

Organophosphate based pesticides and ADHD

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Abstract

Attention-Deficit Hyperactivity Disorder (ADHD) is a neurobehavioural and neurodevelopmental disorder that is caused by a dopamine deficiency in the prefrontal cortex and an overall incomplete dopaminergic functioning. 3 to 5% of U.S. and Dutch children are affected by ADHD. Most children are diagnosed with ADHD by the time they go to school. During their childhood and also throughout adulthood they can suffer from ADHD, which is characterised by social, emotional and cognitive dysfunctioning.

Inherited genetic variation of genes that involve the dopaminergic system result in an increased risk of developing ADHD. These genes encode for dopamine receptors 2 to 4 (DRD2-4), dopamine transporter (DAT), norepinephrine transporter (NET), monoamine oxidase-A (MAO-A) and catechol-O-methyltransferase (COMT). The environment can also lead to an increased risk of developing ADHD. Studies showed that exposure to organophosphate based pesticides (OPs) is associated with the incidence of ADHD. OPs have a high affinity to acetylcholinesterase (AChE), nicotinic acetylcholine receptor (nAChR) and adenylyl cyclase (AC). AChE and nAChR are involved in the cholinergic transmission leading to regulating dopamine neuronal activity. AC is located in the postsynaptic neuron that is stimulated by dopamine neurons. Inhibition of AChE and nAChR result in disrupted cellular response of the dopamine neurons. When OP is bound to AC in the postsynaptic neuron, AC regulation by G-protein coupled DRDs result in disrupted cellular responses of the postsynaptic neuron. Neuropathy target esterase (NTE) is an enzyme that regulates intracellular membrane trafficking in neurons. During neurodevelopment it is a key component in regulating migration and differentiation of neurons. During adulthood, NTE is critical in axonal maintenance. NTE is also inhibited by OPs. OP exposure is associated with ADHD because OPs disrupt key components considering dopaminergic cell signalling and neurodevelopment possibly leading to behavioural disorders such as ADHD.

The aim of this paper is to clarify the exact link between OP exposure during the sensitive phase of neurodevelopment, disorders in neurodevelopment and the incidence of ADHD. It appears that the risk of the occurrence of ADHD can be due to genetic variation leading to a dysfunctional dopaminergic system or to early exposure to OP that causes deregulated cell signalling during the sensitive neurodevelopment phase. This also leads to a dysfunctional dopaminergic system. There were no publications available indicating a relation between NTE inhibition and ADHD. Furthermore, considering that NTE inhibition can lead to Creutzfeldt-Jakob like symptoms and the fact that NTE is located in long axons it appears very unlikely that OP causes ADHD via NTE inhibition. Due to the lack of relevant publications, it can be considered less likely that OP exposure results in ADHD via AC reprogramming. A model is provided showing the possible pathways towards development of ADHD. Pathways that need more investigation are also pointed out. Based on current available publications it can be concluded that it is very likely that exposure to OP increases the risk of developing ADHD. Inhibition of AChE and nAChR by OPs is the most likely pathway leading to ADHD symptoms.

Chapter 1. Introduction

1.1 Attention-Deficit Hyperactivity Disorder in short

Attention-Deficit Hyperactivity Disorder, or ADHD, is a chronic [1], clinically heterogeneous disorder of neurobehavioral and developmental kind [2-4]. The main factors of ADHD are prefrontal dopamine deficiency and an incomplete central dopaminergic functioning [5]. ADHD is one of the most frequent psychiatric disorders of childhood [6]. 3 to 5% of all U.S. and Dutch children are affected by ADHD [4, 7]. Most children are first diagnosed with ADHD at the age when they go to school for the first time [8]. Of the total school attending children, 2 to 16% have been diagnosed with ADHD [9] and around 75% of these children are male [8]. Individuals with ADHD also have more costs on medical care. These costs increase per child by €409,- to €1092,- per year. Adults with ADHD have a cost increase of €2342,- to €2811,- per year on medical care [10].

Symptoms of ADHD can put pressure on various aspects of child development. These aspects include social, emotional and cognitive functioning [6] and continue to affect the individual throughout adolescent life. However, prevalence of ADHD in adults is gradually more recognised [2]. Furthermore, studies confirm that individuals with ADHD symptoms are more likely to commit crimes than non-ADHD individuals. The arrest rate for adults with ADHD is 46% higher (versus 11% control) and for young adults with ADHD that rate is 21% higher (versus 1% in control). ADHD patients are also more likely to cause (more) traffic accidents [11].

The behavioural symptoms of ADHD can be categorised into two primary categories: impulsivity/hyperactivity and inattention but also a combination of both exists [8]. ADHD can be a persistent disorder that can be predicted via family history on ADHD, comorbidity and psychosocial difficulty. Studies on the occurrence of ADHD within families support that this disorder is of a strong inheriting nature. The ADHD disorder is also highly correlated with other behavioural disorders. Other factors that may cause increased risk of ADHD are the natural environment (air pollution including maternal smoking during pregnancy) and family environment [2].

In about 75% of all ADHD cases, genetics can cause increased risk for developing ADHD [12]. A combination of various genes gives rise to the known symptoms in ADHD cases. These genes include dopamine receptors D_2/D_3 and D_4 (DRD2-4) [13-15], dopamine transporter (DAT) [13], norepinephrine transporter (NET) [16, 17], monoamine oxidase A (MAO-A) [18, 19] and catechol-O-methyltransferase (COMT) [20]. DAT, DRD2-4 [13] and NET [17] genes are downregulated and the COMT gene is upregulated [21]. No publications are available mentioning up- or downregulation of the MAO-A gene. Volkow, *et al* (2009) showed that there is an association between reduction of components that consider the dopamine neurotransmission (such as DAT's and D_2/D_3 receptors) and ADHD symptoms [13]. This would indicate that disruptions occur on the presynaptic neuron (DAT1, NET, DRD2 and DRD3) as well on the postsynaptic neuron (DRD1-5, MAO and COMT) [13]. Dopamine synaptic component reduction affects the general dopamine system, which is also involved in the reward system. Persons with ADHD or similar behavioural disorders have lowered dopamine secretion. In order to reduce ADHD symptoms, additional dopamine is required. This results into a low or hypo-dopaminergic trait, where the brain requires more dopamine in order to maintain reduced ADHD symptoms. Ritalin, a commonly used stimulant by ADHD patients, increases the level of available dopamine by blocking the transporter that removes dopamine [2]. A similar effect can be achieved by drugs of abuse. For example, exposure to speed, marijuana and cocaine result in higher dopamine levels in the brain that may circumvent any undesired feelings [22]. This association with the reward pathways implies a greater risk for drug abuse [13].

Environmental factors can also be the cause of ADHD. A possible correlation is found between smoking and alcohol consumption during pregnancy and the incidence giving birth to children that will be diagnosed with ADHD [23]. PCB and DDT exposure has also been positively correlated to neurological impairment/ADHD [24-26]. PCBs can mimic thyroid hormones [24]. This leads to disruption of thyroid hormone signalling [27] that can lead to neurodevelopmental damage [28]. DDTs induce a hyperexcitable state in the brain [29] that might lead to disruption of neurodevelopment. However, DDT and PCB exposure is not the focus of this paper. Exposure to organophosphate (OP) pesticide and the risk of developing symptoms of ADHD have also been positively correlated [30]. OPs can inhibit nicotinic acetylcholine receptors (nAChR) [31] and acetylcholine esterase (AChE) [32-34]. Another target of OPs is neuropathy target esterase (NTE) [34], a lysophospholipase [35], which is an endoplasmic reticulum (ER)-anchored protein and is primary distributed in the nervous system. It is an essential enzyme during neural development. In adults, NTE is more or less restricted to large neurons where the enzyme plays a role in axonal maintenance. Disruption of NTE can lead to the occurrence of neuro-degenerative symptoms [36]. Studies on the effect of NTE inhibition on brain development of children have not yet been published. OPs can also bind to adenylyl cyclase (AC) leading to reprogramming of AC in differentiating neurons [37]. Reprogramming AC during the sensitive state of development might lead to disrupted brain development.

1.2 Aim

As mentioned earlier, 3 to 5% of U.S. and Dutch children are diagnosed for ADHD [4, 7], which can cause severe problems during childhood and adulthood [8] and also have higher cost on medical care [10]. Several associations have been made between OP exposure, neurodevelopment and ADHD [8, 13-15, 30, 36, 38-40] and although the risk of ADHD symptoms can be increased due to genetic origin [13-15], other cases of ADHD might occur due to low OP exposure at an early age, during their sensitive phase of development [30]. The latter cases can also happen due to genetic background but OP exposure might elicit ADHD symptoms. Without OP exposure these cases might not develop (severe) ADHD symptoms. The exact link between OP exposure and the incidence of ADHD is not yet clarified. This paper aims to connect these two via each mechanisms of action.

1.3 Approach, research questions and hypothesis

In order to find a possible connection between ADHD symptoms and OP exposure via each mechanism of action, the individual mechanism of action are to be discussed. Once a clear view on these mechanisms of action is established based on the latest research, a mechanistic connection might be found between ADHD and exposure to OPs.

The research questions addressed in this report are: what mechanisms of action are involved in ADHD symptoms and OP exposure and how can these be connected? What are the mechanisms of action of OP activity on AChE, nAChR, NTE and AC? What relationship exists between ADHD symptoms and neurotransmitters such as dopamine, norepinephrine? Finally, how does OPs and AChE interaction relate to ADHD and neurotransmitters?

As mentioned earlier, ADHD is strongly associated with dopaminergic and noradrenergic system dysfunction [13-15]. OPs are known to inhibit nAChR [31] and AChE [32-34]. nAChR inhibition causes reduced cellular response of dopamine neurons and subsequent AChE inhibition results in accumulation of ACh. This overstimulates the postsynaptic neuron at first [34] but the nAChR become desensitised so cellular response of dopamine neurons is even more reduced. Dopamine

receptors will therefore secrete less dopamine at their synapses [41]. Whether this is the exact cause of ADHD symptoms remains unclear. Another option is the interaction of OPs and NTE, which plays an important role during neurodevelopment [36, 38]. Dysfunction of NTE and AC reprogramming might cause disruptions in neurodevelopment, leading to neurologic disorders, possibly including ADHD.

