantly higher than their respective controls at PND1 and PND21, but not at PND90. The $t_{1/2}$ for PCB 126 elimination was 7 days (Figure 11).

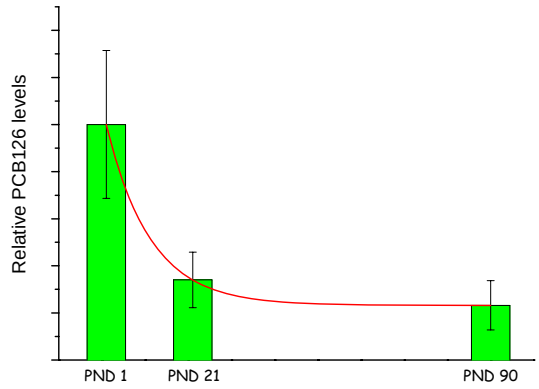

Figure 11. Time-course changes in PCB126 concentrations in the brain of pups developmentally exposed (GD7-PND21) through a maternal oral intake of 100 ng/kg/day.

There were no significant differences in the toxicant distribution between different brain regions (cerebral cortex, cerebellum, striatum and hippocampus) in rat pups at any of the ages examined. Combined exposure to MeHg and either PCB did not influence the regional distribution of the compounds.

Maternal exposure to MeHg resulted in brain Hg levels which were about 10-fold higher in dams than in pups, both at weaning and at puberty. This trend was observed not only in rats exposed to MeHg alone, but also in those co-exposed to MeHg and PCB 153 or PCB 126. Consistent with the data in brain, maternal blood contained higher MeHg levels than that of pups at both time-points (45:1 at PND21 and 38:1 at PND36, respectively). Similar differences in Hg levels between mothers and offspring have previously been found at day 21 post-partum, both in brain and in blood (Coccini et al., 2006).

Cerebral PCB 153 concentrations at PND21 were 40-80% lower in dams than in pups, also after co-exposure with MeHg. The same trend also applied to serum PCB 153 levels at PND21. After the end of exposure (PND36), dams and offspring had similar PCB153 levels in brain and serum.

Altogether, the kinetics of Hg does not seem to be modified by the co-presence of PCBs. This conclusion was supported by Hg measurements in mice exposed to MeHg or MeHg and PCB 153 at PND10. Similarly, MeHg seemed not to affect the kinetics of PCB 153 in the rat offspring's brain or blood at the developmental stages analysed.

In contrast, rat pups developmentally co-exposed to MeHg and PCB 126 displayed markedly higher brain PCB levels than those exposed to PCB 126 only, at all ages examined, namely PND1 (+56%), PND21 (+120%) and PND90 (+49%). The elimination kinetics of PCB 126 was slower in co-exposed animals, than in single-exposed animals.

Hormonal levels in exposed animals

Serum levels of 17-beta estradiol and testosterone were examined in rat offspring at different developmental ages, *e.g.,* PND21 (weaning), puberty (PND36) and adulthood (PND90). The results support that both PCBs, alone or combined with MeHg, alter the levels of circulating steroid hormone in a gender-, age- and hormone-dependent manner.

While MeHg was unable, *per se*, to induce any significant effect on circulating levels of estradiol or testosterone, PCB 153 (alone or in mixture with MeHg) induced long-lasting changes in estradiol levels in both genders, which became apparent at puberty (PND36) and were still present in adulthood (PND90). The extent of changes did not differ between rats treated with PCB 153 alone and those co-treated with MeHg. Testosterone, on the other hand, was not significantly affected by MeHg or PCB 153, in any gender, at any developmental stage.

PCB 126 significantly reduced estradiol levels at puberty (but not at weaning) in males but not females. Additionally, it caused a persistent decrease (both at weaning and puberty) in testosterone concentrations in the male offspring only.

Co-exposure to PCB 126 and MeHg resulted in antagonistic effects on testosterone in PND21 males, synergistic effects on testosterone in PND21 females, and lack of interactive effects on estradiol and testosterone in PND36 males.

Investigations of the PCB-binding Clara cell protein

One of the aims of the DEVNERTOX study was to investigate the role played by the Clara cell protein (CC16) on dioxin and PCB-binding in both humans and animals. This small (16 kD) anti-inflammatory protein is produced in high quantities by the cells lining the airways and can permeate into the general circulation. Since CC16 possesses a hydrophobic pocket, the working hypothesis was that through its capacity to bind certain lipophilic xenobiotics, such as dioxins and PCBs, CC16 might facilitate their excretion via the urinary tract. The biological sequestration and excretion functions of CC16 may therefore influence the neurotoxicity of dioxins and PCBs, also in the developing foetus.

Brain cortex homogenates from rats were analysed for CC16 and the levels were below the limit of detection in all samples, thus, it appears unlikely that CC16 plays a major role within the brain itself. Exposure to MeHg and/or PCB 153 did not affect

CC16 serum levels in rats. However, analysis of human amniotic fluid specimens (obtained from routine amniocentesis samples performed at week 16-36 of pregnancy) showed that the CC16 excretion in human amniotic fluid rises exponentially through pregnancy from week 20 to 30, reaching a plateau of about 300 µg/l, which is at least ten times higher than the normal human plasma levels.

Analysis of PCB 153, PCB 126 and several additional PCBs and polychlorinated dibenzodioxin/dibenzofuran congeners (PCDD/Fs) in human amniotic fluid protein fractions, indicated that the CC16 protein binds PCB 126 with much greater affinity than other protein fractions, whereas PCB 153 was not observed in any of the protein fractions studied. Interestingly, another PCB congener with potential neurotoxic effects, PCB 28, showed major and almost exclusive binding to the CC16 protein fraction of human amniotic fluid.

Studies on the role of estrogen receptors in the developing central nervous system

PCBs are well known to be estrogenic so it is appropriate to ask whether they interfere with estrogen signaling during development. The relative binding affinity of PCBs for ERα and ERβ are roughly 1000-fold lower than that to estradiol (Kuiper et al. 1998). Estrogen via ERα is essential for imprinting of male sexual behavior: At embryonic day 18.5, testosterone secreted from the embryonic testis, is converted to estradiol through the action of the enzyme aromatase in the hypothalamus. This estrogen permanently programs the male brain to be masculine (Naftolin et al. 2001). The fetal brain is protected from maternal estrogen through the extremely high binding affinity of alpha fetoprotein (AFP) for estradiol (Mizejewski 1985). AFP sequesters circulating estrogens and, by so doing, protects the developing female brain from their effects. In alpha fetoprotein -/- mice females, high exposure to estradiol in the embryonic period leads to dysfunction of the gonadal-pituitary axis and these mice are anovulatory (De Mees et al. 2006). Environmental and synthetic estrogens such as PCBs and DES do not bind to alphafeto protein. In both humans and rodents, exposure to DES *in utero* causes multiple deleterious effects which become evident in the urogenital tract of adults (Prins et al. 2001).

Previous studies in β-estrogen receptor knockout (BERKO) mice, demonstrated that as early as E14.5 the β-estrogen receptor (ERß) is necessary for embryonic development of the brain and is involved in neuronal migration (Wang et al. 2001). In the DEVNERTOX project, studies of ERβ expression in the embryonic mouse brain demonstrated that ERβ appears in the brain at E12.5, mainly localized in the wall of midbrain, neuromere, hypothalamus, thalamus and basal plate of pons. At E15.5 and E16.5, ERß expression increased in the hypothalamus, thalamus and midbrain and appeared in the limbic forebrain. At E18.5, ERß expression was strongly and widely expressed throughout the brain including cerebellum and striatum, while there were very few positive cells in the ventricular region. In the paraventricular thalamic nucleus and parafascicular nucleus, most of the calretinin-immunopositive interneurons expressed ERß. In BERKO mice, calretinin expression was markedly lower than in WT mice in the hippocampus, thalamus and amygdala both at E16.5 and at E18.5. Epidermal growth factor receptor expression was lower in the cortex of BERKO than WT mice at E15.5, and unlike WT mice, was absent from the superficial marginal zone. Unlike ERβ, ERα was not expressed in the mouse brain before day 18.5 and its expression was limited to the hypothalamus.

From embryonic day 20 to postnatal day 14 both ERα and ERβ are widely expressed throughout the cortex, thalamus and mid brain. No ERs were detected in the cerebellum. By three months of age the expression pattern of both receptors became much more restricted. Both receptors were expressed in the adult amygdala, hypothalamic preoptic area and brain stem. There was scattered expression in the cortex. These results indicate an extensive period of early postnatal life when estrogens receptors are abundant in the brain and when their function may be disrupted by xenoestrogens.

Surprisingly, an increase in the number of interneurons in the cortex was found in the brains of aromatase -/-mice, *i.e.* in absence of estrogen. This result could be interpreted to mean that estrogen is essential for both the survival and the death of neurons. Since there is excessive neuronal loss in BERKO mice, we can conclude that ERβ is the receptor which is important for neuronal survival. ERα may mediate neuronal loss during development. Thus if PCBs are not selective for ERα or ERβ the outcome of PCB exposure throughout embryonic development may be difficult to understand.

5α-Androstane, 3β, 17 β diol (3βAdiol) is an estrogenic metabolite of dihydrotestosterone (Fan et al. 2006). It is inactivated to nonestrogenic triols through the activity of CYP7B1 a member of the cytochrome P450 super gene family. Cyp 7B1 is unique in that it is highly expressed in the brain and pituitary but not in the liver. When Cyp7B1 is inactivated, the estrogenicity of 3βAdiol is not terminated and a hyperestrogenic state ensues. To study what happens when the brain is continuously exposed to estrogens during development, brains of CYP7B1 –/- mice were examined and found to be very enlarged from embryonic day 18.5. So large are the brains that the ventricles are completely occluded. Thus in the continuous presence of 3βAdiol there is an excessive accumulation of neurons in the brain.

Studies on neurotransmission

The DEVNERTOX project has studied the effects of developmental exposure to MeHg and/or the selected PCBs on three of the main neurotransmitter systems in mammals: the dopaminergic, cholinergic and glutamatergic systems (activated by the neurotransmitters dopamine, acetylcholine and glutamate, respectively). The receptors for each neurotransmitter can be differentially expressed in different brain areas. Moreover, the same neurotransmitter receptor may modulate different cerebral functions depending on the brain area or cell type in which it is expressed. For example, the metabotropic receptors for glutamate in striatum modulate motor function, while the same receptors modulate synaptic plasticity (long-term potentiation) and cognitive function in hippocampus. Thus, a neurotoxic substance affecting a specific neurotransmitter system might disrupt different functions in different brain regions.

Dopaminergic neurotransmission

Effects of MeHg and/or PCB 153 on dopaminergic parameters were studied in both developmentally exposed rats and in cultured cells (the PC12 cell line). Behavioural effects of PCB exposure are frequently ascribed to alterations in the functional state of aminergic systems, mainly dopaminergic neurotransmission. The ability of PCB to affect the dopaminergic system has been shown in *in vitro* (Bemis and Seegal.1999), and *in vivo* (Seegal et al. 2002) studies. Some results to the contrary have also been reported (Zahalka et al. 2001).

The results obtained in DEVNERTOX show that MeHg and PCB 153 affect the dopamine system in a manner that is age, brain area and gender dependent. Dopamine (DA) levels were determined in striatum, hippocampus, cortex and cerebellum from male and female rat pups at PND36 (puberty). MeHg was the only compound that affected the DA content; levels were decreased in the striatum and increased in the cerebral cortex. No effect was detected after co-exposure to MeHg and PCB 153.

Dopamine D_1-like and D_2-like receptor number and affinity were evaluated in the cerebral cortex and striatum of male and female rat pups at PND21 and PND36. Cortical D2-like receptors seem to be the most susceptible target for single or combined exposure to MeHg and PCB 153 while no effects on this receptor could be detected in the striatum. In the cortex, reduced D2-like receptor affinity was observed for both genders and the males also had increased D2 receptor density. Similar results were found at PND21 and PND36. The amount of D1 receptors were transiently decreased (*i.e.* reduced at PND21 but not PND36) in male rats exposed to the toxicants, but there was no change in affinity. The effects were similar in cerebral cortex and in striatum. In female rats, only MeHg exposure gave the same effect as in males, and only in striatum.

DA levels were also measured *in vitro*. High concentrations of either MeHg (>1 μM) or PCB 153 (>100 μM) induced an increase in extracellular DA which was associated with a decrease in intracellular levels, suggesting that both toxicants can induce release of DA from PC12 cells. In addition, similar concentrations of either MeHg or PCB 153 also resulted in a reduction in the tyrosine hydroxylase (TH) activity, one of the key enzymes in the DA synthesis. In line with the cell viability studies in the PC12 cells, competitive interactions were observed after co-exposure to certain doses. For example, 200 μM PCB 153 induced significant reductions in the intracellular DA levels and TH activity that were completely prevented when cells were exposed to the same concentration of PCB 153 combined with 0.5 μM MeHg. However, at most concentrations tested no interactions (competitive or synergistic) were detected.

Cholinergic neurotransmission

The cholinergic system is essential for normal brain development as a modulator of neuronal proliferation, migration and differentiation processes (Hohmann and Berger-Sweeney, 1998). Effects of developmental exposure to MeHg and/or PCB 153 on cholinergic muscarinic receptor (MR) density and affinity were determined in cerebral cortex, striatum, cerebellum and hippocampus of male and female rat offspring at weaning (PND21) and puberty (PND36) by means of saturation binding experiments. While no treatment affected the MR affinity, the density was altered in a brain area, gender and age dependent fashion.

In cerebral cortex, MeHg and/or PCB 153 decreased the amount of MRs to a similar extent (10-20%) and in a delayed fashion (PND36 only) both in female and male rats.

Striatal MRs were decreased by all treatments at PND21 in both genders. At PND36 only females were affected significantly and only by exposure to MeHg alone or in combination with PCB 153.

In cerebellum all exposure regimes decreased the number of MRs at PND36 in males but not in females. In PND21 animals, MeHg decreased muscarinic receptors in both genders, while no effects were detected after PCB 153 alone or the combined exposure.

In hippocampus both MeHg and PCB 153 decreased MRs in males at PND21 and PND36. However, exposure to the mixture of PCB 153 and MeHg did not affect the amount of receptors. In female rats, MeHg and MeHg + PCB153 reduced hippocampal MRs only at PND36.

Based on the analysis of toxicant levels in different brain areas (see above) the regional specificity of the effects on MR density cannot be ascribed to a differential regional distribution of MeHg or PCB 153 in the rat pup brain.

In addition to the studies on muscarinic receptors, cholinergic nicotinic receptors were also investigated in brain tissue from mice exposed at PND10 to MeHg and/or PCB 126. A significant decrease in nicotinic receptors was observed in the cerebral cortex from adult mice neonatally co-exposed to MeHg and PCB 126 (Table 2).

Table 2. Male NMRI neonatal mice were exposed to a single oral dose of PCB 126, MeHg, co-exposure to PCB 126 and MeHg, or a 20% fat emulsion vehicle at a neonatal age of 10 days. The animals were killed at 5 months of age and [^{3}H]-α-Bungarotoxin binding (mean ± SD) was assessed in the P2 fraction. The statistical evaluation was made using one-way ANOVA and pairwise testing using Duncan's test. Capital letters P≤0.05.

Effects on nicotinic receptors in cerebral cortex in 5 months old mice after neonatal exposure to PCB 126 and MeHg

Treatment (mg/kg body weight)	[^{3}H]α-Bungarotoxin binding (pmol/g protein)	
Vehicle (A)	51 ± 18	
PCB 126 0.046 (B)	49 ± 16	
PCB 126 0.46 (C)	42 ± 12	
MeHg 0.4 (D)	44 ± 15	
MeHg 4.0 (E)	39 ± 12	
PCB 126 0.046+MeHg 0 .4	34 ± 12	A,B
PCB 126 0.046+MeHg 4.0	33 ± 12	A,B
PCB 126 0.46+MeHg 0.4	26 ± 8	A,C,D
PCB 126 0.46+MeHg 4.0	26 ± 8	A,C,E

Glutamatergic neurotransmission

Studies of glutamatergic effects of MeHg and/or the selected PCB congeners (both 153 and 126) were performed using exposed animals, rats or mice, and primary cultures of cerebellar neurons from rats (cerebellar granule cells – CGCs). The function of the glutamate-NO-cGMP pathway (see Fig. 12) was analysed by microdialysis in the cerebellum of freely moving rats when they were 3 or 7 months-old. Addition of NMDA through the microdialysis probe activates this pathway and increases extracellular cGMP. The increase in cGMP is a good measure of the function of the pathway in cerebellum *in vivo*.

