UPPSALA UNIVERSITET

Projektrapport från utbildningen i

EKOTOXIKOLOGI

Ekotoxikologiska avdelningen

Nr 120

2,2',4,4',5-pentabromodiphenyl ether (PBDE 99), a polybrominated flame retardant, can interact with DDT in Enhancing Developmental Neurotoxic Defects

Hanna Markström

PREFACE

This work has been done as a degree project, as a part of the graduate studies in Ecotoxicology at the Department of Environmental Toxicology, Evolutionart Biology Center, Uppsala University, Sweden.

First of all I would like to thank my supervisor Professor Per Eriksson for his encouragement, optimism and guidance, and Professor Anders Fredriksson for assisting with the sponeaneous behavior tests. I also would like to thank Dr Henrik Viberg, PhD student Celia Fisher and especially PhD student Niclas Johansson for practical assistance and advice. I would like to thank my boyfriend Per Holmström for valuable comments on my report and finally my friends and colleagues Jesper Säfholm and Tommy “Tierp” Strömberg for assistance, compassion and good company, despite circumstances!

ABSTRACT

In the environment today we are surrounded by numerous toxic substances, most of who we never even notice. Despite their ability to accumulate in the environment, whether they are persistent or not, they can cause irreversible changes in the brain if they are present during a critical phase during development. Dichlorodiphenyltrichlorethane (DDT) is an insecticide formerly used worldwide for agriculture purposes and as protection against vector borne diseases like malaria and typhus. DDT and its metabolites are persistent in the environment and causes disturbances in spontaneous behavior in mice if they are exposed during a critical phase in brain development, referred to as the brain growth spurt (BGS). The same can be seen in studies involving polybrominated diphenyl ethers (PBDEs), substances used in materials to prevent them from catching on fire. The purposes of this study were to establish if co-exposure to DDT and PBDE 99, which is one of the most abundant congener of the PBDEs in the environment, enhances the developmental neurotoxic effects regarding spontaneous behavior, spatial learning abilities and densities of muscarinic receptors in cerebral cortex in the brain.

Ten-day-old male NMRI mice were divided into five treatment groups and were given either 0.1 mg DDT/kg b.wt, 0.5 mg DDT/kg b.wt., 0.8 mg PBDE 99/kg b.wt., a combination of 0.1 mg DDT and 0.8 mg PBDE 99/kg b.wt. or a combination of 0.5 mg DDT and 0.8 mg PBDE 99/kg b.wt. as single oral doses, dissolved in a fat emulsion vehicle and administered via a metal gastric tube. The control group was given 10 ml of the 20% fat emulsion vehicle/kg b.wt. the same manner.

Spontaneous behavior was observed at the age of eight weeks. Mice exposed to combinations of DDT and PBDE 99 differed significantly compared to vehicle treated animals. Those exposed to the combination of 0.5 mg DDT and 0.8 mg PBDE 99/kg b.wt. differed significantly from all other groups, their spontaneous behavior was the most deranged. Mice exposed to the high dose of DDT and PBDE 99 differed significantly from the vehicle treated animals. Mice treated with the combination of 0.5 mg DDT and 0.8 mg PBDE 99/kg b.wt. showed effects on the cholinergic system exhibited as significantly reduced densities in muscarinic binding sites in cerebral cortex.

In conclusion there is an interaction between DDT and PBDE 99 in causing neurotoxic effects during development of the brain but since the dose is higher in the combination group that showed these alterations it is not possible to say whether the effects are more than additive.

SAMMANFATTNING

I dagens samhälle blir vi konstant utsatta för miljögifter, oftast utan att vi ens lägger märke till det. Många av dessa giftiga substanser är persistenta men oavsett ämnens förmåga att ackumuleras i miljön kan de orsaka irreversibla skador i hjärnan om de är närvarande vid en kritisk period under utvecklingen, när den största tillväxten av hjärnan sker och när många vitala signalsystem grundläggs. Diklordifenyltriklormetylmetan (DDT) är en insekticid som tidigare använts över hela världen inom jordbruket och som skydd mot vektorburna sjukdomar som malaria och typhus. Polybromerade diphenyl etrar (PBDEer) är bromerade föreningar som används som tillsatser i material för att hindra dem från att börja brinna. Båda dessa ämnen påverkar utvecklingen av hjärnan hos möss vid exponering dag tio efter födseln vilket sedan visar sig i ett stört spontanbeteende när mössen blir vuxna. Syftet med den här studien var att se om en kombination av dessa två ämnen kan samverka och därmed förvärra utvecklingstoxiska effekter, vilket uttrycker sig genom stört spontanbeteende, försämrad inlärning och minne samt förändrad densitet av muscarina receptorer i cerebral cortex.

Tio dagar gamla hanliga NMRI möss delades in i fem behandlingsgrupper samt en grupp med kontroller. De matades sedan med antingen 0,1 mg DDT/kg kroppsvikt, 0,5 mg DDT/kg kroppsvikt, 0,8 mg PBDE 99/kg kroppsvikt, en kombination av 0,1 mg DDT och 0,8 mg PBDE 99/kg kroppsvikt eller en kombination av 0,5 mg DDT och 0,8 mg PBDE 99/kg kroppsvikt blandat i en fettemulsionslösning. Lösningen gavs som en engångsdos, oralt via en sond av metall. Kontrolldjuren gavs 10ml/kg kroppsvikt av fettemulsionslösningen på samma sätt.

Mössens spontanbeteende testades vid två månaders ålder, med avseende på variablerna horisontell rörelse, vertikal rörelse samt total aktivitet. Möss som exponerades för den högre dosen av DDT, PBDE 99 eller en kombination av DDT och PBDE 99 skiljde sig alla signifikant mot kontrollerna. De som exponerades för kombinationen av 0,5 mg DDT och 0,8 mg PBDE 99/kg kroppsvikt skiljde sig signifikant från alla andra grupper, deras beteende var mest stört och visade avsaknad av habituering till den nya miljön samt hyperaktivitet. Densiteten muscarina receptorer i cerebral cortex var signifikant lägre hos möss som exponerats för kombinationen av 0,5 mg DDT och 0,8 mg PBDE 99/kg kroppsvikt jämfört med alla andra grupper.

Den här studien visar att DDT och PBDE 99 kan samverka vid utvecklingen av hjärnan och orsaka bestående neurotoxiska effekter men det är svårt att säga om effekterna är annat än additiva eftersom kombinationsdosen är högre än doserna för de enskilda substanserna.

