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Towards improved models for treatment of organophosphate poisoning

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door
Maria Johanna Adriana Joosen
geboren te Bergen op Zoom

promotor: prof.dr. A.B. Smit
copromotor: dr. H.H.P.M. van Helden

"If you change the way you look at things,
the things you look at change."
Wayne W. Dyer

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Contents

Chapter 1

General Introduction

1 Organophosphates

The term organophosphate (OP) is used to describe many different types of esters of phosphoric and phosphonic acids. Organophosphates are a class of highly toxic compounds that include nerve agents and pesticides, among others insecticides. OPs represent the largest class of insecticides used worldwide and pesticide poisoning is recognized as a global problem. The risk of human exposure mainly arises from the agricultural use of OPs in developing countries in which OPs regularly lead to incidents, or are even intentionally used to inflict self harm, totalling approximating 3 million exposures and 200 thousand fatalities each year (Eddleston et al., 2005; Eyer, 2003).

Development of OP compounds into insecticides occurred in the late 1930s by the German scientist Dr. Gerhard Schrader. This research led to the discovery and development of nerve agents, such as Tabun, Sarin, Soman, and VX (Fig. 1) (Newmark, 2004).

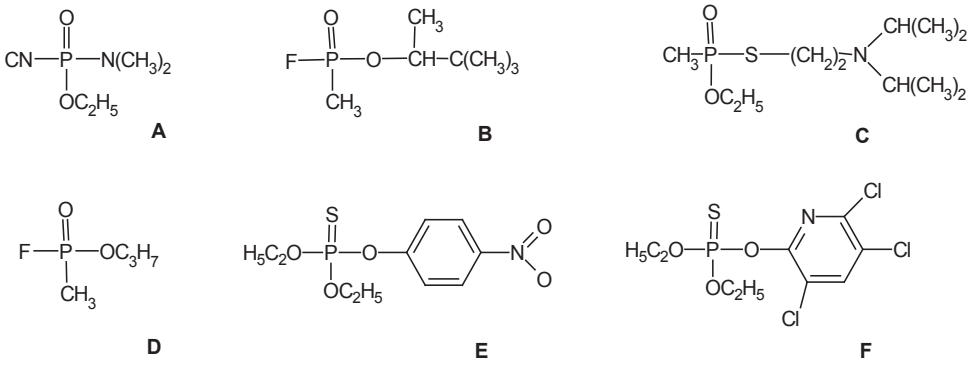

***Figure 1**. Chemical formulas of the most well-known nerve agents (A through D) and pesticides (E and F), all organic esters of phosphoric acid. They possess a central phosphorous atom bound to two alkyl groups, a leaving group and oxygen (A-D) or sulfur (E-F) atom. A. Tabun (O-ethyl-N,N-dimethyl-phosphoramidocyanidate); B. Soman (Pinacolylmethylphosphono-fluoridate);. C. VX (O-ethyl-S-2-diisoprpylaminoethyl-methylphosphonothiolate) D. Sarin (Isopopylmethylphosphonofluoridate); E. Parathion, F. Chlorpyrifos.*

Although weaponized, nerve agents have not been used in World War II. During the 'Cold War' nerve agents were stockpiled on both sides of the 'Iron Curtain', but were again not used (Black and Harrison, 1996). In the Iraq-Iran conflict in the 1980s, however, sarin and tabun were used on a large scale, causing large numbers of casualties (Newmark, 2004). During the First Persian Gulf War in 1991, the threat of Iraq's chemical weapons was perceived, but the conflict was over before Iraq had a chance to use them. In 1995 the world was shocked by a terrorist attack with sarin in the Tokyo subway by the Aum Shinrikyo cult, killing a dozen people and intoxicating of few thousand (Croddy, 1995). Later, it became apparent that this cult had killed various individuals who hindered them using sarin and VX (Vale, 2005).

In 1997 the Chemical Weapons Convention (CWC) has entered into force, prohibiting the development, production, acquisition, stockpiling, retention, transfer or use of chemical weapons by States Parties. Currently, 98% of the global states have ratified the CWC. Two states have signed, but not ratified the convention; five states have neither signed nor ratified the CWC (OPCW.org). The CWC will be more successful because this convention covers the whole chain from development to use of chemical weapons.

Since the recipes for synthesis of among others nerve agents are readily available, there is a reason for concern that such agents will fall into the hands of terrorist organizations and may be used against civilians and military personnel. Consequently, means of protection against nerve agents are needed, both in terms of physical protection (masks and suits) and medical countermeasures. Current medical countermeasures for nerve agents are effective, to reasonable extent, yet there is room for improvement. In order to be able to improve medical countermeasures a thorough understanding of the routes of entry, fate within the body and the mechanism of action is needed.

Due to the similarity in chemical structure (Fig. 1) and in mechanism of action, the insight that is acquired by studying nerve agents can also be used to improve treatment of intoxications with Ops in general, in particular relevant for pesticides, which, as already mentioned above, is a huge and global problem.

Therefore, in order to improve medical management, this thesis focussed on improving the design of animal models to further investigate the mechanism of OP toxicity and treatment efficacy. For that purpose two types of OPs were used: soman, representing a highly toxic volatile OP entering the body via the inhalation route and producing toxic signs very quickly, and VX, representing a much less volatile OP entering the body via the percutaneous route and demonstrating a clinical delay of toxic signs and long persistence.

2 Toxicity of organophosphates

2.1 Exposure risks

Exposure to OPs occurs via the oral, dermal or inhalation route. Oral dosing is mostly the result of accidental ingestion or in case of suicidal intents. The general population may also be chronically exposed to low doses by consumption of OP pesticide residues in food or as contaminant in drinking water. People working in production, transport, mixing and loading and application of pesticides are at highest risk for exposure, mostly via the dermal route (Costa et al., 2008; Costa, 2006). With regard to nerve agents, these may form a risk for exposure both in liquid and in vapour states. At ambient temperatures, nerve agents are liquids. The more volatile agents, soman, tabun and sarin, constitute both a vapour and a liquid hazard upon dispersion. Agents with lower volatility, such as VX, represent primarily a liquid hazard, and therefore skin contamination forms the highest risk. In addition, VX is very persistent, because it hardly degrades and is difficult to wash off (van der Schans et al., 2003; Sidell, 1997).

2.2 Acetylcholinesterase inhibition

The main toxicological action of OPs is exerted through irreversible inhibition of acetylcholinesterase (AChE), the key enzyme involved in cholinergic neurotransmission. The inhibition of AChE leads to excessive buildup of released acetylcholine (ACh) at cholinergic synapses resulting in a failure of neuromuscular transmission and to seizures followed by status epilepticus. Eventually, poisoning with OPs is likely to result in death or long-term neuronal deficits.

AChE is located both in the central nervous system (CNS) and in the peripheral nervous system (Massoulie et al., 1993). This enzyme of the serine hydrolase family is evolutionary highly conserved and controls a key step in cholinergic signalling, that is the breakdown of the neurotransmitter ACh. AChE is one of the most efficacious and fast-acting mammalian enzymes known, capable of hydrolysing 20000 molecules of ACh per second (Rosenberry, 2009). Inhibition of the enzyme at cholinergic synapses leads to death of an organism, due to the uncontrolled action of high levels of ACh. Two cholinesterases very similar to AChE in the nervous system are found in blood, i.e., the erythrocyte-bound cholinesterase and butyrylcholinesterase in plasma. Because these cholinesterases are the first targets for OPs entering the blood stream, they act as initial buffers and scavenge OPs. Inhibition levels of both cholinesterases in blood are considered as measures of exposure and blood AChE activity closely reflects the AChE activity at the neuromuscular junction (NMJ) (Thiermann et al., 2009).

Synaptic AChE exists in tetramer units, which are linked to the synapse by transmembrane anchors in vertebrate fast muscles and in the CNS. In the CNS, a single tetramer is attached to a 20 kD membrane spanning protein, whereas at the neuromuscular endplate one to three catalytic subunit tetramers (AChET) are attached to a triple-helical collagen tail, which anchors them to the basal lamina within the synaptic cleft (Silman and Sussman, 2005; Perrier et al., 2002).

The enzyme has two sites showing affinity for ACh. The active catalytic site is located at the bottom of a deep and narrow gauge. At the top of the gauge, the peripheral anionic site is located, where the bisquaternary moiety of ACh interacts with the indole ring of Trp279. Near the bottom of the gauge, close to the catalytic centre of the enzyme, the quaternary group of choline interacts with Trp 84, and the acyl moiety of ACh is confined by the acyl pocket consisting of Phe 288 and Phe 290. Actual hydrolytic cleavage of ACh into choline and acetic acid is achieved by the catalytic triad of a serine (Ser 200), histidine (His440) and glutamic acid (Glu 327) at the bottom of the gauge (Silman and Sussman, 2005). During hydrolysis, AChE forms a very short-lived (~50 ms) acetyl-enzyme intermediate with ACh. A nucleophilic attack by a water molecule then restores the AChE into its original state (Fig. 2A).

Several carbamates have been developed to act as selective reversible AChE inhibitors for the use as insecticides or as drugs in Alzheimer's disease. Several of these compounds have found their way to nerve agent research to be tested as pretreatment drugs. Carbamates shield a fraction of AChE before entrance of the OP into the body, and AChE decarbamylates while the circulating OP is eliminated (Wetherell et al., 2002; Philippens et al., 1998; Wetherell, 1994; Harris et al., 1990). A carbamate acts as a competitive inhibitor of AChE, by leaving a carbamyl group on

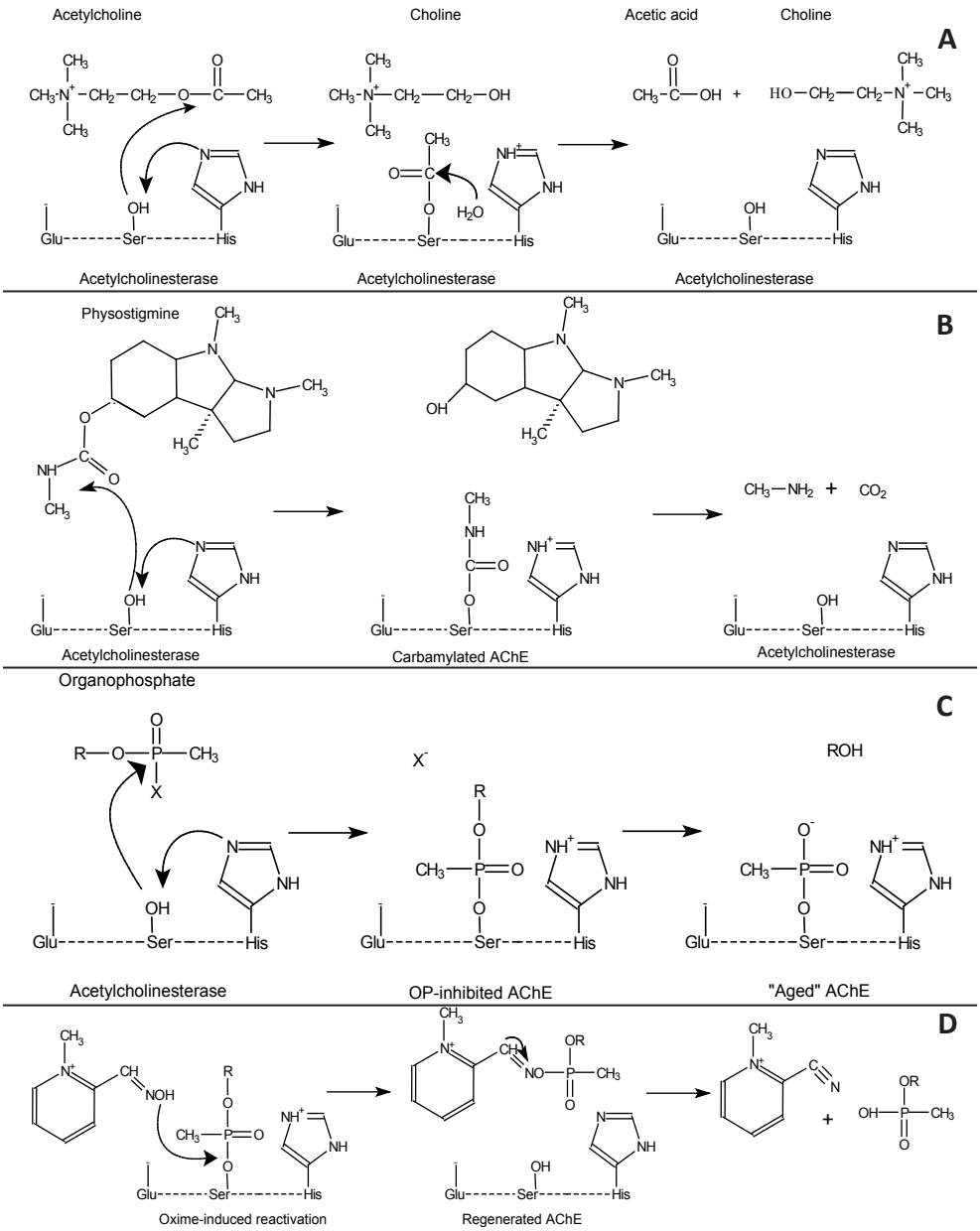

***Figure 2**. A. Schematic presentation of the hydrolysis of ACh by AChE. AChE is represented by the catalytic triad Glu-327; Ser-200 and His-440. A short-lived acetyl-enzyme intermediate is formed by Ser-200 and ACh, which is degraded by a nucleophilic attack by a water molecule, restoring the AChE to its original state. B. The inhibition of AChE by the carbamate physostigmine. Physostigmine acts as a competitive inhibitor, leaving the enzyme carbamylated. This intermediate persists for minutes to hours, after which the enzyme is restored to its original state again. C. Inhibition of AChE by an OP, in which R is an alkyl chain. The OP forms a stable phosphorylated intermediate with AChE, which may persist for hours to days. A secondary reaction termed "aging" occurs when the alkyl chain of the OP acts as leaving group, further complicating reactivation. D. Reactivation of un-aged phosphorylated AChE by an oxime (2-PAM). An oxime can restore enzymatic activity by removing the OP from the Ser-200 residue.*

the enzyme (Fig. 2B). The complex has a decarbamylation rate ranging from minutes to hours depending on the carbamyl group (Wetherell and French, 1991).