Chapter 2. The dopaminergic system

The dopaminergic system or pathways are concerned with transmitting dopamine through the brain. It controls functions such as reward, pleasure and motor functions [41]. The dopaminergic system consists of eight individual pathways. The main four pathways are the mesolimbic pathway, mesocortical pathway, nigrostriatal pathway and the tuberoinfundibular pathway. Most pathways end in regions of the basal ganglia or the prefrontal cortex (PFC) [42]. These pathways are shown in figure 1 [43]. The mesolimbic pathway starts in the midbrain in the ventral tegmental area (VTA). The VTA contains dopamine, GABA and glutamate neurons. VTA dopamine neurons are connected via the nucleus accumbens, the amygdale and the hippocampus to the limbic system. They are also connected to the medial prefrontal cortex (PFC). It is associated with feelings of reward (or motivation) [44]. The mesocortical pathway connects the VTA to the cerebral cortex, mainly the frontal lobes. It is associated with thinking and working memory [44]. The nigrostriatal pathway connects the substantia nigra to the striatum and it is concerned with motory functions. The tuberoinfundibular pathway connects the tuberal region (arcuate nucleus of the hypothalamus) to the median eminence (infundibular region). This pathway controls prolactin secretion from the anterior pituitary gland [45]. Neurotransmitters such as ACh, GABA and glutamate regulate the dopamine neurons in the basal ganglia [41].

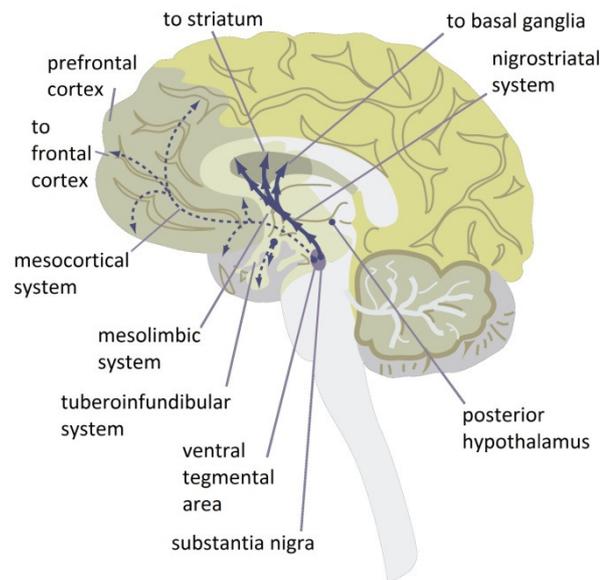


Figure 1. The four major dopaminergic pathways and their associated brain regions. DA neuronal signals originate from the VMA and are connected to various regions of the brain, depending on the type of dopaminergic pathway [42]. Adapted from [43]

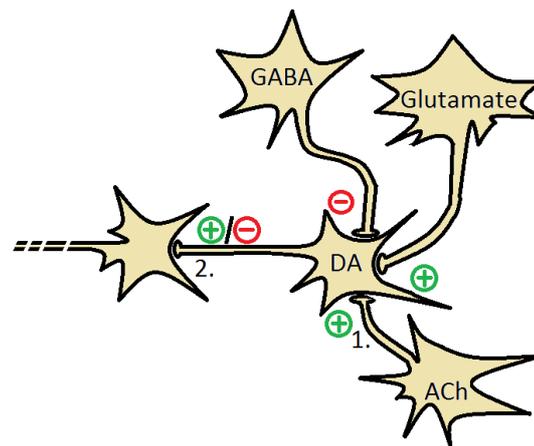


Figure 2. Overview of initiation of dopaminergic transmission. Glutamate and ACh stimulate the dopamine (DA) neuron and GABA inhibits DA neuronal activity. The DA neuron excites when the balance between stimulation by glutamate and ACh neurons and inhibition by GABA neurons exceeds the neurotransmission threshold level [42]. The DA neuron is connected to other neurons in other regions of the brain as described in figure 1. Number 1 and number 2 are explained in figure 3 and figure 4, respectively.

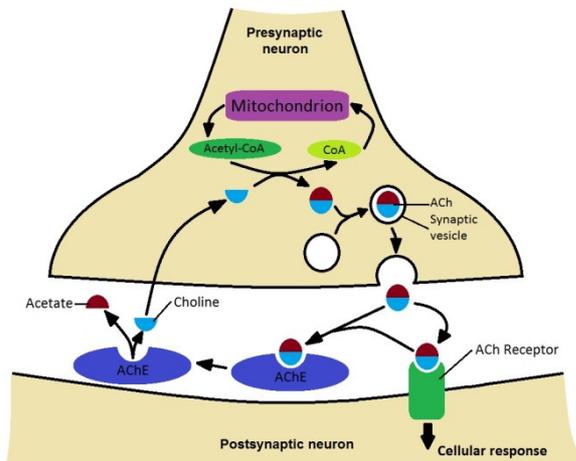


Figure 3 Cholinergic nerve transmission [42] as represented by point 1 in figure 2. Mitochondria secrete Acetyl-CoA that is bound to choline, thereby forming ACh. Upon nerve impulses into the presynaptic neuron, ACh is secreted into the synaptic cleft where binding to ACh-R takes place that results in postsynaptic neurotransmission [41]. ACh is degraded into acetate and choline by AChE. Choline is then transported into the presynaptic neuron [42]. Figure adapted from [46].

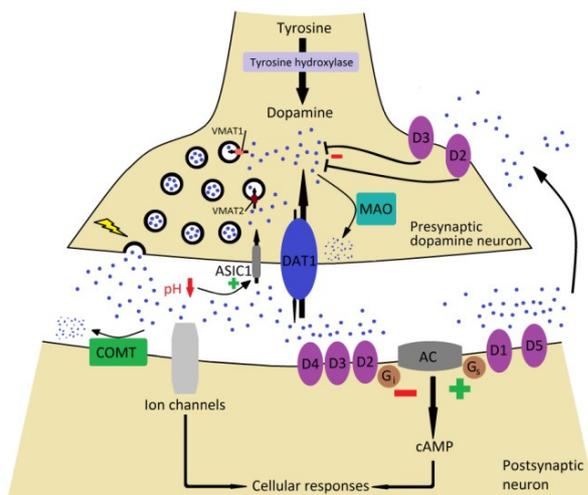


Figure 4. Interactions at the postsynaptic dopamine neuron as indicated at point 2 in figure 2. Tyrosine is converted to dopamine by TH followed by actions of VMAT1, which transports dopamine into vesicles. Upon neural impulses, dopamine is secreted into the synaptic cleft where it can bind to DRD1 to 5 that result in stimulation of AC activity via a receptor bound G-protein. Consequently, AC increased cAMP synthesis. Binding of dopamine to DRD2-4 leads to inhibition of AC via their G-protein. cAMP synthesis is then decreased. Dopamine is then either degraded by MAO or COMT or taken up via ASIC1 or DAT1 into the presynaptic neuron. Non-degraded dopamine is reused by VMAT2 that transports dopamine into vesicles. Dopamine spill outside the synaptic cleft binds to DRD2 and DRD3 located on the presynaptic neuron outside the synaptic cleft. This results in negative feedback on dopamine secretion. Figure adapted from [59].

The dopamine pathway starts by the stimulation of dopamine neurons. Stimulation of these neurons is regulated by a balance between cholinergic, glutamatergic and GABAergic neurotransmission [41] as shown in figure 2. Cholinergic and glutamatergic neurons induce dopamine neurons and GABAergic neurons inhibit dopamine neurons [41]. Figure 3 shows the cholinergic nerve transmission [46]. ACh is synthesised in the presynaptic neuron and is secreted into the synaptic cleft. Upon binding to the acetylcholine receptor (AChR), postsynaptic neurotransmission takes place. The AChR can be a muscarinic receptor (mAChR) or a nicotinic receptor (nAChR). The latter is mainly involved in activation of dopaminergic neurons [42]. AChE hydrolyses ACh into acetate and choline of which the latter is transported into the presynaptic neuron [46]. Glutamic acid, or glutamate is an abundant excitatory neurotransmitter in the vertebrate CNS. In the developing brain, glutamate regulates neocortical growth [47, 48]. Glutamate plays an important role in cognitive functions; learning and memory. The connection between glutamate and the mentioned cognitive functions is associated with the capacity of N-methyl-D-aspartate (NMDA) receptors to stimulate hippocampal long-term potentiation, which is a process related to synaptic plasticity [49]. Glutamate also plays a role in modulating the activity of midbrain dopaminergic neurons [41]. Gamma-aminobutyric acid, or GABA, is the main inhibitory neurotransmitter in the adult mammalian CNS. During the development of the CNS, GABA is an excitatory neurotransmitter before glutamate synapses mature. Thereafter, GABA acts as an inhibitory neurotransmitter [50, 51]. During the developmental stage, GABA acts as a autocrine as well as paracrine signal molecule [52]. GABA is involved in the regulation of the proliferation of neural progenitor cells [47, 48]. Furthermore, GABA regulates migration and differentiation, the elongation of neurons and the development of synapses [51, 53-56].

Dopamine is a catecholamine neurotransmitter that can excite and inhibit postsynaptic neurons, depending on the dopamine receptor profile of these neurons [57]. This neurotransmitter is widespread in the brain and affects sleep, mood, attention and learning [58]. A schematic overview of dopamine neurotransmission is shown in figure 4 [59]. Dopamine is derived from tyrosine and is the precursor to norepinephrine and epinephrine [57]. Tyrosine hydroxylase (TH) is the enzyme that converts tyrosine into dopamine. Furthermore, TH plays an important role in the physiology of adrenergic neurons because it is the limitation to the maximum rate catecholamines can be formed. [60]. Dopamine is released in the striatum and PFC and plays a major part in motor control and attention [61]. In the striatum and PFC, dopamine is released in the synaptic cleft where it binds to DRD1 to 5. After DRD activation, dopamine is deactivated by reuptake through DAT that is located in the presynaptic neuron [62] or broken down by monoamine oxidase (MAO) [63] or COMT [20]. These receptors are differentially distributed within the brain and can initiate a cascade of postsynaptic signalling. Eventually, the signal can lead to altered gene expression via the AC system [62] or regulate movement and cognitive functions.