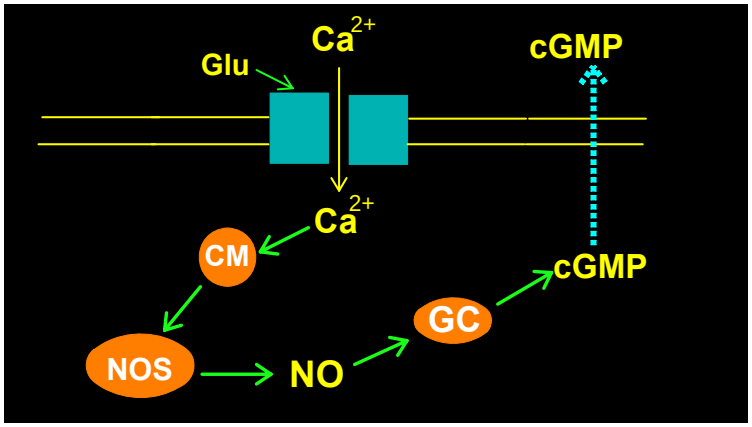

Figure 12. The Glutamate-NO-cGMP pathway. cGMP = cyclic GMP; CM = calmodulin ; GC = guanylate cyclase; Glu = glutamate; NO = nitric oxide; NOS = nitric oxide synthase.

Developmental exposure to MeHg, PCB 153 or PCB 126, significantly reduced the function of the glutamate-NO-cGMP pathway when the rats were 3 but not 8 months old (regardless of gender). The results suggest that the function of the glutamate-NO-cGMP pathway decreases with age in the control animals and that this is associated with decreased learning ability. Rats exposed to mixtures of MeHg and one of the PCBs did not differ from controls. Interestingly, there was an excellent correlation between the effects on the function of the glutamate-NO-cGMP pathway in cerebellum *in vivo* and the ability of the rats to learn the Y-maze task, indicating that the toxicant induced glutamate-NO-cGMP pathway impairments can affect learning and memory function in the rats.

Primary cultures of cerebellar neurons were treated *in vitro* with different concentrations of MeHg, PCB 153, and PCB 126, alone or combined. The aim was to assess whether this model could reproduce the effects on the glutamate-NO-cGMP pathway found in brain *in vivo* and to gain further insights into the mechanisms of action of the neurotoxicants on the function of this pathway.

Long-term (10-14 days) exposure to MeHg, PCB 153 or PCB 126 did indeed disrupt the function of the pathway in the CGCs, thus reproducing the effects found in the cerebellum of 3-month-old rats. The prevention of the impairment of the pathway found *in vivo* after co-exposure to MeHg and either PCB was not generally reproduced *in vitro*. Only some specific combinations of concentrations of PCB and MeHg had antagonistic effects, while others potentiated the effect compared to single exposures with the same concentrations.

The *in vitro* model was also used for studies of separate steps of the glutamate-NO-cGMP pathway, to shed some light on the mechanisms of the different compounds: Results indicated that MeHg impairs activation of guanylate cyclase by NO and, subsequently, the function of the pathway. PCB 153 and PCB 126, on the other hand, increase basal levels of cGMP. Co-exposure with MeHg could attenuate the increase in basal cGMP induced by PCB 153 but not by PCB 126.

Figure 13 shows a comparison of at what concentrations in the cell medium the selected toxicants induced cell death, affected basal cGMP or NMDA-induced activation of the glutamate-NO-cGMP pathway in the CGCs. Estimations of *in vitro* concentrations that would correspond to the brain tissues levels measured in exposed rats are also included.

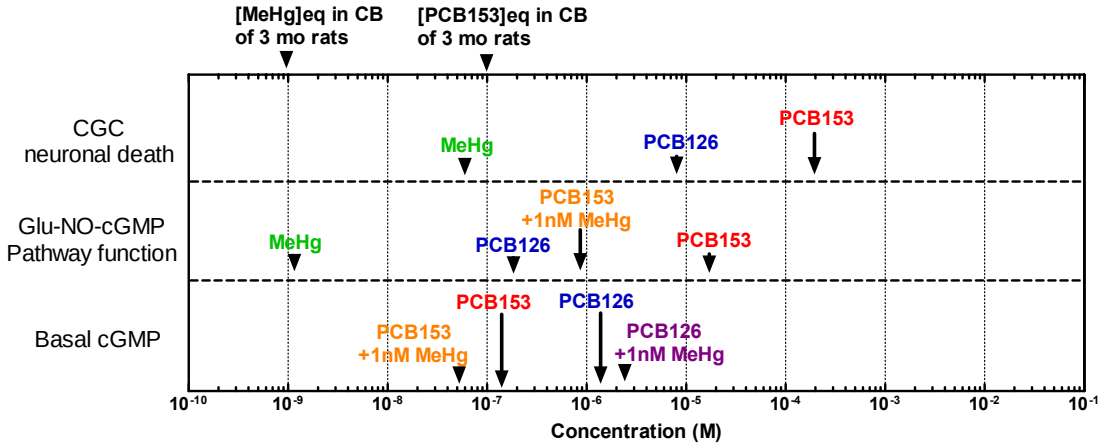

Figure 13. EC_{50} for different effects of MeHg and/or the selected PCBs on primary cultures of cerebellar neurons (CGC). The arrows show concentrations of the toxicant(s) in the cell medium causing a 50% effect. [MeHg/PCB153] eq in CB of 3 mo rats = Concentrations estimated to be equivalent to the levels of the toxicants measured in cerebellum of 3 months old exposed rats.

The effects on glutamatergic neurotransmission were also studied at the level of synaptic plasticity in rats and mice. In rats, long-term potentiation (LTP) in the CA1 area of hippocampus was analyzed *ex-vivo* in hippocampal slices from 2 or 8 months old males and females. Hippocampal LTP is an activity-dependent form of increased transmission efficacy at the synapses that is considered to be the basis for some forms of learning and memory. The LTP studied in the rats is dependent on activation of the NMDA type of glutamate receptors, thus, the same type of receptors that control the glutamate-NO-cGMP pathway. The effects observed were strongly dependent on gender and age.

In young (2 months old) rats, developmental exposure to MeHg, PCB 153, PCB 126, or the combination of MeHg with either PCB, impaired LTP (*i.e.* decreased the magnitude of the potentiation) in male but not in female rats. In the older animals (8 months old), exposure to MeHg alone impaired LTP regardless of gender, while MeHg+PCB 126 only affected the males and MeHg+PCB 153 only the females. No other exposure regime caused significant effects on the LTP magnitude in the mature rats, although PCB 153 impaired the maintenance of LTP in females only.

In addition to the rat studies, LTP measurements were also performed in the dentate gyrus of hippocampus from wild type (WT) and ß-estrogen receptor knockout (BERKO) mice. PND10 treatment with PCB 153 caused a reduction in LTP in adult male and female WT mice (LTP was assessed 2-3 months after exposure). This effect was also detected in male, but not in female, BERKO mice, indicating a possible role for the ß-estrogen receptor in the neurotoxic action of PCB 153 in females (Fig. 14). No effects on LTP were found in WT mice exposed to MeHg via their mothers (GD7-PND21). However, if combining this MeHg exposure with a single injection of PCB 153 *p.o.* at PND10, reductions in the magnitude of the LTP were observed in both males and females, comparable to those caused by PCB 153 alone.

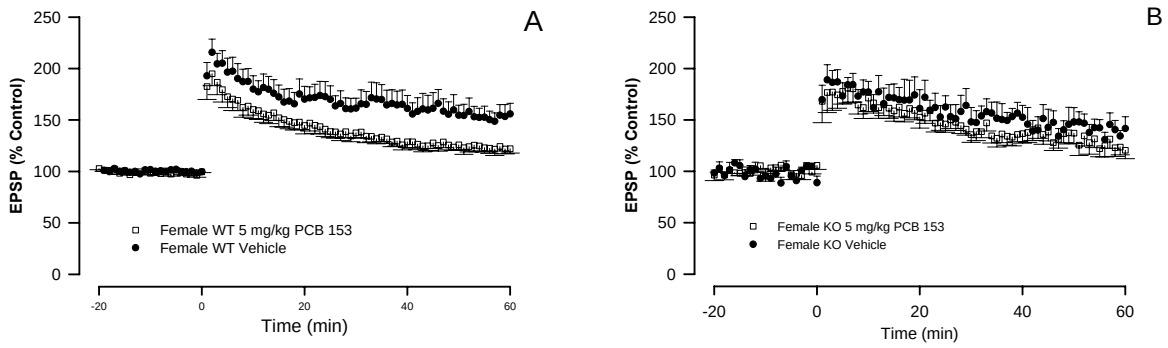

Figure 14. Effects of PCB 153 on LTP of excitatory synaptic transmission in WT and BERKO female mice. A; wild type (WT) and B; ß-estrogen receptor knockout (BERKO) mice. Neonatal mice were orally dosed with 5.1 mg PCB 153/kg b.w. PND10.

Studies on behavioural effects in rats

Behaviour is a major function whereby animals adapt to changes in the environment and changes in behaviour may reveal effects on the nervous system caused by the influence of a toxicant. Behavioural effects of the selected neurotoxicants in rats exposed during gestation and lactation (GD7-PND21) were tested both pre- and post-weaning, taking gender and age into consideration in the analysis.

Pre-weaning tests and observations included measurements and assessments of the following: body weight gain, ano-genital distance, pinna detachment, ear opening, incisor eruption, hair growth, eye opening, negative geotaxis, cliff avoidance, righting reflex, free fall righting, forepaw suspension, pole grasping, homing behaviour, and acoustic startle reflex. Although a few developmental effects (delays or hastenings) were observed in exposed pups, these effects were small and all animals reached criterion by PND21.

Post-weaning behavioural tests were carried out from PND30 up to 20 months of age and included: locomotor activity in an open field, rotarod (testing motor coordination and balance), hot plate response (testing sensitivity to pain), sudden silence test (testing attention), elevated plus maze (testing anxiety-like behaviour), novel object test (testing exploration and memory), Morris water maze, radial arm maze and passive avoidance (all testing learning and memory). In addition, the behavioural (motor) response to an amphetamine challenge dose, given after amphetamine sensitizing treatment, was analysed in rats exposed to MeHg and/or PCB 153.

MeHg seems to affect locomotion in female rats, indicated by a decrease in ambulatory movements at PND30 and a decrease in vertical movements at PND60 and 90. However, at 8 months of age this effect was no longer detected in females and males had significantly increased locomotor activity compared to controls. At the same age, the male rats also displayed reduced motor coordination and balance. Similar to the studies in mice, impaired learning and memory ability was observed in both genders after MeHg exposure when testing at an age of 3 months. This effect was however not detected at 8 months. MeHg also increased sensitization to amphetamine in male but not in female rats.

The observed behavioural effects of developmental exposure to PCB 153 in rats included increased locomotor activity and impaired motor coordination and balance. Effects were detected in both males and females, but with some age differences. In addition, learning and memory was affected in both genders at an age of 3, but not 8, months. Co-exposure to MeHg and PCB 153 resulted in reduced locomotion at 3 months and, in contrast, an increase at 8 months, regardless of gender.

Hyperactivity was observed in male rats developmentally exposed to PCB 126 and tested from PND90. In females the locomotor activity was decreased or unaltered at earlier ages, and increased at 7 months. Decreased performance in the rotarod was found at PND60 in male rats only. Similar to MeHg and PCB 153, and in line with the studies on the glutamate-NO-cGMP pathway, impaired learning and memory was observed in both genders at an age of 3, but not 8, months. Co-exposure to MeHg and PCB 126 also affected locomotion in an age- and gender-dependent manner.

Studies on behavioural effects in mice

Behaviour in mice was studied both after maternally mediated exposure (GD7-PND7 or GD7-PND21) and after single oral exposure during a defined critical period of neonatal brain development (PND10). Both wild type and genetically modified mice, β-estrogen receptor knockout (BERKO) mice, were assessed to investigate a possible role of the β-estrogen receptor in the onset of developmental neurotoxic processes.

The neonatal PND10 model was used to investigate whether PCBs (ortho- or co-planar) together with MeHg, can interact to enhance developmental neurotoxic effects on spontaneous behaviour, habituation, learning and memory. In addition, cholinergic receptors were studied. In traditional behavioural teratology and/or developmental toxicology the chemical to be tested is given to the mother. This implies that effects of direct exposure to chemicals at critical developmental phases can be underestimated. Earlier studies with the PND10 model have found that low-dose exposure to environmental toxic agents such as PCB, DDT, brominated flame-retardants, pyrethroids, organophosphates, and nicotine, can cause persistent developmental neurotoxic effects when administered directly to pups during this defined critical period of neonatal brain development in the mouse (Eriksson et al., 1992; 2000; Eriksson and Fredriksson, 1996a,b, Eriksson, 1997, Eriksson 1998, Eriksson et.al. 2001; Viberg et al. 2003a,b; 2004).

Spontaneous behaviour is especially meaningful to test as it reflects a function dependent on the integration of a sensoric input into a motoric output, and thus reveals the ability of animals to habituate to an environment and integrate new and previously attained information. In this test the animal's ability to habituate to a novel "home-cage" environment was studied (Fredriksson, 1994). The results shows that when PCB 153+MeHg, or PCB 126+MeHg, are given directly to pups on postnatal day 10 in low doses, they can interact and significantly enhance developmental neurotoxic effects, manifested as disrupted spontaneous behaviour and lack of habituation (for PCB 153 results see Fischer et al. 2004).

The ability of adult mice to learn and memorize was studied in several tests: In a swim maze of the Morris water-maze type (Morris, 1981) especially mice co-exposed to MeHg and PCB 126 showed significant impaired learning and memory functions (Fig. 15). The performances in this type of test is also suggested to be correlated to the function of the cholinergic system (Lindner and Schallert, 1988; Whishaw, 1985), and, as described in the section on neurotransmission, a significant decrease in cholinergic

nicotinic receptors in the cerebral cortex was observed in adult mice neonatally co-exposed to MeHg and PCB. The results on exposure to PCB 153 or PCB 126 alone were in accordance with earlier findings testing other PCB congeners (Eriksson and Fredriksson, 1996a,b; Eriksson and Fredriksson, 1998; Eriksson 1998). Neonatal exposure of wild type (WT) and BERKO mice to PCB 153 revealed no difference in spontaneous behaviour between WT and BERKO.

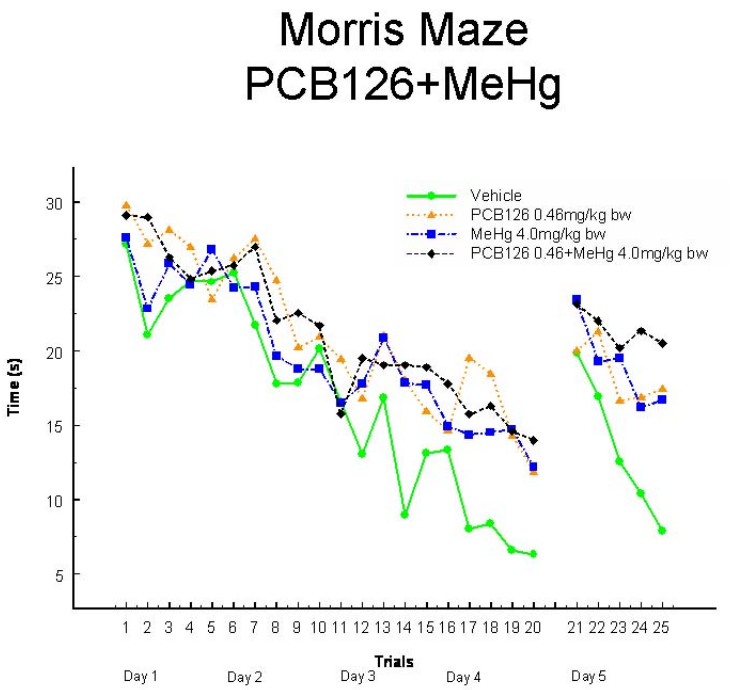

Figure 15. Swim maze behaviour in 5-month-old NMRI male mice exposed to a single oral dose of PCB 126, MeHg, co-exposure to PCB 126 and MeHg or the 20% fat emulsion vehicle at a neonatal age of 10 days. Data from days 1-4 was submitted to an ANOVA using a split-plot design with Duncan's test. Day 5 was analysed by a one-way ANOVA and Duncan's test. The data showed a treatment effect day 1-4, control<PCB126, MeHg, PCB126 + MeHg. Relearning on day five showed treatment effect, control<PCB 126, MeHg <PCB126 + MeHg.

Spontaneous T-maze behaviour is a hippocampal-dependent measure of spatial working memory which was significantly affected after maternally mediated MeHg exposure (GD7-PND21). The addition of single exposure to PCB 153 on PND 10 did not alter this effect. T-maze spontaneous alternation was more sensitive than novel hole board exploration, as a behavioural indicant of neurotoxicity of low dose MeHg. This is indicative of a selective spatial working memory impairment. A decrease was also found in another learning test, patrolling behaviour, in male mice exposed to MeHg GD7-PND7 (Onishchenko et al. 2007).

The shorter duration MeHg exposure (GD7-PND7) also caused higher predisposition to depressive-like behaviour in male mice, as shown in the forced swim test. Thus, this study provided novel evidence that the developmental exposure to MeHg could affect not only cognitive functions but also motivation-driven behaviour (Onishchenko et al. 2007).

The MeHg induced deficit in spontaneous T-maze behaviour was not detected in exposed BERKO females mice exposed to MeHg (GD7-PND21) (Fig. 16). Neonatal exposure to PCB 153 at PND10 also caused a significant deficit in T-maze behaviour in female WT but not BERKO mice. These data indicate that the effects of PCB 153 or MeHg on this endpoint are less pronounced in female mice lacking the β-estrogen receptor. There was a relatively poor correlation between neurotoxicant-induced deficits in hippocampal LTP and T maze performance.