1. INTRODUCTION

1.1 Toxic substances in the environment

Humans have always had the urge to invent new things and make things more effective. Along with this come problems that have to be solved and the goal is to optimize everything. One example is the agricultural use of pesticides/insecticides and another is the incorporation of additives in building and construction materials that gives the materials its wanted properties. Along with the positive effects of the substances used for these purposes come negative effects on living organisms, in the case of pesticides on non-target organisms. Plenty of these environmental toxicants are present in the every-day environment and we are exposed to them continuously. The ways of exposure are numerous but the most important ones are exposure to fetuses via the placenta and neonates via breast milk and direct exposure. The fetuses and neonates are still developing and can therefore be more sensitive to toxic substances (Rogers and Kavlock, 2001).

Insecticides are used to control pests/insects, affecting both the central nervous system (CNS) and the peripheral nervous system (PNS). Scince the CNS of insects is complex and similar to that in mammalians some insecticides are known to affect mammals too. It is and has therefore been of great importance to study these chemicals and the way they affect both insects and mammals (Ecobichon, 2001).

There are different kinds of toxic substances, the persistent ones that can be stored in fatty tissue and accumulate to high concentrations that are toxic to organisms, and those who are short-lived but still cause severe effects if they are present during a vulnerable period of development. Low doses of some toxicants can give irreversible changes in the adult brain (Ahlbom *et al.,* 1994; Eriksson *et al.,* 1992; Eriksson *et al.,*1997; Eriksson *et al.,* 2000; Eriksson *et al.,* 2001a; Eriksson *et al.,* 2002; Fredriksson *et al.,* 1993). The reason is that they are present during the rapid growth and development of the brain, the brain growth spurt, BGS (Davison and Dobbing, 1969).

Threshold values are currently based on single chemicals and therefore the amounts of chemicals in the environment rarely exceed the threshold values and there is no estimated health hazard to humans. However these low amounts should not be ignored because of the many different chemicals in the environment today and the fact that many of them act in similar ways or together to enhance the effects. Until now, research has been focused on

exposure to single chemicals. It is important to determine and understand the mechanism behind different chemicals and to learn about the effects. However it is also important to learn about the interactions that occur in the environment. This is why emphasis of modern research is turning towards interaction-studies.

1.2 Dichlorodiphenyltrichlorethane (DDT)

DDT is an organochlorine insecticide formerly used worldwide for agriculture purposes and as protection against vector borne diseases like malaria and typhus. Today DDT is banned or restricted according to the United Nations Environment Programme (UNEP), the Stockholm Convention on Persistent Organic Pollutants (POPs). Despite these restrictions, developing countries in the tropics are still allowed to use DDT, until there are appropriate substitutes available, due to severe problems with malaria (*Stockholm Convention on Persistent Organic Pollutants (POPs)* homepage). The effectiveness of DDT is striking considering it has been estimated that DDT has saved more lives than penicillin, which was discovered about the same time. The insecticidal properties of DDT were discovered in 1939, although the compound was synthesized the first time long before that, in 1874 by Ziedler (Woolley, 1982).

The chemical formula of DDT is $C_{14}H_9Cl_5$ with a molecular mass of 354.5 (figure 1). The main component of technical DDT, the isomer *p-p* DDT, has a melting point between 108.5 to 109°C and the vapour pressure at 20°C is 2.53×10^{-5} Pa. The high fat solubility and low water solubility of DDT and its metabolites results in bioaccumulation of the compounds in fatty tissues of organisms, the log k_{ow}–value is 7.48. The ways of exposure to DDT is through food and via the surrounding environment. Terrestrial organisms are exposed primary through food and aquatic organisms through water. Low concentrations of DDT in water are sufficient to cause severe effects on aquatic organisms, hence they are more sensitive to DDT than terrestrial ones. Due to the insolubility in water high concentrations are rarely found in natural waters. Bioaccumulation through the food chain is common due to the stability of the compounds, and this resistance of breakdown results in adsorption to soils and sediments. Since the retention time of DDT metabolites is high the stored compounds can practice its toxic effects for a long time after the actual exposure. This also results in occurance of the compounds in a geographically remote point from the site of exposure (WHO, 1989; Ecobichon, 2001).

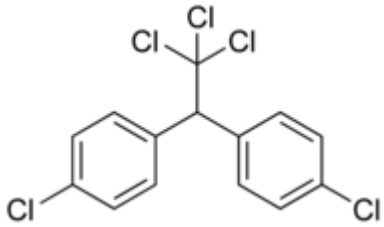

Figure 1. Structural formula of dichlorodiphenyltrichlorethane (DDT).

The result of acute poisoning from DDT in mammals, lower vertebrates, insects and arthropods is hyperactivity and hyperexcitability followed by tremors, convulsive behavior, ataxia and loss of equilibrium (Woolley, 1982; Hrdina *et al.,* 1975). These symptoms are due to the effect of DDT on the voltage-gated sodium channels in the nerve membrane. DDT binds to the receptors in the membranes and causes repetitive firing; ions pass through when they are not supposed to and cause nerve responses (Narahashi, 1992).

1.3 Polybrominated diphenyl ethers (PBDEs)

Brominated flame retardants (BFRs) are a group of halogenated organic flame retardants (FRs), highly brominated organic compounds (WHO, 1997). FRs are used to prevent fires and thereby, save lives. The FRs react with hydroxy radicals and similar species which are responsible for the propagation of fire (Hutzinger and Thoma, 1987). The BFRs include the polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs) (WHO, 1994a), hexabromocyclododecane (HBCDD) and tetrabromobisphenol A (TBBPA) (WHO, 1995). These are all additive flame retardants except for TBBPA that can be used as either an additive or reactive flame retardant. Reactive flame retardant molecules are incorporated into the polymers by chemical reactions whereas physical blending with the polymers incorporates the additive flame retardant molecules. The most striking difference between these two is that the additive ones are more prone to leach out of the polymers and therefore more likely to be found in the environment. The reactive ones can only leach out after extensive degradation of the polymer or by chemical reactions (Hutzinger *et al*., 1976).

The PBDEs are used in a variety of different products such as computers, TVs, household appliances, synthetic textile coatings, wire and cable insulation and much more. The chemical formula is $C_{12}H_{(9-0)}Br_{(10-1)}O$ and there are 209 possible congeners (figure 2) (WHO, 1994).

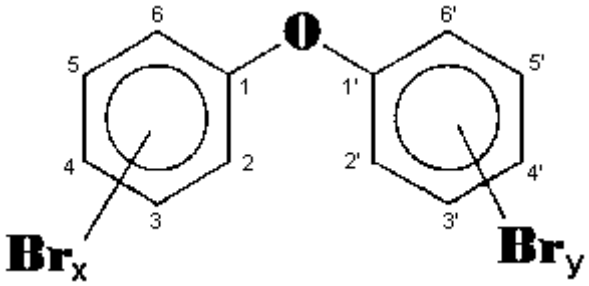

Figure 2. General stucture of Polybrominated diphenyl ethers (PBDEs).