Organophosphates inhibit the hydrolytic activity of AChE by acting as a false substrate (Fig. 2C). The O=P group reacts with the serine residue in the catalytic triade, forming a OP-enzyme complex which may persist for hours or days, in contrast to the short lived intermediate formed with ACh (Silman et al., 1999). "Aging" occurs when the alkyl chain of the OP-enzyme complex acts as leaving group, rendering the AChE molecule truly irreversibly inhibited (Fig. 2C) (Silman et al., 1999; Barak et al., 1997).

Before the "aging" process has taken place, the enzyme can reactivate spontaneously after a few days. The hydrolysis process of an OP can be enhanced by an oxime (Jokanovic and Prostran, 2009; Hobbiger et al., 1960). Several oximes are used in the current emergency treatment regimen, such as HI-6, 2-PAM and obidoxime. These molecules possess a quaternary amine and an oxime (N-OH) moiety, which perform a nucleophilic attack at the phosphorous atom of the OP-AChE complex (Fig. 2D) (Dawson, 1994). Both the source of the enzyme and the nature of the bound OP influence the reactivating efficiency of the oximes (Taylor et al., 1999). When reactivation is impossible, AChE activity can only be restored by de novo synthesis.

2.3 The cholinergic system

2.3.1 The peripheral nervous system

Two primary types of cholinergic receptors exist in the mammalian body, muscarinic and nicotinic receptors, respectively G-protein coupled receptors and acetylcholine-gated ion channels. These receptors exist both in the peripheral nervous system and in the central nervous system. In G-protein coupled receptors the G protein binds to an intracellular loop of the receptor upon activation of the receptor by an agonist, i.e., ACh. In the peripheral nervous system, muscarinic receptors are located on end-organs innervated by postganglionic parasympathetic fibers. Peripheral muscarinic receptors at parasympathetic neurons are stimulated via the nervus vagus, and activation results in smooth muscle contractions and glandular secretion. In these cases, inhibition of AChE may cause miosis, salivation, abdominal cramps and incontinence.

Nicotinic acetylcholine receptors are found at the neuromuscular junction of striated muscles and at all autonomic ganglia, sympathetic and parasympathetic. Nicotinic receptors are members of the superfamily of the cys-loop ligand-gated ion channels. Muscle type and neuronal type nAChRs belong to this family. Along with 5-hydroxytryptamine type 3 (5-HT3) receptors, γ-amino-butyric acid type A (GABAA) and type C (GABAC) receptors, glycine receptors and invertebrate glutamate and histidine receptors (Karlin, 2002; Dani, 2001). Nicotine receptors consist of five membrane-spanning subunits, arranged as a ring structure with a central pore forming the ion channel. The subunit composition of nAChRs differs in skeletal muscle, peripheral nervous system and the central nervous system. The composition of the subunits characterizes the physiological properties of the receptors, such as agonist affinity, ion permeability and desensitization rates.

At the neuromuscular junction, ACh is released from synaptic vesicles of the nerve terminals in response to a presynaptic action potential. When AChE in the synaptic cleft is inhibited by an OP, repetitive activation of postsynaptic nicotinic receptors occurs, resulting in paralysis of striated muscle and spasm of smooth muscles. Paralysis of respiratory muscles, bronchospasm, bronchorrhea and central apnoea may lead to respiratory failure. The presence of nicotinic receptors at the target end-organs of sympathetic fibers may lead to tachyarrhythmias or hypertension (Cannard, 2006; Sidell, 1997).

2.3.2 The central nervous system

A common denominator for the effects of OPs entering the CNS is that they induce seizures via irreversible AChE inhibition causing subsequent ACh accumulation. Cholinergic innervation is present in the entire CNS. Three major cholinergic projection pathways have been identified (Fig. 3). Two pathways originate in the basal forebrain. Neurons in the medial septum, consisting of the medial septal nucleus and vertical diagonal band, project to the hippocampus and the parahippocampal gyrus. Neurons in the nucleus basalis project to all areas of the neocortex, parts of the limbic cortex and to the amygdala. The cholinergic neurons in the pontomesenphalon innervate the hindbrain, thalamus, hypothalamus and basal forebrain (Myhrer, 2007; Woolf, 1996; Woolf, 1991).

Throughout the CNS, a widespread expression of 5 different subtypes of mAChRs is found, pointing to their involvement in many different neuronal processes. A prominent role for the high densities of M1 mAChRs to learning and memory in the cortex and hippocampus has been identified. Attention and sleep have also been shown to be regulated by involvement of mAChRs (Herrero et al., 2008; Hasselmo, 2006; Goutagny et al., 2005; Coleman et al., 2004; Levey, 1996). In OP poisoning, excessive stimulation of mAChRs in the brain leads to seizure development.

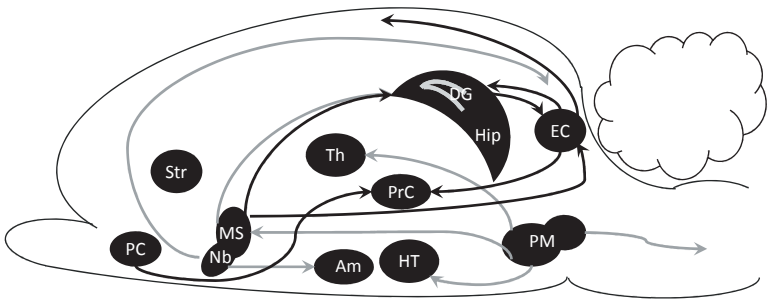

Figure 3. *Schematic presentation of the main cholinergic (gray arrows) and seizure propagating (black arrows) pathways in a lateral view of the rodent brain. Neurons in the medial septum (MS) project to the hippocampus; neurons in the Nucleus Basalis (Nb) project to the Amygdala (Am) and to the Cortical region and neurons in the pontomesenphalon (PM) project to the hindbrain, Thalamus (Th), Hypothalamus (Ht) and basal forebrain (MS and Nb). Upon OP poisoning, the piriform cortex (PC) innervates the Perirhinal Cortex (PrC). The MS activates the hippocampus (Hip) and the Entorhinal Cortex (EC). The EC projects to the hippocampus via the perforant pathway, and to all cortical areas including motor areas. Adapted from Myhrer (2007) and Woolf (1991).*

Neuronal nAChRs are widely distributed in the brain, of which the majority is located presynaptically, where they modulate the release of neurotransmitters such as dopamine, glutamate, and noradrenaline (Albuquerque et al., 2009; Gotti and Clementi, 2004). The nAChRs are involved in many aspects of behaviour, including locomotion, nociception, anxiety and learning and memory (Picciotto et al., 2000; Ross et al., 2000; Decker et al., 1995). Because of the diverse roles of the different nAChRs in the brain, receptor dysfunction is associated with several pathophysiological conditions, among which nocturnal frontal lobe epilepsy, Parkinson's and Alzheimer's disease and schizophrenia (Hogg et al., 2003). The ubiquitous localization of nAChRs and their diverse function, synaptically and extrasynaptically, causes nAChR-mediated CNS dysfunction as a result of OP exposure.

2.4 OP-induced seizures and neurotoxicity

It is proposed that a 3-phased sequential recruitment of different neurotransmitter systems is involved in the initiation and maintenance of seizure activity (Shih and McDonough, Jr., 1997). The neurochemical event initiating seizure activity in susceptible neuronal circuits is the accumulation of ACh, which is characterized as the first, or cholinergic, phase (Bueters et al., 2002; Tonduli et al., 1999; Lallement et al., 1992a; Fosbraey et al., 1990). During a transition phase, excitatory activity rapidly spreads and other neurotransmitter systems are recruited. This is followed by the final phase of seizure progression, which is mainly non-cholinergic. At this time, ACh levels return to normal levels, accompanied by increases in dopamine and GABA concentrations (Shih and McDonough, Jr., 1997; Fosbraey et al., 1990; Liu et al., 1988).

Seizures appear to be generated via specific anatomical routes (Fig. 4). The piriform cortex is the first site to be activated in seizure propagation, and therefore identified as a key site for seizure intitation, followed by the amygdala, hippocampus, thalamus, cortical areas and striatum (Zimmer et al., 1997). These regions show the most severe signs of neuropathology, indicating their involvment in seizure propagation (McDonough, Jr. et al., 1995).

Two brain areas are identified as seizure controlling areas, i.e., the substantia nigra and the area tempestas, which is located in the piriform cortex. Seizure modulation via the substantia nigra is only susceptible to treatment with GABAergic drugs, whereas in area tempestas cholinergic, glutamatergic and GABAergic drugs are effective (Myhrer et al., 2008a; Myhrer et al., 2006; Gale, 1988). In the medial septal area, soman-induced seizures can be controlled by atropine (Denoyer et al., 1992; Lallement et al., 1992b). Selective lesions of the area tempestas or medial septum are also effective in preventing soman-induced seizures (Myhrer et al., 2007). The area tempestas and the medial septum have been proposed to innervate hyperactivity in the hippocampal region in two ways: First, via direct cholinergic input, and second, via cholinergic input into the entorhinal cortex, which in turn activates the glutamatergic perforant path. Via the hippocampus, the entorhinal cortex innervates other cortical areas, including motor areas, thereby propagating epileptiform activity.

The mechanisms underlying neurotoxicity that follow OP exposure are subject to debate. Some evidence exists for direct neurotoxic effects of OPs, both from in vitro and in vivo studies. Several OPs have shown to possess cytotoxic activity in cultured neuroblastoma cells (Carlson and Ehrich, 2008; Carlson et al., 2000), rat hippocampal cell cultures (Bahrami et al., 2009) and in rat cortical neurons (Wang et al., 2008). Other direct effects of OPs are disruption of polymerisation of microtubules (Grigoryan and Lockridge, 2009), an altered sensitivity to glutamate excitotocity, and a decline in synaptic markers, indicating that OPs are able to directly induce synaptopathogenesis (Munirathinam and Bahr, 2004). In vivo, low doses of OP, not causing acute toxicity, have shown to lead to subtle behavioral dysfunctions in rats (Verma et al., 2009; Prendergast et al., 1997; Nieminen et al., 1990). These findings imply that OPs are capable of inducing neurotoxicity, largely independent from AChE inhibition, and this capacitiy is different amongst OPs.

Higher doses of OPs induce seizure activity and excessive excitatory amino acid (EAA) release, which is thought to be the main mediator of OP-induced brain injury. Both studies on epilepsy and OP-induces seizures revealed that 40 to 60 minutes of seizures are required to induce neuronal death (Baille et al., 2005; Fujikawa, 2005; Carpentier et al., 2000; Shih et al., 1991). The duration and intensity of seizures has shown to correlate well with the degree of neuropathology (Baille et al., 2005; Carpentier et al., 2001; Carpentier et al., 2000; McDonough, Jr. et al., 1995). The glutamate released as a result of ongoing seizures activates the postsynaptic NMDA receptors, leading to excessive calcium influx into neurons. This, in turn, triggers activation of proteolytic enzymes, phospholipases, neuronal NOS and it triggers free radical generation, resulting in neuronal damage (Fig. 4).

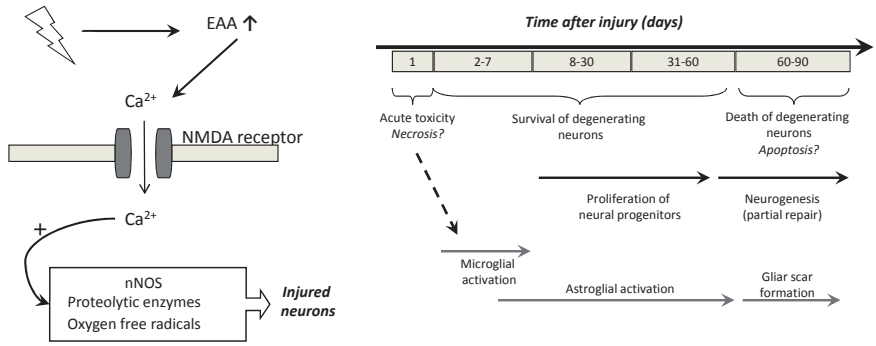

***Figure 4**. Excitotoxicity mediated by OP-induced seizures (left) and an overview of the CNS response to OP-induced neurotoxicity as proposed by Collombet et al. (2007 (right)).*

2.5 Responses to OP-induced neurotoxicity: a role for neurogenesis?

A time course for neuronal and glial responses in the brain upon OP-induced seizures was proposed by Collombet et al. (2007) (Fig. 4). After a period of acute toxicity, in which numerous cells die, microglial cells are activated peaking at day 3, followed by astroglial activation peaking at day 8. These cells produce numerous

cytokines, neutrophic- and growth factors which might contribute to the prolonged survival of degenerating neurons 3 to 60 days after soman exposure (Liberto et al., 2004; Lallement et al., 2002)). After returning to normal glial activity at days 30-60, a period of delayed toxicity is present, in which the damaged neurons slowly die, paralleled by glial scar formation and partial repair by neurogenesis. Although apoptotic cells are mostly absent, probably due to the rapidity and energy dependence of the process (Nicotera and Leist, 1997), degenerating cells resulting from soman-induced seizures show increased expression of proteins related to programmed cell death, indicating the presence of a hybrid form of cell death between apoptosis and necrosis (Baille et al., 2005).

