Developmental neurotoxicity of persistent and non-persistent pollutants

Behavioral and neurochemical assessments of a perfluorinated compound, pesticides and interaction effects

IWA LEE
The focus of this thesis was to investigate developmental neurotoxic effects of different persistent and non-persistent environmental pollutants, alone or in binary mixtures, when exposure occurs during a critical period of brain development, in mice. The compounds investigated included a perfluorinated compound, perfluorohexane sulphonate (PFHxS), and four different pesticides, endosulfan, cypermethrin, chlorpyrifos and carbaryl.

Both persistent and non-persistent pollutants are detected in the environment and in humans, which shows that exposure to these compounds is occurring in real life. Humans can therefore be exposed to various pollutants during their whole lifetime, starting from the gestational period to adulthood. Furthermore, exposure to environmental pollutants is rarely exclusive to a single compound, but rather occurs through combinations of various pollutants present in the environment. Exposure to environmental pollutants during human brain development have been suggested to be a possible cause for neuropsychiatric disorders, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD). Previous studies have shown that chemicals can induce irreversible disorders in brain function when exposure to these chemicals occurs during a critical defined period of the brain development known as the brain growth spurt (BGS). The BGS is characterized by a rapid growth and development of the immature brain. In humans, and mice, this period also overlaps the lactation period indicating that newborns and toddlers can be exposed via mothers’ milk as well.

This thesis has shown that a single oral exposure to PFHxS, endosulfan, cypermethrin, chlorpyrifos or carbaryl can induce developmental neurotoxic effects in mice, when exposure occurs during a critical period of brain development. These effects are manifested as persistent altered adult spontaneous behavior in a novel home environment, modified habituation, altered susceptibility of the cholinergic system and changed levels of neuroproteins in the mouse brain. Furthermore, a single neonatal co-exposure to a binary mixture of carbaryl/chlorpyrifos or PFHxS/endosulfan can interact and exacerbate the adult behavioral effects. These effects were seen at dosages were the single compound did not elicit a response or induced a much weaker behavioral effect. This indicates that risk assessments conducted on single compounds might underestimate interaction effects of mixtures when co-exposed.

Keywords: Brain, Neonatal, Mixtures, Cholinergic system, Organochlorines, Pyrethroids, Organophosphates, Carbamates, Insecticides, PFCs, PFAAs, PFHxS, Endosulfan, Cypermethrin, Chlorpyrifos, Carbaryl

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To my parents
Lee Yuet Ying and Lee Wai Keung
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Additional publications

The following papers were also published during the course of my doctoral studies, but are however not part of the present dissertation.


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Abbreviations

ACh  Acetylcholine
AChE  Acetylcholinesterase
ADHD  Attention deficit hyperactivity disorder
AMPA  Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA  Analysis of variance
ASD  Autism spectrum disorder
bw  Body weight
BGS  Brain growth spurt
BPA  Bisphenol A
CaMKII  Calcium/calmodulin-dependent kinase
CARB  Carbaryl
ChAT  Choline acetyltransferase
CNS  Central nervous system
CPF  Chlorpyrifos
DDT  dichlorodiphenyltrichloroethane
DFP  Diisopropyl fluorophosphate
DTNB  5,5'-dithiobis(2-nitrobenzoic acid)
EU  European Union
GABA  Gamma-amino-butryric acid
GAP-43  Growth associated protein-43
GluR1  Glutamate receptor 1
HSD  Honest significant different
LTD  Long-term depression
LTP  Long-term potentiation
mAChR  Muscarinic acetylcholine receptors
MeHg  Methyl mercury
nAChR  Nicotinic acetylcholine receptors
NMDA  N-methyl D-aspartate
NMRI  Naval medical research institute
OCs  Organochlorines
OPs  Organophosphates
PBDEs  Polybrominated diphenyl ethers
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Introduction

This thesis focuses on developmental neurotoxic effects after neonatal exposure to different environmental pollutants and mixtures during a critical period of rapid brain development in mice.

Exposure to environmental pollutants

In recent years, the awareness of exposure from persistent and non-persistent environmental pollutants has increased, as more studies reveal their ubiquitous spread across the globe. The persistent organic pollutants (POPs) are of particular concern as they can accumulate in the environment and in organisms, and thereby still cause exposure years after their production has stopped or been banned. This means that individuals may be exposed during their entire course of life, from fertilization, through childhood and as adults. However, non-persistent pollutants (e.g. pesticides) are also of concern as they may be present a short time period at higher concentration levels, but harder to detect as they are degradable. These concerns have been proven accurate as many studies have shown the presence of persistent pollutants and pesticides in humans and food products (Tao et al., 2008; D'Hollander et al., 2010; Schecter et al., 2010; Bedi et al., 2013; EFSA, 2013). Furthermore, exposure to environmental pollutants during human brain development has been suggested to be a possible etiological factor for the increasing number of reported cases of neuropsychiatric disorders, such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD) and cognitive impairments (Grandjean and Landrigan, 2006).

In reality, exposure to environmental pollutants is rarely exclusive to a single compound, but rather occurs through combinations of various pollutants present in the environment. Additionally, new chemicals are constantly being produced to improve or replace old ones, making it important to evaluate interaction effects of mixtures as these interaction responses may possibly have more severe exposure consequences. Moreover, risk assessments are only conducted on individual compounds and may therefore overlook possible interaction effects.

Children are more sensitive and susceptible to toxic insult (Grandjean and Landrigan, 2006) as they are not yet fully developed (Davison and Dobbing, 1968), which allows toxicants to enter the brain easier, compared to the adult
Toxicity is also dependent on various factors, such as physical-chemical properties, time of exposure, dose, exposure route and mechanisms of action. Therefore, when assessing toxic effects of chemicals using different animal models, it is essential to keep in mind the different developmental stages and when they occur in different species (Dobbing and Sands, 1979; Rice and Barone, 2000).

Perfluorinated compounds

Perfluorinated compounds (PFCs) consist of a vast group of synthetic chemicals, which have been in production since the 1950’s. The compounds are in principle characterized by a fully fluorinated carbon chain and a functional group, and can be subdivided into different classes. The perhaps most infamous group are the perfluoroalkyl acids (PFAAs), where we find perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). PFCs are used in manufacturing of products that are oil, stain, water and fire resistant, such as nonstick cookware, waterproof and breathable fabrics and clothing, impregnating agents, firefighting foams, floor polishes, but also have other industrial applications within aerospace, automotive, construction, chemical processing and electronics industries (EPA, 2009). The distinctive properties of PFCs come from their molecular composition. The carbon-fluorine bond (C-F) is among the strongest in organic chemistry, and do not occur naturally in the environment. This makes the PFCs stable against oxidation, high temperature, hydrolytic and photolytic degradation (Kissa, 2001), and due to their stability the PFCs are virtually non-biodegradable and extremely persistent in the environment (Key et al., 1997). The PFC’s persistency have led to a worldwide spread and bioaccumulation, and they are found in humans as well as in animal wildlife far away from their production sites (Giesy and Kannan, 2002; Calafat et al., 2007; Lau et al., 2007). The primary route of human exposure is via dietary intake from products that have been in contact with PFCs, and inhalation of indoor air and dust (D'Hollander et al., 2010). Initially, most focus has been directed towards long-chained PFCs, such as PFOS and PFOA, as they are the two most commonly detected PFCs in the environment. However, recently the attention has shifted towards other short-chained PFCs, such as perfluorohexane sulfonate (PFHxS), due to the voluntary phase out of PFOS and PFOA by its primary manufacturer (3M, 2013). PFOS/PFOS-related compounds were listed as persistent organic pollutants in the Stockholm Convention (EPA, 2009).

PFHxS

PFHxS is a short-chained PFAA, as it contains less than eight carbon atoms in its structure, unlike PFOS and PFOA (fig. 1). Many short-chained PFCs
are still in production and are used to replace PFOS and PFOA, as they have similar oil and water repellent properties (Buck et al., 2011). PFHxS is of interest as it is the third most common PFAA found in the environment and in humans, as well as showing an increasing temporal trend of blood serum levels (Kärrman et al., 2007; Tao et al., 2008; Glynn et al., 2012). PFHxS has also been detected in human umbilical cord blood and breast milk (So et al., 2006; Kärrman et al., 2007; Monroy et al., 2008; Sundstrom et al., 2011), which strongly suggests that infants and toddlers are exposed early in life. There are limited studies on the estimated total daily intake of PFHxS; however, one study has suggested a dietary intake of PFHxS ranging from 0.02-0.17 ng/kg bw/day, with the highest intake found in children (in Spain) (Domingo et al., 2012). Additionally, the half-life of PFHxS is estimated to be 8.5 years, which exceeds that of PFOS (5.4 years) and PFOA (3.8 years) (Olsen et al., 2007; EPA, 2009). One study has evaluated the potential reproductive and developmental toxicity of PFHxS in rats (Butenhoff et al., 2009a), and revealed no adverse effects under the study conditions. There are very little published data on the overall toxicity of PFHxS, as most studies conducted have focused on PFOS and PFOA. In previous studies, it has been shown that PFOS and PFOA can induce developmental neurotoxicity in mice and rats. These effects manifested as, inter alia, decreased activity of choline acetyltransferase (ChAT) in prefrontal cortex (Lau et al., 2003), altered cholinergic gene transcription (Hallgren et al., 2015), changed levels of protein markers in the hippocampus and cerebral cortex (Johansson et al., 2009) and aberrant motor activity and habituation (Johansson et al., 2008; Butenhoff et al., 2009b).

Figure 1. Chemical structures of PFCs.
Pesticides

Pesticides are globally used for agricultural and domestic purposes, and are readily found in the environment, as well as in food products (EFSA, 2013). Direct exposure to pesticides can occur from occupational, agricultural and household use, whereas indirect exposure occurs via contaminated food and drinking water. These compounds are often referred to according to the type of pest they target, and are intentionally designed to mitigate, prevent or eliminate pests (EPA, 2014), unlike other environmental pollutants (e.g. PFCs). The most common type of pesticide used today is herbicides, followed by insecticides (EPA, 2011a). The most known or common types of insecticides include organochlorine, pyrethroid and anticholinesterase insecticides. All of these insecticides commonly target the nervous system in their target pests, although through different mechanisms. In many aspects the nervous system in various species share striking resemblances, and as the insecticides are not selective, they may affect non-target organisms and cause similar effects (Ecobichon, 2001).

