Alternative flame retardants:

Is it possible to use protein expression levels as a biomarker for the neurotoxic effect of compounds on learning and memory?

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# Abstract

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in textiles, plastics, wire insulation and automobiles. They are additive flame retardants, which makes them prone to diffusion into the air. Exposure via house dust and diffusion into the air together with the toxicological and persistent characteristics of PBDEs, PBDEs can be a danger to human health. It has been shown that PBDE-exposed animals and children have cognitive dysfunctions and decreased learning and memory. Certain PBDEs have been banned and a search for alternative flame retardants is ongoing. Learning and memory is based on changes in the efficacy and local geometry of synaptic connections and transmissions, called synaptic plasticity. A long-lasting increase in synaptic strength is called long-term potentiation (LTP) and is a measure for learning and memory. Suggested key-proteins involved in LTP are AMPA-R, NMDA-R, calmodulin and ERK1/2.

Currently the neurotoxicity of substances is tested using animals. The animals are exposed to the substance, then sacrificed and have their hippocampus removed. The hippocampus is cut into slices and LTP is measured using electrodes. These steps require time and precision. This thesis will research if it is possible to use changes in certain protein expression as a measure for neurotoxicity (biomarker), like learning and memory.
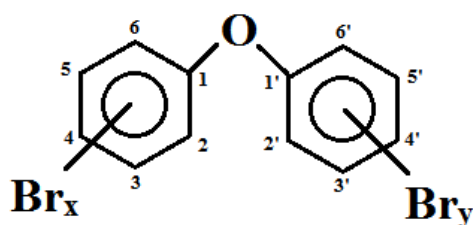
Lead ($Pb^{2+}$), methylmercury (MeHg) and polychlorinated biphenyls (PCBs) are known neurotoxic compounds with adverse health effects on learning and memory. They cause alterations in the neurotransmitter system, calcium homeostasis and enhance oxidative stress in neurons. Alterations in the expression of the suggested key-proteins were studied. No literature was found on AMPA-R, calmodulin and ERK1/2. NMDA-R subtypes were found to be altered by Pb2+ and MeHg but not by PCBs. Besides the suggested key-proteins, PKC was found to be disturbed by all three compounds.

It is possible to assess neurotoxicity of compounds by measuring protein expression levels. A common protein affected by the studied neurotoxins is PKC. Besides PKC, two out of three showed alterations in NMDA-R subtype expression. It is likely not all compounds affect the same protein(s). Further research is needed with more neurotoxic compounds to compose a list of biomarker proteins and to develop a protocol to analyze when a compound affects learning and memory.

# Introduction

In 2010 41000 fires were reported. 65 people died, 1000 were wounded and 600 needed to be saved. To increase the safety of flammable materials, like buildings, furniture, transportation, electronics and textiles, flame retardants are added. Addition of flame retardants give people more time to get to safety. Flame retardants consist of inorganic and organic compounds based on bromine, chlorine, phosphorus, nitrogen, boron, metallic oxides or hydroxides (Esch et al., 1997).

Polybrominated diphenyl ether (PBDEs) (figure 1) were the most commonly used flame retardant in plastics because they were cheap and have a high performance efficiency (Birnbaum & Staskal, 2004). There are 209 different PBDE congeners, most used are penta-, octa- and decabrominated BDEs (Costa et al., 2008). The sale of pentaBDE and octaBDE in concentrations higher than 0,1% and use of pentaBDE, octaBDE and decaBDE in electronic equipment have been banned (RIVM, 2010). Besides bromine, flame retardants can also be phosphorus or metal based, but these are more expensive and can pose manufacturing problems (Rahman et al., 2001). Flame retardants can either be reactive or additive chemicals: reactive flame retardants are covalently bound to polymers, whereas additive flame retardants are dissolved into the polymer. Additive flame retardants can be volatile and diffuse into the air, gradually losing their flame retardancy and polluting the environment. Distribution and toxicity of flame retardants are comparable to polychlorinated biphenyls (PCBs) and the insecticide dichlorodiphenyltrichloroethane (DDT).



BDE-47: 2,2',4,4'-tetrabromodiphenyl ether
BDE-99: 2,2'4,4',5-pentabromodiphenyl ether
BDE-100: 2,2',4,4',6-pentabromodiphenyl ether
BDE-153: 2,2',4,4',5,5'-hexabromodiphenyl ether
BDE-154: 2,2',4,4',5,6'-hexabromodiphenyl ether
BDE-209: Decabromodiphenyl ether

**Figure 1. Polybrominated diphenyl ether (PBDE).** General chemical structure of PBDEs and chemical names of the major PBDE congeners (adapted from Costa et al., 2008).

When flame retardants decompose at lower temperatures than the polymer, preventing the formation of flammable gases (Rahman et al., 2001). Halogen-based flame retardants release hydrogen chloride or bromide. These catch the reactive H and OH radicals formed during combustion, terminating combustion. Flame retardants should decompose at a lower temperature than the host polymer to be effective. Aromatic bromine compounds (figure 1) are used extensively because they have a lower thermal stability than aliphatic bromines.

## *Human exposure*

Exposure to PBDEs can occur via several routes; breast milk, food, air and dust (Costa et al., 2007). The concentration of 15 PBDEs (BDE-17, 28, 32, 35, 37, 47, 49, 71, 75, 85, 99, 100, 119, 153 and 154) were measured in breast milk in of 54 mothers living in Great Britain (Kalantzi et al., 2004). The most common PBDEs were BDE-47 (100% positive samples), BDE-100 (94%) and BDE-99 (92%). The geometric mean of all PBDE congeners was 6,6 ng/g lipid, where PBDE-47 was mostly abundant (3 ng/g lipid). European countries have a lower concentration of PBDE in breast milk when compared to the United States of America (table 1). In the US pentaBDEs are highly used, according to an industry report, 98% of the pentaBDEs world production were used in North America (Sjödin et al., 2008). In Great Britain the concentration of PBDEs is higher than the rest of Europe, because Great Britain has more stringent fire safety regulations (Harrad et al., 2008).

**Table 1. Comparison of PBDE levels in breast milk samples from different countries.**

| Country (n) | Year of sampling | PBDE ng/g (range) | Reference |
|---|---|---|---|
| Canada (10) | 1992 | 5,7 (0,7-28) | Ryan and Patry, 2000 |
| Finland (11) | 1994-1998 | 2,3 (0,9-5,9) | Strandman et al., 2000 |
| Sweden (40) | 1997 | 4 | Norén and Meironyté, 2000 |
| Great Britain (54) | 2001-2003 | 6,6 (0,1-63) | Kalantzi et al., 2004 |
| United States of America (47) | 2001 | 73,9 (6,2-418,8) | Schecter et al., 2003 |

A Spanish study measured PBDEs (tetra-, penta-, hexa-, hepta- and octa-BDE) in 54 samples divided into 11 foodstuffs; vegetables, tubers, pulses, cereals, fruits, fish and shellfish, meat and meat products, eggs, milk, dairy products and fats and oils (Bocio et al., 2003). Products were randomly bought in local markets, big supermarkets and grocery stores from seven different cities. The highest concentration of PBDE was found in oils and fats (587,7-569,3 ng/kg wet weight), fish and shellfish (33,9-325,3 ng/kg wet weight), meat and meat products