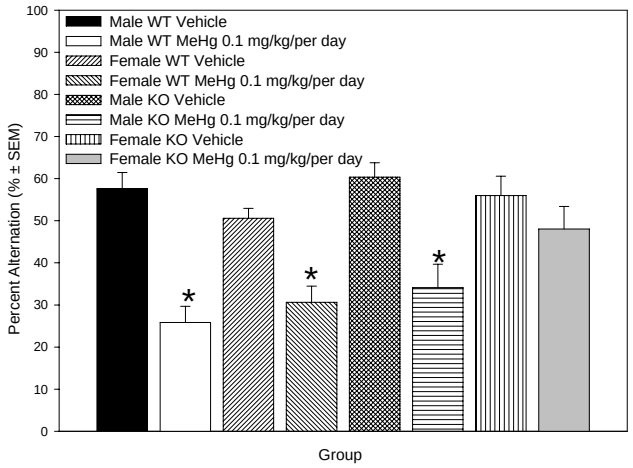

Figure 16. T-maze percent alternations in methylmercury treated wild type (WT) and ß-estrogen receptor knockout (KO) mice at an age of 2 months. Exposure GD7-PND21.

Comparison of *in vivo*- and *in vitro*-models

Toxicant levels in cells and tissue

As described in previous sections, effects on dopaminergic and glutamatergic neurotransmission were observed both *in vitro* and *in vivo* after exposure to the neurotoxicants. One way of evaluating the sensitivity and comparability of different models is to compare the so called actual target doses. Mercury and PCB 153 levels were measured both in exposed cells and in tissues from exposed animals. Figure 17 shows a comparison of Hg levels in cells and in brain tissue after exposure to MeHg, in relation to the suggested lowest observed adverse effect level (LOAEL) in humans (Lewandowski *et al.* 2003). It should be noted that this comparison is based on certain estimations and should be considered as "order of magnitude".

In a similar way, *in vitro* and tissue levels of PCB 153 were compared. For this toxicant, the *in vitro* concentrations that induce significant effects on cell viability in the three cell lines analysed (C17.2, PC12 and SK-N-MC) were at least one order of magnitude higher than the brain tissue levels measured in exposed rats. However, the highest *in vivo* measurements (PND1) were only slightly lower than levels affecting cell viability in primary cultures of embryonic cortical neural stem cells (cNSC exposed to 1 μM PCB 153). For PCB 126, the cNSC intracellular levels were compareable or lower than the rat brain levels.

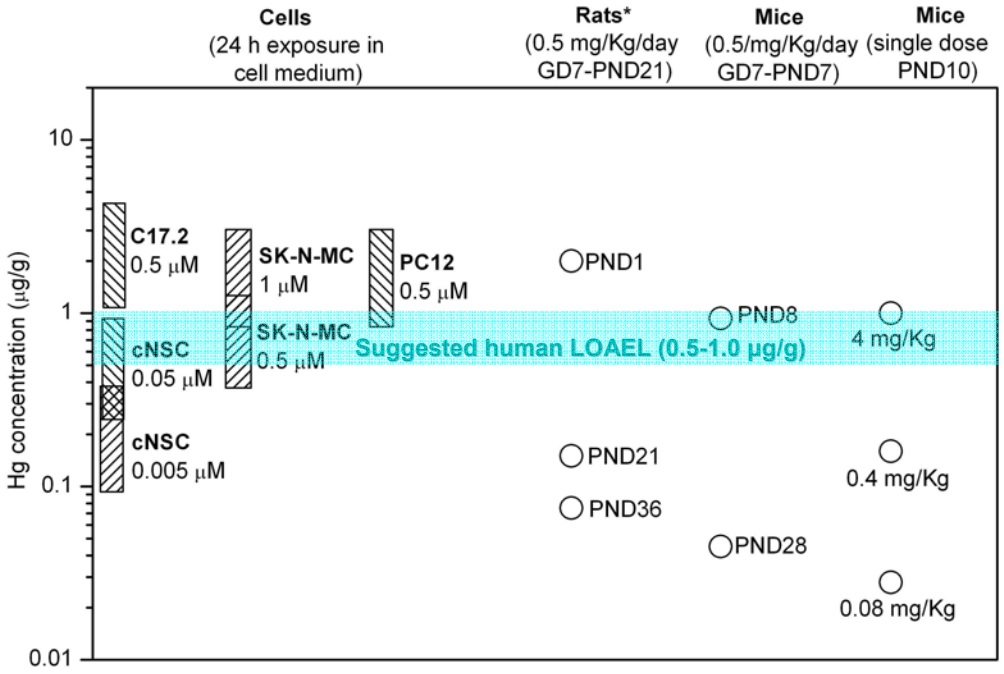

Figure 17. Mercury target doses (expressed as µg/g wet weight) in different DEVNERTOX models. Intracellular Hg levels in three MeHg-exposed cell lines (C17.2, PC12, SK-N-MC) and one primary culture of cortical neural stem cells (cNSC) compared to average target doses in brain tissue from exposed animals and the human lowest observed adverse effect level (LOAEL) suggested by Lewandowski *et al.* 2003. In cNSC, exposure to 5 nM MeHg inhibits spontaneous neuronal differentiation. The other *in vitro* MeHg exposure doses shown are in the same range as the benchmark doses for viability (equal to EC_{10}, *i.e.* doses causing 10% cell death). In mice exposed to a single oral dose of MeHg at PND10 the Hg levels were measured after 24h exposure (PND11).**Values are extrapolated from dry tissue measurements taking into account a factor of 4 between wet and dry tissue.*

Effects on neurotransmission were found *in vitro* and *in vivo*. For example, exposure to MeHg, PCB 153 or PCB 126 impaired the glutamate-NO-cGMP pathway in both model systems and the results also correlated with decreased learning and memory in the exposed rats. The Hg and PCB 153 levels that affect glutamatergic transmission in cerebellar granule cells in culture and in rat cerebellum *in vivo* are estimated to be in the same range (Fig. 13).

Effects of combined exposure to MeHg and PCB

Co-exposure to MeHg and one of the PCBs were studied both *in vitro* and *in vivo*. In general, the results were highly dependent on the model, the endpoint studied and the concentrations of the toxicants (naturally more dose combinations were tested *in vitro*). No clear overall pattern emerges when looking at the entire DEVNERTOX data set.

Discussion

The DEVNERTOX *in vitro* models

During the DEVNERTOX project, nine *in vitro* experimental models have been evaluated to improve neurotoxicity testing methods and to identify biomarkers based on the mechanism of action of MeHg and PCBs. The *in vitro*-models displayed different sensitivity for the selected toxicants (Fig. 3). Doses inducing cytotoxicity after 24 hours of exposure were generally in the micromolar range. Primary cultures of embryonic neural stem cells (cNSC) was the most sensitive model with regard to both MeHg and the PCBs. Cells exposed under serum-free conditions appear to be more sensitive, especially to PCB 153. Therefore, culture conditions should be taken into account when comparing different *in vitro* studies.

Human cord blood levels of both PCBs and MeHg are typically in the nanomolar range (Winneke et al., 1998; Björnberg et al. 2005). However, the comparison of doses used *in vitro* with levels measured in human tissues is a complicated issue. In fact, the amount of toxicant actually taken up by cells in culture might depend on a variety of factors, including the volume of the exposure media, the cell density, the presence of serum and potential binding of PCB to plastic (Meacham et al., 2005).

Nevertheless, cNSC results show that levels of MeHg (2.5-5 nM) lower than those found in the umbilical cord blood of Swedish pregnant women (0.99 µg/L) (Björnberg et al. 2005) can inhibit spontaneous neuronal differentiation (Tamm et al. 2006). Remarkably, a daily exposure to the reference dose of 0.1 µg MeHg/kg bodyweight (NRC 2000), equates to 5.8 µg/L in cord blood, which is 10-fold higher than the concentration used in our studies. In addition, as shown in Figure 17, intracellular Hg levels in cNSC exposed to 5 nM MeHg were lower than the suggested human lowest observed adverse effect level (LOAEL, Lewandowski et al. 2003) and in the same range as what has been detected in brains from normal human infants (Choi et al. 1978; Lapham et al. 1995).

Thus, in light of these results, there seems to be a narrow margin of exposure against the risk of neurodevelopmental effects, and dietary advice for pregnant women is necessary and justified. In addition, considering that neural stem cells are also present in the adult nervous system, where they may have a role in learning, memory and response to injury, exposure to low levels of MeHg may have negative consequences in adulthood as well.

Intracellular target doses constitute a better measure of exposure than the nominal concentration in the cell medium. In our models, the target doses varied depending on the cell type: the PC12 pheocromocytoma cell line accumulated more Hg and less PCB 153 than the SK-N-MC neuroblastoma cell line. Accordingly, the EC_{50} for viability after PCB 153 exposure was about three times lower for SK-N-MC compared to PC12. The C17.2 neural progenitor cell line and the PC12 cells were similar with regard to Hg and PCB 153 uptake, but C17.2 cells were slightly more sensitive than PC12 when investigating cell viability. The cNSC was the most sensitive *in vitro* model, both for MeHg and the PCBs, and was also the cell type were the lowest intracellular levels were measured.

Toxicant levels in the DEVNERTOX *in vitro* and *in vivo* models

Estimated intracellular target doses were compared to the measured brain tissue levels in exposed animals (see Fig. 17 for mercury data). Due to differences in methodology and uncertainties in comparing *in vitro* and *in vivo* results these comparisons should be considered as "order of magnitude". Briefly, Hg brain levels detected in rats at

postnatal day (PND)1 were similar to the levels in C17.2, PC12 and SK-N-MC cells exposed to a nominal MeHg dose that induces significant cell death (0.5 or 1 μM MeHg in the cell medium). The highest Hg levels measured in mice brain (at PND8 or PND11) were in the same range or higher than intracellular levels in SK-N-MC cells and cNSC.

The data on brain Hg levels in children are obviously very limited. Total Hg levels reported in asymptomatic neonates from the Seychelles were below 0.3 μg/g (Lapham et al.1995). In two full-term babies who died from *in utero* exposure to MeHg during the Iraqi outbreak, brain total Hg levels ranged between >1 and 13.7 μg/g, well above the levels of 0.1 to 0.4 μg/g measured in normal infants in the area of Minamata, Japan (Choi et al. 1978). Based on these data Lewandowski et al. (2003) have proposed a human *in vivo* LOAEL in the range of 0.5-1.0 μg/g. Figure 17 shows the measured Hg target doses in different DEVNERTOX models in relation to this level.

The maximal levels of Hg detected in the rat pups' brain (PND1) were higher than the suggested human LOAEL and just below the range of moderate MeHg exposure (brain Hg : 3-11 μg/g, Burbacher et al. 1990). In humans, developmental MeHg exposure resulting in brain Hg doses between 3 and 11 μg/g tissue has been associated to mental deficiency, abnormal reflexes and muscle tone, and retarded motor development. A delayed psychomotor development of children has been shown to result from brain Hg doses below 3 μg/g (Burbacher et al. 1990). Hg levels detected in the mice models and at later time points in rats were in the same range or lower than the suggested human LOAEL.

For PCB 153, the levels in PC12, C17.2 and SK-N-MC cells were higher than those measured *in vivo*, while levels inducing cell death in cNSC were more comparable to the brain levels in rats. Unfortunately, except for adipose tissue, there are very few data available regarding the investigation of PCBs in human tissues and to the best of our knowledge, there is no data available concerning brain PCB 153 levels in exposed children. According to a recent study (Chu et al. 2003) PCB 153 averaged 3.10 ng/g wet tissue in the brain tissue of unexposed Belgian subjects aged 5 to 76 years. In comparison, the PCB 153 levels in our exposed rats were in the μg/g range. The PCB 153 congener accounted for almost 30% of the sum PCB in the human samples (Chu et al. 2003).

The situation is the same for PCB 126; information on levels in brain tissue from exposed humans is totally lacking, and therefore an animal *vs*. human comparison in terms of brain target doses is not feasible. Indeed, PCB 126 levels in the brain of non-exposed individuals are very low, i.e., 0.6 pg/g wet weight (Chu et al. 2003). The intracellular PCB 126 levels in cNSC were similar or lower than those measured in exposed rat brains.

Distribution and kinetics of Hg and PCB 153 in exposed animals

To main types of toxicant exposure regimes were used in the experimental animal studies in DEVNERTOX: Maternally mediated exposure during gestation and lactation in rats and mice (GD7-PND7 or GD7-PND21) and single dose exposure of mice at PND10 (the neonatal 'brain growth spurt' – model). Comparison of toxicant levels in brain tissue from exposed animals to the intracellular in vitro levels and reported human brain levels are discussed in the *Toxicant levels in the DEVNERTOX* in vitro *and* in vivo *models*-section.

The rapid decrease of tissue Hg levels in the rat offspring brain during the suckling period observed by us is consistent with the data of Sakamoto et al. (2002a). These authors reported that in rat offspring brain Hg levels were about 1/5 of those at parturition as early as 10 days after birth and did not further decrease during the following 10 days of lactation. Altogether, the present results and the data of Sakamoto et al. (2002a) support that the MeHg excretion through milk is limited.

In humans Hg is usually measured in whole blood or hair and the results are difficult to directly compare with our data which is expressed as μg/g erythrocytes. In general, the Hg levels estimated in the rat pups studied in the DEVNERTOX project are lower or comparable to blood mercury levels found in intoxicated subjects from Iraq or Japan (Amin-Zaki et al. 1976; Akagi et al. 2000) or in other intoxication cases (Knobeloch et al. 2006).

With respect to the PCB 153 levels in rats, the total dose given to rat dams (180 mg/kg) was similar to the total maternal PCB 153 dose (175 mg/kg) that has previously been shown to cause a significant reduction of LTP in the rat offspring (Hussain et al. 2000). The brain PCB 153 levels measured in the rat pups are comparable with those found in other animal studies (Lyche et al. 200b). The amount of ortho- and co-planar PCBs found in the brain 24 hours after a single oral administration to 10-day-old mice has previously been determined to about 3-5 per mille of the administered dose (Eriksson and Darnerud, 1985; Eriksson, 1988, Eriksson, 1998).

In human studies, PCB 153 levels are usually normalized to the serum lipid content, while we expressed the PCB 153 data as μg/g serum to take into account other possible PCB binding sites, e.g. proteins (Lyche et al., 2004b; Maervoet et al. 2005). Assuming that the human serum lipid content is about 680 mg/dl (Akins, 1989), PCB 153 levels in maternal pregnancy serum (110 ng/g serum lipid, Longnecker et al., 2003) and in preschool Inuit children exposed to both MeHg and PCBs (7-777 ng/g serum lipid, Saint-Amour et al. 2006) are reported to be 10 to 1000 lower than those found in the exposed rats, depending on the time-point considered.

The situation is the same for PCB 126; information on levels in brain tissue from exposed humans is totally lacking, and therefore an animal *vs*. human comparison in terms of brain target doses is not feasible. Indeed, PCB 126 levels in the brain of non-exposed individuals are very low, i.e., 0.6 pg/g wet weight (Chu et al., 2003).

The differences observed between rat dams and pups in terms of PCB 153 brain levels at weaning are consistent with previous findings that have documented that at PND21 cerebral PCB 153 values are 8-10 times higher in the pups than in their mothers after gestational (GD10-GD16) exposure to this congener at 20 mg/kg/day (Coccini et al., 2006). Previous animal studies of PCB 153 found that about 60 % of the body burden was eliminated via milk during the first 5 days of lactation and virtually all by day 20 (Vodicnik and Lech, 1980; Gallenberg and Vodicnik, 1989). Also, Kaya et al. (2002) reported that from parturition to weaning PCB concentrations increase in the brain of suckling rat pups, while they simultaneously decrease in the brain of the lactating dams that were exposed *per os* to a PCB mixture from day 50 before mating until delivery. Taken together, these experimental observations support that, unlike MeHg, PCB 153 is transferred very efficiently from mother to pup through lactation.

Not only the dose but also the timing of developmental exposure to a neurotoxic substance can affect the outcome. For example, it has been suggested that the

developmental effects of MeHg can be underestimated in rodent studies since critical steps of the neurodevelopment that occur *in utero* in humans, when the maternal transfer of MeHg is high, develop postnatally in rats and mice, when the MeHg exposure (via lactation) is limited. Examples of such neurodevelopmental steps include the development of cortical, hippocampal and cerebellar structures and neuronal interconnections (Bayer et al., 1993; Davison and Dobbing, 1968). Thus, differences in outcome between the different *in vivo* models used in the DEVNERTOX project could relate to differences in the type of exposure at certain neur0developmental phases.