Organochlorine insecticides

The organochlorines (OCs) are historically one of the most known types of insecticides with the famous, and infamous, dichlorodiphenyltrichloroethane (DDT) belonging to this group. The OCs can be divided into three chemical classes: the DDT-type, chlorinated cyclodienes and benzenes. They share the common characteristics of being very resistant to degradation, therefore persistent, and have a high ability to bioaccumulate. Their acute neurotoxic effects in animals are principally caused by disruption of signal transduction, leading to hyper-stimulation in the nervous system and subsequent respiratory failure and death (Coats, 1990). While most of the DDT-type compounds have been classified as POPs, some of the cyclodienes still remain as active ingredients in various pest control products.

Endosulfan (fig. 2) is a restricted OC-classed insecticide. It is being voluntarily phased-out and is scheduled for cancellation in 2016, in the United States (U.S.) (EPA, 2010). However, other countries like Sweden have not approved it use since 1997, and it has not been allowed in plant production products in the European Union (EU) since 2007. Even so, it can still be present in small amounts in imported agricultural products and in animal feed (KEMI, 2014). The estimated daily intake of endosulfan during 2007-2009, in three different Asian countries, ranged from 1.42-92.2 ng/kg bw/day in adults. While still at low levels, all three countries showed an increasing temporal trend of endosulfan exposure compared to the early 1990’s (Desalegn et al., 2011). Endosulfan is a non-competitive gamma-amino-butyric acid (GABA) antagonist, and binds to the ionotropic \textit{GABA}\textsubscript{A} receptors linked to chloride channels in the CNS. \textit{GABA}\textsubscript{A} receptors are in-
hibitory receptors, and upon binding, endosulfan inhibits chloride ions from entering the neurons, which results in hyper-stimulation (Silva and Beauvais, 2010).

Figure 2. Molecular structure of endosulfan.

Pyrethroid insecticides

The pyrethroids are synthetic non-persistent insecticides, derived from the naturally occurring pyrethrins from chrysanthemum flower extract. In mammals, the acute toxicity of pyrethrins is low to moderate, due to poor gut uptake and rapid detoxification by liver enzymes, compared to insects where the acute toxic effects are more severe. The pyrethrins are also inherently unstable, which promoted the modification of the structure and production of the more stable pyrethroids (Valentine, 1990). The pyrethroid insecticides account for a major part of the insecticide market, and are often used in mixture combination with other compounds (Shafer et al., 2005). The pyrethroids mainly mediate their toxic effect by disrupting the function of voltage-gated sodium channels in the nervous system. In general, two types of pyrethroids exist (type I and II), classified after their intoxication syndromes and chemical structure (Soderlund, 2012). The type I pyrethroids, which lack an alpha-cyano substituent, induce a prolonged opening of the sodium channels causing a repetitive firing of action potential; while the type II pyrethroids induce an even longer prolongation, resulting in no action potentials (Soderlund et al., 2002). The main symptom of type I intoxication, in mammals, is whole body tremoring, while type II induce choreoathetosis (whole body writhing) and profuse salivation (Ecobichon, 2001).

Cypermethrin (fig. 3) is a type II pyrethroid, commonly used in household applications, and is detected in dust and food products (Morgan, 2012). The acceptable daily intake for cypermethrin is estimated to 0.05 mg/kg bw/day (EFSA, 2011). The estimated daily intake of pyrethroids in the general U.S. population ranges from 0.11-1.85 µg/kg/day (EPA, 2011b). In Sweden, the estimated daily intake of cypermethrin was low (1-2% of the acceptable daily intake) in 2005-2013, with no indication of temporal changes (Naturvårdsverket, 2014). In general, human pyrethroid poisoning is rare, and almost exclusively involves the type II pyrethroids. Although, indications have been made that the type II pyrethroids may involve secondary
targets, such as voltage-gated calcium or chloride ion channels, as well as antagonistic effects on GABA receptors, in mammals (Shafer et al., 2005; Soderlund, 2012). Albeit, being readily biodegradable, there are indications of exposure of pregnant mothers and infants (Berkowitz et al., 2003; Heudorf et al., 2004). Epidemiological studies are few, however there have been reports that associate childhood pyrethroid exposure with impaired neurodevelopment (Rodriguez, 2012); but this is contradicted in other reports (Oulhote and Bouchard, 2013; Quiros-Alcala et al., 2014).

Figure 3. Molecular structure of cypermethrin (type II pyrethroid).

Anticholinesterase insecticides
The substances involved in this class feature a common mechanism of action, i.e. they inhibit the action of the enzyme acetylcholinesterase (AChE) in the nervous system, thereby causing an accumulation of acetylcholine (ACh) in cholinergic synapses and subsequently cholinergic hyper-stimulation of muscarinic and nicotinic receptors. In this class of insecticides, compounds of various structures are found, however most are derived from organophosphoric or carbamic esters, and are commonly referred to as organophosphates (OPs) or carbamates. Exposure to high doses of OPs or carbamates induce similar acute effects, such as increased salivation, lacrimation, gastrointestinal secretions; leading up to muscle and respiratory convulsions and paralysis (Ecobichon, 2001).

Organophosphates
The first OP insecticides were synthesized in the 1930’s and have historically been misused as chemical warfare agents. Although, OPs and carbamates have similar mechanism of action they differ in terms of interaction rate with AChE. The organophosphoric esters react with an active site on AChE (a serine hydroxyl group), forming a covalent bond with the enzyme. The newly formed “OP/AChE”-complex, is largely unreactive, with a very slow reactivation time. Because of the slow reactivation and the tenacious binding, many of the OPs can cause irreversible inhibition of AChE by so called aging (Ecobichon, 2001).

Chlorpyrifos (fig. 4) is one of the most well studied OPs (Burns et al., 2013), and is still commonly used in agriculture, although several countries have restricted or banned its use. Still, in the U.S., the dietary intake of
chlorpyrifos in preschool children has been estimated to be 2.32 µg/kg/day (Morgan and Jones, 2013). In Sweden, chlorpyrifos is banned, however it is still detected in fruit and vegetable samples (NFA, 2013). Furthermore, childhood exposure to chlorpyrifos has been strongly associated with impaired neurodevelopment and ADHD in children (Rauh et al., 2006; Bouchard et al., 2010).

![Molecular structure of chlorpyrifos.](image)

**Figure 4.** Molecular structure of chlorpyrifos.

Carbamates
Carbamates have in general been regarded as a safer option to OPs, as carbamate intoxication largely resolves within a few hours, in contrast to OPs, where the effects may last from days to months. This is because the binding of carbamates to AChE is less stable, compared to the OP/AChE-complex, and the binding is spontaneously hydrolyzed within minutes to hours. Due to this transient inhibition, carbamates are regarded as reversible AChE inhibitors (Colovic et al., 2013).

The majority of the carbamate pesticides are N-methyl carbamates, and they share a common mechanism of action compared to other carbamates. Carbaryl (fig. 5) is an N-methyl carbamate, and is banned within the EU. However, it has been approved for re-registration and use in the U.S., for home, garden and agricultural applications (EPA, 2007). Dietary intake of carbaryl via infant foods has previously been shown to range between 0.2-343 ng/kg bw/day in Canada (Rawn et al., 2006). Despite the widespread use of carbamates, there are few studies concerning the neurotoxic effects of these compounds in young individuals. However, in an animal study, it was shown that there was age-related differences in neurotoxicity, and that young rats were more sensitive to carbaryl than adults (Moser et al., 2010). Furthermore, carbamate poisoning appears to cause similar toxic symptoms as OPs, while the toxic symptoms differ more between children and adults (Lifshitz et al., 1997; 1999).
The developing brain and critical periods

In the relatively new field of developmental neurotoxicity, it is essential to identify critical periods of brain development, when exposure to various environmental toxicants can be harmful. Therefore, the selection of model test system and endpoints are crucial, as neurotoxic effects may differ between the models and endpoints. Neurotoxic effects during these critical periods, independent of causality, may lead to various functional or behavioral distortions and malfunctions, which are featured early or late in life, depending on the timing of exposure.

The fundamental pattern of the development of the central nervous system (CNS) is in principle similar between mammalian species, and the physiological and functional properties of the cellular units of the CNS are much alike. The most notable differences are the timing and onset of the developmental processes and the gross proportions of different brain regions (Davison and Dobbing, 1968). The CNS development can roughly be divided into two stages, with each having its own critical periods, where marked changes are supposed to occur. In the first stage, early embryonic brain development takes place, i.e. the brain acquires its general shape, by organogenesis and neuronal multiplication. However, the brain is not yet fully mature. Notably, the embryonic development in humans occurs during the first two months of gestation, making up 20% of the gestation period, whereas in mice the embryonic period constitutes 80% of the entire gestation. Insults happening during the embryonic period usually lead to morphological malformations.

The second stage is referred to as the “brain growth spurt (BGS)” and is characterized by a rapid increase in size and weight, due to increase of glia cells and biochemical changes in the brain. The major developmental processes in the BGS include growth and maturation of neurites, establishment of neuronal connections, synaptogenesis, gliogenesis, myelination and neuronal pruning (Dobbing and Sands, 1979; Kolb and Whishaw, 1989). During this second stage, mice and rats also acquire many motor and sensory aptitudes (Bolles and Woods, 1964) and their spontaneous behavior peaks (Campbell et al., 1969).
The onset and duration of the BGS differ between species. In humans, the period begins around the third trimester of pregnancy and lasts up to the first two years of life, whereas, in mice this stage is entirely neonatal, with the onset around birth and continuing during the first four weeks of life, with a peak around postnatal day (PND) 10 (Davison and Dobbing, 1968; Semple et al., 2013). In humans and mice, the growth spurt coincides with the lactation period; therefore it is highly possible that newborns and toddlers are exposed, via the placenta or the diet, to toxicants from an early stage.

Previous studies within our group have shown that exposure to low doses of toxic agents (e.g. DDT, OPs, pyrethroids, brominated flame retardants and nicotine) during the neonatal brain development, in mice, can induce irreversible disruptions in brain function as adults (Eriksson et al., 1992; Ahlbom et al., 1995; Eriksson et al., 2000; Eriksson et al., 2002). However, these induced persistent disturbances only occur when exposure is limited to a defined period during the BGS i.e. around PND 10. In these studies, mice were administered a chemical on either PND 3, 10 or 19, and spontaneous behavior was examined at an adult age. Neurotoxic effects were only seen in the mice exposed on PND 3 or 10. However, the effects were more pronounced in the mice exposed on PND 10, compared to PND 3; whereas the mice exposed on PND 19 showed no neurotoxic effect. It has also been shown that not only dose and time of administration is crucial for inducing neurotoxic effects, but also uptake, sufficient retention and metabolism of the compound, during this defined critical period, is equally important (Viberg et al., 2003b; Viberg et al., 2006).