(109,2-102,4 ng/kg) and eggs (64,5-58,3 ng/kg). With the results gathered the following dietary intake of PBDEs were estimated (figure 2).
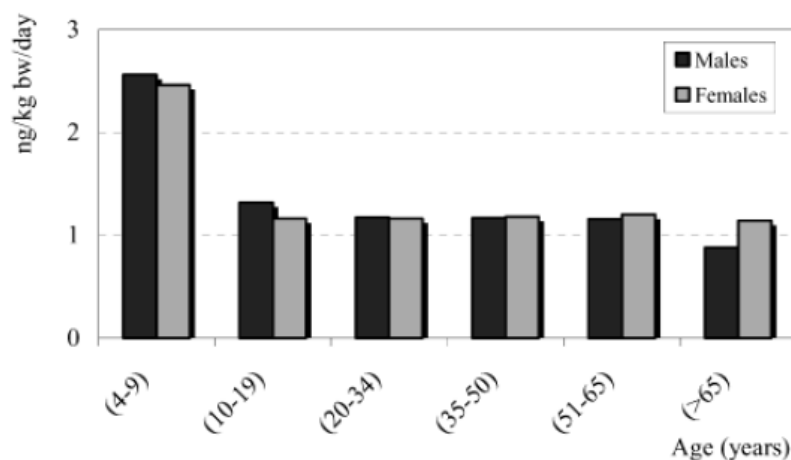


**Figure 2. Estimated PBDE dietary intake.** Estimated dietary intake of PBDEs by the general population in Spain, divided into age and sex (Bocio et al., 2003).

PBDEs have also been found in the air using passive air samplers (3-4 $m^3$/day) (Jaward et al., 2004). 71 air samples were collected over a six week period in 22 countries (mostly West Europe). 25 samples were taken in urban areas and 46 in rural/remote locations. Overall, urbanized areas have a higher airborne concentration of PBDEs. The mean concentration of total PBDE (BDE-28, 47, 49, 75, 99, 100, 153 and 154) was 5,48 ng/sample (0,06-43 ng/sample), where BDE-99 (mean 4,24 ng/sample) and BDE-47 (mean 2,86 ng/sample) were most abundant. From the gathered data the air concentration could be estimated. Total PBDE air concentration were estimated at 0,5-250 pg/$m^3$, BDE-99 at <10-120 pg/$m^3$ and BDE-47 at <8-80 pg/$m^3$. Using the average of the mean daily inhalation values for humans (13,3 $m^3$/day), exposure to PBDEs via inhalation would range between 6,65-3325 pg/day.

Levels of PBDEs are also present in household dust (Sjödin et al., 2005). 40 dust samples (particulate fraction <2mm) were collected in four different countries (Australia, Germany, Great Britain and the United States of America). The samples were tested for BDE-47, 99, 100, 153, 154, 183 and 209. Significantly lower concentration were found in German household dust, total PBDE concentration had a median of 74 ng/g dust (17-550 ng/g dust), when compared to Australia (1200 (500-13000 ng/g dust)), Great Britain (10000 (950-54000 ng/g dust)) or the US (4200 (520-29000 ng/g dust)).

A German study estimated the adult daily intake of PBDEs for the general population after measuring PBDEs in diet, indoor air, house dust and biomonitoring (Fromme et al., 2009).

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The results are shown in table 2. Air samples were taken for 24 hours at a rate of 4 m$^3$/h. The study of Jaward shows a daily exposure of 6,65-3325 pg to airborne PBDEs, while Fromme reports 586 pg/day as high exposure. This is a 5,6 times difference. It is questionable if these two studies can be compared, sample locations, times and flow rate were different. Jaward measured 128-168 m$^3$ air in 6 weeks in 22 outdoor locations in rural and urban areas, while Fromme sampled 96 m$^3$ indoor air in 24 hours.

|  | Intake (pg/day) |  |
|---|---|---|
|  | Average scenario | High scenario |
| **Indoor air** | 154 | 586 |
| **Outdoor air** | 15 | 35 |
| **Dust** | 1955 | 7700 |
| **Diet** | 69,609 | 179,913 |

**Table 2. Estimated total PBDE intake.** Estimated adult daily intake of total PBDEs for the general population in Germany, divided into indoor and outdoor air, dust and diet exposure (Fromme et al., 2009).

From these results an average total daily intake of PBDEs was estimated at 1,2 ng/kg bodyweight. Assuming people spend 90% of their time indoors and 10% outdoors. The average dust intake was 50 mg/day (Fromme et al., 2009).

This study also measured blood PBDEs (BDE-47, 99, 100 and 153) concentrations 47 subjects. The median blood concentrations were 1,8 (0,23-6,44) ng/g lipid, 0,8 (0,19-2,19) ng/g lipid, 0,6 (0,27-2,71) ng/g lipid and 2,4 (0,86-8,19) ng/g lipid respectively.

## *Health effects of PBDEs*

Exposure to PBDEs occurs on a daily basis, therefore the health effects of PBDEs have to be studied. Adverse health effects of PBDEs, like endocrine disruption and developmental neurotoxicity, have been seen in different laboratory animals. The focus of this paper will be on the developmental neurotoxicity of PBDEs, because infants and toddlers have a high body burden by exposure through breast milk and household dust and disturbances during development might result in permanent changes in brain structure or function.

The effects of PBDE exposure have been studied in humans, mostly focusing on prenatal exposure. BDEs were measured in blood of pregnant women in the 35th week of pregnancy (Roze et al., 2009). Neuropsychological functioning, motor performance, cognition and

behavior was determined when the children were 5-6 years old. Brominated flame retardants were associated with reduced fine manipulative abilities and attention, but were also associated with better coordination, visual perception and behavior. An association between increasing PBDE concentrations in colostrum and a worse cognitive development in the first year of life in children is suggested (Gascon et al., 2012).

*In vivo* studies showed serious adverse health effects on spontaneous behavior and learning and memory. Exposure of mice to BDE-153 (Viberg et al., 2003), -209 (Viberg (2) et al., 2003), -203, -206 (Viberg et al., 2006), -47 and -99 (Eriksson et al., 2001) resulted in reduced spontaneous behavior (measure of locomotion, rearing and total activity) and/or a lower score in the Morris water maze test (measure of spatial learning and memory). Neonatal exposure of mice to BDE-153 or -203 results in a decrease in spatial learning and memory and gets worse with age. BDE-47, -99, -153, -203, -206 and -209 exposed mice have decreased spontaneous behavior when compared to control mice.

*In vivo* studies clearly show that PBDEs have adverse health effects of neuronal function. To better understand the molecular effects, *in vitro* studies have been performed. *In vitro* studies showed that BDE-47 reduced long-term potentiation (LTP), which is a neurophysiological substrate for learning and memory, and postsynaptic protein, proteins related to long-term potentiation, levels in mice after a single oral dose on PND 10 (Dingemans et al., 2007). BDE-47 caused reduced glutathione, superoxide dismutase (SOD) and increased DNA damage in primary rat hippocampal neurons at 2,06 µM (He et al., 2008). Primary neonatal rat hippocampal neurons exposed to 10, 30 or 50 µg/ml BDE-209 had decreased cell viability and increased apoptotic cells, reactive oxygen species, calcium concentrations (Chen et al., 2010).

Due to serious adverse health effects caused by brominated flame retardants there is a need for alternative flame retardants. Prenatal exposure disturbs cognitive development and animal studies have showed a permanent decrease in spontaneous behavior, learning and memory. The search for safe alternative flame retardants is still ongoing. Currently, neurotoxic analysis of a compound is tested by decapitating *in vivo* exposed animals. The hippocampus is then extracted and divided into slices. Using high-frequency stimulation, LTP is measured with

electrodes (Dingemans et al., 2007). This is a time-bearing process and a more efficient method, like measuring protein expression, would be beneficial.