The commercially used PBDEs are penta-, octa- and decabromodiphenyl ethers. They are all rather stable compounds with low vapour pressures at room temperature, from 3.85 to 13.3 Pa. The boiling points range between 310 and 425°C. They are lipophilic (log P_{ow} ranging between 4.28 and 9.9), increasing with higher bromination, and their solubility in water is therefore poor. These PBDEs are made by the bromination of diphenyl oxide under certain conditions and this process results in a mixture containing different PBDEs (WHO, 1994b).

The characteristics of PBDEs are similar to those of PCBs but the difference is that the PCBs are decreasing in the environment whereas the PBDEs are steadily increasing (WHO, 1993; Meironyté *et al.,* 1999; Hites, 2003). Since the first of July 2006 it is forbidden to use PBDEs in new electric and electronic products that are to be released on the market in the EU, with an exception for decaBDE (KemI, 2003). In Sweden decaBDE is also forbidden from January the first 2007 (SFS 1998:944, 1998). Studies on breast milk from American, European and Japanese women have shown that the most abundant congeners are PBDE 47, 99, and 153 with women in the U.S. showing a lot higher concentrations than the others (Akutsu *et al.,* 2003; Fängström *et al.,* 2004; Merionyté *et al.,* 1999; Schecter *et al.,* 2003).

1.4 Brain development

Brain development is a complex process that includes several important steps. Since different parts of the brain develop at different stages and independently of each other, there are many things that can go wrong. The development of the brain can roughly be divided into four different stages: the organogenesis and neural multiplication, the “brain growth spurt” (BGS), the mature and adult stage and last the senile regression (Davison and Dobbing, 1969). The most delicate process is the second, referred to as the BGS, when the brain size increases rapidly. It is also during this period that the growth of axons and dendrites occur, as well as increasing neuronal connections, glial multiplication and myelination. The timing of the BGS

differs between species. In humans it starts during the third trimester of pregnancy and continues throughout the first two years after the baby is born. Other species, like the guinea-pig, have their whole BGS *in utero* and in rodents the BGS is neonatal, occurring during the first 3-4 weeks of life, with the peak occurring at day 10 after birth. Mice and other rodents also acquire many of their motor and sensory faculties during this period (Bolles and Woods, 1964).

Small doses of both DDT and PBDE 99 have effects on neonatal brain development in mice and the effects are induced when administered on day 10, the peak of the BGS (Eriksson *et al.,* 1992; Eriksson *et al.,* 2002). Mice neonatally exposed to 8 mg PBDE 99/kg b.wt. on day 10 exhibit significant lack of habituation and significantly hyperactive conditions compared to control animals after four months (Eriksson *et al.,* 2002). PBDE 99 has effects on the cholinergic nicotinic receptors in the brain. Mice neonatally treated with 8 mg PBDE 99/ kg b.wt. on day 10 after birth exhibit increased susceptibility of the cholinergic system, observed as an altered reaction to nicotine as adults compared to control animals (Viberg *et al.*, 2002). A significant decrease in density of nicotinic receptors in hippocampi in brains of mice treated with 12 mg PBDE 99/ kg b.wt. on day 10 after birth has also been seen which supports the theory that the cholinergic system is affected (Viberg *et al.,* 2004).

Mice exposed to DDT 0.5 mg DDT/kg b.wt. exhibit a significant decrease in high affinity muscarinic binding sites and an increase in the low affinity binding sites in the cerebral cortex seven days after treatment in the neonatal mouse (Eriksson and Nordberg, 1986). In the adult mouse the total amount of muscarinic binding sites decrease significantly and there is no longer a difference in the number of high compared to low affinity binding sites. As a response to DDT exposure the spontaneous behavior in adult mice is affected. (Eriksson *et al.,* 1992).

1.5 The cholinergic system

The colinergic system is one of the major transmitter systems in the brain. The system is associated with behavioral properties like memory and learning as well as many physiological properties (Karczmar, 1975; Perry *et al.,* 1999). There are two types of cholinergic receptors, the muscarinic and nicotinic (Dale, 1914). They are both activated by acethylcholine but their origin differs, they belong to different gene families. The names of the receptors derive from the abilities of nicotine and muscarine to bind the receptors and paralyze their functions.

Acethylcholine is synthesized in the terminal endings of the nerve fibers from acethyl coenzyme A and choline, catalyzed by the enzyme choline acethyltransferase. In the brain and muscle cells acethylcholine is packed into vesicles by a specific transporter and released into the synaptic cleft in response to nerve impulses. Acethylcholine can then bind to pre- or postsynaptic receptors. The postsynaptic receptors cause ion channels to open in the muscle fiber membrane allowing ionic currents to pass through; resulting in a change in the postsynaptic membrane potential and the nerve signal is transmitted to the next nerve. The presynaptic receptors act as autoreceptors and can be involved in the release of acetylcholine. A hydrolytic enzyme called acethylcholinesterase break down the excess of acethylcholine in the postsynaptic cleft to choline and acetat. Choline is then transported back to the presynaptic terminal, over the presynaptic membrane via a sodium dependent and a sodium independent system, for re-use in the formation of acethylcholine. (Campbell and Reece 2002; Purves *et al.* 2004)

2. AIMS

Earlier research has shown that low doses of both DDT and PBDE 99 cause irreversible effects in the brain development of mammals. It is interesting to study the interaction of DDT and PBDEs due to the extremely high levels of PBDEs in the USA, and the common migration to the USA from Central America, where DDT still is, or recently has been in use. The aim of this study is to see whether there is an interaction between DDT and PBDE 99 when administered to NMRI mice on neonatal day 10 and what the consequences are on adult behavior and receptor densities in cortex.

3. MATERIALS AND METHODS

3.1 Animals, chemicals and treatment

Pregnant Naval Medical Research Institute (NMRI) mice were purchased from B&K, Sollentuna, Sweden. They were housed individually in plastic cages at a temperature of 22°C with a 12/12 hour light/dark cycle. The mice were provided with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. Once a day, around 4pm, the mice were checked for births and the day of birth was designated day 0. The litters were then adjusted so that they included 8-14 pups, of both sexes. At the age of ten days the mice were weighed and given either 0.1 mg DDT/kg b.wt., 0.5 mg DDT/kg b.wt., 0.8 mg PBDE 99/kg b.wt., a combination of 0.1 mg DDT and 0.8 mg PBDE 99/kg b.wt. or a combination of 0.5 mg DDT and 0.8 mg PBDE 99/kg b.wt. as single oral doses, administered via a metal gastric tube. Control animals were given 10 ml of the 20% fat emulsion vehicle/kg b.wt. the same manner. After 4-5 weeks they were once again weighed and separated. Males were placed in new cages with their male siblings, 4-7 mice in each cage, and the females were euthanized. The males were placed in a room for male mice only.