Neuronal protein markers

The complex developmental processes, which occur during the BGS, involve expression and regulation of various neuroproteins, such as calcium/calmodulin-dependent kinase II (CaMKII), growth-associated protein-43 (GAP-43), glutamate receptor 1 (GluR1), synaptophysin and tau. The accessible use of transgenic mice models have also shown that alterations, such as deficits or over-expression in these proteins, can affect behavioral and cognitive functions (Schmitt et al., 2003; Holahan and Routtenberg, 2008; Hasegawa et al., 2009; Schmitt et al., 2009; Morris et al., 2011). Therefore, many of the different proteins may also serve as markers for developmental toxicity, as they have specific functions and play key roles in maintaining normal development (Mundy et al., 2008).

CaMKII is a calcium activated enzyme, abundantly enriched in the synapses in the brain (Lisman et al., 2002). It plays a central role in the regulation of synaptogenesis, synaptic plasticity, calcium homeostasis, behavior and formation of memory (Silva et al., 1992; Rongo and Kaplan, 1999; Frankland et al., 2001; Lisman et al., 2002). There are two principal isoforms, αCaMKII and βCaMKII, and they have different roles in synaptic
plasticity (Giese and Mizuno, 2013). αCaMKII is required for hippocampus-dependent memory formation, by autophosphorylation mediated by long term potentiation (LTP); whereas, βCaMKII has a non-enzymatic role in memory formation (Lisman et al., 2012). Recently, CaMKII has also been suggested to be implicated in long term depression (LTD), displaying CaMKII’s intricate regulation in synaptic processing and information storage (Coultrap and Bayer, 2012; Coultrap et al., 2014). During the BGS, in mice, the levels of CaMKII continuously increase in the cerebral cortex, hippocampus and whole brain. The increase is most rapid during the BGS, between PND 7 and PND 14 (Viberg et al., 2008).

GAP-43 is a presynaptic protein, found in the growth cone of axons, and is involved in regulating and organizing neuronal networks, synaptic plasticity and LTP. Depending on the strength of calcium signaling, GAP-43 can either modulate endocytosis and vesicle recycling (Neve et al., 1998) or become phosphorylated by protein kinase C (PKC) and subsequently facilitate downstream calcium/calmodulin-dependent, events, triggering CaMKII autophosphorylation and LTP (Hulo et al., 2002). The highest expression of GAP-43 occurs during development, however it decreases after synaptogenesis except in certain brain regions with high plasticity rate, such as hippocampus CA1 region (Jacobson et al., 1986; Benowitz and Routtenberg, 1997; Baumgartel and Mansuy, 2012). Its specific expression has led to the use of GAP-43 as a biomarker for axonal sprouting and progression (Oestreicher et al., 1997). During the BGS in mice, the level of GAP-43 has a bell-shape ontogeny curve, with a distinct increase during the beginning of the neonatal period followed by a decrease during the last part. In the hippocampus the level of GAP-43 peaks around PND 7, while in the cerebral cortex and whole brain the level of GAP-43 peaks around PND 10 (Viberg et al., 2008).

GluR1 is an ionotrophic receptor that belongs to the family of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. It is found throughout the CNS, and is predominantly involved in fast excitatory neurotransmission in synapses and synaptic plasticity (Ozawa et al., 1998). Phosphorylation of GluR1 mainly occurs on serine and threonine residues, by CaMKII, PKC and protein kinase A (PKA), and can enhance currents through AMPA receptor channels (Banke et al., 2000). The presence, as well as phosphorylation and de-phosphorylation, of GluR1 have shown to be involved in LTP and LDP, albeit through different mechanisms (Lee, 2006). During development, GluR1 is found primarily at emergent synaptic junctions of immature neurons (Martin et al., 1998), while almost exclusively in the somatodendritic compartment in the mature brain (Martin et al., 1993). GluR1 is regulated spatiotemporally at regional cellular and synaptic levels during development. The expression of GluR1, in the rat neocortex and hippocampus, markedly increases during early postnatal development (PND 2-15) and reaches adult levels by PND 19-21 (Martin et al., 1998).
Synaptophysin is one of the most abundant transmembrane glycoproteins expressed in presynaptic vesicles and is commonly found in nervous tissue (Wiedenmann and Franke, 1985; Navone et al., 1986; Takamori et al., 2006). The full extent of its functions is still enigmatic; however, it has been shown to be involved in synaptic vesicle (SV) endocytosis and act as an adaptor protein for other SV proteins. Synaptophysin, per se, is not essential for neurotransmission, but it is suggested to modulate the efficiency of SV cycling, and efficient SV cycling is necessary for maintaining functional neurotransmission. Subsequently, this indicates regulation of learning and memory efficacy (Gordon and Cousin, 2014). As synaptophysin is localized in the SVs, it is widely used as a biomarker for presynaptic terminals (Gordon et al., 2011; Kwon and Chapman, 2011). Synaptophysin is mainly phosphorylated by tyrosine kinases, although there is evidence that it is a substrate for CaMKII as well (Rubenstein et al., 1993). The ontogeny of synaptophysin is similar to that of CaMKII. There is a continuous increase of the amount of synaptophysin throughout the BGS in the hippocampus, cerebral cortex and whole brain of mice. The increase was most rapid between PND 7 and PND 14 (Viberg, 2009).

Tau is a microtubule-associated protein, predominantly expressed in the axons throughout the CNS. Its primary function has for long been believed to assemble and stabilize microtubules (Weingarten et al., 1975), and to modulate axonal transport (Dixit et al., 2008). Physiological tau has an inherent disordered structure to facilitate post-translational modifications (e.g. phosphorylation, acetylation, isomerization and ubiquitination), upon direct binding to enzymes, which also indicates that tau can influence cell signaling pathways as a scaffolding protein (Morris et al., 2011). Tau is also known to have other functions in the developing brain compared to the adult brain. It is a main component of neurofibrillary tangles and tauopathies, caused by tau aggregation from abnormal phosphorylation, which is recognized as a hallmark of Alzheimer’s disease (Lee et al., 2001). The ontogenic pattern for tau is similar to that of GAP-43, displaying a bell-shaped form. The level of tau increases during the early neonatal period with a subsequent decrease towards the end of the BGS, in mice. In the hippocampus, the level of tau peaks between PND 3 and PND 7, while in cortex and whole brain it peaks between PND 7 and PND 10 (Viberg, 2009).

Exposure to toxic agents (e.g. polybrominated diphenyl ethers (PBDEs), PFCs and bisphenol A (BPA)) or radiation, during a defined period of critical brain development, has also been shown to affect the levels of these proteins in the brain, as well as causing persistent disturbed adult behavior and reduced cognitive function in mice (Johansson, 2009; Viberg et al., 2011; Viberg and Lee, 2012; Buratovic et al., 2014). This indicates that the neuroproteins may be involved in the persistent behavioral disturbances.
The cholinergic system and behavior

Simultaneously, or parallel, with the CNS development, components of the cholinergic system organize to form an extensive network of cell groups and pathways that project throughout the nervous system (Woolf and Butcher, 2011). The cholinergic components consist, inter alia, of ChAT, ACh, AChE, nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). Each component follows a specific developmental scheme, from the moment they appear in distinct regions of the CNS to the time of adult maturation levels. In mice and rats, the ontogenesis of these components takes place during the BGS. In addition to having functional roles in the neurotransmitter system, they are also involved in developmental processes, such as neurite outgrowth and organization, synaptic plasticity, cell proliferation and neurogenesis (Abreu-Villac et al., 2011).

The cholinergic system has early on been suspected to be involved in cognitive functions, such as learning and memory (Russell, 1982). The link between the cholinergic system and behavior is not surprising, considering the widespread orchestration of the cholinergic pathways and their influence on other neurotransmitter systems (Woolf and Butcher, 2011). Moreover, behavior is a major process whereby animals can adapt to changes in their environment. These environmental changes may disclose the effects of chemical pollutants; for instance, an exposure to an environmental pollutant may result in a behavioral change that is adaptive or modified (i.e. it departs from the normal pattern), or it may result in a complete failure in behavior (Evans, 1994). Therefore, behavior can be useful in detecting potential neurotoxic agents that also might affect the cholinergic system.

Developmental cholinergic lesions or insults caused by neurotoxic agents, such as anticholinesterases, have earlier been shown to affect learning and memory tasks, as well as attention tasks in rats (Ricceri et al., 1999; Levin et al., 2001; Qiao et al., 2002; Ricceri, 2003). However, adult lesions did not affect the performance tasks, which indicate that disturbances of cholinergic development can produce more profound deficits than adult influences (Berger-Sweeney, 2003). Changes in cholinergic receptors have been suggested to affect learning and memory. Furthermore, nicotinic receptors have been shown to be important in neurodevelopment (Levin and Simon, 1998), and behavior tests with different cholinergic agonist and antagonists acting on nicotinic receptors have affected learning and memory in rats (Levin, 2002).

In previous studies, with both cholinergic and non-cholinergic toxicants, it were shown that these substances could affect the developing cholinergic system, and cause permanent effects in adult mice (Eriksson, 1992; Ahlbom, 1995; Eriksson et al., 2000; Viberg et al., 2002). These effects were only seen when exposure occurred during a critical defined period of brain development i.e. around PND 10-14. Moreover, Eriksson et al. (2000) showed
that a low dose of nicotine has no permanent effect when administered before or after this period. In the neonatal mouse brain, nAChR binding sites increase until PND 10, after which they decline to adult values around PND 25 (Fiedler et al., 1987). During this period the nAChR differentiates, and distinct subtypes of nicotinic receptors can be found with low-affinity or high-affinity binding sites for different ligands (Paterson and Nordberg, 2000). A study by Nordberg et al. (1991), showed that neonatal nicotine exposure between PND 10 and 16, prevented the development of low-affinity nicotinic binding sites in the mouse brain, and that this early exposure to nicotine induced different behavior responses in the adult mice. Interestingly, Eriksson et al. (2000) also showed that mice exposed to nicotine on PND 10-14, lacked low-affinity nicotinic binding sites in the adult brain; whereas mice exposed to nicotine on PND 3-7 or PND 19-23 still had low-affinity binding sites in the brain. Furthermore, all these animals showed a normal adult spontaneous behavior, however when challenged to nicotine only mice neonatally exposed on PND 10-14 showed a hypoactive response. This indicates that neonatal exposure during a defined critical period during brain development can alter cholinergic susceptibility in adult mice, which appear to be mediated via changes in nAChR composition.
Objectives

The overall objective of this thesis was to investigate the developmental neurotoxic effects of neonatal exposure to environmental pollutants or binary mixtures of environmental pollutants in NMRI mice. The specific aims of the thesis were:

- To investigate if a single neonatal exposure to a persistent industrial pollutant, PFHxS, during a critical period of brain development, could induce neuroprotein changes in the brain, adult neurobehavioral aberrations and affect the cholinergic system.