# Research aim

This thesis will focus on the proteins involved in learning and memory. The aim is to find proteins affected by neurotoxic compounds disturbing learning and memory. Which proteins are affected by the known neurotoxic compounds, lead, methyl mercury and PCBs? Which pathway(s) is/are mostly affected? Are there any common proteins affected which can be used as a biomarker for the neurotoxic effect on learning and memory?

# Background information

Synaptic plasticity is change in the efficacy and local geometry of synaptic connections and transmission and is a basis of learning, memory and other forms of brain plasticity (Neuroscience, 2004). A long-lasting increase in synaptic strength is known as long-term potentiation (LTP) and a long-lasting decrease in synaptic strength is known as long-term depression (LTD). LTP and LTD arise from molecular mechanisms that vary over time: initial changes in synaptic transmission arise from post-translational modifications of existing proteins, like changes in trafficking of glutamate receptors, while later phases result from changes in gene expression. Alteration in gene expression change synaptic transmission, including growth of synapses (Neuroscience, 2004). The focus of this thesis will be in the formation of LTP.

LTP is dependent of activation of N-Methyl-D-aspartic acid receptor (NMDA-R) and A-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid receptor (AMPA-R), which are, among others, located in the CA1 region of the hippocampus (Breedlove, 2007). The NMDA-R channel is blocked by physiological concentrations of $Mg^{2+}$. This channel is voltage-dependent and will only open when the postsynaptic cell is depolarized. Low-frequency synaptic transmission leads to binding of glutamate to both AMPA-R and NMDA-R, AMPA-R will be permeable to $Na^+$, but NMDA-R will be blocked by $Mg^{2+}$ (figure 3, left). High-frequency stimulation will cause a summation of ESPSs that causes depolarization, removing $Mg^{2+}$ from the NMDA-R channel. This results in an influx of $Ca^{2+}$, increasing the intracellular

$Ca^{2+}$ concentration. $Ca^{2+}$ serves as a second messenger signal that induces LTP (figure 3, right) (Neuroscience, 2004).
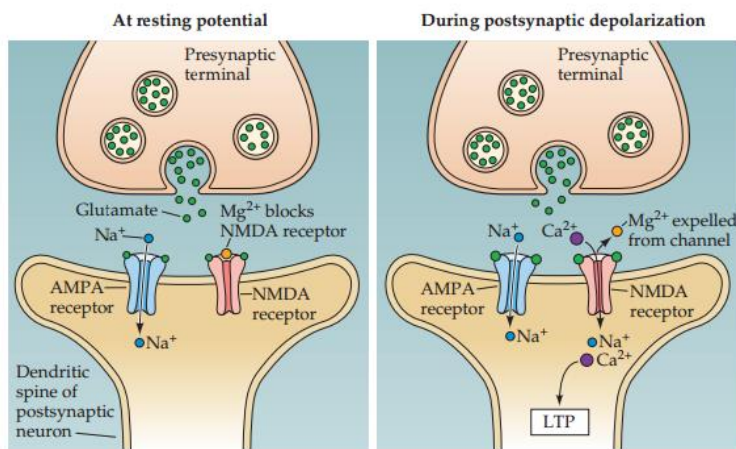


**Figure 3. Triggering of LTP.** At resting potential the NMDA-R channel is blocked by physiological concentrations of $Mg^{2+}$. During postsynaptic depolarization, $Mg^{2+}$ will be expelled and result in an influx of $Ca^{2+}$. The increased intracellular concentrations of $Ca^{2+}$ triggers LTP (Neuroscience, 2004).

$Ca^{2+}$ can bind to calmodulin (CaM) and activate protein kinases, including calcium/calmodulin-dependent protein kinase II (CaMK-II), CaMK-IV and protein kinase C (PKC) (figure 4). Translocation from the cytosol to the membrane results in activation of PKC. CAMK-II phosphorylates GluR1, a subunit of the AMPA-R, resulting in an increased $Na^+$ conductance. CAMK-II also promotes the translocation of intracellular stored AMPA-R to the membrane. $Ca^{2+}$ may also stimulate the release of neurotransmitter from the presynaptic axon. The neurotransmitter, due to the increase in AMPA-R, will exert a stronger response in the postsynaptic neuron (Neuroscience, 2004).
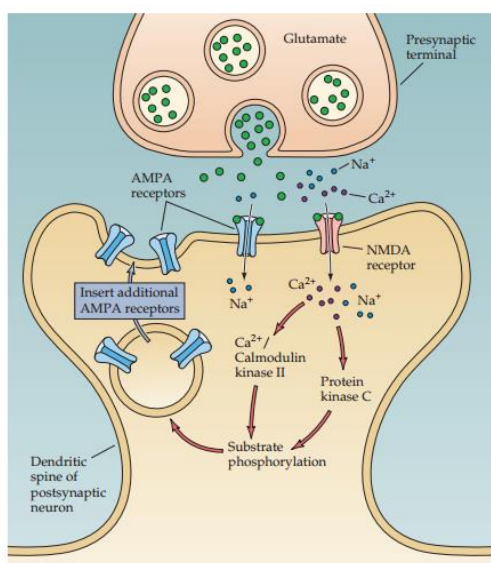


**Figure 4. Proteins involved in the formation of E-LTP.** Activation of AMPA-R results in the influx of $Na^+$, which depolarizes the neuron resulting in the activation of NMDA-R. Activation of NMDA-R results in an influx of $Na^+$ and $Ca^{2+}$. $Ca^{2+}$ binds to CAM and activates protein kinases, like CAMK-II and PKC (Neuroscience, 2004).

Late-phase LTP is dependent on activation of the transcriptional regulator cAMP response element-binding protein (CREB), which is activated by extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) (activated by protein kinase A (PKA) or PKC) or CaMK-IV (figure 5 and 6) (Angenstein et al., 1994; Sadiq et al., 2012). CREB turns on expression of genes that produce long-lasting changes in PKA activity and the synapse structure. One of the transcripts is brain-derived neurotrophic factor (BDNF), BDNF is secreted and binds to the tropomyosin-related kinase receptor B (TrkB) on the presynaptic neuron (Neal et al., 2010). Activation of TrkB results in a positive feedback loop by increasing glutamate vesicles and vesicle release.
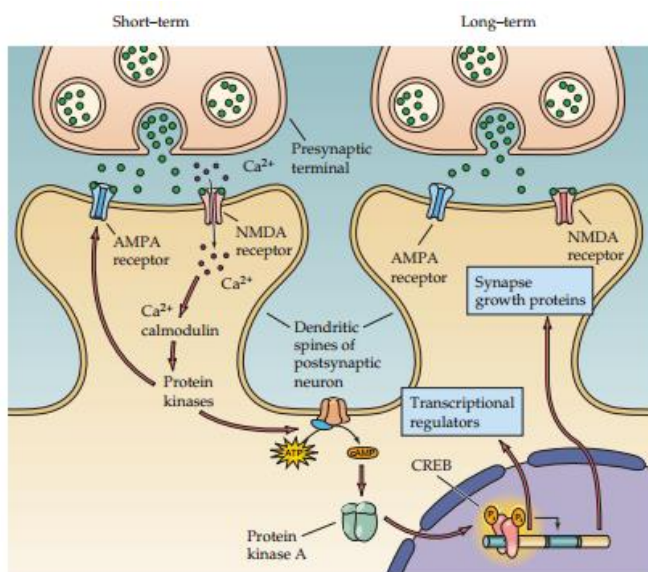


**Figure 5. Proteins involved in the formation of L-LTP.** Protein kinases activate protein kinase A (PKA) which activates CREB, a transcriptional regulator which turns on the expression of genes that produce long-lasting changes in PKA activity and synapse structure (Neuroscience, 2004).