Dopamine in the synaptic cleft can bind to 5 types of dopamine receptors, DRD1 to DRD5. DRD1 is the most abundant dopamine receptor in the CNS. It is a G-protein coupled receptor that stimulates AC thereby activating cAMP-dependent protein kinases. DRD1 activity is associated with neuronal growth and development and the mediation of behavioural responses [64]. DRD2 is also a G-protein coupled receptor but unlike DRD1, it inhibits AC activity thereby inactivating cAMP-dependent protein kinases [65]. DRD3 is a G-protein coupled receptor that inhibits AC activity and is associated with cognitive, emotional and endocrine functions [66]. DRD4 is also a G-protein coupled receptor that inhibits AC activity. [67]. DRD5 is a G-protein linked receptor that stimulates AC activity and is located in the limbic areas of the brain. Its affinity to dopamine is ten times higher than DRD1 [68].

After interaction with dopamine receptors, dopamine is removed from the synaptic cleft. This is done via MAO, COMT, acid-sensing ion channel 1 (ASIC1), DAT1 and NET. MAO catalyses the oxidation of monoamines and is located mainly in the outer membrane of mitochondria [69]. Distribution of MAO is 70% in mitochondria, 24% in microsomes and 1% in soluble form [70]. In humans two types MAO exist; MAO-A and MAO-B. MAO-A degrades, norepinephrine, epinephrine and serotonin. The function of MAO-B is catalysing xenobiotic amines via oxidative deamination [71, 72]. Both MAO types are able to degrade dopamine. COMT methylates catecholamines such as dopamine, norepinephrine and epinephrine resulting in the initiation of a degradative pathway of the catecholamine neurotransmitters. Two forms of COMT are known; a soluble and a membrane-bound form [73]. As dopamine accumulates in the synaptic cleft, the pH lowers. This activates ASIC1 that transports dopamine into the presynaptic neuron. DAT1 is expressed in few neurons in the brain, mostly in striatum and nucleus accumbens, however DAT1 is also found in other brain regions such as the cingulate cortex and the midbrain [74]. DAT1 is a Na^+/Cl^- transmembrane transporter that regulates the amount of dopamine in the synaptic cleft [75]. The function of NET in the adult nervous system is internalisation of norepinephrine from the synaptic cleft [76]. In brain regions with low levels of DAT, such as in the prefrontal cortex (PFC), NET also internalises dopamine [77]. In the developing nervous system, NET initiates neural crest stem cells to differentiate, for example, into noradrenergic neurons [76].

Storage of neurotransmitters in secretory vesicles is established by vesicular neurotransmitter transporters such as Vesicular Monoamine Transporters (VMAT). Overall, VMAT controls trafficking of neurotransmitters along the presynaptic neuron. Altered expression of the

VMAT gene can influence the level of neurotransmitter storage [78]. Another essential component of intracellular membrane trafficking in neurons is NTE [79], which plays an important role during (early) neurodevelopment [29] and axonal maintenance in adults [36]. NTE is not specifically associated with dopamine neurons, yet, since no publications exist discussing such association. However, NTE is also a target of OPs and it could be expected that NTE inhibition by OPs causes disruption of neurodevelopment [79].

Chapter 3. Neuropathy Target Esterase

NTE is a membrane-bound enzyme [33] that plays a vital role in the axonal maintenance in the adult brain by regulation of phospholipids [36]. This is done via a cell-signalling pathway that is involved in interactions between neurons and accessory glial cells in the developing nervous system [80]. In adolescents, NTE is found in all brain regions especially in the cortex, hippocampus and Purkinje cells of the cerebellum [36]. The NTE molecule consists of two distinct functional domains; a regulatory N-terminal and a catalytic C-terminal. A transmembrane segment is located at the end of the N-termini. Several cyclic nucleotide binding domains are located in the regulatory domain suggesting NTE regulation by binding of cyclic AMP. Another sequence in this domain suggest possible binding of ubiquitin by which NTE would be degraded via the ubiquitin-proteasome pathway [36]. Most part of NTE is located on the cytoplasmic side of the ER [81].

NTE has several biological functions. NEST, the recombinant esterase domain of NTE, has the ability to catalyse hydrolysis of naturally membrane-associated lipids suggesting that NTE might be concerned with intracellular membrane trafficking [79] in other words, NTE regulates phospholipid metabolism. A significant part of the total brain lysophosphatidylcholine (lysoPC) is hydrolysed by NTE [82]. Maintenance of lysoPC homeostasis is important since it is assumed that excess lysoPC can prevent Golgi vesicles from fissioning and thus slowing down intracellular trafficking [83]. It appears that NTE is not related to processes involving serine hydrolases such as AChE [80].

NTE is also an important enzyme during early neurodevelopment [84]. In mice, NTE is expressed from embryonic day 7 and all the way through development of the embryo [36]. It appears that NTE is not needed for cell division during early mammalian embryo development. NTE is important for placenta formation [85] and for the formation of the neuronal labyrinth layer [36]. NTE as well as the dopaminergic system can be targeted by OP based pesticides that may result in neurodevelopmental disorders such as ADHD [30, 37, 39, 41, 79, 85, 86].

Chapter 4. Organophosphate based pesticides

The most commonly used insecticides are OP pesticides, carbamates and previously, organochlorines [87]. Since 1972, the latter are largely replaced in the U.S.A. by OP (such as Parathion and Chlorpyrifos) and carbamates (such as Carbaryl and Carbofuran) [88]. This occurred because organochlorines, such as DDT were shown to be highly persistent and potentially bioaccumulative [89] due to their relative low breakdown rate [29] and high hydrophobicity [90]. By 2001, DDT and other organochlorines had been banned for worldwide agricultural use [34]. OPs are the largest group of pesticides that are used worldwide [34]. OP and carbamates are strong inhibitors of

carboxylic ester hydrolases such as acetylcholinesterase (AChE) [33]. This makes them effective insecticides because AChE inhibition results in acetylcholine (ACh) accumulation in the synaptic cleft [29], which leads to neuronal overstimulation resulting in death [34].

Besides application as pesticides, OPs are also used as chemical weapons such as Sarin and VX [91]. Discovered during Nazi Germany, Sarin was accepted as standard chemical weapon by the NATO and was produced by the U.S.A. and the former U.S.S.R. During the war between Iraq and Iran in the 1980s, Sarin was deployed by Iraq. VX was produced by the U.S.A. in large volumes during the 1960s [92]. In 1994 and 1995, Japan suffered from two terrorist attacks by members of the Aum Shinrikyo cult, who used Sarin in a residential area in Matsumoto and in the Tokyo subway system that resulted in a dozen deaths and hundreds injured [93].

Around 40 OP pesticides are registered for use in the U.S.A. with the US Environmental Protection Agency [32]. In 2001, the amount of OPs used in both agricultural and at homes reached 73 million U.S. pounds [30, 94]. However, a decline of more than 10% of OP (and carbamate) use was noticed in 2007 [88]. The reason for this decline is unclear. The major sources of OP exposure include food, drinking water and home-use pesticides [95]. Infants and children are mainly exposed to OPs via their diet [30, 94]. For instance, detectible concentrations of OP malathion was found on 19 to 28% of analysed fruit samples [96].

The general chemical structure of OPs is shown in figure 5 [97]. Organothiophosphates have double bonded sulphur at A. However, they are converted in the liver to OPs, which have double bonded oxygen. R is usually an ethyl or a methyl group. The group at X is the specific group that identifies an OP and is also the primary metabolite [29]. Examples of three OPs, parathion, malathion and dichlorvos, are shown in figure 6 [97] including their respective oral and dermal LD₅₀ in rat.

Absorption of OPs occurs very efficiently via inhalation, ingestion and skin penetration. Many variations exist in the relative absorption considering the LD₅₀. For example, the LD₅₀ of parathion in rats via oral exposure is 3 to 8 mg/kg, which is more or less equal to LD₅₀ of 8 mg/kg via dermal exposure. However, phosalone has a dermal LD₅₀ of 1500 mg/kg as opposed to an oral LD₅₀ of 120 mg/kg [98]. It appears that compounds with a high general LD₅₀ have probably also a high dermal LD₅₀ [29]. This might be due to lipophilic properties of toxic agents. For instance, parathion is more lipophilic than dimethoate [99] and these compounds have a rat dermal LD₅₀ of 8 mg/kg [98] and an average rat dermal LD₅₀ of 350 mg/kg, respectively [100]. These doses differ from the doses stated in figure 6. This is probably caused by differences between studies.

OPs are broken down by hydrolysis in the liver. Hydrolysis ratio depends on the type of compound. Since hydrolysis occurs relatively slow, temporary storage in fatty tissue takes place. Highly lipid soluble OPs tend to cause delayed toxicity due to their late release [90]. Most organothiophosphates are converted from thions (P=S bond) to oxons (P=O bond) in the liver. Both are hydrolysed, yielding alkyl phosphates followed by excretion. Most pesticides such as chlorpyrifos have low potential on inhibiting AChE. These thion OPs generally target nAChR [31]. After

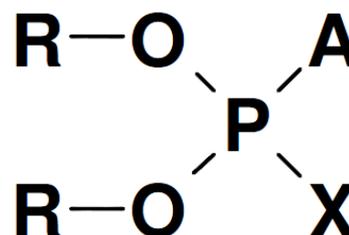


Figure 5. General structure of OP [97].