- To investigate if a single neonatal exposure to persistent or non-persistent pesticides, endosulfan, cypermethrin, chlorpyrifos or carbaryl, during a critical period of brain development, could induce neuroprotein changes in the brain and adult neurobehavioral aberrations.

- To investigate if a single neonatal co-exposure to two different cholinesterase inhibitors, chlorpyrifos and carbaryl, during a critical period of brain development, could interact to induce adult neurobehavioral aberrations, enhance the possible neurobehavioral effects and determine if the cholinergic system was affected.

- To investigate if a single neonatal co-exposure to two different persistent pollutants, PFHxS and endosulfan, during a critical period of brain development, could interact to induce adult neurobehavioral aberrations and enhance possible neurobehavioral effects.
Material and methods

A more detailed description of the material and methods is presented in the respective papers.

Chemicals

PFHxS (CAS number 3871-99-6), endosulfan (CAS number 115-29-7), cypermethrin (CAS number 67375-30-8), chlorpyrifos (CAS number 2921-88-2), carbaryl (CAS number 63-25-2) and (-)nicotine-bi-(+)tartrate were commercially obtained from Sigma Aldrich, Sweden.

All of the investigated compounds were dissolved in an egg lecithin (Merck, Darmstadt, Germany) and peanut oil (Oleum arachidis) mixture (1:10, w:w) and sonicated with water to yield a 20% (w:w) fat emulsion vehicle, with different concentrations or mixtures, depending on the experiment. The use of 20% fat emulsion vehicle is to emulate the fat content of mouse milk (~14%) for a physiologically appropriate absorption and hence distribution (Keller and Yeary, 1980; Palin et al., 1982).

Animals

Pregnant NMRI (Naval Medical Research Institute) mice were purchased from Scanbur, Sollentuna, Sweden or Charles River, Germany, and housed individually in Makrolon® III cages in a room with an ambient temperature of 22°C and 12/12 hour cycle of light and dark. The animals had free access to standardized food pellets (Lactamin, Stockholm, Sweden) and tap water ad libitum. The day of birth was assigned PND 0; the litters were culled to 10-14 pups within 48h after birth. At around 3-4 weeks of age the mice were separated and were kept in their respective treatment groups, together with their siblings. Each litter contained 4-7 mice and was raised in separate rooms, for male mice only, with the same conditions as mentioned above, in the studies where both sexes were investigated. Experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval from the local ethical committee.
(Uppsala University and Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals.

**Treatment**

In the studies, carried out in this thesis, the animals were administered a single oral dose of the investigated compounds or vehicle, respectively, via gavage, on PND 10. The control mice received 10 ml of 20% fat emulsion vehicle/kg bw, for all studies conducted. In study:

I. NMRI mice were exposed to 0.61, 6.1 or 9.2 mg PFHxS/kg bw (1.4, 14 or 21 µmol PFHxS/kg bw).

II. NMRI mice were exposed to 6.1 or 9.2 mg PFHxS/kg bw (14 or 21 µmol PFHxS/kg bw).

III. NMRI mice were exposed to 0.1 or 0.5 mg endosulfan/kg bw (0.25 or 1.25 µmol endosulfan/kg bw); 0.1 or 0.5 mg cypermethrin/kg bw (0.24 or 1.20 µmol cypermethrin/kg bw), respectively.

IV. NMRI mice were exposed to 0.1, 1.0 or 5.0 mg chlorpyrifos/kg bw (0.29, 2.90 or 14.3 µmol chlorpyrifos/kg bw); 0.5, 5.0 or 20.0 mg carbaryl/kg bw (2.5, 25.0 or 99.4 µmol carbaryl/kg bw), respectively.

V. NMRI mice were exposed to 5.0 or 20.0 mg carbaryl/kg bw (25.0 or 99.4 µmol carbaryl/kg bw); 5.0 or 10.0 mg chlorpyrifos/kg bw (14.3 or 28.6 µmol chlorpyrifos/kg bw); 5 + 5, 5 + 10, 20 + 5 or 20 + 10 mg carbaryl + chlorpyrifos/kg bw.

VI. NMRI mice were exposed to 6.1 or 9.2 mg PFHxS/kg bw (14 or 21 µmol PFHxS/kg bw); 0.1 or 0.5 mg endosulfan/kg bw (0.25 or 1.25 µmol endosulfan/kg bw); 6.1 + 0.05, 6.1 + 0.1, 9.2 + 0.05 or 9.2 + 0.1 mg PFHxS + endosulfan/kg bw.

All of the brain samples, used for study I-IV, were immediately frozen in liquid nitrogen and stored in -80°C until analysis.
Behavioral analysis

Spontaneous behavior assay
The spontaneous behavior in a novel home environment test was performed in study I, III, IV, V and VI.

The mice used for this behavioral assay were tested at the age of 2 months, and re-subjected to the spontaneous behavior test at 4 or 5 months of age.

The animals were tested between 8 a.m. and 12 p.m. under the same light and temperature conditions as their housing conditions. A total of 12-18 mice were randomly picked from 3-4 different litters in each treatment group, at each testing occasion. Motor activity was measured for a 60-min period, divided into 3 × 20 min periods (0-20, 20-40 and 40-60 min), in an automated device consisting of cages (40 × 25 ×15 cm) placed within two series of low and high infrared beams (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) (Fredriksson, 1994). Three behavioral variables were measured:

Locomotion: Counting was registered when the animal moved horizontally through the low-level grid of infrared beams (localized 10 mm above the bedding material).

Rearing: Movement in the vertical plane was registered at a rate of four counts per second, when a single high-level beam was interrupted (localized 80 mm above the bedding material), i.e. the number of counts registered was proportional to the time spent rearing.

Total activity: All types of vibration within the cage, i.e. those caused by the animal movements, shaking (tremors) and grooming, were registered by a sensor (a needle mounted on a lever with a counter weight), connected to the test cage.

Nicotine-induced behavior assay
The nicotine-induced behavior test was performed in study I and V.

The mice used for the nicotine-induced behavior assay were tested at the age of 4 months, directly after the second spontaneous behavior test. The mice received saline or nicotine-injections (80 µg/kg bw) and thereafter they were tested for locomotion, rearing and total activity, as described for spontaneous behavior, during three consecutive 20-min periods (60-80, 80-100 and 100-120 min).
Biochemical analysis

AChE inhibition assay
The AChE inhibition assay was performed in study IV and V. The mice used for this assay were euthanized 1, 3, 6, 12, 24 or 36h after administration of an anticholinesterase inhibitor or a binary mixture, and the whole brain (without cerebellum) was dissected out.

AChE activity was measured by a modification of the method of Ellman et al. (1961). Briefly, the whole brains were homogenized to yield a protein content of 2 mg/ml tissue. The homogenates were sonicated and an aliquot was mixed with acetylthiocholine iodide, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and phosphate buffer. The absorbance was measured at 412 nm over a 30-min period.

Slot Blot assay
The Slot Blot assay was performed in study II, III and IV. The mice used for this assay were euthanized 24h, 4 or 5 months after administration of a pesticide or PFHxS, and hippocampi and cerebral cortices were dissected out.

The protein levels of CaMKII, GAP-43, GluR1, synaptophysin and tau in the hippocampi and cerebral cortices from all treatment groups were analyzed. The samples were homogenized and the total protein content was measured using Pierce® BCA Protein assay method.

The specificity of the primary antibodies; CaMKII (Millipore, 05-532), GAP-43 (Chemicon Millipore, AB5220), GluR1 (Millipore, AB1504), synaptophysin (Calbiochem, 573822) and tau (Santa Cruz, 32274) were previously evaluated by Western Blot analysis (Viberg et al., 2008; Viberg, 2009). The antibodies were concluded to be specific for respective protein, as the analysis showed only the presence of one band at the appropriate molecular weight. Therefore, the antibodies were considered suitable for use in Slot Blot analysis. The antibodies recognize both phosphorylated and non-phosphorylated forms of the proteins.

The amount of total protein loaded in the assay and antibodies used were; 4 µg and mouse monoclonal αCaMKII (1:5000) for CaMKII, 4 µg and rabbit polyclonal GAP-43 (1:10000) for GAP-43, 3 µg and rabbit polyclonal GluR1 (1:1000) for GluR1, 3 µg and mouse monoclonal synaptophysin (1:10000) for synaptophysin, and 3.5 µg and mouse monoclonal tau (1:1000) for tau. Immunoreactivity was detected using a horseradish peroxidase-conjugated secondary antibody against mouse (KPL, 074-1806, 1:20000) or rabbit (KPL, 074-1506, 1:20000). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce® Supersignal West Dura) with imaging on a LAS-100 (Fuji Film, Tokyo, Japan). The intensity of
bands was quantified using IR-LAS 1000 Pro (Fuji Film). The protein levels were expressed as a percentage of controls.

**Statistical analysis**

Spontaneous behavior assay
The data from the spontaneous behavior tests in a novel home environment were subjected to a split-plot ANOVA (analysis of variance), and pairwise testing was performed using Tukey’s HSD (honest significant difference) test (Kirk, 1968) for studies I, III, IV, V and VI.

Nicotine-induced behavior assay
The data from the nicotine-induced behavior tests were subjected to a split-plot ANOVA, and pair-wise testing between treated and control groups, using Tukey’s HSD test (Kirk, 1968) for studies I and V.

AChE inhibition assay
The data from the AChE inhibition analysis for study IV were analyzed by either Student’s t-test or one-way ANOVA, and pairwise testing was performed using Newman-Keul’s post hoc test.

The data from the AChE inhibition analysis for study V were analyzed by one-way ANOVA, and pairwise testing was performed using Duncan’s post hoc test.

Neuroprotein assay
The data from the Slot Blot analysis were analyzed by either Student’s t-test or one-way ANOVA, and pairwise testing was performed using Newman-Keul’s or Duncan’s post hoc test, for studies II, III and IV.
Results and discussion

Developmental neurotoxic effects of single compound exposures

In this thesis, it has been shown that a single oral exposure to a persistent pollutant or different pesticides can induce developmental neurotoxic effects in mice, when exposure occurred during a critical period of brain development. The developmental neurotoxic effects may be manifested as altered adult spontaneous behavior in a novel home environment, modified habituation, altered susceptibility of the cholinergic system and/or changed levels of neuroproteins in the mouse brain.