An overview of the molecular signaling pathway of LTP formation is shown in figure 6. Key-events in the formation of LTP start with the induction via AMPA-R and NMDA-R (table A). Other key proteins are calmodulin and ERK1/2, both activate kinases which phosphorylate CREB, inducing late phase LTP.

**Table A. Overview of key-proteins in LTP induction.**

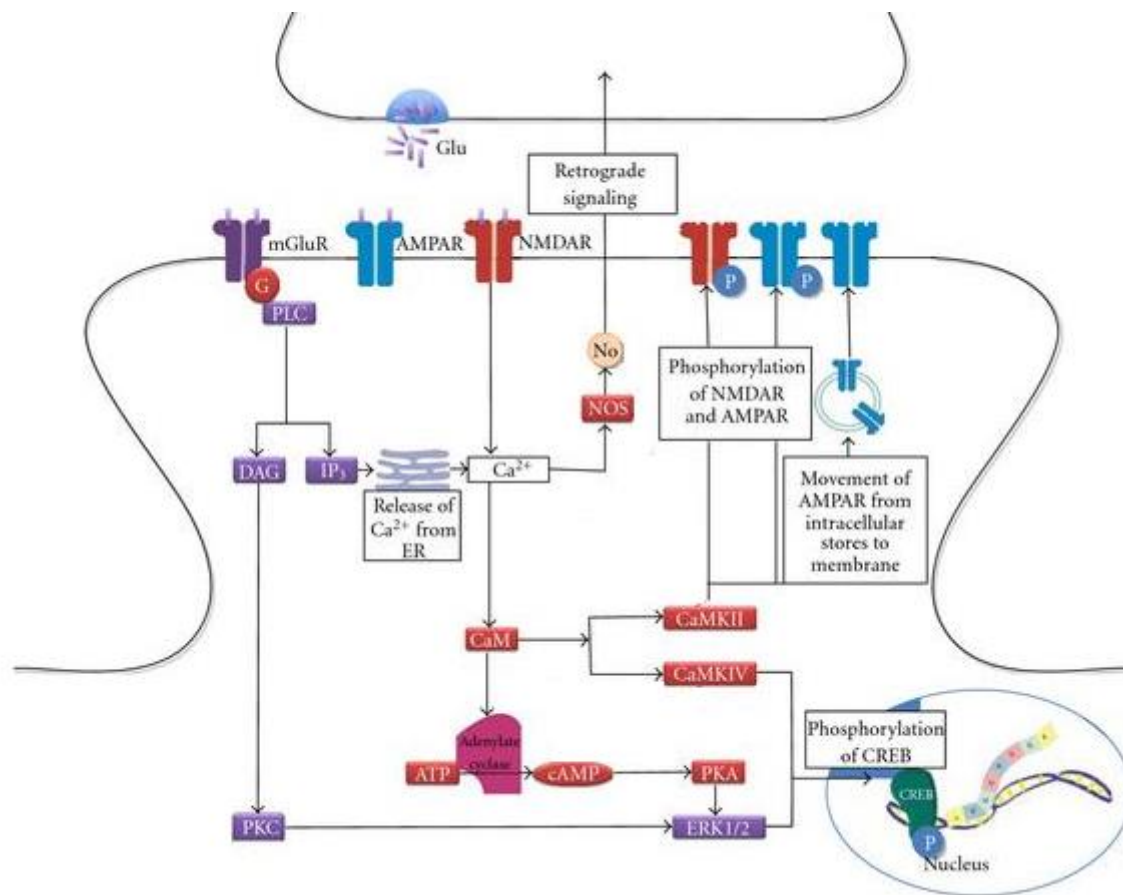| Protein | Alteration disrupts the |
|---------|-------------------------|
| AMPA-R | induction of LTP. |
| NMDA-R | induction of LTP. |
| CaM | activation of<br>- Adenylate cyclase<br>- CaM-KII<br>- CaM-KIV<br>➔➔➔ phosphorylation of CREB → late phase LTP |
| ERK1/2 | phosphorylation of CREB → late phase LTP |

**Figure 6. Overview of the signaling pathways involved in LTP formation.** LTP is dependent on several signaling pathways. AMPA-R and NMDA-R are stimulated by glutamate and results in a depolarization of the membrane, activating AMPA-R and NMDA-R. This results in the influx of $Ca^{2+}$, which activates CaM. CaM phosphorylated and activated CaMK-II and CaMK-IV. CaMK-II phosphorylated NMDA-R and AMPA-R, translocates intracellular stored AMPA-R to the membrane and activates adenylate cyclase. This enhances synaptic stimulation. Another important protein is PKC, which is activated by translocation from the cytosol to the membrane. PKC and PKA activate ERK1/2, which together with CaMK-IV phosphorylates CREB. Phosphorylated CREB is a transcription factor which alters gene expression of proteins needed for the formation of learning and memory (adapted from Sadiq et al., 2012).

# Known neurotoxic compounds

## *Lead (Pb$^{2+}$)*

Lead (Pb$^{2+}$) is a ubiquitous environmental neurotoxin with no biological function. Lead exposure can occur via several routes, but is mostly known via exposure through lead-containing paint in old houses. Pb$^{2+}$ can enter the brain through the blood-brain barrier by acting as a substitute for Ca$^{2+}$ (Lidsky et al., 2003). Adverse health effects of Pb$^{2+}$ can be seen at low concentrations, <10 µg/dL blood. The association between low-level exposure and intelligence was examined in 172 children of 3 and 5 years old (Canfield et al., 2003). Children with high blood lead concentration (>10 µg/dL) showed a linear decrease of 4,6 IQ points/10 µg/dL, whereas children with low blood lead concentration (<10 µg/dL) showed a decrease of 7,4 IQ points (difference 1 and 10 µg/dL). This study shows that low exposure results in a larger loss of IQ points.

*In vivo* experiments have shown that Pb$^{2+}$ affects permanent learning and memory deficits. Prenatal exposure (during gestation) causes long-term learning and memory deficits in young adult rats (Yang, Y. et al., 2003). Pregnant rats were fed with low (0,03%), middle (0,09%) or high (0,27%) Pb$^{2+}$ acetate containing diets during gestation. Blood lead concentration was significantly higher in exposed mice at PND0 (control: 3,25 µg/dL, low: 30,65 µg/dL, middle: 33,05 µg/dL, high: 41,7 µg/dL), but at adult age (PND65) blood lead concentrations were similar as control (1,6-2,1 µg/dL). All male offspring exposed to Pb$^{2+}$ had a significantly impaired memory retrieval, but in the female offspring only the low exposed group showed impaired memory retrieval.