The long-term deficits as a consequence of OP exposure urge for understanding the mechanisms that might stimulate brain repair and/or diminish mental health effects. In the past decade, it has become apparent that in specific parts of the adult brain new neurons are formed, a process termed neurogenesis (Schinder and Gage, 2004; Gross, 2000; Eriksson et al., 1998; Kuhn et al., 1996). Two main regions in the hippocampus, the subgranular zone (SGZ) and the dentate gyrus (DG) show prominent levels of neurogenesis (Fig. 5). In these regions, newborn neurons undergo migration before they eventually maturate (Schinder and Gage, 2004). Neural progenitor cells are distributed along the SGZ. In this region, they proliferate and differentiate, followed by a superficial migration into the GCL. The cell bodies remain in the granular cell layer, with their dendrites projecting through the molecular layer, and the axons projecting to the hilus and CA3 region (Schinder and Gage, 2004; Hastings et al., 2002; Hastings and Gould, 1999).

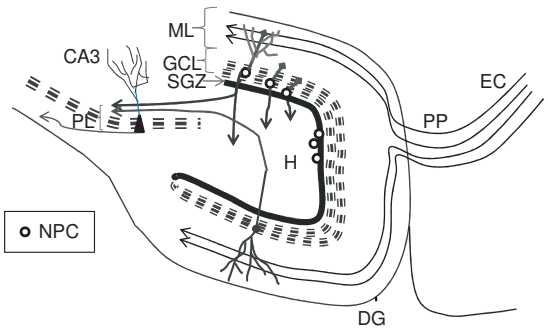

***Figure 5**. Illustration of neurogenesis in the adult dentate gyrus. Neuronal progenitor cells (NPCs) are located in the subgranular zone (SGZ), which separates the granular layer (GCL) from the hilus (H). After differentiation of the NPCs into neurons in the SGZ, the neurons migrate into the GCL. Their dendrites project through the molecular layer (ML) and the axons project towards CA3 and the hilus. The dendrites receive input from the entorhinal cortex (EC) via the perforant path (PP). Adapted from Schinder and Gage (2004).*

The rate of neurogenesis is affected by several factors. Stress and aging suppress the levels of neurogenesis in the hippocampus (Heine et al., 2004; Kempermann et al., 1998) (Heine et al., 2004; Westenbroek et al., 2004). Corticosterone injections also reduce the levels of hippocampal cell proliferation (Cameron and Gould, 1994). Physical activity is associated with increases in neurogenesis (Van Praag et

al., 1999). A role for neurogenesis has been implicated in hippocampal learning and memory (Dupret et al., 2007; Dobrossy et al., 2003). It has been shown that neurogenesis in the SGZ of rodents is induced by brain injury resulting from seizures or ischemia shortly after injury, also in OP poisoning (Wiltrout et al., 2007; Parent, 2007; Collombet et al., 2005; Emsley et al., 2005; Sharp et al., 2002).

Several neurotransmitters, among which glutamate, acetycholine and serotonin, have been implicated in the regulation of neurogenesis, potentially enabling pharmacological invervention. NMDA receptor activation reduces neurogenesis, whereas lesion of the Perforant Path, the glutamatergic input from the EC into the hippocampus, showed to enhance neurogenesis (Nacher et al., 2003; Cameron et al., 1995). Selective serotonin reuptake inhibitors, used as anti-depressants (e.g. fluoxetine), and atypical anti-psychotics (e.g. olanzapine) have shown to increase neurogenesis (Paizanis et al., 2007; Kodama et al., 2004; Wang et al., 2004; Wakade et al., 2002). Damage to the cholinergic circuitry projecting to the hippocampus has been shown to suppress neurogenesis (Kotani et al., 2006; Van der Borght et al., 2005; Cooper-Kuhn et al., 2004), whereas the reversible AChE inhibitor donepezil enhanced neurogenesis (Kotani et al., 2008). So, both brain injury resulting from seizures, as well as from cholinergic impairment following OP poisoning might interfere with neurogenesis and accordingly with cognition. If so, restoration of neurogenesis might be an attractive approach for the treatment of long-term cognitive deficits.

3 Clinical implications of OP poisoning

Since the currently available treatment with atropine and oximes in case of OP poisoning is insufficient, victims might suffer from persisting adverse effects on cognitive processes, mood and sleep-wake rhythm in spite of treatment (van Helden and Bueters, 1999; Lallement et al., 1998; Shih and McDonough, Jr., 1997; Willems et al., 1993). The adverse effects are mostly documented in animal studies and case studies of human subjects accidentally exposed to organophosphate pesticides.

Farmers exposed either intentionally or accidentally to organophosphate pesticides displayed psychological disturbances, such as intellectual impairment, anxiety and disturbed sleep patterns (Jamal, 1995; Steenland et al., 1994; Minton and Murray, 1988; Levin et al., 1976). More recent human data are available from two terrorist attacks with the nerve agent sarin in Matsumoto and Tokyo, Japan, in 1994 and 1995, when over 6000 people were injured and 12 people were killed. Acute and long-term effects in these patients are well-documented (Yanagisawa et al., 2006).

After the terrorist attacks there was a great concern regarding persistent brain dysfunction and cognitive impairment. The distinction between direct effects of the sarin exposure and the effects of uncertainty and fear exerted by the attacks has not been made unambiguously (DiGiovanni, Jr., 1999). Culmination of epileptic seizures occurred within a few hours, and EEG abnormalities were still present for a period of approximately 5 years. Such changes were also found in animal studies and earlier studies in humans (van Helden et al., 2004; Burchfiel and Duffy, 1982; Duffy et al., 1979). Brain function tested by event-related potentials also showed

abnormalities at 6 months and 3 years after the exposure (Nishiwaki et al., 2001; Murata et al., 1997). At 5 years after the sarin attacks in Tokyo, forgetfulness, lack of concentration and depressive moods were observed more frequently in exposed people (Yanagisawa et al., 2006). However, only a small number of these people had suffered from seizures and convulsions. The long-term complaints can therefore not with certainty be ascribed to seizure activity. Both structural changes related to sarin exposure, as found by Yamasue et al. (2007) and stress-related disorders might contribute to long-term psychological problems. Such life-threatening events may induce stress-related disorders such as PTSD, resulting in signs similar to those after OP poisoning at long term (Ursano et al., 2008; Kessler et al., 1995).

4 Animal models in OP poisoning

Because experiments with human subjects regarding the mechanisms of OP poisoning and therapeutic efficacy are obviously not possible, several animal models, including mice, rats, guinea pigs and occasionally primates were used. The choice for a typical animal model depends on, for example, differences in susceptibility to OPs between species. In rats, high levels of carboxylesterase, an endogenous scavenger of OPs, are found, which makes the doses required to induce toxicity higher than in other species (Karanth and Pope, 2000; Dettbarn et al., 1999; Yang and Dettbarn, 1998; Maxwell, 1992). However, neurochemical or cognitive consequences of OP poisoning due to seizure activity are similar in different species, making rats and mice suitable animal models to address research questions in this respect. In guinea pigs the levels of carboxylesterase are much lower than in rats and rabbits, making the guinea pig the preferred model to investigate therapeutic or prophylactic efficacy of carbamates or human butyrylcholinesterase (Leadbeater et al., 1985; Inns and Leadbeater, 1983). In most animal models, relatively high levels of exposure were used, resulting in the appearance of severe toxic signs and death in a short period of time. Exposing animals to lower challenging doses would improve monitoring the pathophysiological process and treatment efficacy; it may reveal new targets for treatment and more effective treatment protocols. The research described in this thesis pays attention to this aspect.

5 Countermeasures against OP poisoning

5.1 Physical protection and pretreatment

Under circumstances of chemical threat several precautions can be taken. First of all, protective clothing and respiratory protection will be employed to prevent physical exposure to the chemical threat. Additionally, pretreatment will be available. The current pretreatment regimen consists of pyridostigmine bromide, a quaternary carbamate, which preserves a residual pool of AChE in the blood. During the first Gulf war (1991), thousands of soldiers used pyridostigmine. Because pyridostigmine is not able to enter the brain, it cannot provide sufficient protection against brain injury. Pretreatment with the carbamate physostigmine, that does enter the brain, showed higher efficacy than pyridostigmine, but

also showed several side effects (Myhrer et al., 2004; Philippens et al., 1996). By combining physostigmine with scopolamine, a muscarinic antagonist, gross side effects could be abolished in guinea pigs (Philippens et al., 2000; Philippens et al., 1998). However, recent work suggests that this combination of drugs does induce cognitive behavioural side effects in rats, probably precluding use of this regimen in humans (Myhrer et al., 2008b). The expected side effects of a carbamate pretreatment, its low efficacy and the difficulty to predict nerve agent exposure, argues for development of an effective post-poisoning treatment that does not rely on pretreatment.

5.2 Current treatment

The current treatment regimen in case of OP poisoning is based on reactivation of inhibited AChE, protection of muscarinic receptors, and suppression of seizures. In case of skin contamination, decontamination is accomplished by swiping the contaminated site with hypochlorite or RSDL pouches. Entrance of the nerve agent into the circulation requires immediate treatment using autoinjectors, containing an oxime such as HI-6, 2-PAM or obidoxime and atropine as anti-muscarinergic agent. Diazepam or pro-diazepam may be added to the autoinjector or in a separate autoinjector for immediate anti-convulsant treatment. Follow-up treatment will require intensive medical care and hospitalisation, to continue reactivation of inhibited AChE and provide anticholinergic treatment and, if required, supportive artificial respiration.

Atropine sulphate is the classical antimuscarinergic, which still is the main drug in counteracting the deleterious effects of excessive ACh build-up to counteract the major cholinergic signs, such as salivation, and, to a lesser extent, the loss of central respiratory drive. Atropine also has some anticonvulsant effects (Shih et al., 2007; Shih and McDonough, Jr., 1999; Capacio and Shih, 1991), in particular during the first phase of poisoning.

Oxime treatment also has several limitations. First of all, the central nervous system is hardly protected by oximes, because of their quaternary structure hampering their entrance into the brain. Although some oximes, such as monoisonitrosoacetone (MINA) and diacetylmonoxime (DAM), showed a high lipid solubility and ability to enter the brain (Cohen and Wiersinga 1960), further research into these compounds was terminated because of the much higher reactivating potency towards phosphorylated AChE of quaternary pyridinium oximes, such as 2-PAM (Hobbiger et al., 1960).

None of the oximes available today has a broad-spectrum efficacy, meaning that efficacy depends of the nerve agent - oxime combination. To overcome this problem, it is proposed to use combinations of oximes to generate a cocktail effective against the known nerve agents (Worek et al., 2007). Although the main therapeutic efficacy is proven to be due to enzyme reactivation (Maxwell et al., 2006), there is also evidence that other effects of oximes contribute to therapeutic effects. Experiments in primates have shown that survival of OP-exposed animals was not associated with a significant recovery of AChE activity (van Helden et al., 1996; Hamilton and Lundy, 1989). Oximes were reported to have direct effects on the ion channel of the nicotinic receptor, which is characterised as an use-dependent

open-channel block (Tattersall, 1993; Alkondon and Albuquerque, 1989; Alkondon et al., 1988). Neuromuscular recovery of OP-poisoned diaphragm by oximes was directly correlated with the level of channel block induced by the oximes. Recently, it was found that non-competitive blocking of the ion channel was responsible for restoration of the decay of the endplate current allowing repolarisation and relief of the depolarisation block (Turner 2007).

Early termination of seizures following OP challenge is crucial in limiting neuropathology. Benzodiazepine anticonvulsants such as diazepam and midazolam have shown efficacy in terminating seizures up to 40 minutes after OP poisoning. Benzodiazepines act by increasing the open time of the chloride channel of the GABAa receptor (Twyman et al., 1989). Chloride influx into the cells decreases the depolarisation probability of the cells by hyperpolarisation of the membrane. This lowers the number of action potentials that can be initiated, thereby lowering the summation of action potentials that would otherwise cause seizure activity. Shih et al. (2007) showed that midazolam is more effective than diazepam in counteracting seizures/convulsions induced by all nerve agents in two animal models. Benzodiazepines might aggravate OP-induced respiratory depression and must therefore be used with great caution (Forster et al., 1980), although in animal studies respiratory depression and neuropathology showed to be reduced by diazepam treatment.

5.3 New approach for treatment improvement

Since carbamates were shown to be effective as a pretreatment against lethal doses of nerve agents, it has been proposed to evaluate whether carbamates could also be effective in post-poisoning therapy. For example, administration of physostigmine 1 min after lethal doses of soman poisoning, appeared to be effective (Wetherell et al., 2007; Wetherell et al., 2006). In the latter studies high doses of the oxime HI-6 were given in combination with scopolamine hydrobromide. Also the reversible AChE inhibitor galantamine showed efficacy when administered shortly after VX or soman poisoning (Hilmas et al., 2009; Albuquerque et al., 2006). The exact mechanism of this treatment is not clear, since both protection of AChE by competition of carbamates with incoming nerve agent for the same binding site, as well as anti-nicotinic properties of, in particular, galantamine might contribute to neuroprotection. An important part of this thesis contributes to elucidate the rationale behind the therapeutic efficacy of a post-poisoning treatment consisting of an oxime and a carbamate.

6 Goal and outline of thesis

The goal of this thesis was to evaluate the treatment protocols in case of poisoning with two OP compounds: soman, a volatile and fast acting cholinesterase inhibitor and VX: a low volatile agent administered by the percutaneous route. Because of the ratification of the CWC to ban the use of OP nerve agents by most countries, the threat of these being used on the battlefield is low, and exposures are most likely to be unexpected or accidental. In that case, people that are exposed will not have received pretreatment, urging the present medical countermeasures to be efficacious and not reliant on pretreatment. Specific aims of the thesis were:

1.) Identify toxicological impact on physiology of the different OPs and different routes of exposure
2.) Identify efficacy of treatment protocols and possible improvements
3.) Investigate long term-effects of OP-exposure and possible interventions

In Chapter 2, the effects of different treatment regimens in a soman-exposed guinea pig model, developed for monitoring biochemical and vital physiological parameters, are investigated. The efficacy of obidoxime and atropine was tested both in at 1 minute after challenge, as well as after appearance of first clinical signs after different doses of soman. In addition, it was investigated whether the treatment regimen could be improved by adding the carbamate physostigmine to the treatment.