Effects observed in the spontaneous behavior assay

In study I, III and IV, spontaneous behavior in a novel home environment was observed in mice neonatally exposed to different doses of PFHxS, endosulfan, cypermethrin, chlorpyrifos or carbaryl, respectively. Interestingly, this behavior assay revealed a similar change in spontaneous behavior pattern and modified habituation, regardless of whether the compound was a persistent or non-persistent pollutant, compared to the control animals.

In control mice, a normal spontaneous behavior to a novel home environment is seen as a distinct decrease in activity for the three test variables locomotion, rearing and total activity, in response to the diminishing novelty of the home environment, over a 60-min test period (fig. 6), which also demonstrates an ability to habituate. This form of habituation can be used as a measure of cognitive function, as it requires the ability to integrate new sensory stimuli from the novel environment with information previously attained to habituate (Groves and Thompson, 1970; Wright et al., 2004; Rankin et al., 2009). It is a form of non-associated learning, and is considered as the most basic form of learning, as it allows for filtering of irrelevant versus relevant stimuli (Poon and Young, 2006). Furthermore, the behavior testing was conducted during forenoon hours, a time period where the mice are more likely to be less active and more prone to habituate.
In study I, both male and female mice were administered a single oral dose of 1.4, 14 or 21 µmol PFHxS/kg bw (0.61, 6.1 or 9.2 mg/kg bw), on PND 10, and the control animals received a 20% fat emulsion vehicle. These animals were subjected to the spontaneous behavior tests in a novel home environment, at 2 months of age, and the animals exposed to the highest PFHxS dose (21 µmol/kg bw) showed a significantly decreased activity during the first 20-min period (0-20 min) and significantly increased activity during the last 20-min period (40-60 min) for all of the three test variables, compared to the controls. At 4 months of age, there was still a significant dose-related change in all three test variables, when these animals were re-subjected to the spontaneous behavior test in a novel home environment (fig. 7). The results, from study I, showed a clear neurotoxic effect in animals exposed to the persistent pollutant PFHxS, manifested as altered adult spontaneous behavior to a new home environment and modified habituation. The neurotoxic effect did not differ between the sexes and was long-lasting, or possibly irreversible; similarly to other studies with persistent pollutants (e.g. PBDEs) (Viberg et al., 2004).

Increased motor activity and reduced habituation have previously also been reported in rats exposed to 1.0 mg PFOS/kg bw/day, from gestational day 0 to PND 20 (Butenhoff et al., 2009b). This is in agreement with our previous study, showing that PFAAs can induce altered persistent adult spontaneous behavior and cognitive impairment, when exposed to a single oral dose on PND 10, in mice (Johansson et al., 2008). The altered behavior-
al effects seen after PFHxS exposure, here in study I, are similar to the behavioral effects induced by PFOS and PFOA in the study by Johansson et al. (2008). Also, the PFHxS doses used in the present study were the same, on a molar basis, as the doses used in the recent studies with PFAAs, PBDEs and BPA (Viberg, 2004; Johansson et al., 2008; Viberg et al., 2011).

Figure 7. Spontaneous behavior in 4 months old male NMRI mice exposed to single oral dose of either 20% fat emulsion, 1.4, 14 or 21 µmol PFHxS/kg bw (0.61, 6.1 or 9.2 mg/kg bw) on PND 10. The data was analyzed by one-way ANOVA with split plot design and the statistical differences are indicated as: (A) significantly different vs. control, p ≤ 0.01; (B) significantly different vs. 1.4 µmol/kg bw, p ≤ 0.01; (C) significantly different vs. 14 µmol/kg bw, p ≤ 0.01; (b) significantly different vs. 1.4 µmol/kg bw, p ≤ 0.05. The data is presented as mean value + SD, n = 12 animals/treatment group.
In study III, male mice were neonatally exposed to two pesticides, one OC, endosulfan (1.25 µmol endosulfan/kg bw (0.5 mg/kg bw)), representing a persistent compound and one pyrethroid, cypermethrin (0.24 or 1.20 µmol cypermethrin/kg bw (0.1 or 0.5 mg/kg bw)), representing a non-persistent compound. A single oral dose was administered. At 2 and 5 months of age, these animals were likewise subjected to a spontaneous behavior test in a novel home environment. At the first behavior testing, at 2 months of age, the animals exposed to 1.25 µmol endosulfan/kg bw showed a significantly decreased activity during the first 20-min period (0-20 min) and significantly increased activity during the last 20-min period (40-60 min) for all of the three test variables, compared to the controls (fig. 8, only locomotion and rearing shown). The animals exposed to 1.20 µmol cypermethrin/kg bw showed a significantly decreased activity during the first 20-min period for the rearing variable, but a significantly increased activity, during the last 20-min period, for all three test variables compared to the controls.

Figure 8. Spontaneous behavior (locomotion and rearing) in 2 months old male NMRI mice exposed to single oral dose of either 20% fat emulsion or 1.25 µmol endosulfan/kg bw (0.5 mg/kg bw) on PND 10. The data was analyzed by one-way ANOVA with split plot design and the statistical differences are indicated as: (A) significantly different vs. control, p ≤ 0.01. The data are presented as mean value + SD, n = 12 animals/treatment group.
At 5 months of age, when the animals were re-subjected to the spontaneous behavior test in a novel home environment, there were still significant changes for all three test variables in the endosulfan exposed animals. The cypermethrin exposed animals still had significant changes for the three variables measured. However, these animals also had a significantly decreased locomotor activity during the first 20-min period of testing (fig. 9, only locomotion shown), which was not observed at 2 months of age.

Figure 9. Spontaneous behavior (locomotion) in 2 (upper panel) and 5 (lower panel) months old male NMRI mice exposed to single oral dose of either 20% fat emulsion, 0.24 or 1.20 µmol cypermethrin/kg bw (0.1 or 0.5 mg/kg bw) on PND 10. The data was analyzed by one-way ANOVA with split plot design and the statistical differences are indicated as: (A) significantly different vs. control, \( p \leq 0.01 \); (B) significantly different vs. 0.24 µmol/kg bw, \( p \leq 0.01 \); (a) significantly different vs. control, \( p \leq 0.05 \); (b) significantly different vs. 0.24 µmol/kg bw, \( p \leq 0.05 \). The data are presented as mean value + SD, \( n = 12 \) animals/treatment group.

The results from study III, showed that exposure to endosulfan or cypermethrin induced neurotoxic effects manifested as altered adult spontaneous behavior to a new home environment and modified habituation. Similarly to
PFHxS, the neurotoxic effects still remained months after the single neonatal exposure, which again suggests that the effects are long-lasting or irreversible. However, in the cypermethrin exposed animals the spontaneous behavior appears to have worsened, as an effect was seen in the locomotor activity at 5 months of age, which was not present earlier. Behavioral alterations in adult animals have previously also been seen in studies with other compounds belonging to the class of OC and pyrethroid insecticides. These studies showed that 4 months old mice, similarly, had an hyperactive condition and a delayed or absent ability to habituate, when neonatally exposed to DDT, bioallethrin (type I) or deltamethrin (type II) (Eriksson et al., 1990; Eriksson and Fredriksson, 1991). A noteworthy aspect is that the non-persistent pyrethroids induced similar irreversible neurobehavioral effects as the persistent OCs, albeit exerting its acute effects by a different mechanism of action, and that OCs are regarded as the more toxic and harmful of the two. The neurotoxic effects induced by endosulfan and cypermethrin are also similar to the effects seen in the PFHxS exposed animals; however, the dosages used differed almost twenty-fold.

In study IV, another two insecticides, belonging to two other classes of pesticides, were tested in the neonatal animal model and male mice were administered a single oral dose of 0.29, 2.90 or 14.3 µmol chlorpyrifos/kg bw (0.1, 1.0 or 5.0 mg/kg bw); 2.5, 25.0 or 99.4 µmol carbaryl/kg bw (0.5, 5.0 or 20.0 mg/kg bw), respectively, on PND 10. At 2 months of age, these animals were subjected to a spontaneous behavior test in a novel home environment, and the mice exposed to the high chlorpyrifos dose (14.3 µmol/kg bw) showed a significantly decreased activity during the first 20-min period (0-20 min), for all of the three test variables, compared to the controls. This type of hypoactivity, during the first 20-min period, was also seen in the animals exposed to the two higher carbaryl doses (25.0 or 99.4 µmol/kg bw); however, the animals exposed to the highest carbaryl dose also showed a significantly increased activity during the last 20-min period (fig. 10). At 4 months of age, the animals were re-subjected to the spontaneous behavior test, which showed a similar behavior outcome as the first test. The results, from study IV, show that both chlorpyrifos and carbaryl can induce altered adult spontaneous behavior in a novel home environment; however, the carbaryl exposed animals had also a modified habituation, which was not seen for the chlorpyrifos exposed animals. In a previous study with other OPs, paraoxon (POX) and diisopropyl fluorophosphate (DFP), altered adult spontaneous behavior was also seen (Ahlbom, 1995). Interestingly, the behavior change induced by chlorpyrifos was more alike the effects seen in POX exposed animals, while carbaryl showed similar effects as DFP, which also affected the habituation. This supports that carbamates can induce similar neurotoxic effects as OPs. The results also indicate that the neurotoxic effects of the non-persistent pesticides, chlorpyrifos and carbaryl, were persis-
tent or irreversible, similarly to the effects seen for the non-persistent pyrethroid, cypermethrin, and the persistent pollutants, PFHxS and endosulfan.

Taken together, this suggests that the neurobehavioral effects may be caused by an alternative mechanism or biological target, than the traditional mechanism of action for acute effects, as the mode of action differs between these toxic agents, but the behavioral outcomes are similar. A possible target could be the cholinergic system, as it is closely related to cognitive functions (Contestabile, 2011; Woolf and Butcher, 2011). This is also supported by previous studies with PFAAs, PBDEs and other pesticides, where effects have been seen in the cholinergic system as altered cholinergic susceptibility (Eriksson, 1992; Viberg, 2004; Johansson et al., 2008).

![Figure 10](image-url)

**Figure 10.** Spontaneous behavior (locomotion) in 2 months old male NMRI mice exposed to single oral dose of either 20% fat emulsion; or 0.29, 2.90 or 14.3 µmol chlorpyrifos/kg bw (0.1, 1.0 or 5.0 mg/kg bw); or 2.5, 25.0 or 99.4 µmol carbaryl/kg bw (0.5, 5.0 or 20.0 mg/kg bw), on PND 10. The data was analyzed by one-way ANOVA with split plot design and the statistical differences are indicated as: (A) significantly different vs. control, p ≤ 0.01; (B) significantly different vs. lowest dose, p ≤ 0.01; (C) significantly different vs. middle dose, p ≤ 0.01. The data are presented as mean value + SD, n = 12 animals/treatment group.
Effects linked to the cholinergic system

In this thesis, we further investigated if the cholinergic system was affected by neonatal exposure to toxic agents, as we in previous studies with other persistent pollutants and pesticides have seen effects in the cholinergic system (Eriksson, 1992; Eriksson et al., 2000; Viberg, 2004; 2011). Furthermore, AChE inhibition is commonly used as a marker for neurotoxic effect of anticholinesterase inhibitors. Therefore, it was of interest to investigate if AChE inhibition can similarly be used as a marker for developmental neurotoxic effects, and see if the effects are related to the classical mechanism of cholinergic hyper-stimulation.