2,2´,4,4´,5-pentabromodiphenyl ether (PBDE 99) and dichlorodiphenyltrichlorethane (DDT) were dissolved, both separately and together, in a mixture of egg lecithin and peanut oil. They were then sonicated together with water to obtain 20% fat emulsion vehicles containing 0.01 mg DDT/ml, 0.05 mg DDT/ml, 0.08 mg PBDE 99/ml, 0.01 mg DDT and 0.08 mg PBDE 99/ml or 0.05 mg DDT and 0.08 mg PBDE 99/ml. The 20% fat emulsion vehicle was used in order to get a physiologically appropriate absorption and distribution.

3.2 Behavior tests

3.2.1 Spontaneous behavior

Spontaneous behavior in male mice was tested at the age of two months. The tests were performed between 8 a.m. and 12 p.m. with the same ambient light and temperature conditions as in their normal housing cages. The motor activity was measured for 3 × 20 minutes in an automated device consisting of cages measuring 40 × 25 × 15 cm. (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden). The cages were placed within two series of infrared beams, one low and one high level beam. The low level beams were sent out 2 cm from the bottom of the cage in a grid pattern with a distance of 73 mm lengthwise and 58 mm crosswise. The high level beams were placed 8 cm above the low beams, only lengthwise, 28

mm apart. The test cages were placed in 12 cm thick sound-proof wooden boxes, with seperate air ventilation to eliminate disturbances in the measurements. The cages were filled with the same type of bedding material used in their normal housing cages (Fredriksson, 1994).

The examined variables were:
Locomotion: Counting took place when the mouse moved horizontally through the low level grid of infrared beams (one count per beam interruption).
Rearing: Movement in the vertical plane was registered at a rate of four counts per second, when a single high level beam was interrupted, i.e., the number of counts obtained was proportional to time spent rearing.
Total activity: All types of vibration within the cage, i.e., those caused by mouse movements, shaking (tremors) and grooming were registered by a pick-up (mounted on a lever with a counterweight), connected to the test cage.

3.2.2 Morris swim maze

At the age of two and a half months the mice's learning and memory abilities were tested in a swim maze, the Morris swim maze (Morris, 1981). 12 animals from three different litters were tested from each treatment group; the control animals, animals exposed to 0.8 mg PBDE 99/kg b.wt., animals exposed to 0.5 mg DDT/kg b.wt. and animals exposed to both 0.8 mg PBDE 99/kg b.wt. and 0.5 mg DDT/kg b.wt. The test was performed using a circular grey tub, diameter 102, depth 15 centimeters from the brim, filled with water at 22°C. In one of the quadrants of the tub a metal platform, 12 cm in diameter was placed one centimeter below the water surface. The mouse's ability to locate the platform was tested five days in a row and each mouse was given five trials each day. Before the first trial each day the mice were placed on the platform for 20 seconds. They were then released in the opposite quadrant of the platform in the tub and were given 30 seconds to find the platform. If they were unable to find the platform within the time frame, they were placed on the platform again and were left there for another 20 seconds, just as those who managed to find the platform on time. The observer had the same position in the room every day. To measure the time the mice spent searching for the platform, the time they spent in the different quadrants and to record their way to the platform, Video Mot 2 was used, a computer program connected to a camera.

3.3 Receptor analysis

The mice were killed by decapitation at the age of three months. Their brains were dissected out on ice-cold glass plates. Cerebral cortex and hippocampus were placed directly in ice-cold sucrose, 0.32 M, about 24 times its own weight, and were then homogenized with a Potter-Elvenhjem homogenizer. The homogenates were then centrifuged for 10 minutes at 1,000g and the supernatants were further centrifuged for 30 minutes at 17,000g. The remaining pellets were then resuspended and homogenized in ice-cold $NaKPO_4$ buffer (0.05M, pH 7.4) to yield a crude synaptosomal P2 fraction (Gray and Whittaker 1962) with a protein content of about 1.0-1.5 mg determind with folin reagent (Lowry *et al*. 1951). The P2 fractions were kept frozen at -80°C until the protein measurements and receptor assays were performed. Measurements of muscarinic binding sites (the M_2 and M_4 subtypes) were performed using ^{3}H-AFDX, (specific activity 4.3TBq/mmol).

Specific binding was measured following the method of Nordberg and Winblad (1981) described by Eriksson and Nordberg (1986) for [^{3}H]QNB with some modifications. Aliquots of the P2 fraction (100 µl, protein content between 0.15 and 0.25 mg) were incubated with 10 µl [^{3}H]AFDX (2 nM in ethanol) for 60 minutes at 25°C in $NaKPO_4$ buffer (pH 7.4) in a total volume of 1000 µl. To measure non specific binding, parallell samples were incubated with atropine (20 µl, 50 µM). Each binding was determined in duplicate. After the incubation the samples were cenrifuged for 5 minutes at 20,000 g. The remaining pellet was washed with ice-cold $NaKPO_4$ buffer and then placed in a scintillation vial and left shaking over night to dissolve in 1 ml of Aquasafe 300+ scintillation liquid (Zinsser Analytic, Ltd., U.K.). The following day 4 ml of scintillation liquid was added to the vials and the radioactivity was determined using a liquid scintillation analyser (Packard Tri-Carb 1900 CA). The analysis was performed after the samples had been kept in the dark for at least eight hours. Specific binding was determined by calculating the differences in the amount of AFDX bound in the precence (or abcence) of atropine.

3.4 Statistical analysis

Spontaneous behavior: data were subjected to a split-plot ANOVA (analysis of variance) and pair wise testing between treatment groups and their corresponding control groups was performed with Turkey HSD (honestly significant difference) test (Kirk 1968).

Receptor analysis: data were subjected to a non-parametric one-way ANOVA; the Kruskall-Wallis test and pair wise testing between treatment groups was performed using Duncan's test.

4. RESULTS

During the time of the experiment no overt clinical signs of dysfunction were detected in the mice treated with DDT, PBDE 99 or combinations of DDT and PBDE 99.