	LD ₅₀ rat (mg/kg)	
	oral	dermal
 Parathion	13	21
 Malathion	1375	>4400
 Dichlorvos	80	107

Figure 6. Examples of OPs and their respective oral and dermal LD₅₀ in rat (mg/kg) [97].

metabolisation, oxon OPs act as highly potent AChE inhibitors [31], which causes ACh accumulation to occur [101, 102]. Bio-inactivation occurs via additional conversions of oxons followed by excretion from the body [29].

OPs (and also carbamates) interact with the same serine residue on AChE where AChE binds to ACh. OP-AChE intermediates are formed [103, 104], as shown in figure 7. Phosphorylated AChE is much more stable and has a lower hydrolysis rate than the AChE-ACh intermediate. The regeneration rate is therefore very slow and for some phosphorylated AChE that rate is so slow that the AChE enzyme can be considered inactive [103, 104]. Overall, compound affinity to AChE, hydrolysis ratio and thus regeneration time is determined by the chemical structure, especially its side groups. This is why certain OPs can be less toxic [33].

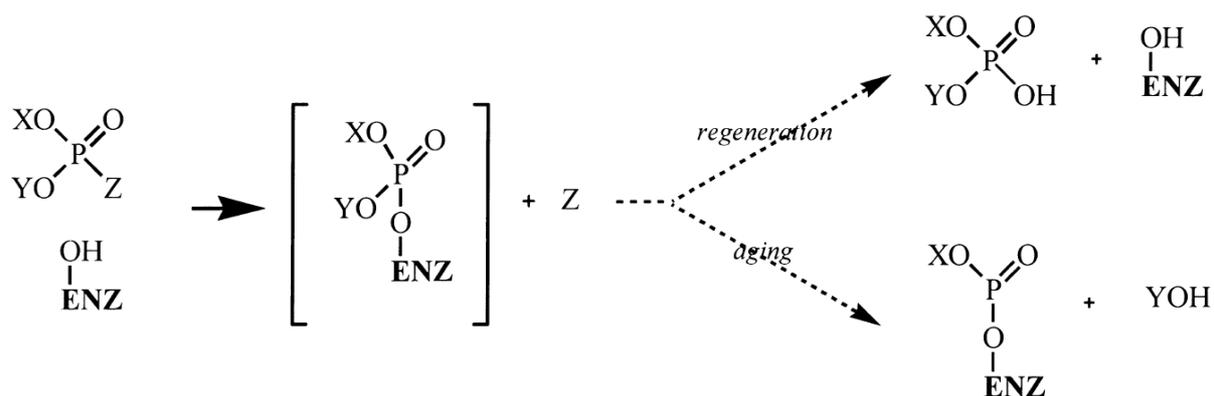


Figure 7. Fates of OP-AChE intermediates. After OP has bound to AChE, interaction is broken via hydrolysis (a process called regeneration), which is a very slow process due to a very stable bond between AChE and OP. Phosphorylated enzymes can also lose an alkyl group within 24 to 48 hours after initial binding. This process, called aging, causes the enzymes to be permanently phosphorylated [33].

Before active enzymes can be regenerated, some phosphorylated enzymes can lose an alkyl group, within 24 to 48 hours after initial binding. This is a process called ageing. Aged enzymes are permanently phosphorylated. Enzyme regeneration is therefore impossible by spontaneous hydrolysis or by an oxime antidote [105]. Neurotoxic agents such as Sarin can be very deadly due to the fact that Sarin-AChE intermediates age very quickly [33, 103].

Exposure to OP can result in acute toxicity, delayed toxicity and chronic toxicity. Which type of toxicity depends on the period of exposure and dose. OPs are specific and very effective inhibitors of AChE, that makes them effective as insecticide [34]. OPs disrupt the acetylcholinergic system. The continuous nerve impulses cause postsynaptic overstimulation that can cause bronchoconstriction, diarrhoea, muscular twitching [34] and paralysis [106]. Death from acute OP intoxication is thought to be by respiratory failure due to inhibition of respiratory centres in the brainstem, bronchial constriction and secretion and paralysis of the respiratory muscles [34, 107]. Since OPs are highly toxic to vertebrates, some types have been replaced by carbamates, which are less toxic [108].

As mentioned earlier, another target of OPs is the NTE [34], a lysophospholipase [35], which is an endoplasmic reticulum (ER)-anchored protein and is primarily distributed in the nervous system. It is an essential enzyme during neural development. In adults NTE is more or less restricted to large neurons where the enzyme plays a role in axonal maintenance. Disruption of NTE can lead to the occurrence of neurodegenerative symptoms (delayed toxicity) [36] as shown in figure 8 [109]. *In vitro* studies suggest that exposure to OPs can also reprogram AC signalling in PC12 cells during critical developmental stages [37]. *In vivo* studies imply CNS cell damage due to alterations of the expression and function of nuclear transcription factors (NTF). NTF dysfunction would lead to errors in cell replication, differentiation and apoptosis. However, the authors suggest more research is

needed on OP exposure and NTF [110], which is not yet been done. More in depth information on NTE and reprogrammed AC signalling will be discussed later in this paper.

Some research groups suggest that low chronic exposure to OPs is associated with impaired neuro-behavioural performance [106] at doses that do not induce cholinergic system disorders [34]. The symptoms caused by this low chronic exposure are Chronic OP-Induced neuropsychiatric Disorders

(COPIND) and are independent on AChE inhibition [111, 112]. The disorder's symptoms appear with a delay and are very persistent indicating possible permanent damage of the CNS. Most common symptoms of COPIND include cognitive deficits (such as impaired memory, problems with concentration/attention and learning), mood changes (such as depression, anxiety and emotional liability), peripheral neuropathy and chronic fatigue [106]. Other symptoms may include reduced tolerance to alcohol, impulsive suicidal thinking, elevated sense of smell and language disorders [113]. COPIND is initiated in peripheral neurons due to NTE inhibition [106] leading to neuropathy, which's process is shown in figure 8.

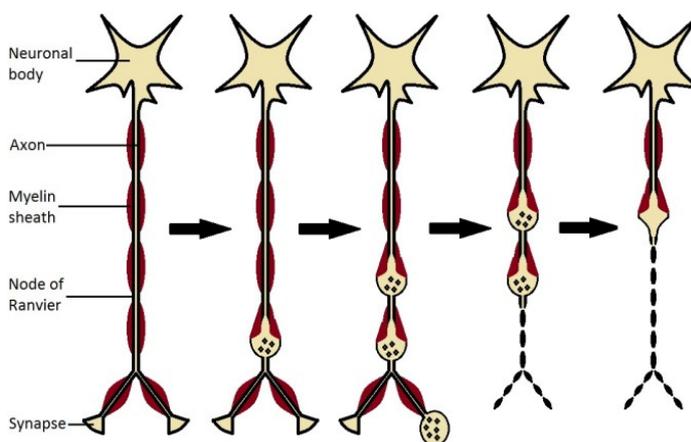


Figure 8. Degeneration of axons. The gradual 'back grow' of axons is often caused by a lack of nutrition [86]. Figure adapted from [109]

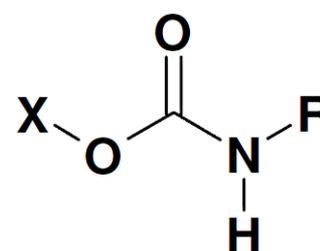


Figure 9. General structure of Carbamate [97].

Carbamates are esters of carbamic acid with various structural diversity in the side chains [33]. The general structure of carbamate is shown in figure 9 [97] and examples of carbamates including their oral and dermal LD₅₀ are shown in figure 10 [97]. Carbamates are also strong inhibitors of AChE and other carboxylic ester hydrolases making them effective insecticides. Since hydrolysis of carbamate-AChE occurs more rapid than OP-AChE, carbamate bioaccumulation is therefore lower than OPs [33].

Association between OP and neurodevelopment has been suggested by many studies on ADHD, neurodevelopment and OP exposure [8, 13-15, 30, 36, 38-40]. It is known that the dopaminergic and norepinegic systems are involved in cognitive deficits related to symptoms of ADHD [14, 40]. Furthermore, in about 75% of all ADHD cases, genetics are a causal factor [12]. A combination of several genes may give rise to symptoms of ADHD, which include dopamine receptors D₂/D₃ and D₄, DAT [13-15] and MAO-A [18, 19]. This would suggest that from a genetic level, the dopaminergic system is adversely affected.

Also, an association between dopamine synaptic marker reduction and ADHD symptoms has also been established [13]. ADHD medication, such as Ritalin inhibits DAT and NET, which appears to decrease symptoms of ADHD. Since DAT and NET facilitate dopamine and norepinephrine transportation from the synaptic cleft into the presynaptic neuron [114] that would suggest that

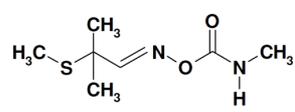
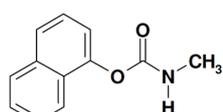
	LD50 rat (mg/kg)	
	oral	dermal
 <p>Aldicarb (Temik)</p>	0.8	3.0
 <p>Carbaryl</p>	850	> 4000

Figure 10. Examples of carbamate and their respective oral and dermal LD₅₀ in rat (mg/kg) [97].

normally dopamine and norepinephrine concentrations in the synaptic cleft are either; 1) too low due to reduced dopamine and norepinephrine secretion by the presynaptic neuron or 2) are secreted at a normal rate by the presynaptic neuron but DAT and NET activity is elevated or DAT and NET concentration is higher than normal or 3) DRD expression or function is altered or 4) MAO/COMT expression or function is altered. OPs are strong inhibitors of AChE and other carboxylic ester hydrolases [33]. This interaction results in ACh accumulation in the synaptic cleft [32] with postsynaptic overstimulation as a result [34].

A correlation has been suggested between symptoms of ADHD and OP exposure [30]. Also, OP exposure causes COPIND symptoms [106], which shows similarities with ADHD symptoms [23, 115]. OPs also inhibit NTE, which is an essential enzyme during neurodevelopment [36]. This may be connected to the development of ADHD symptoms.