In study I, the mice used in the spontaneous behavior test, at 4 months of age, were also subjected to nicotine-induced behavior test, right after the spontaneous behavior test. These mice were administered a single subcutaneous (s.c.) injection of 80 µg nicotine base/kg bw or 10 ml 0.9% saline/kg bw, and were observed for another 60-min period. In the control animals, an increased activity was observed for all three test variables during the first 20-min test period (60-80 min) after the nicotine injection, which is a normal response in mice exposed to this dose of nicotine. A low dose of nicotine is known to induce hyperactivity, whereas a high nicotine dose can induce hypoactivity (Nordberg and Bergh, 1985). However, the mice exposed to 21 µmol PFHxS/kg bw showed an altered response to nicotine compared to the controls. These animals did not show an increase in activity for the locomotor and rearing variables compared to their saline-injected control (fig. 11). This shows that the PFHxS exposed animals expressed an altered response (susceptibility) of the cholinergic system. This is in line with the study by Johansson et al. (2008), where mice neonatally exposed to PFOS or PFOA did not respond to the nicotine injection with an increased activity, albeit for all three variables, as seen in control animals. Also, a recent study showed that neonatal exposure to a single oral dose of PFOS can induce changes in gene transcription of AChE and different nicotinic and muscarinic receptor subtypes in the hippocampus and cerebral cortex, 24h post-exposure (Hallgren et al., 2015). This is also supported by Lau et al. (2003), who observed a decreased activity of ChAT, in the prefrontal cortex in rats, after gestational exposure to PFOS.

Furthermore, altered cholinergic susceptibility has also been observed for animals neonatally exposed to DDT, pyrethroids and PBDEs (Eriksson, 1992; Viberg et al., 2003a). Those studies also showed changes in cholinergic receptor composition, with regional differences within the brain. This change in cholinergic susceptibility suggests that the cholinergic system is affected by the neonatal exposure, which may be a possible mechanism behind the observed developmental neurobehavioral effects.
Figure 11. Nicotine-induced behavior of 4 months old mice male NMRI mice exposed to single oral dose of either 20% fat emulsion, 1.4, 14 or 21 µmol PFHxS/kg bw, on PND 10. The nicotine-induced behavior was studied by using 80 µg nicotine base/kg bw (s.c.) and 10 ml 0.9% saline/kg bw (s.c.). The data was analyzed by one-way ANOVA with split plot design and the statistical differences are indicated as: 
(A) significantly different vs. internal control group i.e. saline-injected, p ≤ 0.01; (B) significantly different vs. saline-saline, p ≤ 0.01. The data is presented as mean ± SD, n = 14-18 animals/treatment group.

In study IV, AChE activity was measured in mice euthanized 1, 3, 6, 12, 24 or 36h after administration of a single oral dose of 14.3 µmol chlorpyrifos/kg bw (5 mg/kg bw); 25.0 or 99.4 µmol carbaryl/kg bw (5.0 or 20.0 mg/kg bw), respectively, on PND 10. Both doses of carbaryl caused a significant AChE inhibition, between 8 and 12%, at 1, 3 and 6h time point, with the highest
inhibition at 3h post-exposure, compared to time-matched controls (fig. 12). Chlorpyrifos did not induce a significant AChE inhibition, at any of the measured time points, however a non-significant 10% inhibition was seen at 3h post-exposure, which is similar to the AChE inhibition from carbaryl exposure.

Figure 12. Brain AChE activity levels in male NMRI mice at different time points after exposure to a single oral dose of 14.3 µmol chlorpyrifos/kg bw; 25 or 99.4 µmol carbaryl/kg bw, on PND 10. The data (moles acetylcholine hydrolyzed/min/g tissue) was subjected to a one-way ANOVA. The statistical differences are indicated as: (*) = 25 µmol carbaryl/kg bw vs. time-matched control, p ≤ 0.05; (#) = 99.4 µmol carbaryl/kg bw vs. time-matched control, p ≤ 0.05. The data is presented as mean ± SD and plotted as percentage of time-matched control, n = 4 animals/treatment/time point.

The AChE assay results, from study IV, show that carbaryl caused a significant AChE inhibition. However, we did not observe any acute neurotoxic symptoms of classical cholinergic hyper-stimulation (e.g. salivation, lacrimation and gastrointestinal secretions) in the mice exposed to any of the insecticides, during the whole experimental period. Generally, symptoms of cholinergic poisoning appear when the cholinesterase inhibition level surpasses ~70% (Slotkin, 2004).

This suggests that if the developmental neurobehavioral effects are caused by effects in the cholinergic system, it is probably induced by an alternative pathway rather than by the classical mechanism of AChE inhibition. Converging indications show that a transient cholinergic inhibition may be sufficient to induce neuronal impairments, considering the vulnerability of the developing brain and the influence the cholinergic system has in neuronal organization and brain function (Berger-Sweeney, 2003; Woolf and Butcher, 2011; Semple et al., 2013). It is, however, possible that the devel-
Developmental neurotoxic effects observed from anticholinesterase inhibitors are induced by alterations in other neurotransmitter systems, such as the monoaminergic or endocannabinoid systems (Aldridge et al., 2005; Carr et al., 2014), as these systems is a part of an intricate conjunction in the brain. In addition, it has become more evident that timing of exposure is a crucial factor to consider, as toxic insults during the BGS may lead to abnormal brain development and cognitive deficits (Berger-Sweeney and Hohmann, 1997; Eriksson, 1997).

Effects on neuroprotein levels in the mouse brain

In addition to the behavior and cholinergic markers, neonatal and adult neuroprotein levels were analyzed in mice neonatally exposed to PFHxS, endosulfan, cypermethrin, chlorpyrifos or carbaryl. The data from neuroprotein assays revealed regional changes in protein levels after exposure to the environmental toxicants. Interestingly, alterations in neuroprotein levels were more pronounced in the neonatal brains compared to the adult brains, which suggests that the neuroprotein changes may manifest differently, depending on the timing of the developmental stage. This also indicates that neonatal changes in protein levels may be another cause for the observed adult neurobehavioral effects.

In study II, neuroprotein levels were measured in mice euthanized 24h or 4 months after administration of a single oral dose of 14 or 21 µmol PFHxS/kg bw, on PND 10. The mice exposed to PFHxS showed significantly altered levels of CaMKII, GAP-43, synaptophysin and tau, 24h post-exposure, with regional differences between the cerebral cortex and hippocampus (fig. 13). At 4 months of age, the only alteration seen was a significant increase of tau in male mice exposed to 14 µmol PFHxS/kg bw, whereas no significant changes were observed in the female mice. A similar pattern of neuroprotein changes was observed in both male and female mice, indicating that there are no sex differences. Changes in neuroprotein levels, and altered spontaneous behavior, after neonatal exposure to PFOS or PFOA have also been previously reported by Johansson et al. (2008; 2009), which supports the results from study I and II. PFHxS, similarly to PFOS and PFOA, showed regional differences in neuroprotein changes. There also appear to be a temporal difference between neonates and adults. Notably, increased levels of tau protein were persistent in the adult brain, which suggests that abnormal phosphorylation can have occurred. Elevated levels of hyper-phosphorylated tau in the brain and progressive loss of cognitive function, attributed to cholinergic dysfunction, is often found in Alzheimer’s disease (Morris et al., 2011). Furthermore, gestational and lactational exposure to PFOS has shown to alter the gene expression of calcium-dependent signaling molecules, such as αCaMKII, calmodulin and a subtype of N
methyl D-aspartate (NMDA) receptor, in the hippocampus in rats (Liu et al., 2010).

**Figure 13.** Neuroprotein levels in neonatal male (upper panel) and female (lower panel) mice, 24h post-exposure, in hippocampus and cerebral cortex after exposure to a single dose of 14 or 21 µmol PFHxS/kg bw, on PND 10. The data was analyzed by one-way ANOVA and the statistical differences are indicated as: (***)) significantly different vs. control, p ≤ 0.001; (**) significantly different vs. control, p ≤ 0.01; (*) significantly different vs. control, p ≤ 0.05. The data are presented as mean + SD and plotted as percentage of control, n = 6-8 animals/treatment group.

In study III and IV, neuroprotein levels were also measured in male NMRI mice euthanized 24h or 4 months after administration of a single oral dose of an insecticide, on PND 10 (table 1). Similarly to PFHxS, more pronounced effects were seen in the neonatal brains, compared to the adult brains, for all
insecticides, except for cypermethrin. However, these effects on neurotransprotein levels appear to decrease or disappear over time, which indicates that the changes in protein level expressions, caused by neonatal exposure, may not be persistent or appear later in life.

Table 1. Summary of neuroprotein effects in male mice, 24h (neonate) or 4 months (adults), after a single neonatal exposure to different insecticides or PFHxS.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Neonates</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hippocampus</td>
<td>Cortex</td>
</tr>
<tr>
<td>Endosulfan (0.25-1.25 μmol/kg bw)</td>
<td>CaMKII ↓</td>
<td>Tau ↑</td>
</tr>
<tr>
<td>Cypermethrin (0.24-1.20 μmol/kg bw)</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Chlordane (1.3 μmol/kg bw)</td>
<td>CaMKII ↓</td>
<td>Synaptophysin ↓</td>
</tr>
<tr>
<td>Carbaryl (2.5-99.4 μmol/kg bw)</td>
<td>CaMKII ↓</td>
<td>GAP43 ↑</td>
</tr>
<tr>
<td>PFHxS (14 and 21 μmol/kg bw)</td>
<td>CaMKII ↑</td>
<td>Synaptophysin ↑</td>
</tr>
</tbody>
</table>

In general, effects in the hippocampus appear to be more adverse than in the cerebral cortex, as more proteins were affected. This could possibly be explained by the fact that these proteins have distinct ontogenic patterns during the BGS, with marked increases occurring around PND 7-14 (Martin et al., 1998; Viberg et al., 2008; 2009). Thus, depending on the timing of exposure, micro-distribution and the inherent chemical properties of the substance, these compounds may affect different regions of the brain differently.