The VX-exposed hairless guinea pig model is presented in Chapter 3. VX is a low volatile nerve agent and therefore mainly constitutes a contact hazard. Exposure via the skin has shown to be unpredictive and variable in terms of toxicokinetics. After a lag period, VX levels continue to rise for several hours. In this chapter, the physiological consequences of central ACh levels, clinical signs and AChE activity were investigated. The model was evaluated for possible indicators for the start and maintenance of treatment.

In Chapter 4 the VX model was extended to investigate toxicokinetics in freely moving animals combined with physiology, e.g. respiration, EEG and heart rate effects. After full establishment of all parameters, a treatment protocol consisting of a high dose, one-shot injection consisting of 3 autoinjector equivalents of atropine, obidoxime and diazepam was tested. In addition, a treatment protocol using repetitive administration of lower doses of atropine, obidoxime and diazepam was tested for improved efficacy compared to the one-shot treatment.

Long-term behavioural deficits of OP-poisoning and a possible intervention by drug-induced neurogenesis were investigated in a soman-poisoned rat model in Chapter 5. A seizure-inducing dose of soman was used, in combination with life saving treatment with atropine and HI-6. After a 4-week chronic treatment with the antipsychotic Olanzapine, a neurogenesis enhancer, behavioural consequences were tested in the Morris Water Maze, and the brains were evaluated for neurodegeneration and levels of neurogenesis.

In Chapter 6, the overall results will be discussed: the different agents and routes of exposure are discussed in terms of expected consequences as well as the most effective treatment protocols and possibilities for further improvement.

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Chapter 2

Treatment efficacy in a soman poisoned guinea pig model: added value of physostigmine?

Marloes J.A. Joosen [1], August B. Smit [2] and Herman P.M. van Helden[1]

1: TNO Defence, Security and Safety, BU CBRN Protection, Rijswijk, The Netherlands
2: Centre for Neurogenomics and Cognitive Research, VU University, Amsterdam, The Netherlands

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Abstract

Current treatment of organophosphate poisoning is insufficient and survivors may suffer from long-lasting adverse effects, such as cognitive deficits and sleep-wake disturbances. In the present study, we aimed at development of a guinea pig model to investigate the benefits of immediate and delayed stand-alone therapy on the development of clinical signs, EEG, heart rate, respiration and AChE activity in blood and brain after soman poisoning. The model allowed the determination of the therapeutic effects at the short-term of obidoxime, atropine and physostigmine. Obidoxime exerted the highest therapeutic efficacy at administration of the lowest dose (3.1 mg/kg i.m.), whereas two higher doses (9 and 18 mg/kg) were less effective on most parameters. Addition of atropine at 0.03 and 3 mg/kg (i.m.) to the treatment did not improve the therapeutic effects of obidoxime alone. Physostigmine (0.8 mg/kg im) at 1 minute after poisoning, increased mortality. Two lower doses (0.1 and 0.3 mg/kg i.m.) showed improvements on all parameters but respiration. The middle dose was most effective in preventing seizure development and therefore assessed as the most efficacious dose. Combined treatment of obidoxime and physostimgine shortened duration of seizures, if present, from up to 80 min to approximately 10-15 min.

In practice treatment will be employed when toxic signs appear, with the presence of high levels of AChE inhibition in both blood and brain. Administration of physostigmine at that moment showed to be redundant or even harmful. Therefore, treatment of OP poisoning with a carbamate, such as physostigmine, should be carefully re-evaluated.

1 Introduction

Organophosphates (OPs) are highly toxic chemicals, which mainly exert their toxicity by irreversible inhibition of acetylcholinesterase (AChE) and the consequent excessive and deleterious buildup of acetylcholine (Maxwell et al. 2006). Nerve agents are the most toxic OPs and might be used in military conflicts or in terrorist attacks, such as those in the Tokyo subway station in 1995 (Suzuki et al. 1995). The buildup of acetylcholine (ACh) following OP exposure in central and peripheral synapses, leads to typical cholinergic signs due to overstimulation of nicotinergic and muscarinergic synapses. This overstimulation evolves into hypersecretions, failure of neuromuscular transmission, seizures, convulsions and eventually death due to respiratory failure (Shih and McDonough, Jr. 1997; van Helden and Bueters 1999). In preclinical studies, the currently available treatment regimens appeared to be ineffective in preventing this, giving way to seizures that might induce brain damage and concomitant deficits in cognition and attention even when they are short-lasting (Joosen et al. 2009; Raveh et al. 2003).

Treatment is generally aimed at three targets. The first one, achieved by oxime treatment, is breaking the covalent bond of the OP from the serine residue in the active site of AChE. However, no broad-spectrum oximes are available to manage the chemistry of all nerve agents (Aas 2003; Maxwell et al. 2008). Moreover, none of the oximes are capable to reactivate inhibited and “aged” AChE, i.e. when the alkyl tail of the OP molecule has gone, thereby rendering the remaining bond virtually irreversible (Berman and Decker 1986). The second target of treatment is directed towards blocking muscarinic overstimulation using compounds such as atropine sulphate. Although rather effective at very high doses, atropine rapidly looses efficacy when administered at low doses or after development of full blown seizures (Shih and McDonough 2000). The final treatment is aimed at terminating seizures by benzodiazepines in order to prevent brain injury. Recent research suggested that seizures lasting no longer than 10 minutes, did not induce visible brain damage, but rather inhibited neurogenesis resulting in impaired cognitive performance (Joosen et al. 2009). These and other findings show the need to advance understanding of the toxic as well as the antidotal mechanisms in order to obtain more effective medical countermeasures.

Therefore the present study was aimed at investigating several features of OP poisoning and treatment. The nerve agent soman was chosen as a model compound because it is expected to have the highest risk of inducing brain damage (McDonough, Jr. et al. 1999; McDonough, Jr. et al. 2000), and after binding to AChE, it ages very rapidly. This makes poisoning with soman less prone to successful treatment with any of the existing oximes, such as 2-PAM, obidoxime and HI-6. It is known that preserving or restoring AChE pools is one of the most efficacious treatments in case of nerve agent poisoning, and most effective when employed very rapidly after poisoning. Although the reactivating capacity of obidoxime towards soman-inhibited AChE activity is known to be very low, this oxime was used in the present experiments for two reasons. The first reason was purely practical in that obidoxime is currently in use as an autoinjector compound. Second, the use of obidoxime would enable identification of other targets for obidoxime efficacy than AChE reactivation alone (Maxwell et al. 2006).

Recently, post-poisoning treatment with carbamates has been proposed. For example, physostigmine, previously shown to be highly effective as pretreatment against OP-poisoning, was effective against 5 LD50 soman in guinea pigs, administered 1 minute post-poisoning in combination with scopolamine and HI-6 (Wetherell et al. 2006; Wetherell et al. 2007). Also galantamine showed efficacy when administered shortly after poisoning with VX or soman (Albuquerque et al. 2006; Hilmas et al. 2009).

In the present study, a soman poisoned guinea pig model was developed to enable investigation of the impact of different doses of soman and subsequent relief of toxic signs by different treatment protocols. The guinea pig is the most widely accepted non-primate model for predicting treatment efficacy for nerve agent poisoning in primate species (Dirnhuber et al. 1979; Gordon et al. 1978). Our approach enabled online monitoring of vital physiological signs and off-line determination of biochemistry in terms of AChE and butyrylcholinesterase (BuChE) activity. Both physiology and biochemistry were investigated for being suitable parameters predictive for clinical outcome. The therapeutic efficacy of several treatment combinations of obidoxime, atropine sulphate and physostigmine, administered at 1 or 10 minutes following poisoning, was tested. The research was focused on identification of the contributing role of physostigmine in the treatment.

2 Methods

2.1 Animals

Male Dunkin Hartley guinea pigs (Crl:HA) obtained from Charles River (Maastricht, The Netherlands) were used in the present study. Prior to the experiments they were housed with 2 animals per cage, and allowed to get accustomed to standard conditions for one week. Room temperature was kept at 19-22 °C and relative humidity was maintained at 55-65% and lights were on from 7 am to 7 pm. Acidified water and standard guinea pig chow (Teklad global diet 2040, Harlan, Horst, The Netherlands) were available ad libitum. The experiments described received prior approval from the TNO Animal Ethical Committee.

2.2 Chemicals

Hypnorm® (fentanyl/fluanisone) was purchased from Janssen Pharmaceutica (Beerse, Belgium) and Dormicum® (midazolam) was delivered by Roche Nederland BV (Mijdrecht, The Netherlands). Soman (O-Pinacolyl methylphosphonofluoridate) was obtained from the stocks of TNO Defence, Security and Safety and of purity ≥98% (GC). The other chemicals used were of standard purity and purchased from renowned companies.

2.3 Study design

The design of the study is presented in Figure 1. After obtaining baseline values of the indicated parameters for 30 minutes, the guinea pigs were poisoned with

soman (30; 37 or 44 µg/kg s.c.; corresponding to approximately 1.2; 1.5 and 1.8 24h LD50 respectively, (Gordon and Leadbeater 1977), followed by i.m. treatment 1 or 10 minutes later. The experiment was divided in 4 parts: establishment of a model dose for soman; efficacy of conventional treatment by obidoxime and atropine; efficacy of physostigmine and the efficacy of postponed treatment (Table 1). A control group was injected with saline (s.c.) followed by an i.m. saline injection. At the indicated time points (downward triangles) blood samples were drawn from the ear vein to assess AChE activity. Following poisoning, the guinea pigs were observed for toxic cholinergic signs such as chewing, tremor, salivation and convulsions. Clear chewing movements involving the entire head were scored as chewing, often accompanied by extensive drooling (salivation) later on. Involuntary shaking of parts of the body or head was scored as tremor. Convulsions were defined as involuntary movements of the entire body, while the animal looks dissociated from its environment. At the end of the experiment, 90 minutes after poisoning, different brain parts (hippocampus, striatum and medulla oblongata) were isolated for AChE level determination.

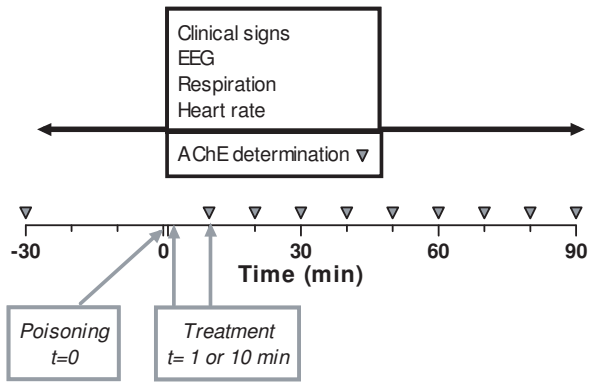

Figure 1. *Overview of the experimental design. Before and after poisoning, EEG, ECG, respiration and clinical signs were continuously monitored. Every 10 minutes after poisoning blood was drawn from the ear vein to determine blood ChE activity.*

2.4 Surgical procedures

Guinea pigs were anaesthetized with 6 ml/kg FFM-mix (1.25 mg/ml midazolam, 2.5 mg/ml fluanisone, 0.079 mg/ml fentanyl citrate) via a single i.p. injection. Premedication with 0.05 mg/kg atropine sulphate (s.c.) was administered to prevent respiratory arrest. Two stainless steel screws (A8.0 and P1.2 mm relative to bregma and 1 mm from the sagittal suture) placed at the dura mater, fixed to a plug, served as EEG electrodes. To obtain an ECG signal, two leads sutured in the superficial muscles under the skin just below the right collarbone and between the second and third rib (Lead II configuration) and also fixed to the plug. The plug and the electrodes were fixed to the skull with dental cement.

***Table 1**. Overview of experimental groups. The left column refers to the corresponding results section. Time and dose of treatments applied, occurrence of seizures and convulsions, and survival rate are shown from left to right.*

Experiment	Exposure	Treatment (mg/kg i.m.)				Signs					
	Soman (µg/kg s.c.)	OBI	PHY	AS	Time of treatment (min after soman)	seizures		convulsions		90 min survival	
						(n)	%	(n)	%	(n)	%
Model	0	-	-	-	-	0/4	0	0/4	0	4/4	100
	30	-	-	-	1	6/6	100	6/6	100	5/6	83
	37	-	-	-	1	6/6	100	6/6	100	3/6	50
	44	-	-	-	1	6/6	100	6/6	100	1/6	17
Conventional	30	3.1	-	-	1	4/6	67	2/6	33	6/6	100
	30	9	-	-	1	5/6	83	4/6	67	6/6	100
	30	18	-	-	1	5/6	83	4/6	67	6/6	100
	30	-	-	3	1	5/6	83	2/6	33	6/6	100
	30	3.1	-	0.03	1	5/5	100	4/5	80	5/5	100
	30	3.1	-	3	1	4/5	80	1/5	20	4/5	80
	44	3.1	-	3	1	6/6	100	6/6	100	5/6	83
Physostigmine	30	-	0.1	-	1	4/4	100	1/4	25	4/4	100
	30	-	0.3	-	1	2/6	33	2/6	33	6/6	100
	30	-	0.8	-	1	1/2	50	2/2	100	0/2	0
	30	3.1	0.1	-	1	3/5	60	1/5	20	5/5	100
	30	3.1	0.3	-	1	2/6	33	1/6	17	6/6	100
	30	3.1	0.8	-	1	4/4	100	4/4	100	2/4	50
	30	3.1	0.3	3	1	1/6	17	0/6	0	6/6	100
Postponed	30	3.1	0.3	-	10	4/6	67	3/6	50	2/6	33
	30	3.1	0.3	3	10	6/6	100	6/6	100	5/6	83
	30	3.1	-	3	10	6/7	86	5/7	71	7/7	100

2.5 Acquisition and analysis of EEG and respiration

Before and after poisoning, the animals were placed in a whole body plethysmograph (Buxco sytems XA, USA) cage to monitor their respiration characteristics. Among other parameters, P-enhanced (Penh), an arbitrary value for bronchoconstriction, and Respiratory Minute Volume were calculated from the signal using IOX software (EMKA technologies, France).