4.1 Effects on spontaneous behavior in mice at the age of eight weeks

The results from the spontaneous behavior in mice treated with fat emulsion vehicle, DDT and PBDE 99, separately or in combination, at the neonatal day 10 are shown in Fig. 3. There were significant group × period interactions [$F_{10, 108}$=116.7; $F_{10, 108}$=321; $F_{10, 108}$=79.6] for locomotion, rearing and total activity variables respectively.

Control animals treated with the fat emulsion vehicle on neonatal day 10 showed high activity during the first 20 minute period in all three variables measured. During the second 20 minute period the activity decreased in all three variables and during the third period the activity was minimal.

Mice treated with 0.1 mg DDT/kg b.wt. as a single oral dose on neonatal day 10 registered a decrease in activity over time in all three variables measured, same behavior as the control animals. Mice treated with 0.5 mg DDT/kg b.wt. as a single oral dose on neonatal day 10 showed significant deviations in behavior compared to controls. During the first period the activity for these animals was significantly ($p \leq 0.01$) lower than that for control animals whereas during the third period the activity was significantly ($p \leq 0.01$) higher for the animals treated with 0.5 mg DDT/kg b.wt. than for the control animals.

Mice treated with 0.8 mg PBDE 99/kg b.wt. as a single oral dose on neonatal day 10 showed significant alterations in behavior compared to control animals in all three variables measured. The activity was significantly ($p \leq 0.01$) lower than for control animals during the first period but significantly ($p \leq 0.01$) higher than for controls during the third period.

Mice treated with a combination of 0.1 mg DDT/kg b.wt. and 0.8 mg PBDE 99/kg b.wt. as a single oral dose on neonatal day 10 showed significant defects on spontaneous behavior. Compared to control animals the activity was significantly ($p \leq 0.01$) lower during the first period and significantly ($p \leq 0.01$) higher during the last 20 minute period.

Mice treated with 0.5 mg DDT/kg b.wt. and 0.8 mg PBDE 99/kg b.wt. as a single oral dose on neonatal day 10 showed significant deviations in behavior compared to control animals and the animals from all other treatment groups, for all three variables measured. During the first 20 minute period the activity is lower (not significantly for all groups) than all other groups and during the last 20 minute period, for all three variables measured, the activity is significantly higher than for all other groups.

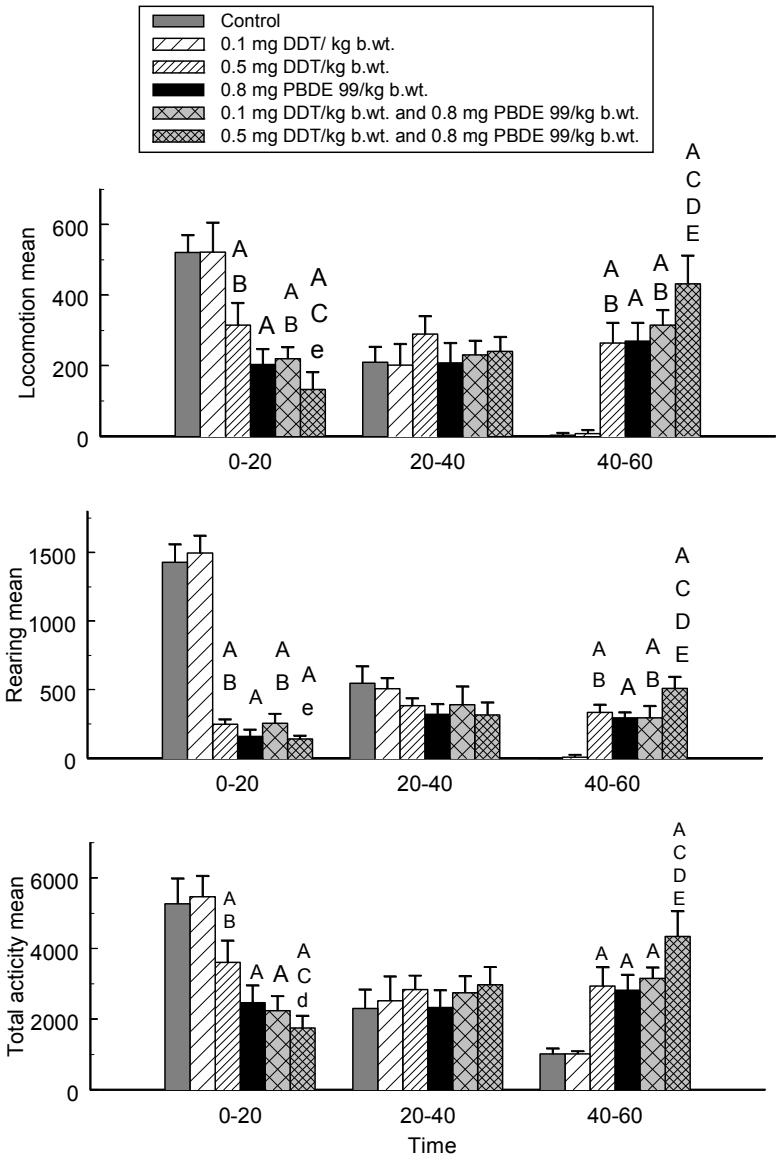

Figure 3. Results from spontaneous behavior tests in 8 weeks old mice regarding the three variables locomotion, rearing and total activity in mice treated with vehicle, 0.1 mg DDT/kg b.wt., 0.5 mg DDT/kg b.wt., 0.8 mg PBDE 99/kg b.wt., 0.1 mg DDT and 0.8 mg PBDE 99/kg b.wt. or 0.5 mg DDT and 0.8 mg PBDE 99/kg b.wt. as single oral doses, administered via a metal gastric tube on neonatal day 10. The heights of the bars represent the mean value ± standard deviation. Statistical analyses of the data were submitted to ANOVA using a split-plot design. Pair wise testing between the treated groups and the control group was performed using the Turkey HSD test. Statistical differences are indicated by capital letters for $p < 0.01$ and lower case letters for $p < 0.05$.
A means that there is a significant difference in behavior compared to control animals, B that there is a significant difference compared to mice treated with 0.1 mg DDT/kg b.wt., C compared to those treated with 0.5 mg DDT/kg b.wt., D compared to those treated with 0.8 mg PBDE 99/kg b.wt. and E compared to the mice treated with 0.1 mg DDT and 0.8 mg PBDE 99/kg b.wt..

4.2 Effects on performance in Morris swim maze in mice at the age of 10 weeks

The results from the Morris swim maze NMRI mice at the age of 10 weeks showed no significant differences between vehicle treated animals and animals treated with DDE, PBDE 99 or combinations of DDT and PBDE 99 in memorizing the platform and therefore minimizing the time spent searching for the platform. However control animals did not display normal behavior and therefore the results are not shown.