Whether early changes in the investigated neuroprotein levels can lead to possible behavior impairment is an intriguing question. These proteins have specific neurological functions during brain development, and a shift in neuroprotein levels may possibly affect fundamental neuronal structure, transmitter systems and/or signal transduction. Interestingly, all of the investigated compounds, except for cypermethrin, induced changed levels of CaMKII. CaMKII is a key regulatory protein, involved in synaptogenesis and learning and memory formation, mediated via LTP/LDP, which facilitates the regulation of synaptic transmission (Lisman et al., 2002). Altered CaMKII expression can result in downstream effects, such as modified levels of GluR1 and synaptophysin, as these proteins can be phosphorylated by CaMKII. GluR1 facilitates excitatory neurotransmissions in synapses (Henley and Wilkinson,
2013) and synaptophysin is indicated in the regulation of synaptic transmission efficacy (Gordon and Cousin, 2014), thus, they are involved in behavioral modifications and learning, through signal transduction (Schmitt et al., 2003; Schmitt et al., 2009). Depending on the calcium level, alternative signaling pathways can also be activated, such as PKC, which regulates GAP-43 functions (Holahan and Routtenberg, 2008). GAP-43 is highly expressed during development and is responsible for neuronal plasticity. PKC and CaMKII signaling pathway may also converge further downstream to affect cytoskeletal proteins, such as tau (Lynch, 2004). Tau is known to have other functions in the developing brain, compared to the adult brain, and effects occurring during development are more related to dysfunctional axonal transport and protein scaffolding (Dixit et al., 2008; Ittner et al., 2010).

The accumulated results from study I to IV, suggest that behavioral impairment caused by changes in neuroprotein levels is plausible, as alterations in protein homeostasis during brain development may lead to structural and organizational alterations in the brain, and subsequent aberrant behavior and cognitive dysfunction. More importantly, the alterations in the neonatal brain are a strong confirmation that a disturbance in normal brain development has occurred.

Developmental neurotoxic effects of co-exposure to a binary mixture

Additional aims of this thesis were to investigate interaction effects of mixtures, combined from the earlier investigated persistent and non-persistent compounds. The results show that a single oral exposure to a binary mixture of two different non-persistent pesticides or two different persistent pollutants can interact to induce developmental neurotoxic effects in mice, when exposed during a critical period of brain development. The developmental neurotoxic effects were manifested as adult neurobehavioral changes and aberrant cognitive function. Also, the developmental neurotoxic effects could be induced at doses where the individual agents did not elicit an effect, and the interaction effects exacerbated the neurobehavioral outcomes.

Interaction effects from two different anticholinesterase inhibitors

In study V, male mice were administered a single oral dose of carbaryl, chlorpyrifos or a mixture, on PND 10. The treatments and dosages are summarized in table 2. As in the experiments with the single compounds, animals at the age of 2 and 4 months were subjected to a spontaneous behavior test in a novel home environment. To assess if the potential neurobehavioral
effects were related to the cholinergic system, a nicotine-induced behavior test was performed directly after the spontaneous behavior test at 4 months, and AChE activity was measured as in study IV.

Table 2. Treatments for mice exposed to a single oral dose of vehicle, carbaryl, chlorpyrifos or a mixtures on PND 10.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosages (µmol/kg bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20% fat emulsion vehicle</td>
</tr>
<tr>
<td>Carbaryl low</td>
<td>25.0</td>
</tr>
<tr>
<td>Carbaryl high</td>
<td>99.4</td>
</tr>
<tr>
<td>Chlorpyrifos low</td>
<td>14.3</td>
</tr>
<tr>
<td>Chlorpyrifos high</td>
<td>28.6</td>
</tr>
<tr>
<td>Carbaryl low + Chlorpyrifos low (Mix1)</td>
<td>25.0 + 14.3</td>
</tr>
<tr>
<td>Carbaryl low + Chlorpyrifos high (Mix2)</td>
<td>25.0 + 28.6</td>
</tr>
<tr>
<td>Carbaryl high + Chlorpyrifos low (Mix3)</td>
<td>99.4 + 14.3</td>
</tr>
<tr>
<td>Carbaryl high + Chlorpyrifos high (Mix4)</td>
<td>99.4 + 28.6</td>
</tr>
</tbody>
</table>

In the animals neonatally exposed to Mix2 or Mix4 a distinct neurotoxic effect was seen, expressed as persistent altered adult spontaneous behavior and a lack of habituation to a new home environment, compared to the control mice and mice exposed to the single compound (fig. 14). The mice exposed to the high carbaryl dose (99.4 µmol/kg bw), both doses of chlorpyrifos (14.3 and 28.8 µmol/kg bw), Mix1 or Mix3, also showed an altered spontaneous behavior, decreased activity during the first 20-min period however, were still able to habituate as the activity was low during the end of the last 20-min period (40-60 min), like the control mice. Interestingly, the pronounced interaction effects, induced by Mix2 or Mix4, appear to be enforced/driven by the high chlorpyrifos dose (28.6 µmol/kg bw), as Mix1 showed similar spontaneous behavior and reduced habituation as the high chlorpyrifos dose; however, Mix2 and Mix4 induced an even more adverse behavioral effect, which appears to be synergistic. In previous interaction studies with PBDEs, methyl mercury (MeHg) and polychlorinated biphenyls (PCBs), exacerbated persistent behavior effects were also seen after neonatal co-exposure to a binary mixture of PBDE + MeHg, PBDE + PCB or MeHg + PCB, in the spontaneous behavior, compared to single exposure (Eriksson et al., 2006; Fischer et al., 2008b; Fischer et al., 2008a). This suggests that persistent and non-persistent compounds similarly can interact to worsen developmental neurotoxic effects, compared to single compound exposure.

Moreover, the aberrant spontaneous behavior outcome induced by the mixtures was probably not caused by cholinergic hyper-stimulation. All of
the binary mixtures induced significant AChE inhibition in the brain at 1 and 3h post-exposure, compared to the time-matched controls, but the inhibition was again low. The AChE inhibition induced by the mixtures was similar to the levels of single carbaryl or chlorpyrifos exposure, seen in study IV i.e. around 10%, indicating that interaction and/or additivity is not a general mechanism for all studied endpoints.

Also, cholinergic susceptibility was assessed, in the nicotine-induced behavior test, at 4 months of age, which showed that the animals exposed to Mix2 or Mix4 did not respond in a normal way to the nicotine injection. This supports the observed aberrant behavior effects, as the mice exposed to the mixtures expressed a worse ability to habituate compared to the other treatment groups, which shows that carbaryl and chlorpyrifos can interact to enhance the neurobehavioral effects. This further supports the hypothesis that an alternative cholinergic pathway is involved, as transient cholinergic inhibition or other alterations in cholinergic components, such as cholinergic receptors, may be sufficient to induce developmental neurotoxic effects (Slotkin, 2004).
Figure 14. Spontaneous behavior (locomotion and rearing) in 4 months old male NMRI mice exposed to single oral dose of either 20% fat emulsion; 25.0 or 99.4 µmol carbaryl (CARB)/kg bw; 14.3 or 28.6 µmol chlorpyrifos (CPF)/kg bw; Mix1 (25 + 14.3 µmol/kg bw), Mix2 (25 + 28.6 µmol/kg bw), Mix3 (99.4 + 14.3 µmol/kg bw) or Mix4 (99.4 + 28.6 µmol/kg bw), on PND 10. The data was analyzed by one-way ANOVA with split plot design. The statistical differences are indicated as upper case (p ≤ 0.01) or lower case (p ≤ 0.05) letters, between the named alphabetic groups. The data are presented as mean value + SD, n = 12 animals/treatment.

Interaction effects between two different types of persistent pollutants

In study VI, interaction effects of co-exposure to different binary mixtures of two persistent pollutants, PFHxS and endosulfan, were studied. Male mice were administered a single oral dose of PFHxS, endosulfan or a mixture, on PND 10. The treatments and dosages are summarized in table 3.
At 2 months of age, these animals were subjected to a spontaneous behavior test in a novel home environment. The animals neonatally exposed to Mix2, Mix3 or Mix4 showed a distinct neurotoxic effect, manifested as altered adult spontaneous behavior and a lack of ability to habituate in a new home environment, compared to the control mice. At 4 months of age, when the animals were re-subjected to the spontaneous behavior test, the behavioral pattern for each group remained the same, meaning that the neurotoxic effects induced by Mix2, Mix3 and Mix4 still persisted (fig. 15).

This altered spontaneous behavior and lack of habituation is similar to the interaction effects seen by the binary mixture exposure in study V. Also, these interaction effects occur at doses where the single compounds have no effect. The interaction effects also enhanced the neurotoxic behavioral effects, compared to study I and III, as the PFHxS or endosulfan exposed mice only had a reduced ability to habituate. Similarly to study V, and the previous interaction studies with PBDEs, MeHg and PCBs, co-exposure to a binary mixture caused persistent deranged spontaneous behavior, altered ability to habituate and cognitive deficits in the adult mice (Eriksson et al., 2006; Fischer et al., 2008a; 2008b). Those studies, together with the studies in this thesis, show that exposure to different types of persistent pollutants, during a critical period of brain development, can induce similar functional behavior effects later on in life, at doses where the single compound were without effect.
Figure 15. Spontaneous behavior (locomotion and rearing) in 4 months old male NMRI mice exposed to single oral dose of either 20% fat emulsion; 14 or 21 µmol PFHxS/kg bw; 0.25 or 1.25 µmol endosulfan/kg bw; Mix1 (14 + 0.25 PFHxS + endosulfan µmol/kg bw), Mix2 (14 + 1.25 PFHxS + endosulfan µmol/kg bw), Mix3 (21 + 0.25 PFHxS + endosulfan µmol/kg bw) or Mix4 (21 + 1.25 PFHxS + endosulfan µmol/kg bw) on PND 10. The data was analyzed by one-way ANOVA with split plot design and the statistical differences are indicated as upper case letters (p ≤ 0.01), between the named alphabetic groups. The data are presented as mean value + SD, n = 12 animals/treatment.

Interestingly, we previously showed that neonatal exposure to PFHxS, or other persistent pollutants, to can induce a cholinergic effect in mice. Recently, exposure to endosulfan during gestation and lactation has been shown to affect the dopaminergic system in rats (Wilson et al., 2014). The study also showed that adult exposure did not affect the dopamine system and that developmental exposure appears to sensitize dopamine neurons to additional insults that occur later in life. Increased adult susceptibility to neurotoxic agents have previously also been shown, after neonatal exposure to nicotine, DDT or pyrethroids (Talts, 1996; Ankarberg, 2003). Taken together, this raises the question if interaction effects may be involved in other neurological and neurodegenerative disorders that manifest later in life, linked to failures in neurotransmitter systems, such as Alzheimer’s and Parkinson’s dis-
ease. Furthermore, the doses used in study V and VI, are comparable on a molar level to previous studies with POPs, thereby indicating that PFHxS and endosulfan can be equally potent in inducing similar developmental neurotoxic effects. Those studies, together with the present studies, show that exposure to different types of persistent pollutants, during a critical period of brain development, can induce similar functional behavior effects later on in life.