Chapter 5. Attention-Deficit Hyperactivity Disorder

A recap on the first chapter; in most cases of ADHD, about 75%, the risk of the occurrence of these symptoms is genetics [12]. Various genes combined would cause the typically ADHD symptoms; DRD₂/D₃ and DRD₄ [13-15], DAT [13], NET [16], MAO-A [18, 19] and COMT [20]. An association between ADHD symptoms and a reduction of DAT and dopamine receptors has been implied [13]. The remained cases are due to environmental factors. OP exposure and increased risks of developing ADHD symptoms has been positively correlated [30].

Overall, it appears that dysfunctional dopaminergic and norepinegic systems are involved in the occurrence of ADHD symptoms [8, 40] and DAT and NET impairment leads to decreased ADHD symptoms [2]. It is also suggested that ADHD can be caused by an imbalance of norepinephrine and serotonin [116]. This is supported by the fact that inhibition of NET decreases ADHD symptoms [2] because the balance between norepinephrine and serotonin is restored. It is possible that secretion of norepinephrine by the presynaptic neuron might be too low, which would cause the imbalance with serotonin. NET inhibition would result in stabilisation.

ADHD's behavioural symptoms consists of two primary categories: impulsivity/hyperactivity and inattention of which three subtypes are formed: the hyperactive-impulsive type, the inattentive type and a combination of both [8]. The predominantly hyperactive-impulse subtype is characterised by (1) restlessness, (2) quick answering without hearing the full question, (3) having difficulty with waiting in a queue or taking turns at a game, (4) unwanted movement while one is supposed to show quiet behaviour [23, 115]. The predominantly inattentive type is characterised by (1) being unsuccessful in giving full attention to details or making sloppy errors during homework, work or any other activities, (2) easily distracted, (3) less responsive when spoken to, (4) chaotic, (5) often starting something new while previous task is left uncompleted [23, 115].

Considering the effect of psychostimulants such as Ritalin on ADHD symptoms, it would suggest that normally an ADHD patient has reduced dopamine and norepinephrine secretion by presynaptic neurons or that DAT and NET activity is either activated or is more available than normal. Dopamine is a neurotransmitter, which appears mostly in the brain and affects sleep, mood, motor functions, attention and learning [58]. Furthermore, dysfunction of the lateral PFC, dorsal anterior cingulate cortex, caudate and putamen [117] and reduced brain volume development, especially of the left-sided PFC, has been associated with ADHD [118]. From these brain regions, nerve impulses lead to dopamine release in the synaptic cleft, where it is received by a group of different dopamine

receptors D₁ to D₅ (DRD1-5). These receptors induce a signalling cascade in the postsynaptic neuron. DAT is part of the major deactivation mechanism that stimulates dopamine reuptake by the presynaptic neuron [62]. By blocking DAT the dopamine receptors are continuously stimulated by dopamine. This leads to a prolonged signalling cascade.

It is suggested that polymorphisms of the DAT are involved in ADHD [62]. As mentioned, ADHD symptoms are associated with brain structure abnormalities, especially in the (fronto)striatal regions [117]. Krause *et al* (2003) found elevated DAT density in the striatal region. However, no differences in DAT density have been found between the left and right side of the striatal region, putamen and caudate nucleus. All types of ADHD, inattentive, hyperactive/impulsive, or a combination of both, showed elevated DAT in the striatal regions [62]. Polymorphism in the DRD4 gene affects receptor function causing disrupted postsynaptic dopamine action [119]. Since genes DRD1 to 4 are also involved in ADHD symptoms [13-15], it might be possible that polymorphisms in the DRD1 to 3 genes also are the cause of disruption.

Identification of the cause of ADHD is most likely to be of neurobiological and genetic origins. Dysfunctional front-sub cortical pathways and unbalanced dopaminergic and noradrenergic systems appear to contribute to the ADHD pathophysiology, that is shown by radiological imaging studies on structure and brain function [2]. Similar implications come forth from genetics studies. Although no single gene has been found to play a major role in ADHD, numerous gene associations have been discovered of which are gene variations involving the dopaminergic system; the DAT [120] and dopamine receptors D₂/D₃ and D₄ [13-15].

Combined data from neuroimaging, neuropsychological, genetics and neurochemical studies suggest that dysfunction of four frontostriatal regions likely contribute to the pathophysiology of ADHD. These regions are the lateral PFC, dorsal anterior cingulate cortex, caudate and putamen. Frontostriatal and prefrontal cortical regions play an important role in motor control and attention [62]. Decreased blood flow in these regions was also detected [117]. Brain volume development of ADHD diagnosed children towards adolescence is reduced by about 3% compared to control. The left-sided PFC shows a greater decrease in volume than the right side [118]. Research also suggests that ADHD is caused by a neurotransmitter disorder in the brain. A disrupted balance between norepinephrine and serotonin can also be cause [116]. Norepinephrine can be both an excitatory as well as an inhibitory neurotransmitter located in the central nerve system (CNS) and peripheral nerve system (PNS). Serotonin can be both excitatory as well as inhibitory and is located in the CNS [58].

Diagnostics on ADHD is conducted via a psychiatric assessment. Comorbidities can be ruled out by physicians and neurologist through physical examination and radiological imaging [23]. However, some research groups suggest that anatomic MRI-scans should be not be used for ADHD diagnosis [118] because it is not an accepted diagnostic tool for ADHD at the moment [117]. Diagnostic assessment for ADHD should include an evaluation of the symptoms, pervasiveness, period of time, consequential impairment and age of onset of these symptoms [8]. Diagnosis is described in the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV), which is mainly used by U.S. psychiatrists [115]. The International Classification of Diseases, 10th Edition (ICD-10) is the World Health Organisation's manual, that is mainly used by non-U.S. psychiatrists and calls the disorder Hyperkinetic Disorder (HKD or HD) [121]. The symptoms of ADHD and HD are very alike. However, the ICD-10 requires all three symptoms (inattention, hyperactivity and impulsivity) to be expressed by the patient in order to make a full diagnosis as opposed to the DSM-IV requirements. This is probably why, according to DSM-IV criteria, diagnosis of ADHD is 3 to 4 times more likely than

by ICD-10 requirements. Besides the use of these two manuals other diagnostic means are used, such as child behaviour checklists and parent interviews [8, 122, 123].

Psychological, behavioural therapies can be effective means to treat ADHD symptoms. Typical behavioural therapies involve parents and teachers to identify behavioural risks in a child's environment, including home and school, thereby trying to modify the child's actions in order to accomplish desired behaviour [124].

ADHD symptoms can also be controlled by medication. The most notable effect of stimulants is improved focus and attention and less impulsive behaviour. Stimulants are also referred to as psychostimulants. Examples of psychostimulants are Adderall, Concerta, Dexedrine, Metadate and Ritalin [125]. Current research suggests that psychostimulants, such as Ritalin, influence the dopaminergic and norepinephrine system by blocking DAT1 and NET leading to increased dopamine levels [114]. This is shown in figure 11. Dopamine and norepinephrine transportation from the synaptic cleft into the presynaptic neuronal cytosol is blocked due to DAT and NET inhibition [2]. This leads to dopamine and norepinephrine accumulation in the synaptic cleft resulting in extended receptor stimulation. This leads to more and/or prolonged cell signalling. Overall, DAT and NET inhibition appears to decrease ADHD symptoms [2].

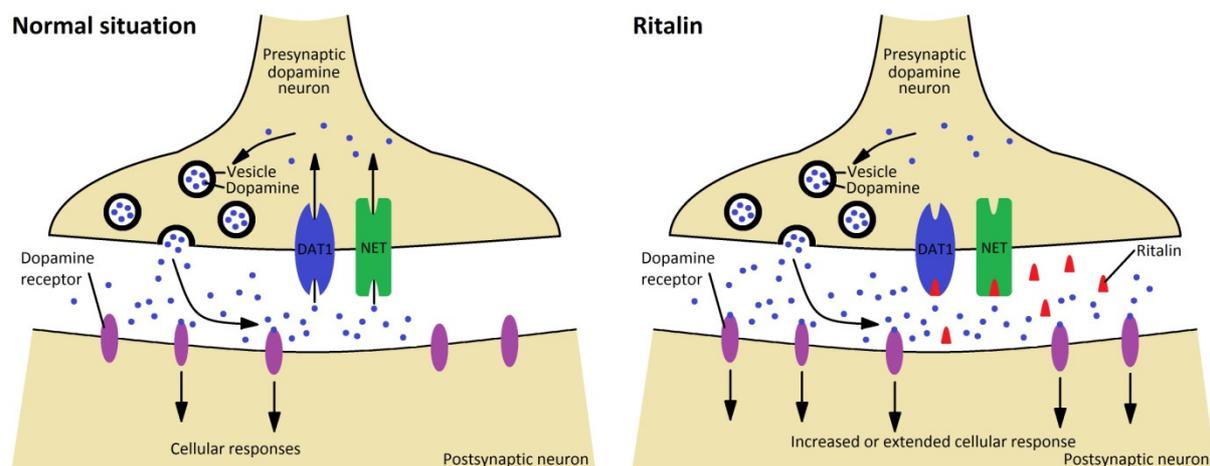


Figure 11. Ritalin interaction with DAT1 and NET. In the normal situation (left), dopamine is secreted into the synaptic cleft where it binds to dopamine receptors that result in cellular responses. ADHD patients have a lowered dopamine level. As shown on the right side, DAT1 and NET activity is blocked by Ritalin. Thereby the level of dopamine is maintained so prolonged interaction between dopamine and dopamine receptors is possible. Cellular response is then increased or is maintained for a longer period of time [114].

Psychostimulants are the first choice concerning ADHD symptom treatment and have been shown to be a more effective treatment than solely behavioural therapy. Medication should be taken regularly in order to remain a steady flow of stimulant and prevent peaks in concentration level. Most children use long-acting medication instead of short-acting medication. Long-acting medication remains effective during 8 to 10 hours and appears to be more effective [8]. Psychostimulant treatment was thought to be relatively safe [114], however recent reports indicate increased risk for cardiovascular effects, growth retardation and possible development of psychiatric disorders such as psychosis [8, 126].