EEG signals were obtained using the PhysioTel telemetry system from Data Sciences Inc. (DSI) using the ML2-11-EET-F40 transmitter. The transmitter was attached to the plug fixed on the guinea pig's skull. A receiver board measured the signal from the transmitter, which was consolidated and stored on an IBM-compatible personal computer via a Data Exchange matrix at 100 Hz sampling frequency. By visual inspection of the EEG, the starting and ending of seizures, defined as amplitudes increasing 3 times over baseline, was scored. Additionally, FFT analysis using 10 second epochs was performed to calculate the total power

in the spectrum, thereby serving as a semi-quantitave measure of seizure activity. Animals were awake during the measurement.

2.6 Enzyme activity

The brain parts were homogenized (900 rpm, 10 % w/v homogenate) in ice-cold TENT buffer, which consisted of 50 mM Tris, 5 mM EDTA, 1 M NaCl and 1% v/v Triton X-100, pH 7.4. The homogenates were centrifuged at 12000 g in an Eppendorf centrifuge at 4 °C and supernatants were immediately frozen in liquid nitrogen and stored at -20 °C until analysis of enzyme activity. Blood samples drawn from the ear vein were diluted 10 times in 1% saponin in MQ and frozen in liquid nitrogen, and stored at -20 °C until analysis.

Tissue supernatants were analyzed for AChE activity using the method by Ellman et al. (1961), modified for analysis in a 96-wel plate reader. Shortly, samples were diluted in 0.8 mM DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)). To 100 µl of diluted sample, 100 µl of 0.8 mM β-methylacetylthiocholine iodide was added, in quadruple. For blood samples, 0.8 mM of butyrylthiocholine (BuSChI) was added to separate portions of 100 µl. The delta OD per min at 415 nm at ambient temperature served as measurement for ChE activity.

Guinea pig AChE does not react with BuSChI, rendering the BuChE activity determined an appropriate representation of BuChE activity. Brain samples showed no reactivity towards BuSChI. However, AChE activity in blood had to be corrected for cross reactivity of β-methylacetylthiocholine with BuChE. The maximum velocity turnover of β-methylacetylthiocholine by BuChE was 47% of that of BuSChI, calculated from separate experiments in which isolated guinea pig AChE and plasma BuChE were incubated with both substrates. Therefore, 47% of the extinction measured with BuSChI as substrate was subtracted from the extinction measured with β-methylacetylthiocholine, which reacts at a similar rate with AChE and BuChE. A similar adaptation has been described by Bosgra et al. (2009). Enzyme activity in the tissue supernatants was normalized versus the average enzyme activity of 6 control animals. AChE and BuChE activity in blood were normalized versus the baseline sample.

2.7 Data presentation and statistical analysis

All data were analyzed using one-way ANOVA or MANOVA followed by Dunnett's or Tukey's post-hoc test using soman or saline as control animals using SPSS statistical software. Results were considered significant for $p<0.05$.

For determination of continuous relationships between blood AChE activity and occurrence of clinical signs logistic regression analysis was performed. AChE activity of similarly treated animals was coupled to presence of a sign (1) or absence of sign (0). To analyze the effects of soman, animals injected with soman only were included in the analysis (n=18). To analyze the effect of separate treatments, animals were grouped into soman poisoned animals treated with obidoxime alone, physostigmine alone, atropine alone or the combinations thereof administered at 1 minute after poisoning. Using logistic regression analysis, probability for each level of AChE activity was calculated.

3 Results

3.1 Soman-poisoned guinea pig model

In the first set of experiments various soman doses (30, 37 and 44 µg/kg) were tested for their induction of clinical signs, and effects on physiology and biochemistry. A dose-dependent effect on survival was found during the 90-min experimental period (See Fig. 2; Table 1). Apart from survival rate no differences regarding the appearance of other clinical signs were found between the different soman doses, albeit that the onset times of clinical signs, if present, were significantly shorter after poisoning with 44 µg/kg of soman (s.c.) compared to the lower doses (Fig. 2).

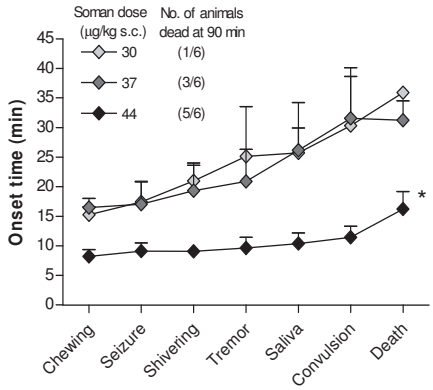

***Figure 2**. Average onset times of clinical signs after different doses of soman. Results are presented as means +/- SEM. Onset times of clinical signs in animals poisoned with 44 µg/kg soman were significantly shorter than in animals poisoned with 30 or 37 µg/kg (One Way ANOVA, within factor signs and between factor dose, p<0.05 followed by Tukey's post-hoc test,*, p<0.001).*

Cholinesterase activity in different brain regions (AChE) at 90 minutes and in blood (AChE and BuChE) at 10 minutes after poisoning was dose-dependently inhibited by soman (MANOVA, $p<0.001$). However, there were no significant differences in inhibition between the different doses (Tukey HSD post-hoc test $p>0.05$ (Table 2)).

Regardless the dose, all animals poisoned with soman developed seizures within 5 to 30 minutes, albeit that animals poisoned with the highest dose tended to develop full blown seizures more rapidly (Fig. 3A). Seizure development is characterized by an increase in total power of the Fast Fourier Transformation of the spectrum of the EEG signal (FFT) compared to seizure-free EEG. In contrast to animals poisoned with 30 or 37 µg/kg, which merely survived the 90-minute observation period, animals poisoned with 44 µg/kg died rapidly after onset of seizures. In surviving animals, seizures continued with similar intensity over the entire 90-min observation period (Fig. 3A).

Additionally, heart rate and respiratory parameters were measured as main vital parameters. There were no obvious and consistent effects of soman on heart rate (not shown), whereas effects on respiration parameters were clearly present.

***Table 2**. Relative residual ChE activity in blood at 10 min and in different brain areas at 90 min post-poisoning. High levels of inhibition of AChE and BuChE are achieved in blood and in brain. Enzyme activity was significantly lower than that of unpoisoned control animals (100% activity), and no difference between soman doses was found (MANOVA followed by Tukey HSD post-hoc test).*

% ChE activity in Blood and brain parts		Soman dose (µg/kg s.c.)					
		30		*37*		*44*	
Blood	AChE	0.9	±1.2	3.9	±2.8	11.0	±16.1
	BuChe	3.5	±0.6	8.4	±1.5	28.6	±7.4
Brain	Medulla oblongata	23.9	±3.3	19.7	±6.0	11.2	±5.2
	Striatum	13.1	±2.6	12.8	±5.7	9.1	±1.5
	Hippocampus	10.0	±3.4	4.7	±2.4	2.0	±1.1

After soman poisoning a clear and significant increase of respiratory minute volume (RMV) compared to control animals was present (Fig. 3B). Soman poisoning also resulted in an increase in bronchoconstriction, which was however not significant (not shown). For these physiological parameters, no dose response effect of soman was observed.

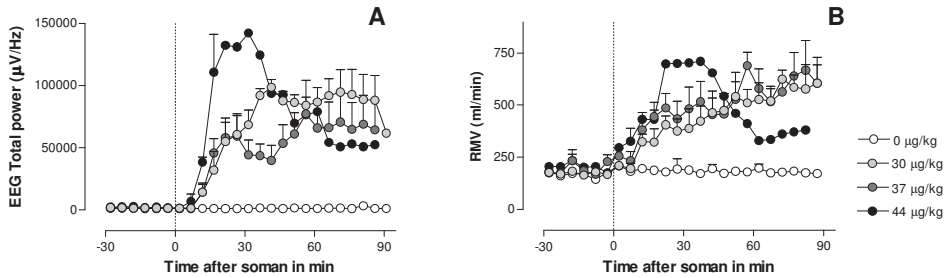

***Figure 3**. A. Effects of different doses of soman on total power in EEG. B. Respiratory minute volume (RMV). Results are presented as mean ± SEM. No significant differences were observed between the different soman doses.*

3.2 Efficacy of conventional therapy

In animals poisoned with 30 µg/kg s.c. soman, components of the treatment currently in use were tested. The effects of different doses and combinations of obidoxime (3.1, 9 and 18 mg/kg) and atropine (0.03 and 3 mg/kg) were tested. None of the obidoxime doses tested induced a significant reactivation of AChE or BuChE in blood at any time point (not shown). Only a dose of 3.1 mg/kg obidoxime had induced a significant increase in AChE activity in the medulla oblongata at 90 minutes after poisoning (Fig. 4A).

Analysis of the occurrence of clinical signs (Table 1) showed that only the lowest dose of obidoxime (3.1 mg/kg) prevented development of convulsions in 67% of animals. Although convulsions were prevented in a high number of animals, seizures were prevented in a low number of animals (33%). Addition of either 0.03 or 3 mg/kg atropine to the treatment did not further improve this. None of these

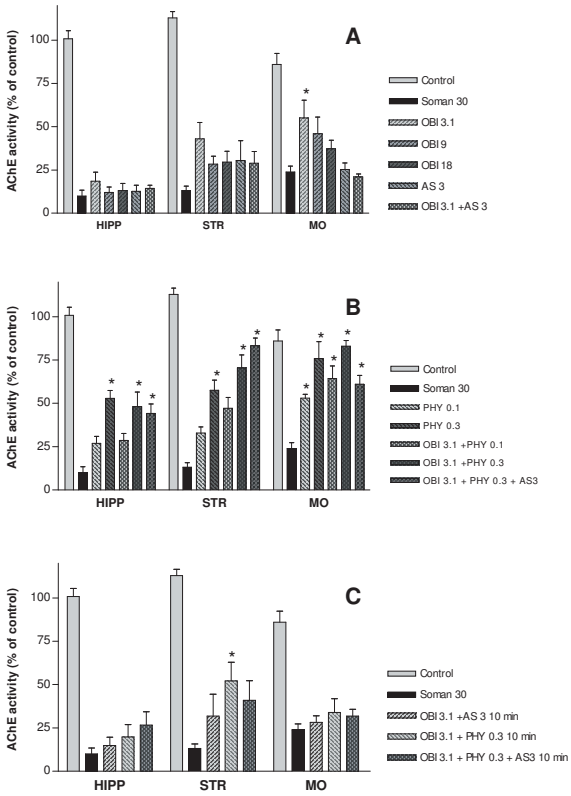

***Figure 4**. A. AChE activity in hippocampus, striatum and medulla oblongata (HIPP, STR and MO) 90 minutes after poisoning with soman and subsequent treatment. The effect of obidoxime (3.1, 9 and 18 mg/kg) and atropine (3 mg/kg) 1 min after poisoning with 30 µg/kg soman. B. Efficacy of addition of physostigmine (0.1 and 0.8 mg/kg). C. the effects of treatments postponed to 10 min. Results are presented as mean ±SEM and analyzed by MANOVA followed by Tukey's HSD post hoc test. * indicates significant (p <0.05) compared to soman alone.*

treatments had a significant effect on the onset time of clinical signs, although the increase in EEG power over time (Fig. 5A) showed some delay in seizure onset. Only the highest dose of atropine (3 mg/kg), either with or without obidoxime, prevented the development of salivation, in contrast to the autoinjector equivalent dose of 0.03 mg/kg.

Regarding the respiration parameters, the lowest dose of obidoxime (3.1 mg/kg) showed a slight and statistically insignificant improvement of respiratory minute volume (Fig. 6A). Addition of atropine to the treatment with obidoxime showed some positive but insignificant effect on RMV (Fig. 6A), whereas atropine alone did not show any improvement at all. In contrast to no treatment, treatment with the highest dose of obidoxime induced a clear effect on heart rate. A pressor effect directly after injection of obidoxime (i.m.) was followed by a depressant effect at approximately 45 minutes after injection (not shown).

To investigate the extent of efficacy against a higher dose of soman, the optimal

obidoxime treatment (3.1 mg/kg) combined with atropine sulphate, was tested against 44 µg/kg of soman s.c.. Although this treatment promoted survival, none of the other physiological effects, such as convulsions or seizure development and respiratory effects, were prevented (data not shown).