Cell death mechanisms induced by MeHg and PCBs

One of the main endpoints studied was cell death and activation of different apoptotic pathways. With regard to cell death mechanisms, MeHg-exposed neural stem cells (cNSC and C17.2) undergoing apoptosis show activation of both caspases and calpains (Tamm et al. 2006). The caspase activation is in line with a study of neuroblastoma SH-SY5Y and glioblastoma U 373MG cell lines (Toimela and Tahti 2004), but in contrast to the results from hippocampal HT22 and pituitary AtT20 cell lines, as well as some other neuronal and glial cells previously studied by us (Johansson et al. 2006; Castoldi et al. 2000; Daré et al. 2000; Daré et al. 2001a), where caspases seem not to play a crucial role in MeHg induced cell death. Activation of the calcium-regulated protease calpain was observed in both neural stem cells and hippocampal HT22 cells after MeHg exposure, and has also been detected in cerebellar granule cells exposed to MeHg (Daré et al. 2000; Sakaue et al. 2005).

ROS formation and oxidative stress in the brain as a result of MeHg exposure has been observed both *in vivo* (Yee and Choi, 1994) and *in vitro* (Sarafian and Verity, 1991), and was also confirmed in our models (***e.g.*** AtT20, PC12). The protective effects of β-estradiol against MeHg-toxicity observed in C17.2 cells could be mediated via estrogen receptor(s) and/or its antioxidant capacity. Unlike some previous studies, ROS formation was not observed after PCB exposure in AtT20 cells (Johansson et al. 2006) and antioxidants did not significantly protect AtT20 or HT22 cells from PCB-induced cell death. In PC12 cells lipid peroxidation was increased by both MeHg and PCB 153 (Vettori et al. 2006). Overall, activation of oxidative stress seems to depend upon both cell type and PCB congener (***e.g.*** Mariussen et al., 2002; Howard et al., 2003; Lee and Opanashuk, 2004).

Both PCBs caused impaired mitochondrial function in AtT20 and HT22. This could be a consequence of alterations of the mitochondrial membrane structure, since PCBs (at least ortho-substituted congeners) are suggested to have membrane disrupting properties (Tan et al., 2004). MeHg and PCBs have been reported to cause elevation of intracellular calcium levels in different cell types (Marty and Atchinson 1997; Marty and Atchinson 1998; Kodavanti and Tilson, 2000; Kang et al. 2004). Proteases (other than caspases) are thought to play a role in neuronal cell death via a chain of events where calcium-induced activation of calpain is suggested to trigger lysosomal rupture and release of cathepsins into the cytosol, leading to further degradation of cellular proteins (for review see Yamashima, 2004). The protective effects of different protease inhibitors in the present study suggest involvement of this ‘calpain-cathepsin cascade’ also in PCB-generated cell death in several of our models.

Induction of similar cell death mechanisms were found after exposure to the dioxin-like PCB 126 and the non-dioxin-like PCB 153. Chemical analysis showed that the PCB 153 stock used was free from contamination of any dioxin-like PCBs. This

suggests that the PCB induced cell death was a result of effects on membrane disruption and calcium homeostasis rather than mediated by binding of the PCBs to specific receptors, such as the aryl hydrocarbon or estrogen receptors.

Hormone levels in exposed animals

There is multiple evidence that PCBs can interfere with the endocrine system of animals and humans (Colborn et al., 1993; Hany et al., 1999; Kaya et al., 2002; Lyche et al., 2004a). In boys prenatally exposed to PCBs and PCDFs during the Yucheng disaster, serum estradiol levels were higher, while testosterone levels were lower, as compared to control boys, at the age of puberty (Hsu et al., 2005). Experimentally, steroid receptors levels and estrogen receptor-mediated functions as well as steroid metabolism and circulating hormone concentrations have been found to be altered after PCB treatment (Hany et al 1999; Lilienthal et al 2000; Lyche et al 2004a; Wojtowicz et al 2005). PCBs have been reported to possess estrogenic or anti-estrogenic properties depending on the congener type and the experimental system used (Jansen et al 1993; Kaya et al 2002). In particular, coplanar PCB congeners *(e.g.,* PCB 126) are suggested to be anti-estrogenic (Jansen et al., 1993).

According to preliminary data obtained in the DEVNERTOX project, PCB 126 reduced uterus weight in adult female rats developmentally exposed to this coplanar congener, possibly as a result of its anti-estrogenic properties. Other authors have reported that PCB 126 delays vaginal opening in female rats after a single maternal exposure to 10 μg/kg b.w. at GD15 (Faqi et al., 1998). In male rats, the latter treatment reduced ano-genital distance shortly after birth, and decreased serum testosterone in adulthood. A longer prenatal treatment (3 μg/kg/day, GD7-GD21), on the other hand, decreased plasma testosterone levels in males at weaning, but not at later ages (Yamamoto et al., 2005*).* Data from the DEVNERTOX studies further confirm the susceptibility of male testosterone to PCB 126, but not PCB 153. With respect to the latter congener, results from the ER-Calux Assay have indicated that it has anti-estrogenic properties *(*Pliskova et al., 2005), whereas other data in rats have suggested that it exerts estrogenic effects (Desaulnier et al., 1999). *In vitro*, both PCB 126 and PCB 153 have been reported to affect the steroid secretion of follicular cells (Wojtowicz et al., 2000). Long-term changes in estradiol levels were indeed found also by us, both in male and female rats.

The role of the PCB-binding Clara cell protein

Both MeHg and PCBs can interact with various proteins in an organism and the CC16 protein is suggested to play a role in PCB-binding and elimination through renal filtration and excretion in urine. Our studies showed that CC16 reaches high levels in human amniotic fluid samples where it binds with high affinity to PCB 126 and another neurotoxic PCB congener, PCB 28. Clearly PCB-sequestration in amniotic fluid is likely to have an impact on the risk for fœtal neurotoxicity, and further studies are underway to investigate the potentially protective role of CC16 in PCB-induced developmental neurotoxicity.

Estrogen receptors in the developing central nervous system

Studies of the β-estrogen receptor (ERß) indicated that ERß is necessary for the development of calretinin-immunoreactive GABAergic interneurons and for neuronal migration in the cortex through modulating epidermal growth factor receptor expression at middle and later embryonic stages (Fan et al. 2006). This suggests that

interference by neurotoxicants with ERα signaling can occur after day 18.5 but interference with ERβ signaling may occur at any time during brain development from day 12.5. Overall the window of opportunity for xenoestrogens to cause disruption of estrogen signaling extends from embryonic day 12.5 to puberty.

A novel player, CYP7B1 has to be factored in when the role of PCBs in the embryonic brain is being considered. PCBs are well known inducers of Cytochrome P450 enzymes and may influence 3βAdiol levels in the brain. If PCBs are estrogenic via ERβ, in the developing brain we should see an increase in the number of neurons. Alterations in interneuron populations may be novel markers for detection of effects of xenoestrogens in the brain.

Some, but not, all behavioural and electrophysiological studies of the β-estrogen receptor knockout (BERKO) mice indicated a possible role for the β-estrogen receptor in the neurotoxic action of MeHg and PCB 153 in female mice.

Effects of MeHg and PCBs on neurotransmission

Dopaminergic neurotransmission

It has been reported that PCBs may alter dopaminergic neurotransmission (receptors, release, metabolism) in the striatum, cortex and substantia nigra (Seegal et al., 1997). A gender differential effect on dopaminergic neurotransmission (dopamine content and its metabolites) has been reported in rats subchronically exposed to PCB 28, PCB 77, PCB 105, PCB 118 and PCB 153 (Chu et al., 1995; 1996 a, b; 1998).

Among the dopaminergic endpoints tested in the DEVNERTOX project, in rats developmentally exposed to MeHg and/or PCB 153, cortical D2-like receptors were most affected and female rats were in general less sensitive than males. As discussed below, this gender difference in susceptibility has also been found in some previous studies.

Functionally, a dysregulation of the DA pathway may result in impairment of several downstream processes involved in cognitive, motoric and sensory processes (Sawaguchi and Goldman-Rakic, 1991; Bozzi and Borrelli, 2006). Cortical D2 receptors are also reported to interact with other neurotransmitter systems *(e.g.,* NMDA-glutamatergic and GABAergic systems) (Takahashi et al., 2006). The alterations in DA content and DA receptors detected in striatum in the present study may contribute to the motor alterations found in exposed rats.

From a mechanistic point of view, the up- or down-regulation of DA receptors observed may be due to direct effects of PCB 153 and MeHg, or may occur to compensate other neurochemical changes, such as altered DA levels. Indeed, non-coplanar PCBs have been shown to reduce cell/tissue DA concentrations *in vitro* (Bemis and Seegal, 1999; Chishti et al., 1996; Shain et al., 1991; this study in PC12 cells), and *in vivo* (see Tilson and Kodavanti, 1997 for a review). In line with our *in vitro* results, ortho-substituted PCBs are suggested to decrease DA synthesis by inhibiting the activity of the rate-limiting enzyme tyrosine hydroxylase (Choksi et al. 1997).

PCB 153 may alter the ontogenesis of DA receptors by means of its endocrine disrupting properties. Estradiol can, in fact, reduce striatal D2 receptor density or induce receptor supersensitivity in developing rats (Ferretti et al., 1992). Of interest in this respect, the rats developmentally exposed to PCB 153 +/- MeHg in the

DEVNERTOX project displayed long-lasting changes in estradiol levels, which became apparent at puberty (PND36) and were still present in adulthood (PND90).

MeHg can affect several parameters of dopaminergic neurotransmission, which may modulate the density or affinity of DA receptors. Striatal dopaminergic neurotransmission is important in locomotor control. Previous *in vivo* studies of developmental exposure to MeHg (0.5 mg/kg/day, GD7-PND7) have shown, at PND21 and at 6 months of age, changes in locomotor activity suggestive of alterations in DA-induced transmission, in the male rats only (Daré et al., 2003; Rossi et al., 1997). Notably, at PND21 the altered motor response to DA agonist was associated with a significant reduction in D2 receptor binding in the caudate putamen (Daré et al., 2003).

Cholinergic neurotransmission

Cholinergic neurotransmission plays a key role in brain development as a modulator of neuronal proliferation, migration and differentiation (Hohmann and Berger-Sweeney, 1998). The cholinergic muscarinic receptors (MRs), in particular, are involved in several CNS functions, including learning and memory (Levine et al., 2001). Here, developmental exposure to MeHg and/or PCB 153 was found to decrease the density of cerebral MRs in rat pups in a brain region, gender, and age dependent fashion: some changes with early onset (weaning) persisted up to puberty, while other modifications became manifested only at the delayed time point, despite the decrease of Hg and PCB 153 brain levels with time.

Delayed MR changes had been previously reported in adult (Coccini et al 2000) and immature rats after repeated MeHg exposure (Coccini et al 2006). However, in the latter study an increased, rather than a decreased, brain MR density was found in PND21 rats after maternal exposure to 1 mg/kg/day MeHg from GD7 to PND7 (Coccini et al 2006). With this shorter exposure period, the dose used in the DEVNERTOX project, 0.5 mg MeHg/kg/day, had no effect on MRs. The first 3 weeks of the postnatal period are critical for the ontogenesis of rat cerebral MRs. In particular, MR binding rapidly increases during the first 2 weeks post-partum, and reaches adult levels between postnatal weeks 3 and 5 (Aubert et al 1996). Thus, markedly different outcomes may ensue if a toxic insult, like MeHg or PCB 153 administration, is given continuously throughout this crucial period (as in the DEVNERTOX study) or if it is interrupted at an earlier stage.

In the present study, hippocampal MRs were reduced early and persistently by PCB 153 (and also by MeHg) in males only. Accordingly, Eriksson (1988) reported long lasting changes in the density of hippocampal MRs in mice exposed once to PCB 77 at PND10: notably, receptor alterations correlated with behavioural deficits (Eriksson et al., 1991). Interestingly, behavioural changes, reduced learning ability and impaired long term potentiation have been observed in rats exposed to PCB 153 during development in the present as well as previous studies (Schantz et al., 1995; Holene et al., 1998; Hussain et al., 2000). Thus, changes in MRs may contribute to the onset of PCB 153-induced neurodevelopmental deficits.

With respect to the effects of the combined exposure on MRs, the present results support the occurrence of antagonistic interactions between MeHg and PCB153. The analytical determinations do not support pharmacokinetic interactions between these chemicals, in that brain Hg and PCB153 levels were comparable in the single- and co-exposed groups.

Glutamatergic neurotransmission

As discussed below, impaired glutamatergic transmission could be involved both in the learning impairments and the effects on motoric function observed *in vivo* after exposure to MeHg or the PCBs. There was an excellent correlation between the effects of the toxicants on the function of the glutamate-NO-cGMP pathway and on the ability to learn the Y maze task. This supports the idea that impairment in the function of the pathway is responsible for the impairment in learning. Our results also suggest that the function of the glutamate-nitric oxide-cGMP pathway decreases with age in unexposed animals and that this is associated with decreased learning ability. Results from the *in vitro* studies of the same pathway also gave indications on possible mechanisms by which PCBs and MeHg impair learning of the Y maze task.

Previously, the function of the glutamate-NO-cGMP pathway has been found to be reduced in rats with chronic hyperammonemia without liver failure (Hermenegildo et al., 1998) and these rats also show reduced ability to learn the Y maze task (Aguilar et al., 2000). The ability of these rats to learn the Y maze task may be restored by increasing extracellular cGMP in brain by intracerebral administration of zaprinast, an inhibitor of the phosphodiesterase that degrades cGMP (Erceg et al., 2005a) or by oral administration of sildenafil, another inhibitor of the phosphodiesterase (Erceg et al., 2005b).

Effects of MeHg and PCBs on rodent behaviour

The behavioural effects of MeHg have been more widely studied than those of PCBs and it has been shown that prenatal exposure to MeHg can induce neurobehavioural impairments both in humans and in laboratory animals (Chang 1977; Burbacher et al., 1990). Developmental exposure of mice, rats or monkeys to commercial mixtures of PCBs, and to single congeners, has also been shown to cause long-term neurobehavioral changes (Eriksson, 1998; Tilson and Harry, 1994, Tilson et al. 1990; Seegal and Schantz, 1994; Seegal 1996). PCBs have induced neurobehavioral deficits in children born at contaminated sites (Carpenter, 1998), including abnormalities on behavioural assessment and increased activity, greater incidence of behavioral problems, lower IQ, and impaired visual recognition (Schantz, 1996). These neurological defects are primarily due to the exposure of the fetus and child through placental and/or lactational transfer (Buck, 1996; Seegal, 1996). Few studied have addressed the neurobehavioural effects of perinatal exposure to MeHg plus commercial mixtures of PCBs and even less is known about the effects of PCB 153 or PCB 126 combined with MeHg.

Alterations of learning ability have been widely reported both in infants and animals prenatally exposed to PCBs or MeHg. In the present studies, learning and memory functions were found to be impaired by exposure to MeHg, PCB 153 or PCB 126, in both rats and mice and by multiple tests. There was an excellent correlation between the effects on the glutamate-NO-cGMP pathway in cerebellum and the ability of rats to learn the Y maze task. This reduction in Y maze performance was detected in 3-month-old rats and appeared to be transient since no effects were observed in 7-month-old rats.

Prenatal exposure to MeHg can induce neurobehavioral alterations both in human and experimental animals (Myers and Davidson, 1998; Goulet et al., 2003). Certain levels of MeHg exposure *in utero* have been associated with irreversible neurological problems and neuropsychological dysfunctions in children in the domains of

attention, visual-spatial ability, motor function, language, and memory, (Grandjean et al., 1997; Gilbert and Grant-Webster, 1995; Grandjean and Landrigan, 2006). The effects on laboratory animals depend on the exposure paradigm and the test used (Weiss et al., 2005). Rats perinatally exposed to MeHg during the same period of gestation and lactation as in some of our experiments showed significant behavioral deficits characterized by hypoactivity and by reduced appetitive, escape, and avoidance learning in adulthood (Schalock et al., 1981).

Several studies have revealed a dose-dependent relationship between total umbilical cord-blood PCB levels and low intelligence tests performance in infants at 12-months (Darvill et al., 2000) and 11-year-old-children(Jacobson and Jacobson, 1996). Jacobson et al. (1996) performed studies on school age children exposed to a slightly higher level of PCBs than the average population and found that prenatal exposure to PCBs is associated with lower full-scale and verbal IQ scores and that the strongest effects were related to memory and attention (Jacobson and Jacobson, 1996). They also reported that there were some deficits associated with transplacental exposure and not breast feeding, suggesting that the developing fetal brain is particularly sensitive to these compounds (Jacobson and Jacobson, 2003). Other authors have stressed the importance of exposure during lactation (Costa et al., 2004). Monkeys postnatally exposed to a mixture of PCBs similar to that found in human milk showed deficits in spatial delayed alternation and retarded learning (Rice, 1999). In our studies learning impairments were observed both in animals exposed throughout gestation and lactation and at PND10 only.

It is very difficult to attribute alterations found in human population to a single compound, since usually PCBs are found in mixtures. Some investigations in animal models have studied the effects of single compound exposures (Schantz et al. 1996; Eriksson 1998; Hojo et al., 2004). Schantz *et al.* (1996) reported alterations in spatial learning ability of rats prenatally exposed to PCB 153 using an eight-arm radial maze working memory task, but, curiously, they found that prenatal exposure to PCB 126 improved performance in the same test. However, Hojo *et al*. (2004) reported deficits in learning a discrimination task in rats prenatally exposed to PCB 126.