4.3 Effects on muscarinic receptors in cerebral cortex in mice at the age of 12 weeks

The densities of muscarinic binding sites in cerebral cortex in NMRI mice at the age of 12 weeks treated with fat emulsion vehicle, DDT and PBDE 99, separately or in combination, on neonatal day 10 are shown in table 1.

There were significant differences in densities of AFDX-binding sites in animals treated with combinations of DDT and PBDE 99 on neonatal day 10 compared to those treated with vehicle, DDT or PBDE 99. There were no significant differences in densities of AFDX-binding sites between the other treatment groups, animals treated with vehicle, DDT or PBDE 99.

Table 1. Densities of AFDX-binding sites (pmol / g protein) in cerebral cortex, in adult male NMRI mice exposed to single oral doses of DDT, PBDE 99, a combination of DDT and PBDE 99 or fat emulsion vehicle on neonatal day 10. Statistical differences are indicated by *.

Treatment group	*(n)*	Median density (pmol/g protein)	Range
Controls	12	96	72
DDT	12	92	68
PBDE 99	12	83	44
DDT + PBDE 99	10	67.5*	35

*Statistical analyses were submitted to a non parametric one-way ANOVA; the Kruskall Wallis-test and differences between treatment groups were determined using Duncan's test, $p<0.05$.

5. DISCUSSION

Previous research in environmental toxicology/ecotoxicology has been focused on the study of single chemicals. In the environment today we are exposed to several chemicals and in addition, combinations of different chemicals. Problem with studies that include more than one chemical is that the chemicals may act in similar ways or on completely different systems. It is difficult to know what causes the different effects and whether the effects are additive or synergistic.

In the spontaneous behavior tests it is of interest to know if treated animals differ from the normal behavior regarding the ability to habituate. Normal behavior is what the vehicle treated animals in this study exhibit. The activity is high during the first period when they are put into a new environment. During the second period they calm down and the activity decreases. During the third period the activity is minimal; they have habituated to the novel environment (Fredriksson, 1994). This normal behavior has earlier been observed in several studies (Ahlbom *et al.,* 1994; Eriksson *et al.,* 1992; Eriksson *et al.,* 2002; Eriksson *et al.,* 2006; Fredriksson *et al.,* 1993; Viberg *et al.,* 2004b). If the test is performed when the mice are normally active it is difficult to know if the activity is due to their treatment or normal behavior. The first and last periods are the most interesting since the behavior in these are well defined, high in the first and low in the third. The second period is in the middle and the difference between control animals and treated animals is often small.

Behavior is an important endpoint when studying the effects of environmental toxicants on the nervous system of mammals. Spontaneous behavior reflects a function dependent on the integration of a sensory input into a motoric output, and thus reveals the ability of animals to habituate to an environment and integrate new information with previously attained, and can thereby be a measure of cognitive function too. Cognitive function is also closely correlated to the function of the cholinergic system (Peterson and Nordberg, 2001).

The results from the spontaneous behavior tests show that the low dose of DDT is too low to cause an effect on the treated animals. The behavior of animals in this group is similar to vehicle treated animals. The dose 0.1 mg DDT/kg b.wt. is extremely low and it is not surprising that there was no effect. 0.5 mg DDT/kg b.wt. is the lowest dose known to cause effects on spontaneous behavior in mice which is also seen in this study (Eriksson *et al.,*

1992). Mice treated with 0.5 mg DDT/kg b.wt. showed significant deviations compared to mice treated with vehicle in all three variables measured for spontaneous behavior, during the first and last 20 minute periods which are the ones of interest.

The dose chosen for PBDE 99 has showed effects on spontaneous behavior in some studies and no effects in others (Eriksson *et al.,* 2001b; Eriksson *et al.,* 2006; Viberg *et al.,* 2004a; Viberg *et al.,* 2004b; Fisher *et al.,* 2007a). In this study the spontaneous behavior differed significantly from those treated with vehicle. It is interesting that this dose does not always have an effect since this means that it is close to the non effective dose. When investigating combinations of chemicals you search for effects caused by concentrations that show no effects when the chemicals are administered alone.

The combination of 0.1 mg DDT/kg b.wt. and 0.8 mg PBDE 99/kg b.wt. did not show any significant deviations in spontaneous behavior compared to those treated with only 0.8 mg PBDE 99/kg b.wt.. This result and the results from the single exposure to 0.1 mg DDT/kg b.wt. show that the low dose of DDT is too low to cause any effects. The combination of 0.5 mg DDT/kg b.wt. and 0.8 mg PBDE 99/kg b.wt. showed significant deviations in spontaneous behavior compared to all other groups. There is an interaction between DDT and PBDE 99. Whether the effect is synergistic or additive is hard to say, the dose is higher when combined and both doses give effects when administered alone. The results show that the effects are dose-response dependent, if you increase the dose the effects become more severe. It would be interesting to combine lower doses of both chemicals, although not as low as 0.1 mg DDT/kg b.wt., to see if there are any synergistic effects. Co-exposure to PBDE 99 and methyl mercury has previously been shown to cause effects on spontaneous behavior that appears to be more than additive (Fisher *et al.,* 2007a). Similar results have been seen in a co-exposure study with PCB 153 and methyl mercury (Fisher *et al.,* 2007b). The result from both of these studies showed effects when combining doses that alone gave no effects. Co-exposure to PBDE 99 and PCB 52 showed that effects on spontaneous behavior were more pronounced when the chemicals were combined even if the dose was fivefold higher, on a molar basis, when the chemicals were administered alone (Eriksson *et al.,* 2006). If this is the case for PBDE 99 in combination with DDT is not possible to determine based on this study since the highest dose was the combination between PBDE 99 and the high dose of DDT.

Neuropsychological defects in humans due to neurotoxicants have been observed in epidemiological studies from Faeroe Islands. Comparisons to studies on children from the Seychelles have shown that there is a combination of neurotoxicants that cause the neuropsychological defects in children from the Faeroe Islands. Both these populations have a high consumption of MeHg-contaminated fish. The difference is the consumption of whale meat and blubber in the Faeroe Island population, a diet that has been shown to contain PCBs (Myers and Davidson, 1998; Grandjean *et al.*, 2001; Davidson *et al.,* 2006). Children from Central America that migrate to the USA might experience a similar co-exposure of DDT and PBDEs. Therefore the results from this study are interesting; low doses of both DDT and PBDE 99 can interact and enhance the neurophysiological defects in mice and it is likely that the effects in humans are similar.