Chronic exposure to Pb$^{2+}$ leads to altered NMDA-R subunit composition, which has an effect on downward signaling in rats (Toscano et al., 2002). As a result, rats have reduced CREB phosphorylation, affecting the changes in gene expression, like BDNF, necessary for LTP. The CREB pathway can be shut-off by activation of NR1/2B at the extrasynapse by glutamate (Hardingham et al., 2002). Inhibition of the NMDA-R will disturb the formation of LTP. Rats exposed to 750 ppm (blood lead concentration 23,72-59,87 µg/dL) Pb$^{2+}$-acetate through diet showed lower NMDA-R-2A (NR2A) protein expression on PND 14, 21 and 28 (Nihei et al., 1999). An increase in NR2A levels is part of synapse maturation and is called the developmental switch (Liu et al., 2004). Inhibition of the increase in NR2A caused by Pb$^{2+}$

suggests a delay in the developmental switch and thus synapse maturation. Primary cell cultures of rat hippocampal neurons to 5 μM $Pb^{2+}$ significantly decreased protein expression of NR1 and NR2B (Lau et al., 2002). $Pb^{2+}$ changes NMDA channel physiology decreasing its activity and can activate the CREB shut-off pathway. This impairs formation of long-term potentiation and synaptic maturation.

Rats were chronically exposed (starting at GD1) to 1500 ppm of PbAC2 (mean blood lead concentration 31,9±μg/dL) (Nihei et al., 2001). At PND50 the rats showed lower cytosolic (32%) and membrane (25%) PKCγ protein expression. The same effect was seen in fetal rat neurons, total PKCα, PKCβ, PKCγ and PKCε levels were significantly lowered in a dose-dependent manner (Xu et al., 2006). It has been shown that inhibition of PKC disturbs the induction of LTP (Lovinger et al., 1987). This will lead to reduced LTP induction and disturbed learning and memory.

*In vitro* testing showed several effects of $Pb^{2+}$ on learning and memory. It has been shown that $Pb^{2+}$ interacts with NMDA-R as a non-competitive antagonist (Uteshev et al., 1993; Ujihugaram et al., 1992). $Pb^{2+}$ exposure resulted in a fast reversible and a slow irreversible effect. Administration of aspartate/glycine resulted in an inward current, which decreased when $Pb^{2+}$ was simultaneously (asp/gly/$Pb^{2+}$) applied. This effect was reversed within 2 to 5 second of washing (fast reversible effect). Prolonged exposure to asp/gly/$Pb^{2+}$ resulted in a permanent decreased current and amplitude in response to asp/gly when compared to control asp/gly current. This suggest that $Pb^{2+}$ modulates the NMDA channel resulting in a decreased current, disturbing the induction of LTP and learning and memory.

$Pb^{2+}$ also interferes with calcium homeostasis by substituting $Ca^{2+}$, activating calmodulin and PKC (Habermann et al., 1983; Markovac et al., 1988). Picomolar concentrations of $Pb^{2+}$ can activate PKC while micromolar concentrations of $Ca^{2+}$ are needed (Markovac et al., 1988). Nanomolar concentrations of $Pb^{2+}$ inhibit the cation-dependent activity and micromolar concentrations inhibit the kinase activity of PKC (Sun et al., 1999). Activation of calmodulin and PKC will eventually lead to CREB phosphorylation and activation, resulting in altered gene expression (late phase LTP). $Pb^{2+}$ can also increase $Ca^{2+}$ levels, causing influx of $Ca^{2+}$ into mitochondria (Sidhu et al., 2003). This results in opening of membrane transition pores and the production of free radicals, causing oxidative stress. $Pb^{2+}$ increases oxidative stress by

binding to sulfhydryl (SH) groups, decreasing the activity of enzymes with SH groups (Gurer et al., 2000). The antioxidant glutathione contains a SH group and its activity is decreased by $Pb^{2+}$, increasing reactive oxygen species. Neurons are sensitive to oxidative stress, it can lead to neuronal cell death. Co-exposure of $Pb^{2+}$ and glutamate leads to an increased oxidants production and lead-induced cell death (Loikkanen, et al., 2003). Maternal exposure to $Pb^{2+}$ decreases glutamate concentrations in the cerebral cortex, hippocampus and cerebellum (Leret et al., 2002). Glutamate plays an important role in the regulation cognitive processes, learning and memory, so a decrease in glutamate will disturb these processes.

Another protein affected by $Pb^{2+}$ is CamK-IIβ, low long-term exposure decreased cytosolic CaMK-IIβ expression (Toscano et al., 2005). As a result NMDA-R and AMPA-R will not be phosphorylated and the intracellular stored AMPA-R will not be moved to the membrane, which impair the enhancement of postsynaptic currents.

Below a schematic scheme (figure 7) of the adverse molecular effects of $Pb^{2+}$ on synapse formation and plasticity.
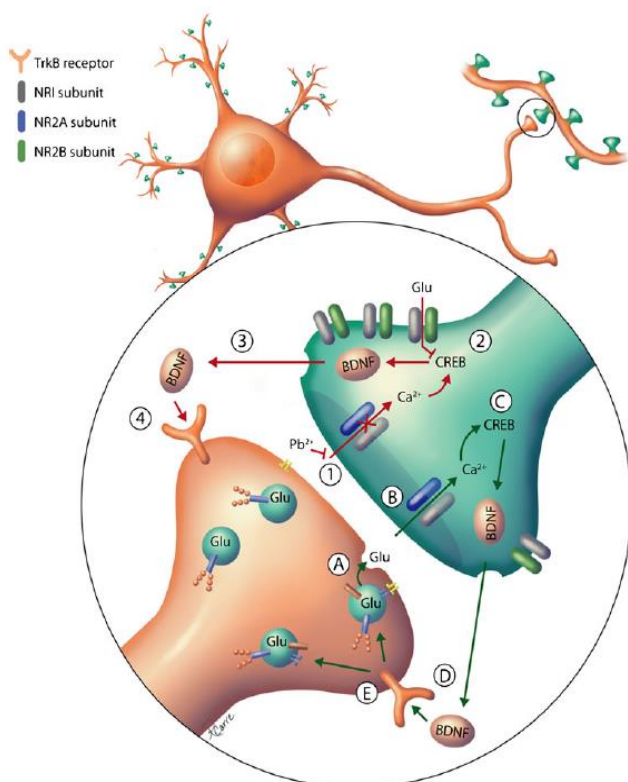


**Figure 7. Overview of the effects of Pb2+ exposure to synapse formation and plasticity in developing neurons.** Presynaptic signaling leads to release of glutamate vesicles (A) and activates NR1/2A (B). Activation of NMDA-R leads to an influx of $Ca^{2+}$, activating CAMK-II and PKC which phosphorylate CREB (C). CREB activates gene expression of BDNF, which is secreted and binds to TrkB receptor on the presynaptic neuron (D). Activation of TrkB enhances glutamate-containing vesicles and release (E). Exposure to $Pb^{2+}$ inhibits activation of NMDA-R and the developmental switch, leading to less abundant NR2A expression (1). Activation of NR2B at the extrasynapse leads to a CREB shut-off pathway (2). Reduced CREB signaling results in lower BNDF levels (3), resulting in reduced activation of TrkB receptors (4) and interrupts the enhanced stimulation (Neal et al., 2010).

Lead was found to affect IQ, learning and memory retrieval. Protein levels of NMDA-R subtypes were altered after $Pb^{2+}$ exposure (table B1). No literature was found on effects on AMPA-R, CaM nor ERK1/2, instead changes in CaMK-II and PKC were found. CAMK-IIβ is activated by CaM (one step later in signaling pathway) and PKC activates ERK1/2 (one step earlier in signaling pathway). Protein levels of CaMK-IIβ and all PKC isoforms (α, β, γ and ε) were decreased.