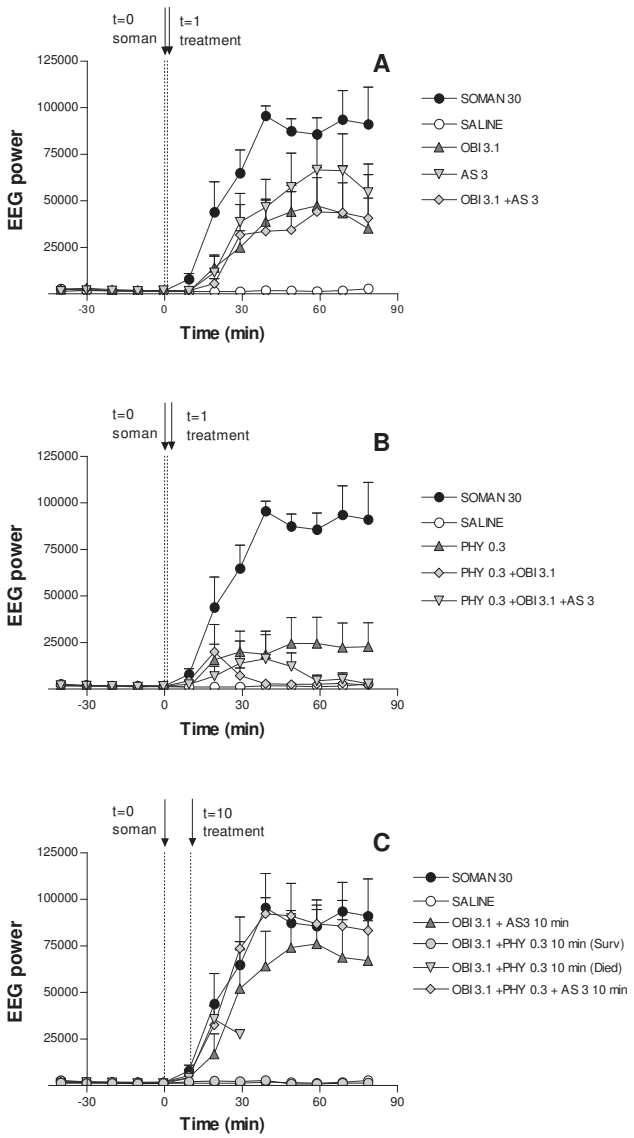

***Figure 5**. A. Treatment effect on total power in EEG before and after soman poisoning. Results are presented as 10 minute averages +/- SEM. The effect of obidoxime (3.1 mg/kg) and atropine (3 mg/kg) administered at 1 minute after soman poisoning. B. The effect of physostigmine (0.3 mg/kg) alone or as an addition to obidoxime and/ or atropine. C. The effect of similar treatments administered at 10 minutes after poisoning.*

3.3 Efficacy of physostigmine

The therapeutic efficacy of physostigmine as immediate treatment in case of organophosphate poisoning was tested in three different doses: 0.1; 0.3 and 0.8 mg/kg i.m., administered at 1 minute after soman poisoning. In particular the middle dose (0.3 mg/kg) showed a major improvement on most parameters. Only a small proportion of the animals treated with this dose of physostigmine showed development of seizures and convulsions (2/6 and 1/6 respectively, see Table 1). Additionally, soman-induced effects on respiration were prevented in animals

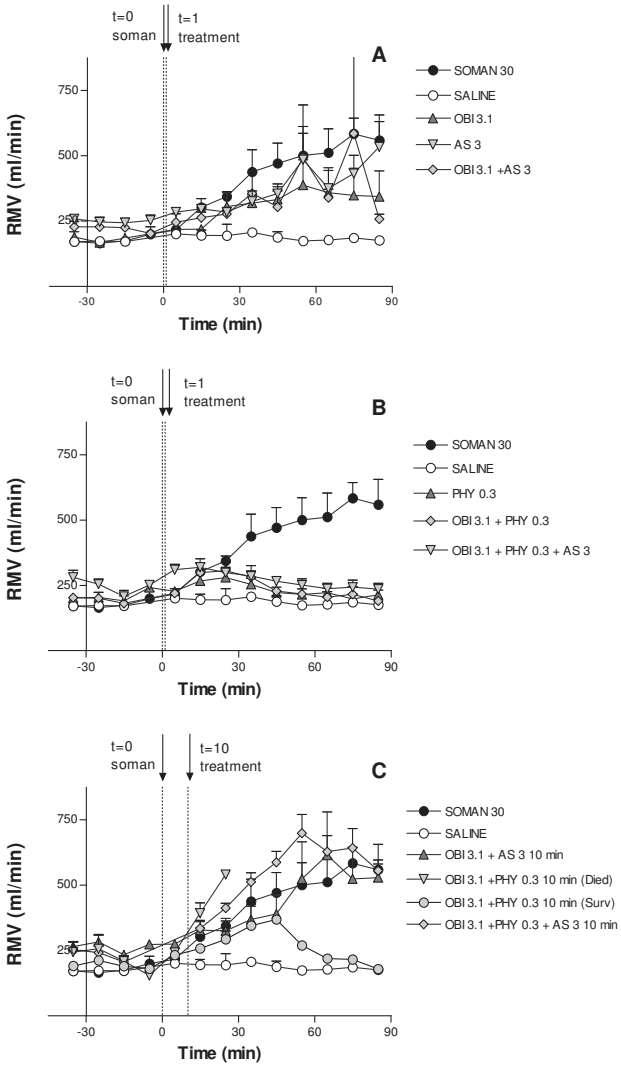

***Figure 6**. A. Treatment effect on respiratory minute volume (RMV) before and after soman poisoning. Results are presented as 10 minute averages +/- SEM. The effect of obidoxime (3.1 mg/kg) and atropine (3 mg/kg) administered at 1 minute after soman poisoning. B. The effect of physostigmine (0.3 mg/kg) alone or as an addition to obidoxime and/ or atropine. C. The effect of similar treatments administered at 10 minutes after poisoning.*

treated with 0.3 mg/kg of physostigmine, but not by the other doses (Fig. 6B). This was accompanied by high residual AChE activity in blood and brain regions (Fig. 4B). Treatment with the highest dose of physostigmine, 0.8 mg/kg, accelerated development of clinical signs. In addition to an increase in RMV, these animals showed high levels of bronchoconstriction. In contrast to untreated animals, these animals died within 30-45 minutes after poisoning, in spite of residual AChE activity similar to that of animals treated with 0.3 mg/kg. The lowest dose of physostigmine, 0.1 mg/kg, prevented the development of convulsions in 5 out of 6 animals, but not seizures. Residual AChE activity in the brains of these animals showed to be lower than in the animals treated with other physostigmine doses (Fig. 4B). In particular AChE activity showed to be preserved by physostigmine, and no residual BuChE activity was detected in blood.

When different physostigmine doses were combined with the most efficacious dose of obidoxime (3.1 mg/kg), again the dose of 0.3 mg/kg physostigmine showed the highest efficacy. Seizure development was prevented in most animals, and if present, they only lasted for a maximum of 15 minutes (Fig. 5B). The highest dose of physostigmine (0.8 mg/kg) combined with obidoxime also aggravated signs, including survival (3/6 animals died within 90 minutes), but the effects were less deleterious compared to physostigmine (0.8 mg/kg) treatment alone. Physostigmine 0.1 mg/kg combined with obidoxime showed some efficacy on residual enzyme activity, and accordingly on convulsions, seizures and respiration. In contrast to physostigmine alone, seizures were prevented in 2 out of 6 animals. Residual enzyme activity was not different between animals treated with physostigmine alone and physostigmine combined with obidoxime (Fig. 4B).

The addition of atropine (3 mg/kg) to the most efficacious physostigmine/obidoxime combination showed no improvements compared to the combination without atropine (Fig. 5B). One animal developed seizures for a short period of approximately 30 minutes, which was similar to the result shown in the group of animals treated with physostigmine and obidoxime. In all animals RMV increase and bronchoconstriction were prevented, but to a similar extent as in animals treated with the combination of physostigmine and obidoxime (Fig. 6B).

3.4 Efficacy of postponed treatment

As in practice treatment will be postponed until overt signs are present, we also administered the most efficacious combination of obidoxime (3.1 mg/kg) and atropine (3 mg/kg) at 10 minutes after poisoning with 30 µg/kg soman, when toxic signs were already visible. These experiments showed that in that case the therapeutic efficacy of the combination nearly completely lost its effectiveness on all parameters. Salivation was prevented in all animals, but RMV increase (Fig. 6C) and seizure development were not affected (Fig. 5C). In 2 out of 6 animals convulsions were prevented (Table 1), whereas the onset time of convulsions in the remaining animals tended to be later ($p>0.05$).

The combination physostigmine (0.3 mg/kg) and obidoxime (3.1 mg/kg), showing outstanding efficacy when administered at 1 min after poisoning, showed full efficacy in 2 out of 6 animals, but in 4 out of 6 animals lethality was accelerated when the combination was administered at 10 minutes.

The full combination of obidoxime, atropine and physostigmine also completely lost efficacy, in that seizure development (Fig. 5C) and increase in RMV (Fig. 6C) were similar to that of untreated animals. Accordingly, no improvements in residual AChE activity were found in the different brain regions, except the physostigmine-obidoxime combination, which showed some improvement in the striatum (Fig. 4C).

4 Discussion

In the present experiments, a guinea pig model was developed to study soman poisoning and therapeutic efficacy of immediate and postponed treatment. The guinea pig soman model doses showed to be suitable for investigating different levels of therapeutic efficacy. First of all, the lowest dose, 30 µg/kg, showed to be most suitable to investigate therapeutic effects on physiology, whereas the highest dose allowed assessment of efficacy regarding survival. Seizure development, accompanied by increased RMV and to a lesser extent to an increase of bronchoconstriction, was present in all animals at all soman doses. The increase in RMV was primarily mediated by an increase in tidal volume, rather than by increased respiratory frequency. Although this finding was unexpected, a pronounced involvement of in particular the M1 and M3 mAChRs in the control of respiration, might explain the increased tidal volume and RMV observed after soman exposure (Boudinot et al. 2008). In addition, bronchorrhea and bronchoconstriction mediate lower pO2 levels in blood, thereby stimulating chemoreceptors in the respiratory centers (Wiener and Hoffman 2004). Bradycardia expected to be present due to cholinergic overstimulation was absent, probably due to a sympathetic response. In contrast with AChE activity in blood at 10 min post-poisoning, a soman dose response effect on AChE activity was found in all brain regions analyzed, resulting in 80-90% inhibition at 90 min post-poisoning. Similar results were reported by Shih et al. (2005), who investigated the differential inhibition of various nerve agent types in several organs.

AChE activity in blood mainly serves as biomarker for exposure and not for toxicity (Lotti 1995). It was shown previously, that AChE activity in blood is a bad predictor for cholinergic clinical signs due to acute OP poisoning (Bueters et al. 2003). Logistic regression analysis of the present results, using lower doses of OP compared to that study, revealed the inhibition of AChE in blood to be a good predictor for the probability of chewing, shivering, seizures, convulsions and tremor, but not for respiratory distress and death (Fig. 7A). This shows that seizures are accompanied and not necessarily preceded by mild clinical signs. It also shows that poisoning with lower doses does not ameliorate the chance of development of severe clinical signs. The most severe sign well predicted by blood AChE activity was seizure development, mostly held responsible for adverse effects at the long term. The toxicokinetics of soman are not linear, higher doses lead to relatively much higher AUC and less rapid clearance than following lower doses (Van der Schans et al. 2008). This implicates that at higher doses, binding sites will more rapidly be saturated, leading to a more rapid development of clinical signs. In contrast, at lower doses, as used in the present study, clearance is higher, leading to a more

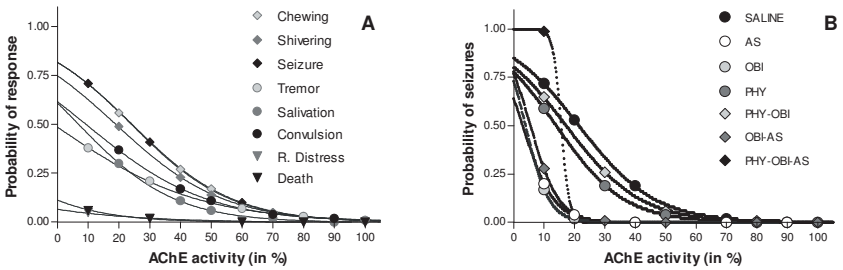

***Figure 7**. Continuous relationships of the occurrence of soman-induced signs obtained with logistic regression analysis. A. The relationship between AChE activity in blood and clinical signs of animals poisoned with 3 doses of soman but no treatment (n=18). B. The relationship between the probability of seizures and the residual AChE of animals poisoned with 30 ug/kg soman treated at 1 minute post-poisoning with single drugs or their combinations (n=6-18 per treatment).*

gradual and slower pattern of poisoning, and AChE activity in blood might more accurately reflect the levels of neuronal AChE activity, resulting in a better prediction of development of clinical signs.

These findings underscore that protection of AChE activity is a primary target for treatment in case of OP exposure. Only at the lowest dose of 3.1 mg/kg and not at both higher doses of 9 and 18 mg/kg, obidoxime showed significant reactivation in the medulla oblongata, but not in blood or other brain parts. The efficacy of the lowest dose of obidoxime on the development of convulsions, seizures and respiratory deterioration might be explained by the higher level of AChE activity found in the medulla oblongata. It might be speculated that some reactivation of soman-inhibited AChE occurs in the first minutes after administration of obidoxime, before our first measuring point and ageing of soman-inhibited AChE. The higher number of binding sites for soman in blood probably reduced the soman load that entered the brain, causing less inhibition in the medulla oblongata. The decline of therapeutic efficacy at higher doses of obidoxime might be ascribed to the inhibitory actions that phosphonylated obidoxime exerts on AChE (Harvey et al. 1986; van Helden et al. 1994). The slight delay of complete inhibition of AChE in the blood and probably also in the brain by obidoxime might explain its therapeutic efficacy, as only little amounts of active AChE in the brain are sufficient to improve the above parameters (Bueters et al. 2003). In addition, suspected direct pharmacological actions of obidoxime might facilitate adaptive processes (van Helden et al. 1994; van Helden et al. 1996). Obidoxime is capable of increasing ACh release 2- to 3 fold in the absence of AChE inhibitors, which can contribute to accelerated adaptation of cholinergic synapses to increased ACh levels and thereby to protection against the toxic load of soman (Oydvin et al. 2005). Second, obidoxime is known to allosterically modulate the mAChR2 receptor (Gnagey and Ellis 1996; Grossmuller et al. 2006; Kloog et al. 1985), which is involved in cholinergic feedback mechanisms (Segal 1989).

Addition of atropine sulphate to obidoxime in exactly one autoinjector equivalent (0.03 mg/kg) had no added value at all. A higher dose of atropine, 3 mg/kg, a dose similar to that often used in OP-research, revealed some protection, in particular

against extensive salivation and convulsions, and to a lesser extent to seizure development and respiration. In contrast to low autoinjector-equivalent doses, only such higher doses showed to be an effective support for anti-convulsant treatments (Shih et al. 2007). In addition, we show that the combined treatment with atropine and obidoxime enhances survival in case of poisoning with 44 µg/kg of soman. Other researchers have showed higher efficacy using relatively high doses of the much more potent mAChR antagonist scopolamine. It might therefore be advisable to replace atropine by scopolamine, although extrapolation of the dose from animal research to the human situation remains difficult (Wetherell et al. 2007).