Locomotor activity was reduced in female rats at certain ages after MeHg exposure, while the PCBs mainly caused hyperactivity in both genders. In the literature there are several studies reporting reduced, increased or no changes in the motor activity following acute, perinatal or developmental exposure to MeHg, depending on the dose, duration of treatment, methods to study motor activity, age and sex of the animals (Buelke-Sam et al., 1985; Fredriksson et al., 1993 and 1996; Rossi et al., 1997). A reduced locomotor activity following developmental MeHg exposure was also observed in other studies, but in this case it was present in both genders (Vorhees 1985) or only in males (Rossi et al. 1997). Increased locomotor activity in males exposed to PCB has also been suggested by some previous studies (Holene et al. 1999; Carpenter et al. 2002). Motor activity is regulated by cortical-basal ganglia-thalamic-cortical loops and dopamine and glutamate play a pivotal role in regulation these neuronal circuits. A differential impairment of dopaminergic neurotransmission in between female and male rats after developmental exposure to PCB 153 or PCB 126 could contribute to the reduced motor activity observed in our work in female but not male rats.

In our studies, motor activity decreased progressively with age in control rats. This decrease was lower in rats perinatally exposed to the neurotoxicants. These results are

in agreement with the report of Holene et al., (1998) showing that exposure to PCB 153 or PCB 126 through the mother's milk induces hyperactivity in the offsprings.

Impaired motor coordination and balance (analysed by the rotarod test) was primarily observed in PCB exposed animals and is consistent with suggestions that motoric function is the main domain which is being affected by PCB exposure (Roegge 2006).

It is hypothesised that reduced performance in the rota-rod test is mainly due to alterations in cerebellum. Thyroid hormones are some of the endogenous substances in cerebellum that regulate motor coordination. Congenital hypothyroidism in humans causes severe alterations in motor coordination (Bargagna et al., 2000). It has been shown that acute exposure of adult rats to PCB 126 or PCB 153 decreases the concentration of the thyroid hormone T4 (Craft et al., 2002). Thus, it is possible that the effect of these PCBs on motor coordination could be due to reduced content of thyroid hormones in the cerebellum.

At two months of age, perinatal exposure to MeHg did not significantly reduced motor coordination in male or female rats although a trend towards a reduction was observed. Similarly, Roegge et al., (2004) found a tendency to a reduction in motor coordination in rats perinatally exposed to MeHg. Sakamoto et al., (1993) found a significant reduction of motor coordination in the rotarod test after perinatal exposure to doses of MeHg 5-20-fold higher than those used in our work. In mice perinatal or developmental exposure to MeHg also impaired rotarod performance at doses higher than those used in our study (Elsner et al., 1988; Spyker et al., 1972).

In addition to these behavioural effects, we also report for the first time higher predisposition to depressive-like behaviour in MeHg exposed male mice (Onishchenko et al. 2007). In MeHg exposed male rats an increased behavioral response to amphetamine (AMPH) was found after a sensitizing AMPH treatment. It has been shown in numerous studies that developmental low-level MeHg exposure results in an increased behavioural sensitivity to direct or indirect dopaminergic agonists (Eccles and Annau 1982, Hughes and Sparber.1978, Rossi et al. 1997, Rasmussen and Newland 2001, Gimenez-Llort et al. 2001, Dare et al. 2003, Wagner et al. 2006). Such effects have been observed in very young (Dare et al. 2003), as well as in mature animals (*e.g.* Rasmussen and Newland 2001). Studies in which both genders were investigated suggest that this effect is more likely to occur in males (Dare et al. 2003), *i.e.* the same gender difference as observed in the DEVNERTOX AMPH sensitization study.

There is some evidence showing that in laboratory animals behavioural sensitization to psychostimulants goes along with an increased propensity to self-administration of the psychostimulant (Robinson and Berridge 2000, Vezina et al. 2002). Thus, for humans, one could speculate that there is a link between dietary exposure of mothers to MeHg during gestation and lactation and overuse of psychostimulants in the developmentally exposed children later in life. So far this issue has not been addressed in epidemiological studies.

Certain behavioural analyses were performed at different ages and in general aging appears not to exacerbate the behavioral effects of perinatal exposures to MeHg and PCBs, alone or in combination.

Effects of co-exposure to MeHg and PCB 153 or PCB 126

Much of the contemporary concern about chemical mixtures stems from the possibility of compounds having synergistic or more than additive effects (Carpenter et al., 2002). PCBs and MeHg can be found in the same food sources, in particular fish and other seafood, and apparent interactive effects have been reported in *in vitro* (Bemis and Seegal, 1999; 2000), *in vivo* (Roegge et al., 2004; Fischer et al. 2004) and epidemiological studies (Grandjean et al., 2001; Stewart et al., 2003).

In general, the kinetics of Hg does not seem to be modified by the co-presence of PCBs. Accordingly, in a previous study by Coccini et al. (2006) concerning developmental exposure to MeHg (1 mg/kg/day, GD7-PND21) and PCB153 (20 mg/kg/day, GD10-GD16), brain Hg retention was not affected by the co-exposure to PCB153 in PND21 offspring. MeHg co-exposure in this previous study seemed to increase both fat and brain PCB 153 concentrations in pups as compared to the group exposed to PCB 153 only. These results differ from the DEVNERTOX results which might be explained by the different exposure protocols.

In our *in vitro* models, the effects of combined exposures to MeHg and one of the PCB congeners varied depending on both cell type and concentration. A common finding was that exposure to doses that were not cytotoxic by themselves did not induce significant amounts of cell death when combined. An antagonistic effect was detected in some models (*e.g.* PC12 and HT22) at certain combinations of doses. Additive or slightly synergistic effects were seen in others (*e.g.* AtT20 and CGC).

The antagonistic effect of MeHg and PCB 153 in PC12 was supported by similar effects on other toxicological parameters shown to be modified in single exposure experiments to the selected neurotoxicants (lipid peroxidation and levels of intracellular dopamine (DA), see Vettori et al. 2006). Based on data from asynchronous exposures, we hypothesize that the antagonism between MeHg and PCB 153 in PC12 might be explained by an activation of cellular defence and/or detoxification mechanisms induced by relatively low concentrations of MeHg, and thereby reducing the toxicity of PCB 153. However, further studies are necessary to understand the mechanistic bases of MeHg and PCB 153 interactions.

With regard to dopaminergic effects, a previous *in vitro* study of concomitant treatment of rat brain striatal slices with PCBs and MeHg found synergistically reduced tissue DA concentration and elevated media DA content (Bemis and Seegal, 1999), suggesting that co-exposure to these pollutants results in a greater change in neurochemical function than single exposure to either chemical. In DEVNERTOX, co-exposure to MeHg and PCB 153 sometimes attenuated the effects on dopaminergic neurotransmission induced by each toxicant alone, both *in vitro* and *in vivo*, suggesting antagonistic effects. In brain tissue from rats, the individual neurochemical effects of MeHg and PCB 153 were never exacerbated following combined developmental exposure. Similar *in vivo* results were found when studying the function of the Glutamate-NO-cGMP pathway These findings are consistent with previous studies of PND21 rats suggesting lack of synergistic effects elicited by gestational and lactational exposure to MeHg and PCB 153 on other brain neurochemical parameters, namely muscarinic receptors (Coccini et al., 2006), monoamine oxidase B (MAO-B) activity and the regional content of biogenic amines (Castoldi et al., 2006).

Overall, results from the behaviour studies in rats indicated no major synergistic interactions following developmental exposure to MeHg in combination with PCB

153 or PCB 126. The effects of co-exposure were no greater than those observed following each compound alone, and in some case, the combined exposure appeared to have an antagonistic effect. However, in female rats exposed to MeHg and PCB 126 hyperactivity was noted, which was not present in animals treated with either compound alone, suggesting a gender-specific synergistic effect.

In contrast to most of the observations in rats exposed GD7-PND21, co-exposure of mice during a critical stage of neonatal brain development (PND10) enhanced the neurotoxic effects. The results indicate that both the ortho-substituted PCB 153 and the co-planar PCB 126 can interact with MeHg and significantly enhance developmental neurobehavioural defects in mice, manifested as deranged spontaneous behaviour and habituation, and decreased learning and memory functions. These results support the hypothesis that co-exposure to both MeHg and PCBs could explain the different results found in epidemiological studies on MeHg and neurological development in children from the Seychelles Islands and the Faroe Islands, since the Faroe Islands population in addition to MeHg are also exposed to PCBs **(**Dourson et al. 2001**)**.

Considering the environmental relevance of co-exposure studies, further investigations are warranted clarifying possible interactions between MeHg and PCBs. We believe that our mathematical approach (Vettori et al. 2006) can be a useful analysis tool for experimentally investigations of the effects of different environmentally relevant mixtures of toxic compounds.

Conclusions

In contrast to other fields of toxicology, there is still limited knowledge about the exact mechanism(s) of action of most environmental neurotoxicants, and this is one of the reasons why the identification of end points for neurotoxic effects has been progressing slowly. Moreover, the anatomical and functional complexity of the nervous system and the consequent multiplicity of adverse effects create additional difficulties. DEVNERTOX studies suggest that a winning strategy to identify neurotoxic substances is to use *in vitro* methodologies to assess their toxic potential and characterize the mechanisms of actions. Changes in relevant endpoints were found *in vitro* at concentrations comparable to those critical for human neurodevelopment. Notably, similar effects on neurotransmission were found both in exposed animals and in cell systems exposed *in vitro*.

The complexity of the nervous system requires the use of multiple cellular models that can represent different neural targets potentially affected *in vivo*. In this respect, neural stem cells seem to be a promising model to investigate developmental neurotoxicity. Neural cells exposed to the selected toxic agents can undergo different types of cell death and, in turn, toxicants can trigger multiple death pathways that may cross-talk. The elucidation of the cell death pathways activated in different neural targets is highly relevant for our understanding of the mechanisms of action of toxic agents that can harm the nervous system.

The analysis of behaviour is a first choice approach to detect subtle modifications *in vivo*, mainly because it offers the possibility to analyze the same animals at different stages of development. Impaired learning ability has been observed both in humans and animals prenatally exposed to PCBs or MeHg. In the present studies, learning and memory functions were found to be impaired in both rats and mice and by multiple tests. Studies on depressive-like behaviour indicate that the developmental exposure

to MeHg could affect not only cognitive functions but also motivation-driven behaviour in mice.

Neurochemical, hormonal and neurobehavioural alterations were found, sometimes with species differences. For example, toxicant exposure could impair a specific neurotransmission system in cultured cells as well as in rat brain, the rat results being correlated with decreased learning and memory.

In general, aging did not to worsen behavioural effects. Gender differences were detected for a number of end points, including hormonal levels and some changes in behaviour and neurotransmission. Similar to the *in vitro* studies, effects of concomitant exposures to MeHg and either PCB differed between the different *in vivo* models.

To summarise, *in vitro* studies are important complementary approaches to studies in experimental animals. A battery of well characterized *in vitro*-models, like those used in the DEVNERTOX project, allows for a better mechanistic-based assessment of potentially neurotoxic substances. Neural stem cells are highly relevant to study neurodevelopmental effects and we propose that they can be used as sensitive *in vitro*-models for the evaluation of toxic effects on both differentiation and cell survival.

Our results also show that to compare *in vivo* and *in vitro* results it is important to measure actual levels in tissues and cells, since cellular levels can vary between different cell types exposed to the same concentrations in the cell medium. Due to differences in methodology and uncertainties in comparing *in vitro* and *in vivo* results such comparisons should be considered as "order of magnitude". The data on brain Hg levels in children are very limited and even less is known about human PCB 153 and 126 levels.

The lack of information that usually justifies experimental studies calls for the use of batteries of tests and multiple cell systems, which may lead to identification of the most sensitive cell type(s) and potential biomarkers. The most critical issue in risk assessment remains exposure characterisation, which is often based on rough surrogates (e.g. occurrence by intake calculations) of relevant target (cellular) doses experimentally determined, A biomonitoring approach using validated and relevant biomarkers seems to be promising to characterise the risk of adverse neurodevelopmental effects and to calculate the margin of exposure resulting from food and other sources in target populations. Overall, DEVNERTOX studies suggest that such an approach is feasible.

Further studies are necessary to address the issue of combined or complex exposures. With simple binary combinations of MeHg and PCB 153 and 126, we have been unable to demonstrate that sub-threshold exposure can result in effective cumulative doses. However, we found significant effects at doses close to those found in epidemiological studies. At these levels of exposure, we noted that even the sequence of asynchronous exposures to the same doses of the same toxicants may produce different effects, which calls for additional efforts to develop a conceptual framework where available human and animal data can be integrated with much cheaper and equally sound *in vitro* results.

Materials and methods

PCB 153 purity

The PCB 153 stock compound was chemically analysed by the Laboratory of Environmental Hygiene and Industrial Toxicology, at the Salvatore Maugeri Foundation (Pavia, Italy), to exclude potential contamination by dioxin-like PCBs. The results indicated that the whole 0.01% fraction of chemical impurities detected in the PCB 153 consisted of PCB 180 (2,2',3,4,4',5,5'-heptachlorobiphenyl), a non-dioxin-like PCB congener devoid of any known neurotoxic effects.

In vitro studies

References describing cell culturing conditions are found in the results section. MeHg was diluted in sterile water and the PCBs in DMSO before addition to the cell medium. Cell viability was assessed by studying cell membrane permeability to Trypan blue or propidium iodide (Johansson et al. 2006), apoptotic nuclear morphology after staining with Hoechst 33342 (Tamm et al. 2006) or the MTT assay for mitochondrial integrity (Mossman, 1983). Studies of neuronal differentiation and lipid peroxidation were performed as described previously (Tamm et al. 2006 and Vettori et al. 2006, respectively).

Different doses and durations of exposure were tested to find the optimal exposure conditions to study toxicant induced effects in our models. Cell death mechanistic studies were performed using doses that caused apoptosis but not necrosis in the NSCs. For the AtT20 and HT22 cells only doses that induced concomitantly apoptosis and necrosis could be identified.The type of cell death (apoptosis or necrosis) was assessed by morphological and biochemical analyses to detect presence or absence of high molecular weight DNA fragments, membrane integrity and nuclear staining. Mitochondrial function was tested by measuring ATP-levels, mitochondrial Ca^{2+}-uptake capacity and membrane potential. Involvement of different cell death pathways was studied using various protease inhibitors, caspase activity and cleavage of caspase- and calpain-substrates (α-fodrin and PARP). Critical apoptotic events, such as oligomerization of Bax and release of cytochrome c and AIF, were also assessed. The potential involvement of lysosomes was investigated by acridine orange staining, cathepsin B detection and pre-treatment with the cathepsin D inhibitor pepstatin. Antioxidants (*e.g.* Trolox and MnTBAP) and a reactive oxygene species (ROS) detection kit were used to study the role of oxidative stress. For details regarding the methods used see Tamm et al. 2006 and Johansson et al. 2006.

Analysis of Hg and PCBs in cells

Mercury: 10^6 cells (<5 mg) were treated for 24h with known concentrations of Me-Hg. Cellular pellet was digested with 1 ml of Nitric acid (70%) and 1ml of water at 65 °C for 3 hours. The digested solution was normalized with water and HCl (final concentration about 5%) at 10 ml. Total Mercury was determined with atomic absorption using the cold vapour method adding stannous chloride reductant specific for mercury. Mercury concentration was normalized for the number of cells.

PCB153: After 24 h of incubation, pellet cells were collected in 4 mL SPME vials containing 500 ml of water and 0.25 g of NaCl to decrease the PCB water solubilization for "salting out" effect and hence promote their extraction by SPME fiber. 2,2',5,5'-PCB in water solution was added to each sample as internal standard.

Extraction by SPME was carried out using a 30-mm PDMS fiber (Supelco, Bellefonte, PA, USA). The sample was pre-heated for 5 min at 100°C to facilitate the transfer of PCB toward the gas phase; then the fiber was exposed in the headspace of samples for 40 min at 100°C under stirring and, after the sampling, immediately desorbed into the GC injection port at 280°C for 10 min. In this condition no carry-over was observed. The sampling was performed using a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland).

The analyses were carried out on a HP 6890 gas chromatograph equipped with a 5973 mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). Separation was performed on a Equity™ –1 column from Supelco (30 m x 0.25 mm d.i., film 1.0 mm). The temperature program was: rate 30°C/min from 100°C to 280°C, hold for 10 min. Hydrogen was used as the carrier gas (1.3 mL/min). The chromatographic run was complete in about 16 min. The acquisition was performed in selected-ion monitoring (SIM) mode, by acquiring the signals of the following ions *m/z* (dwell time in parentheses): 360 (40), 359.50 (40), 360.50 (40), 362 (50), 361.50 (50) and 362.50 (50) for PCB 153 and 220 (40), 222 (60), 292 (40), 290 (50) and 294 (65) for I.S.. A solvent delay of 5 min was necessary to protect the filament from the solvent vapours.

Calibration was carry out in water and linear range were observed for three order of magnitude. The LODs of the method was about $5*10^{-9}$ M and the RSD% was 5-10%.