The receptor study show that there is a significant decrease in density of muscarinic receptors in cerebral cortex in mice treated with the combination of DDT and PBDE 99 compared to the other treatment groups. The densities in mice treated with vehicle were higher than those treated with one of the chemicals PBDE 99 or DDT but the differences were not significant. Earlier studies involving DDT has shown both an increasing amount in neonates and a decreasing amount in adults of muscarinic binding sites in cerebral cortex (Eriksson and Nordberg, 1986; Eriksson *et al.,* 1992). In those studies [^{3}H]QNB was used instead of [^{3}H]AFDX. [^{3}H]QNB is a non selective mAChR antagonist; the affinity is equal to all subtypes of the mAChRs. [^{3}H]AFDX has an affinity to the subtypes m_2 and m_4 (Falk *et al.,* 2005). Studies involving PBDEs has focused on the nicotinic receptors in hippocampus (Viberg *et al.,* 2004b; Viberg *et al.,* 2003; Fisher *et al.,* 2007). The reason for that are the findings that neonatal exposure to PBDE 99 alters the response to the cholinergic agent nicotine in adult mice (Viberg *et al.,* 2002). However, recent studies show that PBDE 99, at higher doses than used in the present study, can decrease the density of muscarinic receptors in the brain, measured using QNB (Viberg *et al.* 2005). Taken together, these studies indicate that PBDE 99 and DDT, alone or in combination, affect the cholinergic system that has implications to cognitive function.

In conclusion, this study shows that co-exposure to PBDE 99 and DDT on neonatal day 10 can enhance the neurotoxic effects manifested as deranged spontaneous behavior as adults and a decrease in cholinergic muscarinic receptors in cerebral cortex. Whether the effects are more than additive is hard to say based solely on this study.

6. REFERENCES

Ahlbom, J., Fredriksson, A., and Eriksson, P. (1994). Neonatal Exposure to a Type-I pyrethroid (bioallethrin) Induces Dose-response Changes in Brain Muscarinic Receptors and Behaviour in Neonatal and Adult Mice. *Brain research* **645,** 318-324.

Akutsu, K., Kitagawa, M., Nakazawa, H., Makino, T., Iwazaki, K., Oda, H., and Hori, S. (2003). Time-trend (1973-2000) of polybrominated diphenyl ethers in Japanese mother's milk. *Chemosphere* **53,** 645-654.

Bolles, R. G., and Woods, P. J. (1964). The ontogeny of behaviour in the albino rat. *Animal behaviour* **12,** 427-441.

Campbell, N. A., and Reece, J. B. (2002). *Biology 6th ed.,* pp 1037. Pearson Education, Inc., Benjamin Cummings.

Dale, H. H. (1914). The actions of certain esters and ethers of choline, and their relation to muscarine. *J. Pharmacol. Exp. Ther.* **6,** 147-190.

Davidson, P. W., Myers, G. J., Weiss, B., Shamlaye, C. F., and Cox, C. (2006). Prenatal methyl mercury exposure from fish consumption and child development: a review of evidence and perspectives from the Seychelles Child Development Study. *Neurotoxicology* **27,** 1106-1109.

Davison, A. N., and Dobbing, J. (1968). *Applied Neurochemistry.* Blackwell, Oxford, pp 179-311.

Ecobichon, D. J., (2001) Toxic effects of pesticides. Chapter 22 in Cassarette and Doull's Toxicology – The basic science of poisons. (C. D Klaassen, 6th ed.), pp 769-7848. McGraw-Hill.

Eriksson, P. (1997). Developmental Neurotoxicity of Environmental Agents in the Neonate. *NeuroToxicology* **18,** 719-726.

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 in Behaviour in Adult Mice. *Brain research* **582,** 277-281.

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 research* **853,** 41-48.

Eriksson, P., Ankarberg, E., Viberg, H., and Fredriksson, A. (2001a). The Developing Cholinergic System as Target for Environmental Toxicants, Nictine and Polychlorinated Biphenyls (PCBs): Implications for Neurotoxicological Processes in Mice. *Neurotoxicity Research* **3,** 37-51.

Eriksson, P., Falkeborn, , Y., Nordberg, A., and Slanina, P. (1984). Effects of DDT on muscarine- and nicotine-like binding sites in CNS of immature and adult mice. *Toxicology Letters* **22,** 329-334.

Eriksson, P., Fischer, C., and Fredriksson, A. (2006). Polybrominated Diphenyl Ethers, a Group of Brominated Flame Retardants, can Interact with Polychlorinated Biphenyls in Enhancing Developmental Neurotoxic Defects. *Toxicological sciences* **94(2),** 302-309.

Eriksson, P., Jacobsson, E., and Fredriksson, A. (2001b). Brominated Flame Retardants: a Novel Class of Developmental Neurotoxicants in our Environment? *Environmental Health Perspective* **109**, 903-908.

Eriksson, P., and Nordberg, A. (1986). The effects of DDT, DDOH-palmitic acid and a chlorinated paraffin on muscarinic receptors and the sodium-dependent choline uptake in the central nervous system of immature mice. *Toxicology and Applied Pharmacology* **85,** 121-127.

Eriksson, P., Viberg, H., Jacobsson, E., Örn, U., and Fredriksson, A. (2002). A Brominated Flame Retardant, 2,2',4,4',5-Pentabromodiphenyl Ether: Uptake, Retention, and Induction of Neurobehavioral Alterations in Mice During a Critical Phase of Neonatal Brain Development. *Toxicological sciences* **67,** 98-103.

Falk, L., Nordberg, A., Seiger, Å., Kjaelgaard, A., and Hellström-Lindahl, E. (2005). Smoking during early pregnancy affects the expression pattern of both nicotinic and muscarinic acetylcholine receptors in human first trimester brainstem and cerebellum. *Neuroscience* **132**, 389-397.

Fisher, C., Fredriksson, A., and Eriksson, P. (2007a). Co-exposure of neonatal mice to flame retardant PBDE 99 (2,2',4,4',5-pentabromobiphenyl ether) and methyl mercury enhances developmental neurotoxic defects.

Fisher, C., Fredriksson, A., and Eriksson, P. (2007b). Developmental neurobehavioral defects in neonatal mice co-exposed to an ortho-PCB (PCB 153) and methyl mercury.

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

Fredriksson, A., Fredriksson, M., and Eriksson, P. (1993). Neonatal Exposure to Paraquat or MPTP Induces Permanent Changes in Stratium Dopamine and Behavior in Adult Mice. *Toxicology and Applied Pharmacology* **122,** 258-264.