Physostigmine was previously proposed as alternative immediate treatment against 5 LD50 of soman (Wetherell et al. 2006; Wetherell et al. 2007). In case of poisoning with soman, physostigmine, at a similar dose as used in our study, was rather effective and its efficacy increased when high doses of HI-6 were used, which was ascribed to the competition with incoming soman for binding sites (Wetherell et al. 2007). In the present study, the therapeutic window of physostigmine appeared to be very small. We showed that 0.1 mg/kg physostigmine provided some protection when administered at 1 minute after poisoning and that 0.3 mg/kg showed to be most effective, but 0.8 mg/kg appeared to aggravate clinical signs and to diminish survival. Residual AChE activity was measured using a modified Ellman reaction, revealing the AChE fraction that was inhibited by physostigmine in vivo. During sample preparation, physostigmine inhibited AChE was likely to fully decarbamylate because of its short half life of approximately 32 min in vitro (Wetherell and French 1991). Therefore, the inhibited fraction most likely reflects the portion of AChE inhibited by soman, because without treatment, the dose of soman used was sufficient to fully inhibit AChE activity in blood. Measurements of residual AChE activity, showed that the lowest dose (0.1 mg/kg) provided the lowest protection of AChE by physostigmine, whereas the higher doses 0.3 and 0.8 mg/kg inhibited a similar fraction of AChE (approximately 50% of activity). These findings indicate that the small therapeutic window is probably due to the carbamylation rate of physostigmine, being too low at a dose of 0.1 mg/kg, whereas the carbamylation rate of physostigmine exceeded decarbamylation at a dose of 0.8 mg/kg. Only at the middle dose tested, 0.3 mg/kg, carbamylation and decarbamylation were balanced leading to protection of sufficient amounts of AChE to exert protection. Although the measurements of residual AChE activity in animals receiving postponed treatment with physostigmine did not reveal a significant decline or protection of AChE activity, our results show that postponed treatment with a dose of 0.3 mg/kg physostigmine clearly led to a lower survival rate and loss of therapeutic efficacy in these animals. These results indicate that postponed treatment with physostigmine induces an additional increase of AChE inhibition in blood and brain to that already brought about by soman. This implies that administration of physostigmine can only be protective when a considerable fraction of AChE is not yet occupied by soman, which was accomplished by the 1 minute treatment.

To compare all treatments for their mechanism of action, logistic regression analysis was performed to estimate the probability of seizure development at certain levels of blood AChE activity in animals receiving different treatments after

soman poisoning (30 µg/kg) (Fig. 7B). This analysis showed that when atropine or obidoxime or their combination was used as a treatment, the seizure probability dramatically decreased at similar AChE activity, confirming the mechanism of atropine and obidoxime in this study to be largely independent from AChE reactivation. However, when physostigmine treatment without atropine was employed, seizure probability at similar AChE activity remained comparable to that in untreated animals. This indicates that the efficacy of physostigmine is probably only due to its ability to prevent soman from binding to AChE, thereby reserving a decarbamylating pool of AChE while the OP is degraded, and is not due to suspected effects on nicotinic receptors (Harris et al. 1990).

In conclusion, the present results show that the combination atropine and obidoxime is hardly effective against soman poisoning, even at a fairly low toxic load as used in the present study. However, for nerve agents that are more prone to reactivation than soman, this regimen is expected to show higher efficacy. Although physostigmine significantly improved therapeutic outcomes, there might be some drawbacks to adding physostigmine to the current autoinjector components. In practice, treatment will only be administered at appearance of toxic signs, indicating inhibition of the major fraction AChE by nerve agent. In particular at high levels of nerve agent poisoning, carbamates will fully inhibit the small remaining pool of AChE, thereby further pushing cholinergic overstimulation and worsen the clinical condition. Treatment of OP poisoning with a carbamate, such as physostigmine, should therefore carefully be re-evaluated.

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Chapter 3

Percutaneous exposure to VX: clinical signs, effects on brain acetylcholine levels and EEG

Marloes J.A. Joosen, Marcel J. van der Schans and Herman P.M. van Helden

TNO Defence, Security and Safety, BU CBRN Protection, Rijswijk, The Netherlands

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Abstract

The nerve agent VX has a variable and delayed absorption through the skin, which may have implications for treatment regimens. In the present study, central and peripheral effects of percutaneous VX intoxication were investigated in hairless guinea pigs. Although onset times of clinical signs varied considerably, the relative onset times of signs of poisoning were shown to have a predictive value for survival time. All animals showed elevation of brain choline levels. Only 2 out of 6 animals demonstrated seizure activity on EEG, which was accompanied by acetylcholine accumulation. The non-seizing animals displayed only marginal increases of acetylcholine levels, but significant changes in all EEG bands. Acetylcholinesterase activity was highly inhibited in brain and diaphragm. The increases in choline levels and EEG effects observed in non-seizing animals probably reflected those of ischemia induced by peripheral effects leading to cardiorespiratory compromise. In conclusion, clinical signs will mainly serve as indicators for the onset and maintenance of treatment in subsequent studies.

1 Introduction

In contrast to volatile nerve agents of the so-called G-type such as sarin and soman, which act primarily via the respiratory route, nerve agents of the V-type are several orders of magnitude less volatile due to their physicochemical properties and act primarily as a liquid via the percutaneous route (Maynard and Beswick 1992). The most important representative of the V-agents is VX ([O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothioate), which is several orders of magnitude more lethal via the skin than for example sarin, but also highly toxic as a vapor via the respiratory and ocular route (National Research Council 1997; Reuter et al. 2000). Several nations have VX-stockpiles awaiting destruction. The threat of using nerve agents in military conflicts (Macilwain 1993) and in terrorist attacks (Nagao et al. 1997) urges the necessity for development of effective treatment regimens.

Following exposure, nerve agents induce several cholinergic signs, such as salivation, miosis, bradycardia, bronchoconstriction, seizures and respiratory depression due to irreversible inhibition of acetylcholinesterase (AChE). VX also showed direct pharmacological effects on several receptor types, for example muscarinic (Bakry et al. 1988; Pittel et al. 2006; Silveira et al. 1990) and nicotinic acetylcholine receptors (Bakry et al. 1988), inhibited evoked GABA and glutamate release and enhanced spontaneous release of the latter transmitters (Rocha et al. 1998).

Since the percutaneous route of exposure is less predictive in terms of onset times and severity of clinical signs, Van der Schans et al. (2003) have determined the toxicokinetics of VX after percutaneous exposure in anaesthetized hairless guinea pigs. Maximum plasma levels of VX were not reached until several hours after exposure, followed by a slow elimination phase. In spite of the use of anaesthetized animals, the variability in toxicokinetics was considerable. The latter variable and delayed absorption of VX from the skin may have important implications for military or civilian first responders with respect to triage, decontamination and therapeutic drug regimens (Dalton et al. 2006; Hamilton et al. 2004). The present study was designed to investigate the central and peripheral effects of exposure to percutaneous VX in hairless guinea pigs, in order to broaden the scope for possible treatment. The hairless guinea pig is considered to be a more appropriate model in this respect than the common guinea pig since its skin structure is more comparable to that of man (Panchagnula et al. 1997; Sueki et al. 2000).

Most previous experiments evaluating physiological effects of VX after percutaneous exposure were conducted in anaesthetized animals. In the present study, the effects of percutaneous exposure to VX (500 µg/kg, ~ 4LD50) were studied in conscious freely moving animals. Acetylcholine (ACh) and choline (Ch) levels were measured in the striate body of the brain using microdialysis. Simultaneously, cortical EEG and cholinergic signs of poisoning were recorded. At the end of the experiment, AChE activities in different brain regions, liver and diaphragm were determined.

2 Methods

2.1 Animals

Male hairless guinea pigs [Crl:IAF(HA)BR] (375 ± 10 g), obtained from Charles River (Maastricht, The Netherlands) were used in the present study. Prior to the experiments they were housed with 2 animals per cage, and allowed to get accustomed to standard conditions for two weeks. Room temperature was kept at 19-22 °C and relative humidity was maintained at 55-65% and lights were on from 7 am to 7 pm. Acidified water and standard guinea pig chow (Teklad global diet 2040, Harlan, Horst, The Netherlands) were available ad libitum. The experiments described received prior approval from the TNO Animal Ethical Committee.

2.2 Chemicals

Acetylcholinesterase, choline oxidase, acetylcholine, choline and Triton-X100 were obtained from Sigma Chemical Co. (Zwijndrecht, The Netherlands). Kathon CG (1.5% 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was provided by Rohm and Haas (Croyden, UK). LiChrosorb® NH_2 was purchased from E. Merck (Amsterdam, The Netherlands). Hypnorm® (fentanyl/fluanisone) was purchased from Janssen Pharmaceutica (Beerse, Belgium) and Dormicum®(midazolam) was delivered by Roche Nederland BV (Mijdrecht, The Netherlands). (±) VX [O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate] was obtained from the stocks of TNO Defence, Security and Safety and of purity ≥98% (GC). The other chemicals used were of standard purity and purchased from renowned companies. For HPLC analysis the highest purity grade was used. All solutions were prepared with water tapped from a Milli-Q system (Millipore SA, Molsheim, France).

2.3 Surgical procedures

Guinea pigs were anaesthetized with 6 ml/kg FFM-mix (1.25 mg/ml midazolam, 2.5 mg/ml fluanisone, 0.079 mg/ml fentanyl citrate) via a single i.p. injection. Premedication with 0.05 mg/kg atropine sulphate (s.c.) was administered to prevent respiratory arrest. A concentric microdialysis probe was stereotactically inserted into the ventral striatum (P1.8, L 2.8, V 8.3 mm relative to bregma and the dura mater). Two stainless steel screws (A8.0 and P1.2 mm relative to bregma and 1 mm from the sagittal suture) placed at the dura mater were fixed to the skull with dental cement and fixed to a plug to serve as EEG electrodes. A heating pad was used to maintain body temperature. The microdialysis probes used were constructed in-house and made of a polyacrylonitryl/sodiummethyl sulfonate copolymer dialysis membrane (Filtral 12, Hospal BV, Breda, The Netherlands), of which 4 mm was exposed.

2.4 HPLC Instrumentation and acetylcholine analysis

Microdialysate samples were assayed for acetylcholine and choline using a HPLC system previously described by Damsma et al. (1987) with some modifications. The system consisted of a LC-10AD VP pump (Shimadzu, Den Bosch, The Netherlands), a pulse damper (SSI, Alltech, Breda, The Netherlands), a refillable guard column (silica pellicular packing material, Alltech, Breda, The Netherlands), an EC 125/2

Nucleosil 100-5 C18 AB analytical column (Aurora Borealis Control, Schoonebeek, The Netherlands), preloaded with 0.5% sodium lauryl sulfate, an enzyme reactor, an electrically actuated injector (VALCO VICI AG, Schenkon, Switzerland) with a 20 ul sample-loop, an INTRO potentiostate equipped with a VT03 flow cell with Pt work electrode (Antec Leyden BV, Hazerswoude, The Netherlands) and a chromatography data acquisition system (Chromeleon©, Gynkotek, Germering, Germany). The post-column enzyme reactor contained immobilized acetylcholinesterase (80 U), which converted acetylcholine to choline, and choline oxidase (40 U), which converted choline to hydrogen peroxide that was electrochemically detected at + 450 mV. The mobile phase consisted of a 166 mM potassium phosphate buffer (pH 8.5) containing 1 mM tetramethylammonium chloride, 0.79 mM EDTA and 250 µl/l Kathon CG. The system was run at 0.35 ml/min and temperature was maintained at 30 °C. Limit of quantification was 1 pmol/ml. Linear calibration curves were obtained in the range from 5 – 1000 pmol/ml ($r > 0.990$). The intra-assay coefficients of variation for 10 and 100 pmol/ml were 2.9 % and 4.1 %, respectively. Inter-assay variability could not be determined due to variable enzyme activities in the post-column reactor between days.

2.5 Microdialysis procedure for acetylcholine determination

Following surgery, the guinea pigs were individually placed in a perspex cage (25 x 25 x 40 cm) with free access to food and water. Experiments started 14-20 hours after recovery from the surgical procedure, between 9 and 10 AM. At the beginning and end of each experiment a 10 nM acetylcholine/choline calibration standard was injected in duplicate, and if necessary corrections for reduced sensitivity of the enzyme reactor during the experiment were made. The microdialysis probe was perfused with a Ringer solution at 2.0 µl/min delivered by a microinjection pump (CMA 100, CMA microdialysis AB, Solna, Sweden). To obtain detectable quantities of acetylcholine in the dialysate, 100 nM neostigmine was added to the perfusate. The guinea pig was connected directly to the sample loop, allowing on-line analysis of the microdialysates. The injection valve was automatically activated every 10 min, resulting in an injection volume of 20 µl.

2.6 Clinical signs

After obtaining one hour of baseline data of EEG and microdialysis samples, the guinea pig was gently held by hand, and a solution of VX in Isopropylalcohol (32 mg/ml), or IPA alone (control animals), was applied at the level of the lumbar 3 plexus on the belly, on an area of about 2 cm^2, at a dose of 500 µg/kg (16 µl/kg), which corresponds to 4LD50 (van der Schans et al. 2003). After absorbance of the fluid, the spot was covered with a stainless-steel cup of 2 cm2, which was strapped to the skin with surgical tape and removed after one hour. The area was not decontaminated.

Following exposure, the guinea pigs were monitored and the onset of the following cholinergic signs were registered: Chewing: A clear chewing-like movement of the guinea pig in which the entire head is involved as a consequence of increasing saliva production; Shivering: Continuous shivering of the entire body. The animal is able to move around; Salivation: Extensive drooling from the mouth, which is often accompanied by tears fluid; Tremor: Extensive shivering combined

with involuntary movements in which the entire body is involved. The guinea pig looks mentally dissociated from the environment and has lost control over its movements; Respiratory Distress: Low respiratory rate and heavy breathing.