Chapter 6. ADHD, OP exposure and the dopaminergic system

As the previous chapters suggest, it appears that ADHD, OP exposure and the dopaminergic system are related. In this chapter, important steps in the dopaminergic system will be discussed that are associated with either OP exposure, induction of ADHD symptoms, or both. The first is the stimulation of the dopaminergic neuron by ACh, glutamergic and GABAergic neurons. The second will be the interaction within the dopaminergic presynaptic neuron and the associated post synaptic neuron. The third step is associated with the downward signalling cascade from the postsynaptic neuron to eventual symptoms specific to ADHD.

6.1 Effects of OP on components related to stimulation of dopamine neurons

As mentioned in chapter 2, ACh is formed in the presynaptic neuron and is secreted into the synaptic cleft upon nerve impulses where it binds to an ACh-R. This can be a muscarinic receptor (mAChR) or a nicotinic receptor (nAChR). The latter is mainly involved in stimulation of dopaminergic neurons [42]. ACh is then hydrolysed by AChE into acetate and choline of which the latter is transported into the presynaptic neuron [127].

OP and carbamates are able to inhibit acetylcholine receptors (AChR) (muscarinic and nicotinic) [31, 128] and AChE [32, 128]. The inhibiting effect is positively correlated to the concentration of OP [31]. Some OP types have a higher affinity for nAChR than for AChE. The thion type of OPs bind to nAChR [31], while the oxon types can bind to mAChR [129, 130] and AChE [31]. Some thions need to be metabolised to oxons in order to inhibit AChE. For example, thion OPs like chlorpyrifos and parathion-ethyl can also inhibit AChE while parathion-methyl, fenthion and disulfoton cannot. In theory, OP exposure results primary in nAChR inhibition and after OP metabolism, AChE is targeted [31].

As shown in figure 12 (on next page), in the normal situation is ACh degraded by AChE after activation of AChR. Upon OP exposure, nAChR is inhibited by mostly thion types [31], leading to decreased cellular responses. AChE is inhibited by oxon OP resulting in ACh accumulation in the synaptic cleft [32]. This causes overstimulation of the AChRs [33]. This overstimulation may result in desensitisation of AChRs leading to an overall decreased neuronal signalling, which leads to dysfunction of the acetylcholinergic system. Acute high-dose exposure can result in death. Decreased or disrupted neuronal signalling can lead to a dysfunctional dopaminergic system [34].

As mentioned in chapter 4, ACh binds to AChE via its hydroxyl group of serine residue 203 in the enzyme's active centre forming an enzyme intermediate. When this enzyme intermediate is broken down, AChE is reactivated (regenerated) and ACh is hydrolyzed in the process [103]. This serine residue is also targeted by OPs (and carbamates). An OP-AChE intermediate is formed [103, 104] that is more stable than the AChE-ACh intermediate because its hydrolysis rate is lower. This rate is so slow, the AChE can be considered as inactive [103, 104].

Glutamate plays vital roles during neurodevelopment [47, 48] and in the adult brain directs learning and memory [49]. Very few studies conduct research on the potential relation between ADHD and glutamate/glutamatergic neurotransmission. Turic *et al* found evidence that polymorphisms in the glutamate-receptor gene (GRIN2A) might result in increased risk for developing ADHD [131].

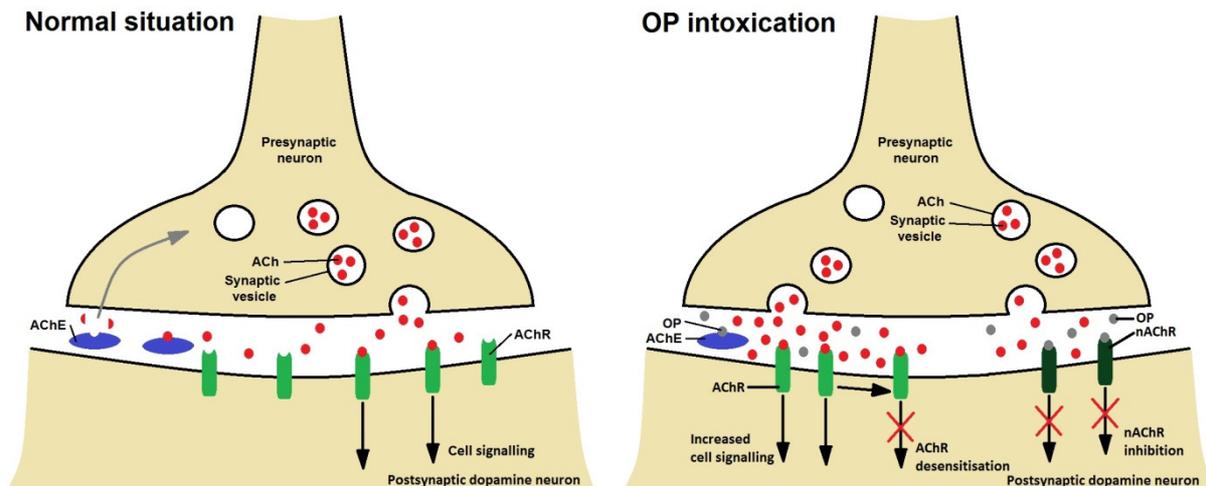


Figure 12. OP intoxication at the synaptic cleft. Left displays the normal situation where ACh is hydrolysed by AChE [103]. On the right side, the situation during OP intoxication is shown. nAChR is being inhibited by mainly OP thion types [31] and AChE is by mainly OP oxon types [31, 33, 103, 104]. nAChR inhibition causes decreased cell signalling whereas AChE inhibition leads to overstimulation of AChRs which eventually result in desensitisation of these AChRs leading to dysfunctional neuronal cell signalling [32, 33].

GABA is an excitatory neurotransmitter during neural development [51, 53-56] and in the adult mammalian brain, GABA has been converted into an inhibitory neurotransmitter [50]. Elevated plasmaGABA (pGABA) levels were found in patients with behavioural disorders with comorbid ADHD. Increased pGABA levels suggests increased inhibition of dopamine neurons leading to lowered dopamine levels; a symptom of ADHD. pGABA levels lowered after pharmacotherapy. In patients with ADHD only no altered pGABA levels were found [132]. This suggests that pGABA does not play a (key) role in ADHD. pGABA levels were elevated in children with Autistic Disorder with comorbid ADHD in the age of 5 to 15 years. Psychotropic medication did not help. Levels of pGABA decreased with age. Dhossche *et al* could not clarify the relation between increased pGABA and Autistic Disorder [133]. Overall, no clear relation between altered (p)GABA levels and ADHD only could be shown.

Dopamine neurons in the dorsal and ventral striatum and PFC are regulated via neurotransmitters such as ACh, GABA and glutamate within the substantia nigra and the VTA [41]. The dorsal and ventral striatum, substantia nigra and the VTA are all part of the basal ganglia. Decreased volume and shape of the basal ganglia has been associated with ADHD [134].

6.2 Effects of OP on dopaminergic neurotransmission

ADHD symptoms are associated with dysfunctional front-subcortical pathways and unbalanced dopaminergic and noradrenergic systems [2]. It is suggested that a combination of gene variations cause the unbalance. These genes include, DAT [120] and DRD2-4 [13-15]. A key component in dopamine neurotransmission is AC, which is a target of OPs. However, AC is not inhibited by OP but reprogrammed, which leads to altered responses [37]. This paragraph discusses the various components in neurotransmission at the synaptic cleft at the postsynaptic dopamine neuron. These components and their interactions are shown in figure 12, except AC, which is shown in figure 4 on page 10.

As mentioned earlier, TH plays a key role in the synthesis of dopamine in the presynaptic neuron [60]. Studies using Spontaneously Hypersensitive Rats (SHR), which are the commonly used animal model for ADHD studies, had lowered TH gene expression [135]. Hypoexpression of TH can

result in lowered dopamine synthesis. However, lowered dopamine synthesis is not necessarily associated with ADHD.

DRD1 to 5 are all G-protein linked receptors that interact with AC. DRD1 and DRD5 are AC inducing receptors whereas DRD2, DRD3 and DRD4 are inhibiting receptors of AC activity [64-68]. Increased transcription levels of the DRD1 gene in the medial PFC in rats have been associated with impulsiveness, which in turn is linked to ADHD [136]. This receptor is also associated with GABA release enhancement in the substantia nigra pars reticulata (SNr) in rats [137].

DRD2 subtypes mediate hyperactivity and the response to amphetamine. Deletion of the DRD2 gene only results in the elimination of hyperactivity and will lead to eventual depletion of the excess dopamine overflow in the synaptic cleft. This would suggest DRD2 as an interesting target for novel drugs for ADHD symptom treatment [138]. The presence of one risk allele in the DRD2 gene will increase the risk of ADHD. Homozygotes of these alleles will increase the risk of ADHD even further [139]. On DRD3 appears to be no data that confirms potential to increase risk for ADHD [139].

Variations in the DRD4 gene have been related to several behavioural disorders such as autonomic nervous system dysfunction and ADHD [67, 140]. It is also associated with schizophrenia and Parkinson disease since DRD4 is a target for drugs that treat these disorders [67]. Becker *et al* indicated that it appeared that the DRD4 genotype modulates the relation between regulatory problems during childhood and the later development of ADHD. It is however necessary to conduct a second study to confirm this [141]. Another study indicated an association between polymorphisms in the DRD4 gene and the risk of developing ADHD [142]. Allelic variants of the DRD4 gene are well characterised with increased risk factors for ADHD. It also seems that foetal hypoxia results in increased DRD4 gene promoter activity. This effect due to hypoxia on the dopaminergic neurotransmission could be a considerable factor that leads to ADHD symptoms [5]. DRD4 receptor is also related to GABA release inhibition in the SNr in rats. Low DRD4 signalling may lead to decreased inhibition of impulse traffic through the thalamus. Acosta-García *et al* expect that malfunction of DRD4 might be associated with ADHD symptoms [137]. The DRD5 gene transcription level is increased in the medial PFC in rats. This has been associated with impulsiveness, that in turn is related to ADHD [136].