**Table B1. Overview of key-proteins in LTP induction affected by $Pb^{2+}$.**

| Protein | Effect |
|---------|--------|
| AMPA-R | Not found |
| NMDA-R | NR1↓, NR2A↓, NR2B↓ |
| CaM | Not found, instead CaMK-IIβ↓ |
| ERK1/2 | Not found, instead PKCα↓, PKCβ↓, PKCγ↓ and PKCε↓ |

## *Methylmercury (CH₃Hg, MeHg)*

Methylmercury (MeHg) can be found in organisms and bio-accumulates in the food chain, exposure mostly occurs via the intake of contaminated fish, shellfish and sea mammals (Edoff & Ceccatelli, 2012). Prenatal exposure to mercury results in severe neurological symptoms of infants (Engelson & Herner, 1952). Children of women who ate grains treated with Panogen (a MeHg-containing fungicide) had neurodevelopmental defects and impairments, including seizures, blindness, deafness, ataxia, spasticity, loss of coordination and mental retardation, while the mothers had no symptoms of intoxication (Grandjean et al., 2001). A follow-up study showed learning disabilities and developmental delay in these children (Grandjean & Herz, 2011).

MeHg is transported over the blood-brain barrier (BBB) mainly via the L-type large neutral amino acid transporter (LAT1) (Farina, Rocha, & Aschner, 2011). MeHg is bound to cysteine forming a MeHg-Cys complex, mimicking the substrate, methionine, of LAT1.

*In vivo* testing showed adverse effects of MeHg on behavior, learning and memory. The effect of MeHg exposure on behavior, basic activities and learning has been studied in mice. Pregnant mice were exposed to 0,5 mg MeHg/kg bw/day via drinking water from gestational

day 7 until 7 days after delivery (Onishchenko et al., 2007). Behavior was monitored at 5-15 week (young) and 26-36 week (adult) old mice. Behavioral changes and depressive-like behavior were only found in male mice. MeHg exposed mice had normal motor function, but the exploratory activity was lower than control.

Exposure of MeHg resulted in inhibition of NMDA-R in both neonatal and adult rat brain, disrupting the formation of LTP (Rajanna et al., 1997). Different effects are observed with the duration and time of exposure. Rats exposed to MeHg for seven days starting on PND7 or 14 showed reduced spatial learning and memory (Liu et al., 2009). NR2A and NR2B mRNA levels were measured in the same rats three days after the last administration. Differences were only seen in PND14 rats; hippocampal NR2A and NR2B mRNA levels were reduced when compared to control. Prenatal exposure of rats to a single oral dose of 8 mg/kg methylmercury chloride (MMC) (GD8) resulted in an increase of NR2B mRNA expression and cognitive impairment at PND60 (Baraldi et al., 2002). Exposed rats had a significantly increased mRNA expression of NR2B when compared to control rats, but no difference was seen in NR2A expression. Exposure during the postnatal brain growth spurt (PND15-17) to 1 or 3 mg/kg/day MMC leads to learning deficits at PND45 (Gao et al., 2008). Protein levels of NR1, NR2A and NR2B were measured at PND46 and were all significantly increased. Alterations in NR2A and/or NR2B levels can disrupt the developmental switch, delaying or even inhibiting synaptic maturation. Disruption of the ratio NR2A/NR2B can lead to activation of the CREB shut-off pathway. Both effects will have a negative effect on the induction and formation of LTP.

Prenatal exposure to 2 mg/kg/day MeHg (GD6-15) changes PKC isoforms levels (Haykal-Coates et al., 1998). PKC isoform $\alpha$, $\beta$, $\varepsilon$, and $\gamma$ were measured on PND1, 4, 10, 21 and 85. At PND1-21 MeHg causes a decrease of total PKC$\alpha$ and PKC$\varepsilon$, whereas at PND85 all PKC isoforms returned to the same level as control rats. This indicates that MeHg alters PKC isoform ontogeny during development which can disturbing synaptic development.

Another neurotoxic effect of MeHg is by increasing oxidative stress. Daily MMC-exposed mice showed increased reactive oxygen species in the brain after three days (Yee et al., 1994). MeHg affects (SOD), an enzyme that catalyzes the dismutation of superoxides into oxygen and hydrogen peroxide. Exposure to MMC resulted in decreased activity of SOD in the

homogenate, mitochondria and cytosol. MeHg also disrupts the glutathione antioxidant system (Stringari et al., 2008). These effects of MeHg on antioxidants lead to increased reactive oxygen species in neuronal cells, possibly leading to neuronal cell death.

*In vitro*, MeHg reduced the formation of LTP. MeHg affected excitatory postsynaptic potentials (ESPSs) in CA1 rat neurons (Yuan et al., 1993). After induction of LTP by HFS, MeHg caused a biphasic increase followed by a decrease or complete block in ESPS amplitude. MeHg also blocks population spike amplitude in the formation of LTP. Long-term exposure of hippocampal CA1 neurons to MeHg results in a block of ESPSs and LTP, disrupting membrane excitability, synaptic transmission and learning and memory.

Impaired learning and memory is also a secondary effect of astrocyte disruption by MeHg (Aschner et al., 2000). One of the function of astrocytes is the uptake and metabolism of neurotransmitters, including glutamate. MeHg causes swelling of astrocytes, disrupting the uptake of glutamate. This results in an increase of glutamate in the synapse, causing a continual stimulation of NMDA-R and influx of $Ca^{2+}$. This can lead to excitotoxicity and result in delayed cell death, caused by mitochondrial calcium overload. The influx of $Ca^{2+}$ disturbs ion homeostasis, swelling and outer membrane rupture of mitochondria leading to release of apoptogenic proteins, like cytochrome c (Pivovarova et al., 2004).

Prenatal exposure to MeHg leads to neurodevelopmental impairment, developmental delay and learning disabilities in children. MeHg affects learning and memory by altering NMDA-R subtypes NR1, NR2A and NR2B (table B2). No literature was found on effects on AMPA-R, CaM nor ERK1/2, instead changes in PKC isoforms were found. Both PKCα and PKCε protein expression was decreased.

**Table B2. Overview of key-proteins in LTP induction affected by MeHg.**

| Protein | Effect |
|---------|--------|
| AMPA-R | Not found |
| NMDA-R | NR2A↓, NR2B↓<br>NR1↑, NR2A↑, NR2B↑ |
| CaM | Not found |
| ERK1/2 | Not found, instead PKCα↓, PKCε↓ |

## *Polychlorinated biphenyls (PCBs)*

Polychlorinated biphenyls (PCBs) are man-made organic chemicals which are highly fat-soluble. There are 209 individual congeners and they are used in industrial and commercial applications like electrical, heat transfer and hydraulic equipment, as plasticizers in paint, plastics and rubber products, in pigments and dyes (EPA, 2013). PCBs were banned in the US in 1979 and Western-European countries between 1977-1980 due to their toxicity, persistency and accumulation in the environment. They can have adverse health effects on the immune system, reproductive system, nervous system, endocrine system and cause cancer.