Primary neuronal cultures CGC:According to Minana MD., Montoliu C., LLansola M., Grisolía S., Felipo V.(1998).Nicotine prevents glutamate-induced proteolysis of the microtubule-associated protein MAP-2 and glutamate neurotoxicity in primary cultures of cerebellar neurons. Neuropharmacology 37(7):847-57

Cell viability studies of CGCs According to: Felipo V., Minana MD., Grisolía S. (1993). Inhibitors of protein kinase C prevent the toxicity of glutamate in primary neuronal cultures. Brain Res. 604(1-2):192-6.

Determination of intracellular calcium in cultured neurons According to:Marcaida G., Miñana MD., Burgal M., Grisolía S., Felipo V. (1995) Ammonia prevents activation of NMDA receptors by glutamate in rat cerebellar neuronal cultures.
Eur J Neurosci. 7(12):2389-96.

Immunoblotting According to: Felipo V., Miñana MD., Azorín I., Grisolía S (1988) Induction of rat brain tubulin following ammonium ingestion. J Neurochem. 51(4):1041-5

In vivo-studies

Exposure of rats
Female rats (Sprague-Dawley or Wistar strain) were orally treated from gestational day 7 (GD7) to postnatal day 21 (PND21) with methylmercury (MeHg), PCB153, and PCB126, alone or combined, according to this scheme: 1) vehicle (corn oil and DMSO); 2) MeHg (Methylmercury Chloride from Sigma-Aldrich. 0.5 mg/kg/day dissolved in DMSO). 3) PCB153 (2,2',4,4',5,5'-esachlorobiphenyl from Fluka. 1 or 5 mg/kg/day dissolved in corn oil); 4) PCB126 (3,3',4,4',5-Pentachlorobiphenyl from Larodan. 100 ng/kg/day dissolved in corn oil); 5) PCB153 + MeHg (1 or 5 mg mg/kg/day + 0.5 mg/kg/day) 6) PCB126 + MeHg (100 ng/kg/day + 0.5 mg/kg/day). MeHg was administered to the dams *via* the drinking water and the PCBs *via* a sweet jelly bit (Transgel® from Charles River Laboratories) or intragastrically (*via* gavage).

Measurements related to the rat studies
Analysis of PCB153 in brain samples: The analyses were performed in whole brains as well as in brain regions (cerebellum, cerebral cortex, striatum and hippocampus). Brain samples were first weighed and homogenized in a KCl solution, contaminated with 2,2',5,5'-PCB used as the Internal Standard (1µl/10 mg of tissue), vortexed for about 1 min and left at room temperature for at least 10 min. The extraction of PCB153 from tissues was performed using a Solid Phase Extraction (SPE) technique. The organic solution obtained after extraction was concentrated to about 200 µl under a gentle stream of air and thus analyzed by gas chromatography mass spectrometry (GC/MS). The chromatographic run was complete in about 16 min. The acquisition was performed in selected-ion monitoring (SIM) mode, by acquiring the signals of the specific ions m/z related to each analyzed compound.

Analysis of PCB126 in brain samples: The analyses were carried out both in whole brains and in dissected brain areas (cerebellum cortex, striatum and hippocampus). Brain samples were

first weighed and homogenized in a KCl solution, contaminated with 2,2',5,5'-PCB used as the Internal Standard (1μl/10 mg of tissue), vortexed for about 1 min and left at room temperature for at least 10 min. Each sample was extracted twice with 2 ml of n-pentane and thus vortexed for 1 min. The phases were then separated by centrifugation at 2000 g for 10 min and the organic phase extract was concentrated to about 100 μL under a gentle stream of nitrogen and analyzed by GC/MS. The chromatographic run was complete in about 16 min. The acquisition was performed in selected-ion monitoring (SIM) mode, by acquiring the signals of the specific ions *m/z* related to each analyzed compound.

Analysis of PCB153 and PCB126 in serum samples: Serum (150 μl-aliquots) was placed in vials containing NaCl, distilled water and 2,2',5,5'-PCB used as the Internal Standard. Samples were mixed for about 1 min and then stored at -20°C until analysis. Serum samples were heated for 5 min at 80°C in order to facilitate PCBs evaporation and then PCB153 and 126 were extracted using the SPME technique by concentration on a fibre covered with adsorbent material. After sampling, the fibre was immediately desorbed into the GC and analyzed in GC/MS as previously described.

Analysis of total mercury in brain samples: Total Hg levels were determined both in whole brain samples and in brain sections (cerebellum cortex, striatum and hippocampus). The risk of contamination during sampling and sample preparation was carefully avoided. The extracted tissue samples were stored in clean plastic containers in liquid nitrogen, and subsequently stored at –80°C, until analysis.
Before digestion, tissue samples were heated at 100°C for 3 hours to dryness and then weighed. Then, a solution of 65% HNO_3 was mixed with an equal volume of water and the final solution was incubated overnight at 65°C. Hence, 37% HCl and distilled water were added and total Hg in samples was measured by cold vapour atomic absorption spectrometry using electrothermal Atomic Absorption Spectroscopy (ET-AAS). Calibration was performed in matrix and Hg in tissue was expressed as μg/g dry tissue.

Analysis of total mercury in erythrocytes: Erythrocytes were weighed, dissolved in 500 μl of H_2O and digested adding 3 ml of 65% HNO_3. The obtained solution was heated overnight at 65°C. One ml of this final solution was diluted with 37% HCl and H_2O and thus analyzed by ET-AAS.

Data analysis for dose measurements: The significance ($p<0.05$) of the differences in Hg or PCB (153 and 126) levels in each brain area after single and combined exposure was calculated using unpaired *t*-student for the comparisons of two groups and ANOVA test followed by Tuckey's post-hoc comparisons for more than two groups. The SPSS 14.0 software (SPSS inc. Chicago, IL,USA) was used.

Serum sex hormones were quantified by radioimmunoassay (RIA) by means of the testosterone DSL-400 RIA kit (Diagnostic System Laboratories Inc, USA; test sensitivity: 8 ng/dL) and the Spectra estradiol RIA kit (Orion Diagnostica, Finland; assay detection limit: 5 pmol/L=1.362 pg/ml) accordino to the manufacturers'instructions. The data were analysed by means of the Kruskal-Wallis test followed by the Dwass-Steel-Chritchlow-Fligner multiple comparison test (Statsdirect program). In addition some data were analysed by ANOVA followed by the Tukey multiple comparison (HSD) test.

In vivo brain microdialysis According to: Hermenegildo C, Montolir C., LLansola M., Munoz MD., Gaztelu JM, Miñana MD., Felipo V (1998). Chronic hyperammonemia impairs the glutamate-nitric oxide-cyclic GMP pathway in cerebellar neurons in culture and in the rat in vivo. Eur J Neurosci. 1998 Oct;10(10):3201-9.

Y maze learning test According to:Aguilar MA, Minarro J.,Felipo V. et al., (2000)Chronic moderate hyperammonemia impairs active and passive avoidance behavior and conditional discrimination learning in rats. Exp Neurol161(2):704-13

Long-term potentiation in hippocampal slices According to: Monfort P., Munoz MD., Kosenko E., Felipo V. (2002) Long-term potentiation in hippocampus involves sequential

activation of soluble guanylate cyclase, cGMP-dependent protein kinase, and cGMP-degrading phosphodiesterase. J Neurosci. 22(23):10116-22.

Exposure of mice
Wild type (WT; C57/BL6J) and ß-oestrogen receptor knockout (BERKO) mice were orally dosed with 0.1 mg/kg/day p.o. methyl mercury on GD 7- PND 21 (or in some cases GD7-PND7). Some MeHg-exposed pups also received a single injection of PCB153 (5.1 mg/kg) on postnatal day (PND) 10.

In addition, sets of experiments have been performed on NMRI mice as well as wild type (C57/BL 6J) and BERKO mice exposed to the toxicants at a specific stage of postnatal development (PND10). MeHg and PCBs were administered alone or combined at the following doses: for MeHg: 0.4, 4.0 mg/kg body weight; for PCB153 0.51-5.1 mg/kg body weight; for PCB 126: 0.046-0.46mg/kg body weight.

Behavioural and neurochmical analyses of mice

For mice exposed GD7-PND7, see Onishchenko et al. 2007.

For mice exposed PND10, see text.

BERKO mice:

Tissue Preparation ERß$^{-/-}$ mice were generated as from heterozygous mice breeding. ERß+/ female mice were mated overnight with ERß+/ males and inspected at 9:00 a.m. on the following day for the presence of vaginal plug. Noon of this day was assumed to correspond to E0.5. All animals were housed in the Karolinska University Hospital Animal Facility (Huddinge, Sweden) in a controlled environment on a 12-h light-12-h dark illumination schedule and fed a standard pellet diet with water provided ad libitum. To obtain embryos, pregnant mice were anesthetized deeply with CO_2 and perfused with PBS followed by 4% paraformaldehyde (in 0.1 M PBS, pH 7.4). Embryos were taken out and put on ice, and heads or brains were dissected and postfixed in the same fixative overnight at 4°C. For the adult group, mice were perfused individually with PBS followed by 4% paraformaldehyde, and brains then were removed and postfixed overnight. Sex was determined after direct visual inspection of the gonads with a dissecting microscope, and the tail and limbs were removed from each embryo for genotyping. After fixation, brains were processed for either paraffin (6 μm) or frozen (35 μm) sections. All animal experiments were approved by Stockholm's Södra Försöksdjursetiska Nämnd.

Immunohistochemistry Paraffin sections were deparaffinized in xylene, rehydrated and then processed in the same manner as the frozen sections. Sections were retrieved by boiling in 10 mM citrate buffer (pH 6.0) for 2 min. The sections were incubated in 0.5% H2O2 in PBS for 30 min at room temperature to quench endogenous peroxidase and then incubated in 0.5% Triton X-100 in PBS for 30 min. To block nonspecific binding, sections were incubated in 3% BSA for 1 h at 4°C. For ERß staining, retrieval was improved by incubating the sections with 0.1 units of ß –galactosidase for 1h. Sections were then incubated with anti- ERß 1:100, anti-Calretinin 1:2000, in 1% BSA and 0.1% Triton 100 for 48 h at 4°C. BSA replaced primary antibodies in negative controls. After washing, sections were incubated with the corresponding secondary antibodies in 1:200 dilutions for 2 h at room temperature. The Vectastain ABC kit (Vector Laboratories) was used for the avidin–biotin complex (ABC) method according to the manufacturer's instructions. Peroxidase activity was visualized with 3,3_diaminobenzidine (DAKO). The sections were lightly counterstained with hematoxylin, dehydrated through an ethanol series to xylene, and mounted. For immunofluorescence, slides were directly mounted in Vectashield antifading medium (Vector Laboratories). The sections were examined under a Zeiss fluorescence microscope with filters suitable for selectively detecting the fluorescence of FITC (green), and Cy3 (red), or under a light microscope. For colocalization, images from the same section but showing different antigen signals were overlaid.

Data Analysis. Stained brain sections (10–12 sections per area for each mouse) were examined under a light microscope, and images were captured under x20 magnification. Calretinin-immunopositive cells were counted manually on the captured images by using a 200 x 200-µm template Estimates of the number of calretinin-immunoreactive cells per region were made based on the counts of the 10 images showing the highest number of labeled nuclei. Statistical analysis was performed by using Student's *t* test.

Chemicals and Antibodies. We purchased ß–galactosidase from Sigma–Aldrich. The following antibodies were used: rabbit polyclonal anti-calretinin from Swant, Bellinzona, Switzerland, monoclonal anti calbindin from Sigma-Aldrich, PDGF beta (C-20, P-20 Santa Cruz Biotechnology), goat monoclonal anti-EGFR from Santa Cruz Biotechnology. The chicken polyclonal anti- ERß 503 was produced in our laboratory (43), Cy3 anti-chicken and FITC anti-rabbit antibodies were from Jackson Immuno Research, and biotinylated

References

Aguilar MA, Minarro J, Felipo V (2000) Chronic moderate hyperammonemia impairs active and passive avoidance behavior and conditional discrimination learning in rats. Exp Neurol 161: 704-713.

Akagi H., Grandjean P., Takizawa Y., Weihe P. (1998) Methylmercury dose estimation from umbilical cord concentrations in patients with Minamata disease. Environ Res 77(2):98-103.

Akins J.R., Waldrep K., Bernert J.T. Jr. (1989) The estimation of total serum lipids by a completely enzymatic 'summation' method. Clin Chim Acta 184(3):219-26.

Amin-Zaki L., Elhassani S., Majeed M.A., Clarkson T.W., Doherty R.A., Greenwood M.R., Giovanoli-Jakubczak T. (1976) Perinatal methylmercury poisoning in Iraq. Am J Dis Child 130(10):1070-6.

Aubert I, Cécyre D, Gauthier S, Quirion R. Comparative ontogenic profile of cholinergic markers, including nicotinic and muscarinic receptors, in the rat brain. J Comp Neurol 1996;369:31-55.

Bargagna S, Canepa G, Costagli C, Dinetti D, Marcheschi M, Millepiedi S,

Bayer, S.A., Altman, J., Russa, R.J., and Xhang, X. (1993). Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. Neurotoxicology, 14: 83-144.

Bemis, J.C., Seegal, R.F., 1999. Polychlorinated biphenyl and methylmercury act synergistically to reduce rat brain dopamine content *in vitro*. Environ Health Perspect. 107, 879-885.

Bemis, J.C., Seegal, R.F., 2000. Polychlorinated biphenyls and methylmercury alter intracellular calcium concentrations in rat cerebellar granule cells. Neurotoxicology 21, 1123-34.

Berenbaum M.C., 1985. The expected effect of a combination of agents: the general solution. Journal of Theoretical Biology 114, 413-431.

Björnberg K. A., Vahter M., Berglund B., Niklasson B., Blennow M. and Sandborgh-Englund G. (2005) Transport of methylmercury and inorganic mercury to the fetus and breast-fed infant. Environ. Health Perspect. 113, 1381–1385.

Buck GM. Epidemiologic perspective of the developmental neurotoxicity of PCBs in humans. Neurotoxicol Teratol. 1996 May-Jun;18(3):239-41

Buelke-Sam J, C.A. Kimmel, J. Adams, C.J. Nelson, C.V. Vorhees, D.C. Wright, V. St Omer, B.A. Korol, R.E. Butcher and M.A. Geyer *et al.*, Collaborative behavioral teratology study: results, ***Neurobehav. Toxicol. Teratol.*** 7 (1985), pp. 591–624.

Burbacher T.M., Rodier P.M., Weiss B. (1990) Methylmercury developmental neurotoxicity: a comparison of effects in humans and animals. Neurotoxicol Teratol 12: 191-202.

Carpenter, D.O., Arcaro, K., Spink, D.C., 2002. Understanding the human health effects of chemical mixtures. Environ Health Perspect. 110, 25-42.

Castoldi, A.F., Barni, S., Turin, I., Gandini, C., Manzo, L., 2000. Early acute necrosis, delayed apoptosis and cytoskeletal breakdown in cultured cerebellar granule neurons exposed to methylmercury. J. Neurosci. Res. 60, 775-787.

Castoldi AF, Blandini F, Randine G, Samuele A, Manzo L, Coccini T., 2006. Brain monoaminergic neurotransmission parameters in weanling rats after perinatal exposure to methylmercury and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153). Brain Res. 1112(1), 91-98.

Chang LW. Neurotoxic effects of mercury--a review. Environ Res. 1977 Dec;14(3):329-73.

Chishti, M.A., Fisher, J.P., Seegal, R.F., 1996. Aroclors 1254 and 1260 reduce dopamine concentrations in rat striatal slices. Neurotoxicology 17, 653-660.

Choi B.H., Lapham L.W., Amin-Zaki L., Saleem T. (1978) 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 37: 719-733.

Choksi, N.Y., Kodavanti, P.R.S., Tilson, H.A., Booth, R.G., 1997. Effects of polychlorinated biphenyls (PCBs) on brain tyrosine hydroxylase activity and dopamine synthesis in rats. Fundam Appl Toxicol. 39, 76-80.

Chu I, Villeneuve DC, Yagminas A, Lecavalier P, Hakansson H, Ahlborg UG, Valli VE, Kennedy SW, Bergman A, Seegal RF, et al. Toxicity of PCB 77 (3,3',4,4'-tetrachlorobiphenyl) and PCB 118 (2,3',4,4'5-pentachlorobiphenyl) in the rat following subchronic dietary exposure. Fundam Appl Toxicol. 1995 Jul;26(2):282-92.

Chu I., Villeneuve D.C., Yagminas A., Lecavalier P., Poon R., Hakansson H., Ahlborg U.G., Valli V.E., Kennedy S.W., Bergman A., Seegal R.F., Feeley M. 1996a Toxicity of 2,4,4'-trichlorobiphenyl in rats following 90-day dietary exposure. J Toxicol Environ Health. Oct 25;49(3):301-18.

Chu, I., Villeneuve, D.C., Yagminas, A., Lecavalier, P., Poon, R., Feeley, M., Kennedy, S.W., Seegal, R.F., Hakansson, H., Ahlborg, U.G., Valli, V.E., Bergman, A., 1996b. Toxicity of 2,2',4,4',5,5'-hexachlorobiphenyl in rats: effects following 90-day oral exposure. J Appl Toxicol. 16, 121–128.