MAO-A and MAO-B both catalyse the degradation of several important neurotransmitters [71, 72]. Several significant associations have been found between variations in the MAO genes and the incidence of ADHD. This way, MAO gene polymorphisms can be used as prediction on the outcome of ADHD during adulthood [18]. A dysfunction of the dopamine homeostasis can be a result due to malfunctioning MAO [143]. Dopamine homeostasis might lead to ADHD symptoms [5]. A proposition has been suggested to study the relation between MAO-A dysfunction and serotonergic system dysfunction including potential risk of ADHD. Also, higher prevalence of comorbid disruptive behavioural disorder could be expected [63]. Promotor polymorphisms in the MAO-A gene seem to be related to ADHD and anxiety with autism spectrum disorder [19]. An association has been found between lowered platelet MAO-B and unmedicated children with ADHD symptoms, especially in boys [144, 145].

COMT can be of importance considering the risk of ADHD since it is involved in degradation of dopamine and norepinephrine [73]. COMT has been related to impulsive behaviours such as violent schizophrenia and drug abuse [21, 146]. Recently it was found that a single nucleotide polymorphism (SNP) in the COMT gene leads to a three to four times lower COMT activity. This was associated with increased risk of ADHD, severity of ADHD symptoms and comorbid conduct disorder

[147]. COMT gene polymorphisms were related to anxiety and ADHD in children with autism spectrum disorder [20].

DAT1 gene polymorphisms can be related to hyperactive dopamine re-uptake from the synaptic cleft [15]. Rat studies indicated that ADHD symptoms have been associated with elevated DAT [62]. Pathological differences were found between the ADHD inattentive type and ADHD combination type in rat studies because it appears that the inattentive type has a lower density of DAT1 than the ADHD combination type [148]. Hypoexpression of DAT1 will result in reduced dopamine uptake at the synaptic cleft.

NET regulates neural differentiation during neurodevelopment and in adults, NET internalises norepinephrine from the synaptic cleft in the adult nervous system. Inhibition of NET would block noradrenergic differentiation because critical pathways in neural crest formation and noradrenergic cell differentiation is halted [76]. It appears that polymorphisms in the NET gene are associated with ADHD symptoms [16]. No studies could be found considering ASIC1 and VMAT function and its potential to disrupt the dopaminergic system and thereby increasing the risk for ADHD.

Signal transduction cascades that are involved in the regulation of cell replication, differentiation and function are very sensitive to OP exposure. OPs can affect the generation and utilisation of cAMP by binding to AC [37]. AC is normally regulated by G-protein coupled receptors that determine the level of cAMP synthesised. Next, PKA is activated that moves into the nucleus and phosphorylates a cAMP Response Element-Binding Protein (CREB). CREB recruits the coactivator CBP, which stimulates gene transcription [149]. Binding to AC would therefore alter gene expression.

In an *in vitro* study, PC12 cells were exposed to OPs chlorpyrifos, diazinon or parathion. It was shown that differentiating cells are sensitive to OP exposure as opposed to undifferentiated cells. A two day exposure to OP caused deficits in the AC signalling cascade; supranormal AC activity, which lasted beyond the two day exposure. This finding suggests that exposure to OP during a critical developmental stage causes the AC pathway to reprogram. The direct effect of OPs on AC pathway reprogramming indicates that this process is unrelated to AChE inhibition [37]. Schuh *et al* showed *in vivo* that chlorpyrifos caused an increase of neuronal plasma CREB (pCREB). CREB is considered a critical component in brain development and cognitive function. Alterations to pCREB (most likely to altered AC activity) could therefore result in disrupted neurodevelopment and changes in behaviour [150]. Early postnatal exposure to chlorpyrifos caused deficits in several components of the AC signalling cascade such as AC itself, G-protein functionality and hormone receptors [151]. Reprogramming of AC in differentiating neurons [37] during a critical phase in neuro-development can cause a disrupted development of the brain [152]. Carbamate also appears to have a high affinity to [153]. Carbamate binding to AC might lead to similar deficits as OP binding.

The dorsal and ventral striatum are part of the basal ganglia [41]. The basal ganglia shows structural and functional differences in patients with diagnosed ADHD, especially the caudate nucleus. Structural differences of the basal ganglia also affect the wider frontostriatal networks that have been reported dysfunctional in ADHD [134, 154, 155]. Dysfunction of four frontostriatal regions is likely to contribute to the pathophysiology of ADHD. These regions are the lateral PFC, dorsal anterior cingulate cortex, caudate and putamen [62]. Decrease blood flow in these regions was detected [117] as well as reduced brain volume development. Towards adolescence, brain volume is reduced by about 3% in ADHD diagnosed patients as compared to control. The left-sided PFC shows a greater decrease in volume than the right side [118]. Maldevelopment of the brain can be caused by multiple factors. An essential component during neurodevelopment is NTE, which is also a target of OP [85].

Chapter 7. ADHD, OP exposure and NTE

NTE plays vital roles during brain neuronal development and in axonal maintenance [36]. As shown in *in vivo* as well in *in vitro* studies, NTE can be targeted by OPs and is very sensitive to direct-acting of OP [82]. Inhibition of NTE can lead to neurodegenerative symptoms [34-36], that affects long axons in the spinal cord and peripheral nerves. The distal part of a neuron swells followed by neurodegeneration from the synapse towards the neural cell body, a process called distal sensory-motor axonopathy. The neural cell body itself remains unaffected. These symptoms are not noticeable until 1 to 3 weeks after initial OP exposure [85]. NTE acts via its active site serine residue, which is also the site at which OPs bind to NTE. OPs can act as pseudo-substrates for NTE. Hydrolysis of the formed organophosphorylated intermediate occurs very slowly, which renders the enzyme inactive. Inhibition of NTE results in axonal degeneration and paralysis in adults [85], that is associated with COPIND [111, 112]. Symptoms of COPIND include cognitive deficits (such as impaired memory, problems with concentration/attention and learning), mood changes (such as depression, anxiety and emotional lability), peripheral neuropathy and chronic fatigue [106]. Other symptoms may include reduced tolerance to alcohol, impulsive suicidal thinking, elevated sense of smell and language disorders [113].

How NTE inhibition exactly leads to delayed neuropathy is not clear [82, 156]. However some hypotheses suggest that distorted homeostasis of membrane phospholipid and disrupted ER functions will cause dysfunction in axonal transport and glial-axonal interaction. These support functions are especially important to the distal parts of long axons, which will therefore be most vulnerable. Impaired nutrition of distal axons might result in neuropathy [86]. Local alterations in lysoPC metabolism or signal transduction pathways may also lead to neurodegeneration [82]. In mice, NTE is expressed from gestational day 7 throughout the neurodevelopment, particularly in the developing lens, along the spinal cord and also in several non-neural cells like kidney and liver [36].

NTE dysfunction causes embryonic growth retardation leading to eventual abortion [36]. NTE knockout mice develop a spongiform pathology of the CNS that resembles the spongiform encephalopathy in Creutzfeldt-Jakob (CJ) patients [157]. This symptom is not associated with ADHD. Adversely affected brain development has also been associated with ADHD symptoms [117, 118, 134, 154, 155]. Combining these findings would suggest a potential association between NTE inhibition and ADHD symptoms. However, considering that NTE inhibition results in symptoms similar to CJ that would make the NTE inhibition as a (possible) cause of ADHD less likely. Also, no studies have been published yet considering possible association between NTE inhibition and ADHD. Nevertheless, no studies have yet been published that suggest no association.

Chapter 8. Conclusion and discussion

This paper tries to clarify the exact link between OP exposure and the incidence of ADHD. What is known is that numerous studies point to an association between OP exposure, neurodevelopment and ADHD [8, 13-15, 30, 36, 38-40]. The risk of developing ADHD has been associated with inherited genetic variations on key components of the dopaminergic system [13-15] and to OP exposure during the sensitive phase of neurodevelopment [30].

The factors behind induction of ADHD symptoms are prefrontal dopamine deficiency and an incomplete central dopaminergic functioning. PFC dopamine deficiency indicates that ADHD is related to lowered dopamine levels [5]. Dysfunctional frontostriatal regions including the PFC contribute to ADHD pathophysiology [62]. The adult brain of an ADHD patients shows a decrease in volume of the PFC, particularly the left-side PFC [118]. The PFC is connected via a dopaminergic pathway to the basal ganglia including the frontostriatal regions [41]. Reduced PFC volume and dysfunction might be explained by various malfunctions in components of the dopaminergic neurotransmission. Malfunctions of these components are related to polymorphisms and altered gene expression.

Altered gene expression, in relation to ADHD has been found in SHR, which is a rat model used in ADHD studies. TH gene expression in SHR is lower than in wild type rats [59]. Gene expression of DRD1 and DRD5 is up-regulated in the medial PFC in rats. Increased DRD1 and DRD5 may lead to increased AC activity [64, 68] and is associated with impulsiveness that is related to ADHD [135]. DRD1 and DRD5 are the only ones with altered gene expression. Other components are mainly dysfunctional due to polymorphisms in their corresponding gene. DRD2 and DRD4 gene variations are associated with increased risk for ADHD [65, 67, 138, 139, 141]. MAO gene polymorphisms are related to development of ADHD [142]. Malfunctioning of MAO due to gene variation leads to imbalances in dopamine homeostasis [18] that has been related to ADHD [5]. Dysfunction of any of both sub types of MAO are also related to symptoms of ADHD [19, 144]. SNP in the COMT gene can also lead to an increased risk for ADHD [21]. Variations in the DAT gene are also considered to increase the likelihood to develop ADHD [15]. Overall, it appears that elevated DAT1 is related to increased risk for ADHD [62]. NET gene variations have been associated with increased risk for ADHD. However, it is not clear if that causes the NET level to increase or decrease [16].