PCBs cross the blood-brain barrier by disrupting tight junction proteins in the cerebrum, cerebellum and hippocampus (Selvakumar et al., 2012). Exposure to PCBs can occur by breathing in contaminated air, eating contaminated food stuffs and skin contact. Prenatal exposure to PCBs was measured in 236 children and their cognitive function was tested at the age of 4 (Jacobson et al., 1990). Postnatal exposure via breast milk was also measured. The mean cord serum PCB was 2,5 ± 2 ng/ml, maternal serum was 5,9 ± 3,6 ng/ml and maternal milk 835,9 ± 338,4 ng/ml. The PCB blood concentration of children who were breast fed ≥6 months was 5,1 ± 3,9 ng/ml, <6 months 1,2 ± 1,6 ng/ml and for non-breast fed children 0,3 ± 0,7 ng/ml. Postnatal exposure through breast milk is higher than prenatal exposure, but exposure from nursing was found to be unrelated to cognitive performance. Prenatal PCB exposure had an inverse dose-dependent relationship with short-term memory function on both verbal and quantitative tests. At the age of 11 the children were again assessed by measuring blood concentrations and IQ, spelling, arithmetic and reading tests (Jacobson et al., 1996). High exposure to PCBs resulted in a 6,2 points lower IQ when compared to lower exposed children. Prenatally exposed children also had poorer word comprehension, overall reading comprehension, antonyms, synonyms and analogies word comprehension. These studies show that prenatal exposure to PCBs results in decreased cognitive function in 4 year old children and at the age of 11 these children still have impairments in learning and memory.

Prenatal exposure results in decreased IQ, learning and memory, but exposure during adulthood also affects learning and memory. Consumption of fish (>24 lb/year = 11 kg/year) results in an increased blood concentration of PCB when compared to non-fish (<6 lb/year = 2,7 kg/year) eaters (Schantz et al., 2001). Fish consumption increases the PCB blood

concentrations with time, suggesting accumulation in the body (table 3). Female blood concentrations are lower than males, this might be because females have more adipose tissue, so the free concentration is lower, and PCBs are excreted via breast milk.

**Table 3. PCB blood concentration in fish and non-fish eaters.** Blood concentration of PCB in fish and non-fish eating male and female per age category (Schantz et al., 2001).

| | | Fish eater | Non-fish eater |
|---|---|---|---|
| **Sex** | **Age (years)** | **Mean (ng/g) ± SD (n)** | **Mean (ng/g) ± SD** |
| **Male** | <60 | 15, 88 ± 14,8 (16) | 6,11 ± 4,2 (11) |
| | 60-69 | 23,03 ± 19,1 (16) | 6,82 ± 3,5 (12) |
| | ≥70 | 24,69 ± 19,3 (14) | 5,66 ± 2,0 (7) |
| **Female** | <60 | 8,67 ± 4,6 (21) | 4,64 ± 2,2 (19) |
| | 60-69 | 14,04 ± 6,8 (17) | 8,71 ± 8,5 (16) |
| | ≥70 | 13,52 ± 10,8 (17) | 5,05 ± 3,8 (13) |

Memory and learning was measured in the same subjects (Schantz et al., 2001). The tests used were Wechsler Memory Scale (WMS (immediate and delayed recall)) and the California Verbal Learning (CVL) Test (immediate and short delay recall). PCB exposure results in a significantly lower WMS verbal delayed recall and CVL test semantic cluster ratio and list A, trial 1. Semantic clustering requires active organization of the words by their meaning. This is a more efficient way to encode to long-term memory. List A and B are fictional shopping lists with each 16 common household items from four general categories. List A is read aloud five times by the examiner and the subject has to recall the items. The results of this study show that adult exposure to PCBs negatively affects learning and memory.

*In vivo* studies showed several effects of Aroclor 1254 (commercial PCB mixture of congeners found in human tissue) on the hippocampus. Adult male albino rats injected with Aroclor 1254 showed a significant increase in $H_2O_2$, hydroxyl radicals and a significant decrease in glutathione levels in the hippocampus (Venkatamaran et al., 2008). Both effects increase the level of oxidative stress in the hippocampus. Adult rats exposed to Aroclor 1254 showed a significant decrease in mRNA expression of SOD and glutathione peroxidase (GPx) (Venkataraman et al., 2010). Both enzymes are involved in protecting the cell from free radicals and thus oxidative damage.

Prenatal exposure of rats to Aroclor 1254 decreased hippocampal PKC ($\alpha$, $\gamma$ and $\epsilon$) protein expression (Yang, J. et al., 2003). Measurements were done on PND4, 7, 14, 21 and 60. During development (PND4-21) all PKC isoforms in the cytosol were lower than control animals. Particulate PKC$\alpha$ and PKC$\gamma$ were increased during development, while PKC$\epsilon$ showed no difference. Both cytosolic and particulate PKC$\alpha$, PKC$\gamma$ and PKC$\epsilon$ levels restored to normal at adult age (PND60). Aroclor 1254 alters PKC isoform ontogeny during development, which can disturb synaptic development and maturation.

*In vitro* testing showed neurotoxic effects of PCBs. Young male rat brain slices exposed to Aroclor 1016 or 1254 resulted in a decreased LTP (Niemi et al., 1998). Aroclor 1016 inhibits LTP in a dose-dependent (0,1-1 $\mu$M) trend, exposure to 10 $\mu$M Aroclor 1254 resulted in a decrease in LTP while higher (100 $\mu$M) concentrations caused a general depression of synaptic transmission. Aroclor 1254 administered to rat neocortical cells disturbed the intracellular $Ca^{2+}$ homeostasis (Inglefield et al., 2001). PCBs cause an increase in inositol 1, 4, 5-triphosphate ($IP_3$) resulting in $Ca^{2+}$ release from the ER. It has been shown that prenatal exposure to Aroclor 1254 inhibits the uptake of $Ca^{2+}$ by mitochondria and microsomes in the hippocampus (Sharma et al., 2000). The released $Ca^{2+}$ cannot be buffered by mitochondria, resulting in elevated cytosolic calcium levels. Aroclor 1254 inhibits the uptake of neurotransmitters by synaptosomes (isolated synaptic terminals) (Mariussen et al., 2001). Aroclor 1254 inhibits the uptake of dopamine, glutamate, GABA and serotonin. Inhibition of the uptake of glutamate can lead to excitotoxicity, which can result in cell death.

Prenatal exposure and exposure during adulthood to PCBs leads to impaired cognitive function, learning and memory. The only key-protein affected by PCBs are PKC isoform $\alpha$ and $\gamma$ (Table B3).

**Table B3. Overview of key-proteins in LTP induction affected by PCBs.**

| Protein | Effect |
|---------|--------|
| AMPA-R | Not found |
| NMDA-R | Not found |
| CaM | Not found |
| ERK1/2 | Not found, instead PKC$\alpha$↑, PKC$\gamma$↑ |

# Discussion and conclusion

Learning and memory is an important cognitive process, especially in children. Short term memory is transitioned into long-term memory via long-term potentiation. There are several neurotoxic compounds known that disturb learning and memory in animals and humans, e.g. polybrominated diphenyl ethers, lead, methylmercury and polychlorinated biphenyls. The purpose of this thesis was to investigate if there is a common cellular pathway affected or alterations in protein expression by known neurotoxic compounds ($Pb^{2+}$, MeHg, PCBs) on learning and memory.

Two studies showed a difference in outcome between female and male rats. In both cases, male rats had worse result than females. This suggests a gender-related difference which may be due to different factors. This should be taken into account.

A common cellular pathway by which the known neurotoxic compounds lead ($Pb^{2+}$), methylmercury (MeHg) and polychlorinated biphenyls (PCBs) cause neurotoxicity was not found. Although they do have common cellular effects: disturbing the neurotransmitter system, calcium homeostasis, and increasing oxidative stress (leading to neuronal cell death).