Chu I., Poon R., Yagminas A., Lecavalier P., Hakansson H., Valli V.E., Kennedy S.W., Bergman A., Seegal R.F., Feeley M., 1998 Subchronic toxicity of PCB 105 (2,3,3',4,4'-pentachlorobiphenyl) in rats. J Appl Toxicol. 18(4):285-292.

Chu S., Covaci A., Schepens P. (2003) Levels and chiral signatures of persistent organochlorine pollutants in human tissues from Belgium. Environ Res 93:167-176.

Coccini T, Randine G, Candura SM, Nappi R, Prockop LD, Manzo L. Low-level exposure to methylmercury modifies muscarinic cholinergic receptor binding characteristics in rat brain and lymphocytes: physiologic implications and new opportunities in biological monitoring, Environ Health Perspect 2000;108:29-33.

Coccini T, Randine G, Castoldi AF, Grandjean P, Ostendorp G, Heinzow B, Manzo L (2006). Effects of developmental co-exposure to methylmercury and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) on cholinergic muscarinic receptors in rat brain. Neurotoxicol 2006, 27:468-477.

Colborn T., vom Saal F.S., Soto A.M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101: 378-384.

Costa LG, Aschner M, Vitalone A, Syversen T, Soldin OP (2004) Developmental neuropathology of environmental agents. Annu Rev Pharmacol Toxicol 44: 87-110.

Craft ES, DeVito MJ, Crofton KM. Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. Toxicol Sci. 2002 Aug;68(2):372-80.

Daré, E., Götz, M. E., Zhivotovsky, B., Manzo, L., Ceccatelli, S., 2000. Antioxidants J811 and 17beta-estradiol protect cerebellar granule cells from methylmercury-induced apoptotic cell death. J. Neurosci. Res. 62, 557–65.

Daré, E., Gorman, A. M., Ahlbom, E., Götz, M., Momoi, T., Ceccatelli, S., 2001. Apoptotic morphology does not always require caspase activity in rat cerebellar granule neurons. Neurotox. Res. 3, 501–14.

Daré, E., Fetissov, S., Hokfelt, T., Hall, H., Ogren, S.O., Ceccatelli, S., 2003. Effects of prenatal exposure to methylmercury on dopamine-mediated locomotor activity and dopamine D_2 receptor binding. Naunyn Schmiedebergs Arch Pharmacol. 367, 500-508.

Darvill T, Lonky E, Reihman J, Stewart P, Pagano J (2000) Prenatal exposure to PCBs and infant performance on the fagan test of infant intelligence. Neurotoxicology 21: 1029-1038.

Davison, A. N., and Dobbing, J. (1968). Applied Neurochemistry. Blackwell, Oxford.

De Mees C, Laes JF, Bakker J, Smitz J, Hennuy B, Van Vooren P, Gabant P, Szpirer J, Szpirer C Alpha-fetoprotein controls female fertility and prenatal

Desaulniers D., Leingartner K., Wade M., Fintelman E., Yagminas A., Foster W.G. (1999). Effects of acute exposure to PCBs 126 and 153 on anterior pituitary and thyroid hormones and FSH isoforms in adult Sprague-Dawley male rats. Toxicol Sci 47: 158-169.

Dourson M. L., A. E. Wullenweber, K. A. Poirier. (2001). Uncertainties in the Reference Dose for Methylmercury. NeuroToxicology, 22, 677-689.

Eccles C.U., Annau Z. (1982) Prenatal methylmercury exposure: II Alterations in learning and osychotropic drugs sensitivity in adult offspring. Neurobehav Toxicol Teratol 4:377-382

Elinder F, Akanda N, Tofighi R, Shimizu S, Tsujimoto Y, Orrenius S, Ceccatelli S. 2005. Opening of plasma membrane voltage-dependent anion channels (VDAC) precedes caspase activation in neuronal apoptosis induced by toxic stimuli.Cell Death and Differentiation 12:1134-40.

Elsner J, Hodel B, Suter KE, Oelke D, Ulbrich B, Schreiner G, Cuomo V, Cagiano R, Rosengren LE, Karlsson JE, et al. Detection limits of different approaches in behavioral teratology, and correlation of effects with neurochemical parameters. Neurotoxicol Teratol. 1988 Mar-Apr;10(2):155-67.

Erceg S, Monfort P, Hernandez-Viadel M, Llansola M, Montoliu C, Felipo V (2005a) Restoration of learning ability in hyperammonemic rats by increasing extracellular cGMP in brain. Brain Res 1036: 115-121.

Erceg S, Monfort P, Hernandez-Viadel M, Rodrigo R, Montoliu C, Felipo V (2005b) Oral administration of sildenafil restores learning ability in rats with hyperammonemia and with portacaval shunts. Hepatology 41: 299-306.

Eriksson., P. (1988). Effects of 3,3',4,4'-tetrachlorobiphenyl in the brain of the neonatal mouse. Toxicology, 49: 43-48.

Eriksson, P. (1997). Developmental neurotoxicity of environmental agents in the neonate. Neurotoxicology 18: 719-726.

Eriksson, P. (1998). Perinatal developmental neurotoxicity of PCBs, p. 56. Swedish Environmental Protection Agency, Stockholm.

Eriksson P, Lundkvist U, Fredriksson A. Neonatal exposure to 3,3',4,4'-tetrachlorobiphenyl: changes in spontaneous behaviour and cholinergic muscarinic receptors in the adult mouse. Toxicology 1991;69:27-34.

Eriksson, P., Ahlbom, J., and Fredriksson, A. (1992). Exposure to DDT during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice. Brain Res 582: 277-81.

Eriksson, P., Ankarberg, E., and Fredriksson, A. (2000). Exposure to nicotine during a defined period in neonatal life induces permanent changes in brain nicotinic receptors and in behaviour of adult mice. Brain Res 853: 41-48.

Eriksson, P., Jakobsson, E., and Fredriksson, A. (2001). Brominated flame retardants: a novel class of developmental neurotoxicants in our environment? Environ Health Perspect 109: 903-8.

Eriksson, P., and Darnerud, P. O. (1985). Distribution and retention of some chlorinated hydrocarbons and a phthalate in the mouse brain during the pre-weaning period. Toxicology 37: 189-203.

Eriksson, P., and Fredriksson, A. (1996a). Developmental neurotoxicity of four ortho-substituted polychlorinated biphenyls in the neonatal mouse. Environ Toxicol Pharmacol 1: 155-165.

Eriksson, P., and Fredriksson, A. (1996b). Neonatal exposure to 2,2',5,5'-tetrachlorobiphenyl causes increased susceptibility in the cholinergic transmitter system at adult age. Environ Toxicol Pharmacol 1: 217-220.

Eriksson, P., and Fredriksson, A. (1998). Neurotoxic effects in adult mice neonatally exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,3',4,4'-pentachlorobiphenyl. Changes in brain nicotinic receptors and behaviour. Environmental Toxicology and Pharmacology 5: 17-27.

Faqi A.S., Dalsenter P.R., Merker H.-J., Chahoud I. (1998). Effects on developmental landmarks and reproductive capability of 3.3',4,4,'-tetrachlobiphenyl and 3,3',4,4',5-pentachlorobiphenyl in offspring of rats exposed during pregnancy. Human Exp Toxicol 17: 365-372.

Fan, Xiaotang, Hyunjin Kim, Margaret Warner, and Jan-Åke Gustafsson Estrogen receptor β expression in the embryonic brain regulates development of calretinin-immunoreactive GABAergic interneurons. PNAS Nov 2006.

Ferretti C, Blengio M, Vigna I, Ghi P, Genazzani E., 1992. Effects of estradiol on the ontogenesisi of striatal dopamine D1 and D2 receptor sites in male and female rats Brain Res. 571, 212-217.

Fischer, C., Fredriksson, A., Eriksson, P., 2004. Co-exposure to an ortho-substituted PCB (PCB 153) and methylmercury enhances developmental neurotoxic effects. Organohal. Compounds 66, 3160-2.

Fredriksson, A., 1994. MPTP-induced behavioural deficits in mice: Validity and utility of a model of parkinsonism. Uppsala University, Uppsala.

Fredriksson A, A.T. Gardlund, K. Bergman, A. Oskarsson, B. Ohlin, B. Danielsson and T. Archer, Effects of maternal dietary supplementation with selenite on the postnatal development of rat offspring exposed to methyl mercury in utero, *Pharmacol. Toxicol.* 72 (1993), pp. 377–382.

Fredriksson A, L. Dencker, T. Archer and B.R. Danielsson, Prenatal coexposure to metallic mercury vapour and methylmercury produce interactive behavioural changes in adult rats, *Neurotoxicol. Teratol.* 18 (1996), pp. 129–134.

Gallenberg, L. A., and Vodicnik, M. J. (1989). Transfer of persistent chemicals in milk. Drug Metab Rev 21: 277-317.

Gimenez-Llort L, Ahlbom E, Dare E, Vahter M, Ogren S –O, Ceccatelli S. (2001) Prenatal exposure to methylmercury changes dopamine modulated motor activity during early ontogeny: age and gender-dependent effects. Environ Toxicol Pharmacol 9: 61-70.

Gilbert SG, Grant-Webster KS (1995) Neurobehavioral effects of developmental methylmercury exposure. Environ Health Perspect 103 Suppl 6: 135-142.

Goldoni M., Vettori M.V., Alinovi R., Caglieri A., Ceccatelli S., Mutti A., 2003 Models of neurotoxicity: extrapolation of benchmark doses in vitro. Risk Analysis 23, 505-514.

Gorman, A.M., Hirt, U.A., Orrenius, S., Ceccatelli, S., 2000. Dexamethasone pre-treatment interferes with apoptotic death in glioma cells. Neuroscience 2, 417-425.

Goulet S, Dore FY, Mirault ME (2003) Neurobehavioral changes in mice chronically exposed to methylmercury during fetal and early postnatal development. Neurotoxicol Teratol 25: 335-347.

Grandjean P, Weihe P, White RF, Debes F, Araki S, Yokoyama K, Murata K, Sorensen N, Dahl R, Jorgensen PJ (1997) Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. Neurotoxicol Teratol 19: 417-428.

Grandjean, P., Weihe, P., Burse, V.W., Needham, L.L., Storr-Hansen, E., Heinzow, B., Debes, F., Murata, K., Simonsen, H., Ellefsen, P., Budtz-Jorgensen, E., Keiding, N., White, R.F., 2001. Neurobehavioral deficits associated with PCB in 7-year-old children prenatally exposed to seafood neurotoxicants. Neurotoxicol. Teratol. 23, 305-17.

Grandjean, P. and Landrigan P.J, 2006, Developmental neurotoxicity of industrial chemicals. Lancet, published online November 8, 2006.

Greco W.R., Bravo G., Parsons J.C., 1995. The search for synergy: a critical review from a response surface perspective. Pharmacological Reviews 47, 331-385.

Groten J.P., Feron V.J., Suhnel J., 2001. Toxicology of simple and complex mixtures. Trends in Pharmacological Sciences 22, 316-322.

Hany J., Lilienthal H., Serasin A., Roth-Harer A., Fastabend A., Dunemann L., Lichtensteiger W., Winneke G. (1999). Developmental exposure of rats to a reconstituted PCB mixture or Aroclor 1254: effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behaviour. Toxicol Appl Pharmacol 158: 231-243.

Hermanson O., Jepsen K. and Rosenfeld M. G. (2002) N-CoR controls differentiation of neural stem cells into astrocytes. Nature 419, 934–939.

Hermenegildo C, Montoliu C, Llansola M, Munoz MD, Gaztelu JM, Minana MD , Felipo V (1998) Chronic hyperammonemia impairs the glutamate-nitric oxide-cyclic GMP pathway in cerebellar neurons in culture and in the rat in vivo. Eur J Neurosci 10: 3201-3209.

Hohmann CF, Berger-Sweeney J. Cholinergic regulation of cortical development and plasticity. New twists to an old story. Perspect Dev Neurobiol 1998;5:401-425.

Hojo, Rieko, Kakeyama, Masaki, Yonemoto, Junzo, and Tohyama, Chiharu. Perinatal exposure to dioxins perturbs learningperformance of the rat in a dose-specific fashion. Congress DIOXIN 2004. Organohalogen Compounds 66, 3143-3147. 2004.

Holene E, Nafstad I, Utne Skaare J, Sagvolden T. Behavioural hyperactivity in rats following postnatal exposure to sub-toxic doses of polychlorinated biphenyl congeners 153 and 126. Behav Brain Res 1998;94:213-224.

Howard, A.S., Fitzpatrick, R., Pessah, I., Kostyniak, P., Lein, P.J., 2003. Polychlorinated biphenyls induce caspase-dependent cell death in cultured embryonic rat hippocampal but not cortical neurons via activation of the ryanodine receptor. Toxicol. Appl. Pharmacol. 190, 72-86.

Hsu P.-C., Lai T.-J., Guo N.-W., Lambert G.H., Guo Y.L. (2005) Serum hormones in boys prenatally exposed to polychlorinated biphenyl and dibenzofurans. J Toxicol Environ Health part A 68: 1447-1456.

Huang, P., Tofighi, R., Emgard, M., Ceccatelli, S., 2005. Cell death induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) in AtT-20 pituitary cells. Toxicology 207, 391-9.

Hughes J.A., Sparber S.B. (1978) d-Amphetamine unmasks postnatal consequences of exposure to methylmercury in utero: methods for studying behavioral teratogenesis. Oharmacol Biochem Behav 8:365-375

Hussain RJ, Gyori J, DeCaprio AP, Carpenter DO. In vivo and in vitro exposure to PCB 153 reduces long-term potentiation. Environ Health Perspect 2000;108:827-831.

Jacobson JL, Jacobson SW (1996) Intellectual impairment in children exposed to polychlorinated biphenyls in utero. N Engl J Med 335: 783-789.

Jacobson JL, Jacobson SW (2003) Prenatal exposure to polychlorinated biphenyls and attention at school age. J Pediatr 143: 780-788.

Jansen H.T., Cooke P.S., Porcelli J., Liu T.-C., Hansen L.G. (1993). Estrogenic and antiestrogenic actions of PCBs in the female rat: in vitro and in vivo studies. Reprod Toxicol 7: 237-248.

Johansson, C., R. Tofighi, C. Tamm, M. Goldoni, A. Mutti, and S. Ceccatelli, 2006, Cell death mechanisms in AtT20 pituitary cells exposed to polychlorinated biphenyls (PCB 126 and PCB 153) and methylmercury: Toxicol Lett. Sep 22; [Epub ahead of print]

Johe K. K., Hazel T. G., Muller T., Dugich-Djordjevic M. M. and McKay R. D. (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. 10, 3129–3140.

Jursa S., Chovancova J., Petrik J., Loksa J. (2006) Dioxin-like and non-dioxin-like PCBs in human serum of Slovak population. Chemosphere 64(4):686-91.

Kang, J.H., Park, I.S., Oh, W.Y., Lim, H.K., Wang, S.Y., Lee, S.Y., Choi, K.H., Kim, J.I., Jung, S.Y., Suh, C.K., Kim, D.S., 2004. Inhibition of aroclor 1254-induced depletion of stored calcium prevents the cell death in catecholaminergic cells. Toxicology 200, 93-101.

Kaya H., Hany J., Fastabend A., Roth-Harer A., Winneke G., Lilienthal H. (2002). Effects of maternal exposure to a reconstituted mixture of polychlorinated biphenyls on sex-dependent behaviours and steroid hormone concentrations in rats: dose-response relationship. Toxicol Appl Pharmacol 178: 71-81.

Knobeloch L., Steenport D., Schrank C., Anderson H. (2006) Methylmercury exposure in Wisconsin: A case study series. Environ Res 101(1): 113-22.

Kodavanti, P.R.S., Tilson, H.A., 2000. Neurochemical effects of environmental chemicals: in vitro and in vivo correlations on second messenger pathways. Ann. N. Y. Acad. Sci. 919, 97-105.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JAInteraction of estrogenic chemicals and phytoestrogens with estrogen receptor beta.Endocrinology. 1998 139(10):4252-63.

Lapham L.W., Cernichiari E., Cox C., Myers G.J., Baggs R.B., Brewer R., Shamlaye C.F., Davidson P.W., Clarkson T.W. (1995) An analysis of autopsy brain tissue from infants prenatally exposed to methymercury. Neurotoxicology Winter 16(4): 689-704.

Lee, D.W., Opanashuk, L.A., 2004. Polychlorinated biphenyl mixture Aroclor 1254-induced oxidative stress plays a role in dopaminergic cell injury. Neurotoxicology 25, 925-39.

Levine RR, Birdsall NJM, Nathanson NM, eds. Proceedings of the Ninth International Symposium on Subtypes of Muscarinic Receptors. Life Sci 2001;68:2449-2642.

Lewandowski T.A., Ponce R.A., Charleston J.S., Hong S., Faustman E.M. (2003) Effect of methylmercury in midbrain cell proliferation during organogenesis: potential cross-species differences and implications for risk assessment. Toxicol Sci 75: 124-133.