Lastly, in study V and VI, we observed a discrepancy compared to study I and IV, concerning the exposure to the single compounds. In study I, an altered spontaneous behavior and modified habituation was observed in the animals exposed to the 21 µmol PFHxS/kg bw dose, which was not seen in study VI. In study IV, the mice exposed to 25 µmol carbaryl/kg bw dose showed a changed spontaneous behavior, which was not seen in study V. Furthermore, this deviation in exposure response was also seen in another previously conducted interaction study with MeHg and chlorpyrifos (not included in this thesis, unpublished). These three studies taken together, suggest that the reason behind these discrepancies is probably due to a change of suppliers of NMRI mice, from a breeding in Scanbur, Sweden, to a breeding in Charles River, Germany. This indicates a difference in susceptibility between the NMRI mice from the different suppliers. However, the 14 µmol PFHxS/kg bw dose did not show any behavioral effect in any of the studies, and mice exposed to the 14.3 µmol chlorpyrifos/kg bw dose showed a decreased activity in all three variables in study IV, compared to one variable (rearing) in study V. This would also suggest that the dosages investigated are around the threshold for inducing a developmental neurotoxic response, which could be toxicologically relevant, as there can be differences in susceptibility between individuals in a population or between populations in real life.
Concluding remarks

The objectives of this thesis were to investigate the developmental neurotoxic effects of persistent and non-persistent environmental pollutants, and study the interaction effects of binary mixtures combined from these compounds, in mice.

This thesis has revealed that exposure to persistent or non-persistent pollutants, such as PFHxS, endosulfan, cypermethrin, chlorpyrifos or carbaryl, can induce developmental neurotoxic effects, when administered as a single oral dose during a defined critical period of the brain growth spurt. The animals showed distinct neurotoxic effects manifested as persistent aberrant spontaneous behavior in a novel home environment and reduced ability to habituate, which also indicates impaired cognitive function. Developmental effects on cholinergic susceptibility and changed neuroprotein levels may be possible explanations for the observed adult neurobehavioral effects. Still, due to the dynamic and probable compensatory mechanisms of the developing brain, it is unlikely that a single change in protein expression can be responsible for all of the functional neurotoxic effects. However, also considering that the cholinergic system is likewise developing during this period, it is likely that cholinergic components may have been affected, which is also indicated by the altered response to adult nicotine exposure. Taken together, this may have contributed to the observed developmental neurobehavioral aberrations. This further shows that the timing of exposure is a critical factor to consider when assessing developmental neurotoxic effects, and that altered levels of neuroproteins can serve as potential markers for developmental neurotoxicity.

This thesis also revealed that a single neonatal co-exposure to persistent pollutants or non-persistent pollutants can interact and exacerbate developmental neurotoxic effects. These neurotoxic effects were seen at dosages, where the individual pollutants did not elicit a response or induced a much weaker effect. Moreover, there are still concerns regarding the interaction effects of the binary mixtures of carbaryl and chlorpyrifos. The co-exposure of the binary mixture appear to have induced a synergistic effect on the behavior, however this was not reflected on the AChE measurements. This suggests that additivity is not a general mechanism for the interaction effects of anticholinesterase inhibitors. Therefore, the use of AChE as a biomarker for studying functional effects or mechanism of effects might be re-evaluated.
The results from this thesis are in line with reported epidemiological studies, indicating that developmental exposure to environmental toxicants can be a possible etiological factor for neuropsychiatric disorders, such as ASD and ADHD (Bouchard et al., 2010; Burns et al., 2013; Shelton et al., 2014). Additionally, animals exposed to PFHxS, showed elevated adult levels of tau protein, as well as a cholinergic effects, which are recognized characteristics of Alzheimer’s disease. There may be a probable involvement, as the thesis also shows that developmental co-exposure to environmental pollutants can enhance adult behavioral neurotoxic effects and cause changes in the cholinergic system. However, it also raises the question if individual environmental pollutants are involved in other neurological and neurodegenerative disorders too, such as Parkinson’s disease, and if interactions from environmental pollutants can further enhance these disorders.

The animal model used in the present thesis, has also shown to be a suitable test model for developmental neurotoxicity assessments, as direct exposure can be applied for detection of changes in the neonatal and adult animals induced by environmental pollutants. However, future research perspective should also involve understanding the link between functional behavioral outcomes and molecular mechanisms. Therefore, it is also important to include in vitro models for studying cellular processes in the developing nervous system, and the effects caused by exposure of these cell cultures. This would not only increase the knowledge and understanding of other future environmental compounds and their interaction effects, but also facilitate the finding of potential prediction or prevention models for developmental neurotoxicity.
Svensk sammanfattning (Swedish summary)

Utvecklingsneurotoxikologiska effekter av persistenta ämnen och pesticider efter neonatal exponering i möss

Denna avhandling avser att undersöka utvecklingsneurotoxiska effekter av persistenta ämnen som perfluorerade (hög fluorerande) ämnen samt olika typer av bekämpningsmedel (pesticider) efter neonatal exponering under ett kritiskt stadium av hjärnans utveckling hos nyfödda möss. De ämnen som specifikt undersöks är: perfluorohexansulfonat (PFHxS), endosulfan, cypermetrin, klorpyrifos och karbaryl.


Hjärnans utveckling kan delas in i olika mognadsfaser, och karaktäriseras av olika processer, vilka sker vid specifika tillfällen. Den neonatala utvecklingsperioden (kallad ”brain growth spurt” på engelska) hos däggdjur, inklusive människan, karaktäriseras av mycket snabb tillväxt och utveckling av synapser, grundläggande signalsubstanssystem, samt fastläggande av grundläggande beteenden. Tidigare studier har visat att exponering för olika xenobiotika under den neonatala utvecklingsperioden kan ge upphov till bestående effekter i det centrala nervsystemet, som kan yttra sig som beteendeförändringar senare i livet. Den neonatala utvecklingsfasen sker vid olika tidpunkter under utvecklingen och är annorlunda hos olika däggdjursarter. Hos möss börjar denna fas vid födseln och pågår under de första 3-4 veckorna. Hos människan börjar denna fas under den sista trimestern av graviditeten och fortlöper under de två första levnadsåren. För båda arterna överlappar utvecklingsfasen laktationsperioden, vilket gör att avkomman kan exponeras för toxiska föreningar via modersmjölken.
Hjärnans tillväxt och mognad under denna utvecklingsfas involverar komplexa förändringar och processer som regleras och kontrolleras av olika neuroprotein. Dessa proteiner är nödvändiga för normal neurittillväxt och synaptogenes, och uppvisar ett specifikt ontogenetiskt mönster under den neonatala utvecklingsfasen hos möss. Under denna period utvecklas även delar av det kolinerga systemet, vilket är starkt kopplat till beteende och kognitiva funktioner.

De första studierna i denna avhandling syftade till att undersöka huruvida de enskilda föroreningarna (PFHxS, endosulfan, cypermetrin, klorpyrifos och karbaryl) kan orsaka effekter i spontanbeteende hos vuxna möss som utsätts för en ny hemmiljö, efter att ha exponerats för en oral dos av respektive substans, under den neonatala utvecklingsperioden. Spontanbeteende kan användas som ett mått på kognitivfunktion, då det mäter mössens förmåga att bearbeta sensoriska intryck från en ny hemmiljö som de placeras i. En normal respons till en ny hemmiljö är gradvis minskning av aktivitet över tid, allteftersom hemmiljön blir mer familjär, vilket visar en förmåga att kunna anpassa sig.

Resultaten från de första studierna visade att alla de undersökta substansen gav upphov till förändrat spontanbeteende hos vuxna djur. De effekter som observerades var en hyperaktivitet och minskad förmåga att anpassa sig i den nya hemmiljön, gentemot kontrolldjur. Beteendeffekterna var även bestående, då samma beteendemönster upptäcktes när de exponerade djuren åter testades några månader senare. Beteendeffekterna var även kopplade till förändrade nivåer av neuroprotein i hjärnan och förändrad respons/reaktion mot nikotin. Förändringar på proteinnivå, under den neonatala utvecklingsperioden, kan leda till organisatorisk omprogrammering av hjärnans strukturella och funktionella processer, vilket i sig troligen leder till avvikande hjärnuttveckling. Förändrad respons mot nikotin tyder på att det kolinerga systemet kan ha blivit påverkat, vilket också skulle kunna vara en bakomliggande faktor till de observerade beteendestörningarna. Andra studier har visat att skador på det kolinerga systemet under utvecklingsfasen, kan ge upphov till störningar i beteende och kognitiva funktioner. Detta tyder på att förändringar i neuroprotein-nivå och i det kolinerga systemet kan vara möjliga orsaker till beteendestörningarna.

Då miljöföroreningar sällan förekommer enskilt i miljön, var det även av intresse att studera huruvida dessa substanser kan orsaka interaktionseffekter. Inom denna avhandling undersökte jag därför huruvida två persistenta föreningar (PFHxS och endosulfan) eller två icke-persistenta pesticider (klorpyrifos och karbaryl), kan samverka och orsaka en så kallad ”cocktaileffekt” på spontanbeteende hos vuxna djur, när de exponerats för en binär blandning under den neonatala utvecklingsperioden. Resultaten från interaktionsstudierna visade att dessa substanser kan samverka och ge förstärkta beteendeförändringar i vuxen ålder, efter neonatal exponering. Dessa effekter sågs inte hos djuren som exponerades för de enskilda substansen.
Sammanfattningsvis visar denna avhandling att olika typer av miljöför-oreningar, oavsett ifall de klassas som persistenta eller icke-persistenta, kan orsaka utvecklingsneurotoxikologiska effekter, då exponering sker under den neonatale utvecklingsperioden. Interaktionseffekter av persistenta eller icke-persistenta föroreningar kan även orsaka kraftigare beteendestörningar i vuxen ålder, vid doser som enskilt inte ger upphov till någon neurotoxisk störning. Detta är en angelägenhet som måste belysas mer, då vi i dagens samhälle exponeras för mer en substans i taget, och doser vi ansett som säkra därför kan vara missvisande. Trots svårigheterna krävs mer forskning på interactionseffekter för att förstå de eventuella risker som miljöföroringarna utgör, samt deras samverkan.
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