Neurotransmitter system
Glutamate is a neurotransmitter that is important in the induction of LTP. Prenatal exposure to lead reduces the glutamate concentration in the hippocampus, cerebral cortex and cerebellum and can lead to disruption of cognitive processes, learning and memory. Methylmercury and polychlorinated biphenyls increase the glutamate concentration in the brain by inhibiting the uptake. MeHg inhibits the uptake of glutamate by astrocytes and PCB inhibits the uptake of glutamate by synaptosomes. This will increase the glutamate concentration in the synapse which leads to continual stimulation of NMDA-R. Overstimulation can lead to excitotoxicity and cell death.

Calcium homeostasis
All three neurotic compounds increase intracellular calcium levels in neurons. Elevated calcium levels are normally buffered by mitochondrial uptake, but too much can lead to a mitochondrial calcium overload. The mitochondrial membrane can rupture and release

reactive oxygen species and cytochrome c. Cytochrome c can induce apoptosis by activating caspases.

Lead disturbs calcium homeostasis by elevating $Ca^{2+}$ or substituting $Ca^{2+}$. Lead can directly and indirectly activate calmodulin and PKC. Directly by mimicking calcium and binding to calmodulin or PKC or indirectly by increasing calcium levels. The increased glutamate concentration in the synapse by MeHg causes continual stimulation of NMDA-R and an influx of $Ca^{2+}$, leading to elevated intracellular calcium levels. PCBs increase $IP_3$ which leads to $Ca^{2+}$ release from the ER.

Oxidative stress

As described above, excessive calcium in the cytosol are taken up by the mitochondria which can lead to mitochondrial calcium overload resulting in rupture of the membrane and release of reactive oxygen species. Another way of increasing oxidative stress is by inhibiting the activity of antioxidants. This is not directly related to LTP, but increasing neuronal cell death will affect learning and memory.

Lead binds to the SH-group of glutathione, which decreases its activity. Methylmercury inhibits the activity of SOD and disturbs the glutathione antioxidant system. Aroclor 1254 increases oxidative stress by increasing $H_2O_2$ concentrations and inhibiting glutathione activity. PCBs decrease SOD and GPx expression. Both proteins have antioxidant activities. Neurons are vulnerable to oxidative stress, which can induce apoptosis, so antioxidants are very important.

Protein expression

Protein expressions were altered by lead, methylmercury and polychlorinated biphenyls. A common protein is PKC. PKC is involved in the phosphorylation of ERK1/2, an enzyme which phosphorylates and activates CREB. PKC is activated by translocation from the cytosol to the membrane. Rats chronically exposed to $Pb^{2+}$ showed lower levels of PKCγ in both the cytosol and membrane fractions at adult age. Primary rat cell culture exposed for 14 hour to $Pb^{2+}$ led to decreased concentration of PKC isoforms α, β, γ and ε. Prenatal exposure of rats to MeHg resulted in lower concentrations of PKC isoforms α and ε during development (PND1, 21), but all PKC isoforms returned to normal levels at adult age (PND85). Prenatal exposure

of rat to PCBs resulted in a decrease in cytosolic PKCγ and PKCε during development, while particulate PKCα and PKCγ were increased during development. At adult age, all isoforms levelled back to normal (PND60). Altered PKC isoform ontogeny during development can disturb synaptic maturation, induction of LTP and thus learning and memory.

Both $Pb^{2+}$ and MeHg alter NMDA-R subtypes protein expression. $Pb^{2+}$ exposure decreases NMDA-R-1 (NR1), NR2A and NR2B protein levels. MeHg exposure shows different results, depending on the age. In younger rats (PND24), MeHg exposure leads to decreased NR2A and NR2B expression. At PND45 an increase is seen in NR1, NR2A and NR2B and at PND60 only an increase is seen in NR2B. Alterations in NMDA-R subtypes protein levels might disturb intracellular signaling. An increase in NR2B will activate the CREB shut-off pathway, inhibiting LTP induction.

$Pb^{2+}$ also inhibits CAMKIIβ expression, leading to decreased phosphorylation of NMDA-R and AMPA-R and translocation of intracellular stored AMPA-R to the membrane. This results in inhibition of increased synaptic stimulation and thus LTP induction.

Of the key-proteins suggested at the beginning, none were altered in all three compounds. NR1, NR2A and NR2B expression were found to be different in two out of three (Table C). An increase in NR2A levels is part of synaptic maturation called the developmental switch. Inhibition of the NR2A increase inhibits the synaptic maturation. An increase in NR2B can cause activation of the CREB shut-off pathway, inhibiting the formation of LTP.

A different protein was found to be affected by the neurotoxins; PKC. All three compounds altered protein expression of PKC isoform α. Besides α, $Pb^{2+}$ altered β, γ and ε, MeHg altered ε and PCBs altered γ. PKC is a protein that activates ERK1/2 in the LTP induction pathway. Alterations in PKC levels will disturb LTP formation and thus learning and memory.

**Table C. Overview of earlier suggested key-proteins involved in LTP induction.**

| Protein | Lead | Methylmercury | Polychlorinated biphenyls |
|---|---|---|---|
| AMPA-R | No effect found | No effect found | No effect found |
| NMDA-R | NR1↓, NR2A↓, NR2B↓ | NR2A↓, NR2B↓ NR1↑, NR2A↑, NR2B↑ | No effect found |
| CaM | No effect found, instead CaMK-IIβ↓ | No effect found | No effect found |
| ERK1/2 | No effect found, instead PKCα↓, PKCβ↓, PKCγ↓ and PKCε↓ | No effect found, instead PKCα↓, PKCε↓ | No effect found, instead PKCα↑, PKCγ↑ |

Neurotoxic compounds do affect expression levels of proteins of which LTP is dependent on, like NMDA-R subtypes and PKC isoforms. It is possible to use protein levels as a biomarker for neurotoxicity. This study only found one common protein, PKC, but basing neurotoxicity on only one protein is not reliable enough. NMDA-R subtypes were found to be altered in two out of three compounds. A wider arrange of proteins have to be selected for analysis (besides PKC and NMDA-R), mostly those involved in LTP induction and formation. Which proteins exactly needs to be researched further.

# List of abbreviations

AMPA-R: A-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid receptor

BBB: blood-brain barrier

BDE: brominated diphenyl ether

BDNF: brain-derived neurotrophic factor

CaM: calmodulin

CaMK: calcium/calmodulin-dependent protein kinase

CREB: cAMP response element-binding protein

CVL: California Verbal Learning test

DDT: dichlorodiphenyltrichloroethane

ERK1/2: extracellular signal-regulated protein kinase 1 and 2

ESPS: excitatory postsynaptic potentials

GD: gestational day

GPx: glutathione peroxidase

HFS: high-frequency stimulation

IP3: inositol 1, 4, 5-triphosphate

LAT1: L-type large neutral amino acid transporter

LTP: long-term potentiation

LTD: long-term depression

MeHg: methylmercury

MMC: methylmercury chloride

NMDA-R: N-Methyl-D-aspartic acid receptor

NRx: NMDA receptor subtype x

$Pb^{2+}$: lead

PbAC2: lead acetate

PBDE: polybrominated diphenyl ether

PCB: polychlorinated biphenyl

PKA: protein kinase A

PKC: protein kinase C

PND: postnatal day

SH group: sulfhydryl group

SOD: superoxide dismutase

TrkB: tropomyosin-related kinase receptor B

WMS: Wechsler Memory Scale

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