Fängström, B., Strid, A., Athanassiadis, I., Grandjean, P., Weihe, P., and Bergman, Å. (2004). A retrospective time trend study of PBDEs and PCBs in human milk from the Faroe Islands. *Organohalogen compounds* **66,** 2829-2833.

Förordning 1998:944 om förbud m.m. i vissa fall i samband med hantering, införsel och utförsel av kemiska produkter. http://www.notisum.se/rnp/sls/lag/19980944.HTM

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., and White, R. F. (2001). Neurobehavioral deficits associated with PCB in 7-year-old children prenatally exposed to seafood neurotoxicants. *Neurotoxicol Teratol* **23,** 305-317.

Grey, E. G., and Whittaker, V. P. (1962). The isolation of nerve endings from the brain: An electron-microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* **96,** 79-87.

Hites, R. A. (2003). Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environmental Science Technology* **38**(4), 945-56.

Hrdina, P. D., Singhal, R. L., and Ling, G. M. (1975). DDT and related chlorinated hydrocarbon insecticides: Pharmacological basis of their toxicity in mammals. *Adv. Pharmacol. Chemother.* **12,** 31-88.

Hutzinger, O.,11 Sundström, G., and Safe, S. (1976). Environmental chemistry of flame retardants. Part. I. Introduction and principles. *Chemosphere* **1**, 3-10.

Hutzinger, O., and Thoma, H. (1987) Polybrominated dibenzo-p-dioxins and dibenzofurans: The flame retardant issue. *Chemosphere* **16**, 1877-1880.

Kirk, R. E. (1968). *Experimental design: Procedures for the Behavioural science.* Brooks/Cole, Belmont, CA.

Karczmar, A. (1975). Cholinergic influences on behaviour, in: Cholinergic Mechanisms. (*P. G. Waser, ed.) Raven Press, New York,* 501-529.

KemI (2003) Europaparlamentets och Rådets direktiv 2002/95/EG av den 27 januari 2003 om begränsning av användningen av vissa farliga ämnen i elektriska och elektroniska produkter.

Lowry, O. H., Rosen rough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with folin reagent. *J. Biol. Chem.* **193,** 265-275.

Merionyté, D., Norén, K., and Bergman, A. (1999). Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972-1997. *Journal of Toxicology and Environmental Health Part A* **58**, 329-341.

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

Myers, G. J., and Davidson, P. W. (1998). Prenatal methylmercury exposure and children: neurologic, developmental, and behavioral research. *Environmental Health perspective* **106 Suppl 3**, 841-847.

Narahashi, T. (1992). Nerve membrane Na^+-channels as targets of insecticides. *TiPS Rewiews* **13,** 236-240.

Nordberg, A., and Winblad, B. (1981). Cholinergic receptors in human hippocampus – regional distribution and variance with age. *Life sci.* **29,** 1937-1944.

Perry, E., Walker, M., Grace, J., and Perry, R. (1999). Acethylcholine in mind: a neurotransmitter correlate of consciousness? *Trends Neurosci.* **22,** 273-280.

Peterson, D., and Nordberg, A. (2000). Neuronal nicotinic receptors in the human brain. *Prog. Neurobiol.* **61,** 75-111.

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A.-S., McNamara, J. O., and Williams, S. M. (2001). *Neuroscience.* Sinauer Associates, Inc. Sunderland, Massachusetts.

Rogers, J. M., and Kavlock, R. J. (2001). Developmental toxicology. Chapter 10 in Cassarette and Doull's Toxicology – The basic science of poisons. (C. D Klaassen, 6th ed.), pp 356-360. McGraw-Hill.

Schecter, A., Pavuk, M., Papke, O., Ryan, J.J., Birnbaum, L., and Rosen, R. (2003). Polybrominated diphenyl ethers in U.S. mother's milk. *Environmental health perspective* **111,** 1723-1729.

Stockholm Convention on Persistent Environmental Pollutions (POPs) homepage: http://www.pops.int/documents/registers/ddt.htm, 2007-11-06. http://www.pops.int/documents/pops/default.htm, 2007-11-06.

Viberg, H., Fredriksson, A., and Eriksson, P. (2002). Neonatal Exposure to the Brominated Flame Retardant 2,2',4,4',5-Pentabromodiphenyl Ether Causes Altered Susceptibility in the Cholinergic Transmitter System in the Adult Mouse. *Toxicological Sciences* **67,** 104-107.

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. *Toxicology and Applied Pharmacology* **192,** 95-106.

Viberg, H., Fredriksson, A., and Eriksson, P. (2004a). Investigations of Strain and/or Gender Differences in Developmental Neurotoxic Effects of Polybrominated Diphenyl Ethers in Mice. *Toxicological Sciences* **81**, 344-353.

Viberg, H., Fredriksson, A., and Eriksson, P. (2004b). Neonatal Exposure to the Brominated Flame Retardant , 2,2',4,4',5-Pentabromodiphenyl Ether, Decreases Cholinergic Nicotinic Receptors in Hippocampus and Affects Spontaneous Behaviour in the Adult Mouse. *Environmental Toxicology and Pharmacology* **17,** 61-65.

Viberg, H., Fredriksson, A., and Eriksson, P. (2005). Deranged spontaneous behaviour and decrease in the cholinergic muscarinic receptors in hippocampus in the adult rat, after neonatal exposure to the brominated flame retardant 2,2'4,4',5- pentabromodiphenyl ether (PBDE 99). *Environmental Toxicology and Pharmacology,* **20,** 283-288.

WHO (1989). DDT and its derivates – Environmental aspects. Environmental Health Criteria Document, 83. WHO, Geneva.

WHO (1993). Polychlorinated Biphenyls and Terphenyls (Second edition). WHO IPCS Environmental Health Criteria Document, 140. WHO, Geneva.

WHO (1994a). Polybrominated Biphenyls. WHO IPCS Environmental Health Criteria Document, 152. WHO, Geneva.

WHO (1994b). Brominated Diphenyl Ethers. WHO IPCS Environmental Health Criteria Document, 162. WHO, Geneva.

WHO (1995). Tetrabrombisphenol A and derivates. WHO IPCS Environmental Health Criteria. WHO, Geneva.

WHO (1997). Flame retardants: a general introduction. WHO IPCS Environmental Health Criteria Document, 192. WHO, Geneva.

Wolley, D.E. (1982). Neurotoxicity of DDT and possible mechanisms of action. In *Mechanisms of Actions of Neurotoxic Substances.* Prasad, K.N. and Vernadakis, A. (eds.) Plenum press, New York, pp. 95-141.