Lilienthal H., Fastabend A., Hany J., Kaya H., Roth-Harer A., Dunemann L., Winneke G. (2000). Reduced levels of 1,25-dihydroxivitamin D3 in rat dams and offspring after exposure to reconstituted PCB mixture. Toxicol Sci 57: 292-301.

Lindner, M.D., and Schallert, T. (1988). Aging and atropine effects on spatial navigation in the Morris water task. Behav Neurosci 102: 621-34.

Longnecker M.P., Wolff M.S., Gladen B.C., Brock J.W., Grandjean P., et al. (2003) Comparison of polychlorinated biphenyl levels across studies of human neurodevelopment. Environ Health Perspect 111(1):65-70.

Lyche J.L., Oskam I.C., Skaare J.U., Reksen O., Sweeney T., Dahl E., Farstad W., Ropstad E. (2004a). Effects of gestational and lactational exposure to low doses of PCBs 126 and 153 on anterior pituitary and gonadal hormones and on puberty in female goats. Reprod Toxicol 19: 87-95.

Lyche J.L., Skaare J.U., Larsen H.J., Ropstad E. (2004b) Levels of PCB 126 and PCB 153 in plasma and tissues in goats exposed during gestation and lactation. Chemosphere 55(4):621-629.

Maervoet J., Beck V., Roelens S.A., Covaci A., Voorspoels S., Geuns J.M., Darras V.M., Schepens P. (2005) Uptake and tissue-specific distribution of selected polychlorinated biphenyls in developing chicken embryos. Environ Toxicol Chem 24(3):597-602.

Mariussen, E., Myhre, O., Reistad, T., Fonnum, F., 2002. The polychlorinated biphenyl mixture aroclor 1254 induces death of rat cerebellar granule cells: the involvement of the N-methyl-D-aspartate receptor and reactive oxygen species. Toxicol. Appl. Pharmacol. 179, 137-44.

Marty, M. S., and W. D. Atchison, 1997, Pathways mediating Ca2+ entry in rat cerebellar granule cells following in vitro exposure to methyl mercury: Toxicol Appl Pharmacol, v. 147, p. 319-30.

Marty, M. S., and W. D. Atchison, 1998, Elevations of intracellular Ca2+ as a probable contributor to decreased viability in cerebellar granule cells following acute exposure to methylmercury: Toxicol Appl Pharmacol, v. 150, p. 98-105.

Meacham, C.A., Freudenrich, T.M., Anderson, W.L., Sui, L., Lyons-Darden, T., Barone Jr, S., Gilbert, M.E., Mundy, W.R., Shafer, T.J., 2005. Accumulation of methylmercury or polychlorinated biphenyls in in vitro models of rat neuronal tissue. Toxicol. Appl. Pharmacol. 205, 177-87.

Miñana, M.D. ; Montoliu, C. Llansola, M. ; Grisolía, S. and Felipo, V. (1998). Nicotine prevents glutamate-induced proteolysis of the microtubule-associated protein MAP-2 and glutamate neurotoxicity in primary cultures of cerebellar neurons. Neuropharmacology. 37 847-857.

Mizejewski, G. J. 1985. New insights into AFP structure and function: potential biomedical applications, p. 5-34. *In* G. J. Mizejewski and I. H. Porter (ed.), Alpha-fetoprotein and congenital disorders. Academic Press, Orlando, Fla.

Morris,R. (1981). Spatial localization does not require the presence of local cues. Learning Motivation 12, 239-260.

Mossman T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods 65: 55-63.

Myers GJ, Davidson PW (1998) Prenatal methylmercury exposure and children: neurologic, developmental, and behavioral research. Environ Health Perspect 106 Suppl 3: 841-847.

Naftolin F, Horvath TL, Balthazart J Estrogen synthetase (aromatase) immunohistochemistry reveals concordance between avian and rodent limbic systems and hypothalami.Exp Biol Med (Maywood). 2001 226(8):717-25.

Pliskova M., Vondracek J., Fernandez Canton R., Nera J., Kocan A., Petrik J., Trnovec T., Sanderson T., van den Berg M., Machala M. (2005) Impact of polychlorinated biphenyl contamination on estrogenic activity in human male serum. Environ Health Perspect 113, 1277-1284.

Prins GS, Birch L, Couse JF, Choi I, Katzenellenbogen B, and Korach KS. Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor alpha: studies with alphaERKO and betaERKO mice. *Cancer Research* 61: 6089-6097, 2001

Onishchenko N, Tamm C, Vahter M, Hökfelt T, Johnson JA., Johnson DA., Ceccatelli S. Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice. Toxicological Sciences , in press.

Rasmussen E.B., Newland M.Ch. (2001) Developmental exposure to methylmercury alters behavioral sensitivity to d-amphetamine and pentobarbital in adult rats. Neurotoxicol Teratol 23:45-55.

Rice D, Barone S Jr. 2000. Critical periods of vulnerability for the developing nervous system: evidence from human and animal models. Environ health Perspect 108: 511- 531.

Rice DC (1999) Behavioral impairment produced by low-level postnatal PCB exposure in monkeys. Environ Res 80: S113-S121.

Robinson T.E., Berridge K.C. (2000) The psychology and neurobiology of addiction: an incentive-sensitization view. Addiction 95 (Suppl 2):91-117.

Roegge CS, Wang VC, Powers BE, Klintsova AY, Villareal S, Greenough WT , Schantz SL (2004) Motor impairment in rats exposed to PCBs and methylmercury during early development. Toxicol Sci 77: 315-324.

Roegge C.S., Schantz S.L. (2006) Motor function following developmental exposure to PCBS and/or MEHG. Neurotoxicol Teratol 28:260-277.

Roelens SA, Beck V, Aerts G, Clerens S, Vanden Bergh G, Arekens L, Darras VM, van der Geyten S. Neurotoxicity of polychlorinated biphenyls (PCBs) by disturbance of thyroid hormone-regulated genes. Ann NY Acad Sci 2005;1040:454-456.

Rossi, A.D., Ahlbom, E., Ogren, S.O., Nicotera, P., Ceccatelli, S., 1997. Prenatal exposure to methylmercury alters locomotor activity of male but not female rats. Exp Brain Res. 117, 428-436.

Saint-Amour D., Roy M.S., Bastien C., Ayotte P., Dewailly E., Despres C., Gingras S., Muckle G. (2006) Alterations of visual evoked potentials in preschool Inuit children exposed to methylmercury and polychlorinated biphenyls from a marine diet. Neurotoxicology 27(4):567-78.

Sakamoto M, Nakano A, Kajiwara Y, Naruse I, Fujisaki T. Effects of methyl mercury in postnatal developing rats. Environ Res. 1993 Apr;61(1):43-50.

Sakamoto M., Kakita A., Wakabayashi K., Takahashi H., Nakano A., Akagi H. (2002a) Evaluation of changes in methylmercury accumulation in the developing rat brain and its effects: a study with consecutive and moderate dose exposure throughout gestation and lactation periods. Brain Res 949: 51-59.

Sakamoto M., Kubota M. , Matsumoto S., Nakano A., Akagi H. (2002b) Declining risk of methylmercury exposure to infants during lactation. Environ Res 90(3):185-189.

Sakaue M, Okazaki M, Hara S. 2005, Very low levels of methylmercury induce cell death of cultured rat cerebellarneurons via calpain activation.Toxicology 213(1-2):97-106.

Sarafian, T., Verity, M.A., 1991. Oxidative mechanisms underlying methyl mercury neurotoxicity. Int. J. Devl. Neuroscience 9, 147-53.

Sawaguchi T, Goldman-Rakic PS., 1991. D1 dopamine receptors in prefrontal cortex: involvement in working memory. Science 251, 947-950.

Schalock RL, Brown WJ, Kark RA, Menon NK (1981) Perinatal methylmercury intoxication: behavioral effects in rats. Dev Psychobiol 14: 213-219.

Schantz SL (1996) Developmental neurotoxicity of PCBs in humans: what do we know and where do we go from here? Neurotoxicol Teratol 18: 217-227.

Schantz SL, Seo BW, Moshtaghian J, Peterson RE, Moore RW. (1996) Effects of gestational and lactational exposure to TCDD or coplanar PCBs on spatial learning. Neurotoxicol Teratol.18(3):305-13.

Schantz SL, Moshtaghian J, Ness DK (1995) Spatial learning deficits in adult rats exposed to ortho-substituted PCB congeners during gestation and lactation. Fundam Appl Toxicol 26: 117-126.

Schantz SL. Developmental neurotoxicity of PCBs in humans: what do we know and where do we go from here? Neurotoxicol Teratol. 1996 May-Jun;18(3):217-27

Seegal RF, Brosch KO, Okoniewski RJ. Effects of in utero and lactational exposure of the laboratory rat to 2,4,2',4'- and 3,4,3',4'-tetrachlorobiphenyl on dopamine function. Toxicol Appl Pharmacol. 1997 Sep;146(1):95-103.

Seegal RF. Can epidemiological studies discern subtle neurological effects due toperinatal exposure to PCBs? Neurotoxicol Teratol. 1996 May-Jun;18(3):251-4.

Seegal, R. F. (1996). Epidemiological and laboratory evidence of PCB-induced neurotoxicity. Crit Rev Toxicol 26: 709-37.

Seegal, R., and Schantz, S. L. (1994). Neurochemical and behavioural sequele of exposure to dioxins and PCBs. Plenum Press, New York.

Seegal R.F., Okoniewski R.J., Brosch K.O., Bemis J.C. (2002) Polychlirinated biphenyls alter extraneuronal but not tissue dopamine concentration in adult rat striatum: an *in vivo* microdialysis study. Environ Health Perspect 110 (11): 1113-1117.

Shain, W., Bush, B., Seegal, R.F., 1991. Neurotoxicity of polychlorinated biphenyls: structure-activity relationship of individual congeners. Toxicol Appl Pharmacol. 111, 33-42.

Sleeper E., Tamm C., Frisen J., Zhivotovsky B., Orrenius S. and Ceccatelli S. (2002) Cell death in adult neural stem cells. Cell Death Differ 9, 1377-1378.

Snyder E. Y., Deitcher D. L., Walsh C., Arnold-Aldea S., Hartwieg E. A. and Cepko C. L. (1992) Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell 68, 33–51.

Snyder E. Y., Yoon C., Flax J. D. and Macklis J. D. (1997) Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. Proc. Natl Acad. Sci. USA 94, 11 663–11 668.

Soderstrom S. and Ebendal T. (1995) In vitro toxicity of methyl mercury: effects on nerve growth factor (NGF)-responsive neurons and on NGF synthesis in fibroblasts. Toxicol Lett 75, 133-144.

Spyker JM, Sparber SB, Goldberg AM. Subtle consequences of methylmercury exposure: behavioral deviations in offspring of treated mothers.

Stewart, P.W., Reihman, J., Lonky, E.I., Darvill, T.J., Pagano, J., 2003. Cognitive development in preschool children prenatally exposed to PCBs and MeHg. Neurotoxicol. Teratol. 25, 11-22.

Takahashi H, Higuchi M, Suhara T., 2006. The role of extrastriatal dopamine D2 receptors in schizophrenia. Biol Psychiatry 59, 919-928.

Tamm C., Duckworth J., Hermanson O., Ceccatelli S. (2006) High susceptibility of neural stem cells to methylmercury toxicity: effects on cell survival and neuronal differentiation. J Neurochem. 97(1):69-78.

Tan, Y., Chen, C.H., Lawrence, D., Carpenter, D.O., 2004. Ortho-substituted PCBs kill cells by altering membrane structure. Toxicol. Sci. 80, 54-9.

Tilson, H. A., and Harry, G. J. (1994). Developmental neurotoxicology of polychlorinated biphenyls and related compounds. In The vulnerable brain and environmental risks (R. L. Isaacson and K. F. Jensen, eds.), Vol. 3, pp. 267-279. Plenum Press, New York.

Tilson, H. A., Jacobson, J. L., and Rogan, W. J. (1990). Polychlorinated biphenyls and the developing nervous system: cross- species comparisons. Neurotoxicol Teratol 12: 239-48.

Tilson, H.A., Kodavanti, P.R.S., 1997. Neurochemical effects of polychlorinated biphenyls: an overview and identification of research needs. Neurotoxicology. 18, 727-744.

Toimela T, Tahti H., 2004, Mitochondrial viability and apoptosis induced by aluminum, mercuric mercury and methylmercury in cell lines of neural origin. Arch Toxicol 78(10):565-74.

Vettori, M.V., Caglieri, A., Goldoni, M., Castoldi, A.F., Daré, E., Alinovi, R., Ceccatelli, S., Mutti, A., 2005. Analysis of oxidative stress in SK-N-MC neurons exposed to styrene-7.8-oxide. Toxicol. In Vitro 19, 11-20.

Vettori, M.V., Goldoni, M., Caglieri, A., Poli, D., Folesani, G., Ceccatelli, S., Mutti, A., 2006. Antagonistic effects of methyl-mercury and PCB153 on PC12 cells after combined and simultaneous exposure. Food Chem. Toxicol. 44, 1505-12.

Vezina P., Lorrain D.S., Arnold G.M., Austin J.D., Suto N. (2002) Sensitization of midbrain dopamine neuron reactivity promotes the pursuit of amphetamine. J Neurosci 22(11):5654-4662.

Viberg, H., Fredriksson, A., and Eriksson, P. (2003). Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. Toxicol Appl Pharmacol 192: 95-106.

Viberg, H., Fredriksson, A., Jakobsson, E., Örn, U., and Eriksson, P. (2003). Neurobehavioural derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. Toxicological Sciences, 76: 112-120.

Viberg, H., Fredriksson, A., and Eriksson, P. (2004). Investigations of strain and/or gender differences in developmental neurotoxic effects of polybrominated diphenyl ethers in mice. Toxicological Sciences, 81: 344-353

Virgili M, Saverino O, Vaccai M, Barnabei O, Contestabile A. Temporal, regional and cellular selectivity of neonatal alteration of the thyroid state on neurochemical maturation in the rat. Exp Brain Res 1991; 83:555-561.

Vodicnik, M. J., and Lech, J. J. (1980). The transfer of 2,4,5,2',4',5'-hexachlorobiphenyl to fetuses and nursing offspring. I. Disposition in pregnant and lactating mice and accumulation in young. Toxicol Appl Pharmacol 54: 293-300.

Vorhees Ch. Behavioral effects of prenatal methylmercury in rats: A parallel trial to the Collaborative Behavioral teratology study. Neurobehav Toxicol Teratol 7: 717-725, 1985.

Wagner GC, Reuhl KR, Ming X, Halladay AK. (2006) Behavioral and neurochemical sensitization to amphetamine following early administration of methylmercury (MeHg). Neurotoxicology (epub ahead of print).

Wang J, O'Donnell P., 2001. D(1) dopamine receptors potentiate NMDA-mediated excitability increase in layer V prefrontal cortical pyramidal neurons. Cereb Cortex 11, 452-462.

Wang L, Andersson S, Warner M, and Gustafsson JA. Morphological abnormalities in the brains of estrogen receptor beta knockout mice. Proceedings of the National Academy of Sciences of the United States of America 98: 2792-2796, 2001.

Weiss B, Stern S, Cox C, Balys M (2005) Perinatal and lifetime exposure to methylmercury in the mouse: behavioral effects. Neurotoxicology 26: 675-690.

Whishaw, I. Q. (1985). Cholinergic receptor blockade in the rat impairs locale but not taxon strategies for place navigation in a swimming pool. Behav Neurosci 99: 979-1005.

Winneke, G., Bucholski, A., Heinzow, B., Krämer, U., Schmidt, E., Walkowiak, J., Weiner, J.-A., Steingrüber., 1998. Developmental neurotoxicity of polychlorinated biphenyls (PCBs): cognitive and psychomotor functions in 7-month old children. Toxicol. Lett. 102-103, 423-8.

Wojtowicz A.K., Gregoraszczuk E.L., Lyche J.L., Ropstad E. (2000). Time dependent and cell-specific action of polychlorinated biphenyl (PCB153 and PCB126) on steroid secretion by porcine theca and granulosa cells in mono- and co-culture. J Physiol Pharmacol 51: 555-568.

Wojtowicz A.K., Goch M., Gregoraszczuk E.L. (2005). Polychlorinated biphenyls (PCB153 and PCB126) action on conversion of 20-hydroxylated cholesterol to progesterone, androstenedione to testosterone, and testosterone to estradiol 17beta. Exp Clin Endocrinol Diabetes 113: 464-470.

Yamamoto M., Narita A., Kagohata M., Shirai M., Akahori F., Arishima K. (2005). Effects of maternal exposure to PCB126 or PCB169 on testicular steroidogenesis and spermatogenesis in male offspring rats. J Androl 26: 205-214.

Yamashima T., 2004. Ca2+-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. Cell Calcium 36, 285-93.

Yee, S., Choi, B.H., 1994. Methylmercury poisoning induces oxidative stress in the mouse brain. Exp. Mol. Pathol. 60, 188-96.

Zahalka E.A., Ellis D.H., Goldey E.S., Stanton M.E., Lau Ch. (2001) Perinatal exposure to polychlorinated biphenyls Aroclor 1016 or 1254 did not alter brain catecholamines nor delayed alternation performance in Long-Evans rats. Brain Res Bull 55:487-500.