Exposure to OP (and carbamates) can lead to inhibition of nAChR and AChE that will eventually result into decreased cell signalling [31-34]. When dopamine secretion is elevated during early neurodevelopment, nAChR is downregulated by negative feedback. When the dopaminergic system is allowed to recover (no OP exposure) then dopamine neuronal stimulation leads to low dopamine secretion since nAChR are reduced on the dopamine neuron. Increased dopamine secretion due to increased and/or prolonged dopamine neuron stimulation can be the result of AChR overstimulation. This is caused by increased ACh levels in the synaptic cleft. OPs inhibition of AChE causes ACh levels to increase [33]. Overstimulation of AChR will ultimately lead to desensitisation of the receptor. nAChR can be inhibited by OPs. Both processes result in reduced cell signalling of the dopamine neuron causing lowered dopamine secretion. As mentioned earlier; this lowered dopamine secretion is a characteristic of ADHD [5]. Considering the mechanism of OPs inhibiting AChE and nAChR makes this the most likely mechanism of OPs to result in increased risk of ADHD.

OPs (and carbamates) also appear to have a high affinity to AC [37, 153]. Binding of OP (but not carbamate) to AC can reprogram the AC pathway [37] and affect components of the total AC signalling pathway [151]. This can cause distorted brain development [152] when OP exposure occurs during sensitive neurodevelopmental phases (like in young children) [37]. This has been confirmed in an *in vivo* and an *in vitro* study [37, 152]. Nevertheless, it is not clear whether this disrupted brain development leads to a dysfunctional dopaminergic system leading to ADHD. Based on current data it appears that it is less likely that OP exposure leads to ADHD by interfering with AC activity.

NTE is important in axonal maintenance and CNS development [36]. NTE is located in large axons [36]. OP binds to NTE's active site rendering it inactive. This can lead to axonal degeneration and paralysis in adults [85]. This condition is referred to as COPIND [111, 112]. NTE inhibition during embryonic development in mice leads to growth retardations followed by abortion [36]. Inhibition of NTE also causes CF-like symptoms in the brain [157]. On inhibition of NTE in the developing brain of children, no publications were available. It appears that it is currently not clear how NTE inhibition leads to delayed neuropathy [82, 156]. Also no reports on the association between NTE inhibition and ADHD symptoms have been submitted. However, it can be expected that delayed neuropathy leads to behavioural disorders [158], but not necessarily ADHD because firstly, DA neurons do not have large axons since they are located in the brain and secondly, ADHD symptoms are not similar to CF-like symptoms. Based on this data it can be said that it is very unlikely that inhibition of NTE by OPs leads to ADHD symptoms.

Other neurotransmitters and their corresponding receptors that are involved in dopaminergic neuron stimulation might also play a role in overall postsynaptic dopamine neuron overstimulation. Polymorphisms in GRIN2A, the gene for glutamate-receptor, are associated with increased risk for developing ADHD [131]. pGABA was elevated in ADHD patients but no clear relation between increased risk for ADHD and increased pGABA level could not be established [132, 133]. Further research should clarify these unclear relations. It can be assumed that altered levels in GABA and altered response of glutamate-receptors to glutamate can cause postsynaptic dopamine neuron overstimulation and it cannot be ruled out that these processes are more determining on overstimulation than ACh does. It is a fact that ACh overstimulation is caused by OP and potential overstimulation in the glutamatergic and GABAergic neurotransmission does not.

A model has been made on the discussed pathways that might cause an increased risk of developing ADHD. A schematic overview of this model is shown in figure 13. The pathway of the inherited genetic variation (green) indicates that genetic variation is associated with dysfunction of

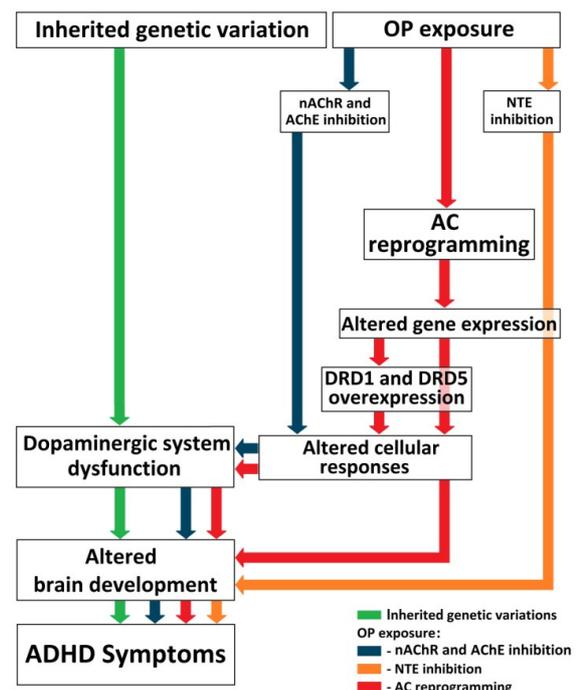


Figure 13. Model of pathways towards development of ADHD symptoms. The proposed model consists of four pathways. All pathways meet at the altered brain development step, which is associated with symptoms of ADHD[62]. The pathway of inherited genetic variation is shown in green. OP exposure is divided into three pathways; nAChR and AChE inhibition (blue), AC reprogramming (red) and NTE inhibition (orange). At the component of "Altered gene expression" the AC reprogramming pathway is split up into two pathways: one via the "DRD1 and DRD5 overexpression" component and one past that component. A light coloured pathway indicates that the pathway goes past a component thus it is not associated with that component. The most likely pathway of OP exposure leading to ADHD is via nAChR and AChE inhibition.

the dopamine system [15, 16, 18, 19, 21, 62, 65, 67, 138, 139, 141, 142, 144] that might result in altered brain development. Abnormal brain development, particularly the PFC is associated with symptoms of ADHD [62]. The OP exposure pathway is divided into three pathways. These are the AChE inhibition pathway (blue), the AC reprogramming pathway (red) and the NTE inhibition pathway (orange). OPs can inhibit nAChR and AChE that results in cholinergic system distortion (altered cellular responses) [31-34]. These abnormal cellular responses of dopaminergic neurons could lead to disruption of the dopaminergic system [65, 67, 138, 139, 141]. This pathway is most likely the mechanism by which OP exposure results in increased risk of ADHD.

The AC reprogramming pathway; OPs has a high affinity to AC leading to reprogramming of AC in differentiating cells [37]. This would alter gene expression [149] that could be the cause of DRD1 and DRD5 over expression discussed earlier. This sub pathway can, via DRD1 and DRD5 over expression, be associated with the dysfunctional dopaminergic system. The second sub pathway shows that changed gene expression results in altered cellular responses, which can act on the regulation of the dopaminergic system [65, 67, 138, 139, 141] and/or on brain development [37, 152]. The AC reprogramming pathway as mechanism of OP exposure leading to increased risk of ADHD is less likely, mainly due to the lack of relevant available publications.

The final pathway is NTE inhibition pathway that indicates that OP can inhibit NTE leading to altered brain development. However, symptoms caused by NTE inhibition are not similar to ADHD symptoms and NTE is possibly not associated with dopamine neurons. This makes NTE inhibition by OPs very unlikely to lead to increased risk of ADHD. This model is just an indication on the possibilities on how ADHD can be developed. Certain steps in this model still need to be studied such as the exact role of DRD1 and DRD5 over expression and AC reprogramming.

The exact link between OP exposure, neurodevelopment and ADHD cannot be not fully understood, yet. According to current data it seems that this link might act through the dopaminergic system. Altered stimulation of the postsynaptic dopamine neuron might lead to dysfunctional signal transduction. When this occurs during neurodevelopment that may lead to dysfunctionality of the brain that is associated with symptoms of ADHD. If this dysfunction can be associated with the disruptive dopaminergic system causing it to lower the general dopamine level, then a relation to ADHD can be established: OP exposure eventually leads to lowered dopamine levels, just as ADHD symptoms are associated with a lower dopamine level. The exact mechanism from postsynaptic overstimulation towards brain maldevelopment is still to be clarified. Disrupted brain development and potential consequential dysfunctional dopaminergic system can also be caused due to AC reprogramming, since AC is essential for signalling cascades during neurodevelopment. However, as mentioned earlier, this mechanism is unlikely to cause ADHD considering current available publications. In conclusion, based on current publications on OP exposure, neurodevelopment and ADHD it can be said that it is very likely that early OP exposure leads to an increased risk of developing ADHD via the mechanisms of AChE and nAChR inhibition by OPs.

Nomenclature

AC	Adenylyl Cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine Receptor
ASIC1	Acid-Sensing Ion Channel 1
ADHD	Attention-Deficit Hyperactivity Disorder
CJ	Creutzfeldt-Jakob
CNS	Central Nerve System
COPIND	Chronic Organophosphate-Induced neuropsychiatric Disorders
DRD1-5	Dopamine Receptors D ₁ to D ₅
DAT	Dopamine Transporter
ER	Endoplasmatic Reticulum
lysoPC	Lysophosphatidylcholine
mAChR	Muscarinic Acetylcholine Receptor
MAO	Monoamine Oxidase
MAO-A	Monoamine Oxidase A
MAO-B	Monoamine Oxidase B
nAChR	Nicotinic Acetylcholine Receptor
NET	Norepinephrine Transporter
NMDA	N-Methyl-D-Aspartate
NTE	Neuropathy Target Esterase
NTF	Nuclear Transcription Factors
OP	Organophosphate
PFC	Prefrontal Cortex
PNS	Peripheral Nerve System
SHR	Spontaneously Hypersensitive Rats
SNc	Substantiana Nigra Pars Compacta
SNr	Substantia Nigra Pars Reticulate
SNP	Single Nucleotide Polymorphism
TH	Tyrosine Hydroxylase
VTA	Ventral Tegmental Area

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