Immediately after obtaining the last microdialysis sample after registration of a flat EEG, organs were quickly removed and homogenized for determination of enzyme activity, as described below.

2.7 EEG acquisition and analysis

EEG was obtained using PhysioTel telemetry system from Data Sciences Inc. (DSI) using the TA11ETA-F40 transmitter body, which was attached to the plug fixed on the guinea pig's skull. A receiver board measured the signal from the transmitter, which was consolidated and stored on an IBM-compatible personal computer via a Data Exchange matrix at 100 Hz sampling frequency. After storage, the data were converted into EDF format. Every 10 seconds power spectra were calculated using Fast Fourier Transformation (FFT). A low cut off filter at 1.6 Hz was used to filter eye movement artifacts and high digital filter at 50 Hz was used. Animals were awake during the measurement.

Relative powers of EEG bands (Delta 1.6-3.5 Hz, Slow Theta 4.0-6.5 Hz, Fast Theta 7.0-8.5 Hz, Alpha 9.0-14.0, Beta1 14.5-24 Hz, Beta2 24.5-30 Hz, Gamma1 30.5-35 Hz and Gamma2 35.5-50 Hz) were calculated by dividing the sum of the absolute power in the frequency range by the total power of the spectrum according to Timofeeva and Gordon (2001). EEG data were synchronized with acetylcholine determinations using 10 minute averages.

2.8 Enzyme activity

Diaphragm, liver and different brain parts (Fig. 2) were homogenized (900 rpm, 10 % w/v homogenate) in ice-cold TENT buffer, which consisted of 50 mM Tris, 5 mM EDTA, 1 M NaCl and 1% v/v Triton X-100, pH 7.4. The homogenates were centrifuged at 12000 g in an Eppendorf centrifuge at 4 ^{o}C and supernatants were immediately frozen in liquid nitrogen and stored at -20 ^{o}C until analysis of enzyme activity, within one month.

Samples were analyzed for AChE activity using a modification of the method by Ellman et al. (1961). Shortly, after appropriate dilution, 10 µl samples were incubated with 0.8 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich B.V.) and 0.8 mM acetylthiocholine iodide. The delta OD per µl homogenate per min at 415 nm at ambient temperature served as measurement for AChE activity. Activities were normalized versus enzyme activities of 6 control animals.

2.9 Study design, data presentation and statistical analysis

Two groups of 6 animals each were used. This group size is based on expected ACh accumulation of approximately 750 ± 300% (mean ± SD) at time of seizure development/ death (Bueters et al. 2002) compared to control animals, resulting in a statistical power of 90% for detecting a scientifically significant ACh accumulation. Whenever appropriate, microdialysis and EEG data were analyzed using Repeated measures one-way ANOVA followed by Dunnett's post-hoc test. Enzyme activities were analyzed using Student's t-test. Results were considered significant if $p<0.05$.

3 Results

3.1 Clinical signs

Cholinergic signs were registered upon percutaneous application of 500 µg/kg VX. Although all animals received the same dose of VX, in particular the time course in which the cholinergic signs appeared showed great variability (Table 1).

***Table 1**. First occurrence of a cholinergic sign following percutaneous exposure to VX (500 ug/kg). The time points at which a sign was observed are indicated in minutes.*

Animal no.	*Chewing*	*Shivering*	*Salivation*	*Tremor*	*R. Distress*	*EEG seizure*	*Death*
1	124	374	320	414	467		518
2	60		180	180		180	232
3	104	344	490		463		670
4		70	70		92	75	107
5	101	120	101	190	262		287
6		55			80		92
Mean	*97*	*232*	*193*	*261*	*273*	*128*	*318*
SEM	*13*	*78*	*69*	*76*	*85*	*53*	*95*

Figure 1 shows the first appearance of the cholinergic signs versus the % of time elapsed between VX application and death (100%). It is clear that, on average, the onset time to the first occurrence of each sign is predictive for the time to the occurrence of more severe signs. The symptom chewing occurs at 25±4 % of the total survival time, followed by shivering and salivation at 58±5% and 63±7% of survival time, respectively. On average, animals start showing tremor at 75±4% and the most severe symptom, respiratory distress, is only seen shortly before death at 85±4% of total time. Remarkably, only 2 out of 6 animals showed seizure activity on their EEG.

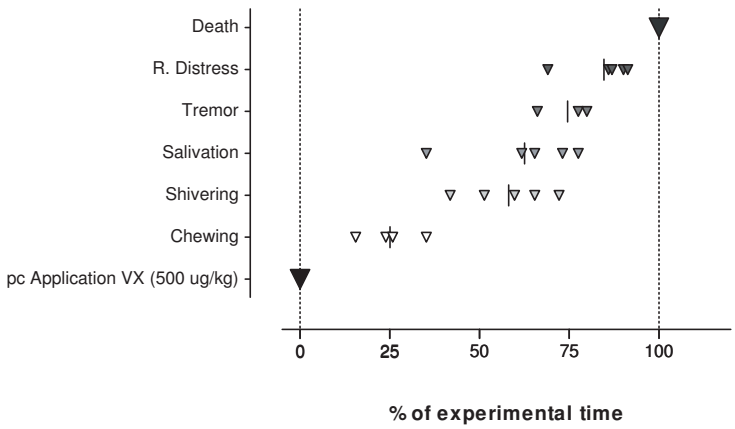

***Figure 1**. First appearance of cholinergic signs in individual animals (vertical axis) at percentages of total experimental time (horizontal axis) between VX application (0%) and death (100%). The small triangles show individual first appearances of signs, vertical lines show average first appearance (n=6). For absolute times see Table 1.*

3.2 Enzyme activities

In figure 2 the relative inhibition of AChE in several brain regions as well as in liver and diaphragm is shown. In animals showing no seizures, AChE activity was significantly inhibited by approximately 85-90% in all organs, except for the striate body, in which inhibition was only 75%. Two animals with seizures showed higher levels of AChE inhibition, to approximately 90-95% of that of control animals, in particular in rest of brain, striate body, liver and diaphragm. However, because of the small number of animals displaying seizures, no statistics were calculated.

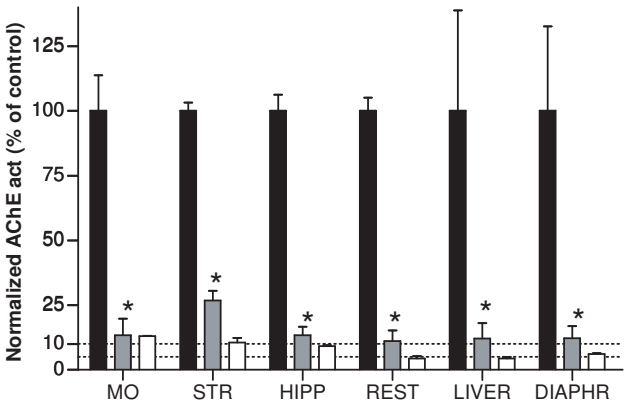

***Figure 2**. Relative AChE activity in brain parts, liver and diaphragm. The AChE activities of 4 animals after pc intoxication with VX (500 ug/kg) not displaying seizures (grey bars) and 2 animals displaying seizures (open bars) after VX were calculated as percentage from AChE activities from control animals (n=6, black bars). Enzyme activities from Medulla Oblongata (MO), striate body (STR) , hippocampus (HIPP), rest of brain (REST), liver and diaphragm (DIAPHR) are displayed. AChE activity is significantly different in all organs of intoxicated animals. Only the 4 non-seizing animals were compared statistically to controls (Student's t-test, *, p<0.05)*

3.3 Acetylcholine, choline levels and total EEG power

Figure 3 shows microdialysis results and EEG total power changes at several experimental time points. Out of 6 animals in the VX-exposed group, only two animals exhibited seizure activity on their EEG. The ACh level in one of these 2 animals had increased early in the experiment (at 50% of experimental time), ending at a very high level at the end of the experiment (Panel A). The seizures started at approximately 65% of experimental time, indicating that the primary moderate increase in ACh (in this animal) did not induce generalized seizures. The ACh measurement in the other animal showing seizures, failed due to a malfunctioning probe. The 4 animals without seizures showed very slight and not significant increases in ACh levels. In contrast to the early increase in EEG power in the 2 animals with seizures, the EEG power in the non-seizing animals gradually decreased, which became significant at 90% of experimental time (Panel C). Both seizing as well as non-seizing animals showed a gradual increase in Choline levels (Panel B).

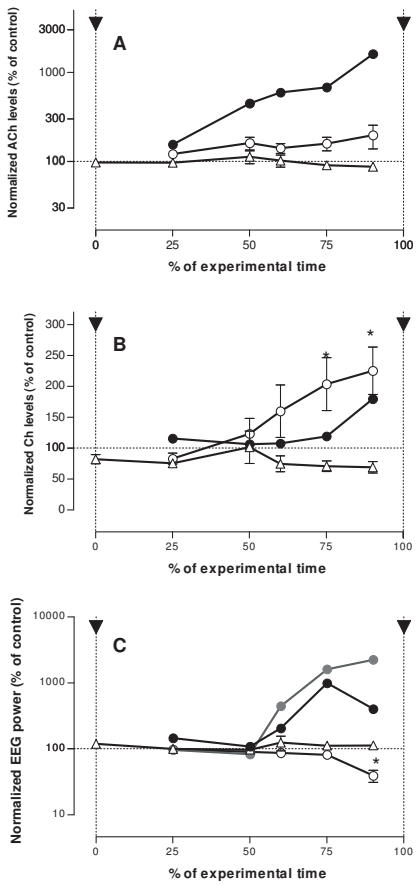

***Figure 3**. Microdialysis and overall EEG effects in hairless guinea pigs after percutaneous intoxication with VX (500 ug/kg). A. Normalized ACh levels. B. Normzalized choline levels and C. Normalized total power of EEG. The open dots represent the average +/- SEM for 4 animals that did not have seizures. The closed dots represent individual values of animals having seizures. Open triangles represent the averages of 6 control animals. Data were considered significantly different from control values if p<0.05 (*, Repeated measures ANOVA followed by Dunnett's post-hoc test, only conducted for non-seizing animals). The changes in control animals were not significantly different (p>0.05, Repeated measures ANOVA).*

3.4 EEG bands

The changes in relative powers in different EEG bands following percutaneous intoxication with VX are shown in figures 4 and 5. For each animal, the 30 minutes of recording before intoxication served as individual baseline value for EEG, and were set at 100%. At 4 experimental time points, the average relative power per band was normalized to the baseline value. Figure 4 shows the EEG parameters for the 4 non-seizing animals. At 25% of experimental time no overt changes were observed in any of the EEG bands. At 50% of experimental time, however, the relative power in some bands had started to change. The relative power in the Delta and Gamma2 band transiently increased and had returned to baseline value

by the end of the experiment. In contrast to the increase in the latter bands, most other bands (Theta1, Theta2, Alpha, Beta2 and Gamma1) showed a significant and in some bands persistent decrease compared to baseline. The Beta1 band showed a less consistent effect, but towards the end of the experiment its relative power had increased significantly.

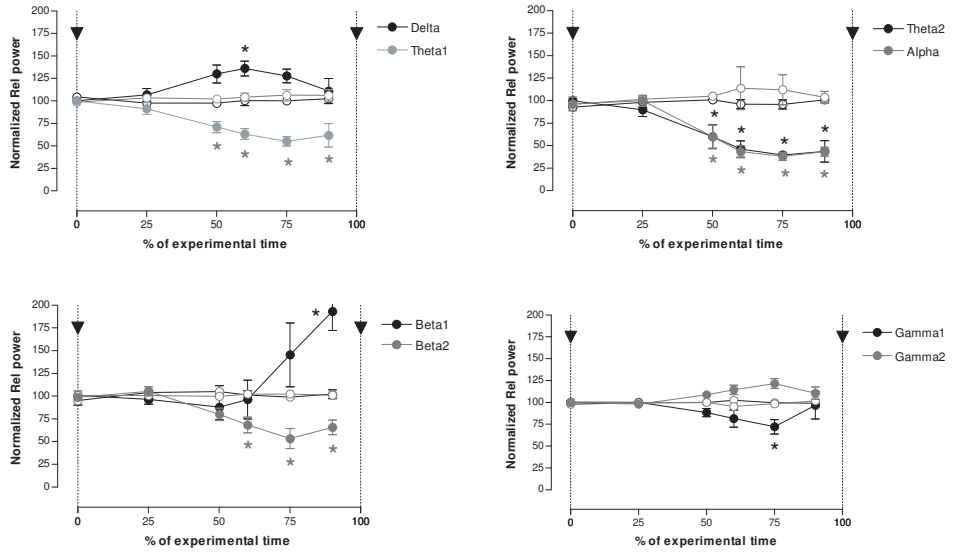

***Figure 4**. Normalized changes in relative powers of EEG bands after 500 ug/kg VX p.c. of animals without seizures (n=4, closed dots) and control animals (n=6, open dots) at several time points during the experiment (% of experimental time, horizontal axis). In VX-intoxicated animals, the powers in Delta, Beta1 and Gamma2 show an increase over time upon intoxication, whereas Theta1, Theta2, Alpha, Beta2 and Gamma1 show increases. EEG bands in control animals remain unaffected over time (p>0.05, Repeated measures ANOVA). Results are presented as mean +/- SEM. Results were considered significantly different if p<0.05 (*, Repeated measures ANOVA followed by Dunnett's post-hoc test).*

The EEG changes in the 2 animals with seizures are shown in figure 5. The time courses in relative EEG-band powers are to some extent similar to those of animals that did not seize. Because of the low number of animals showing seizures, statistics regarding the deflections from baseline could not be calculated. However, the relative power returned to baseline values approximately at 75% of the experimental time.

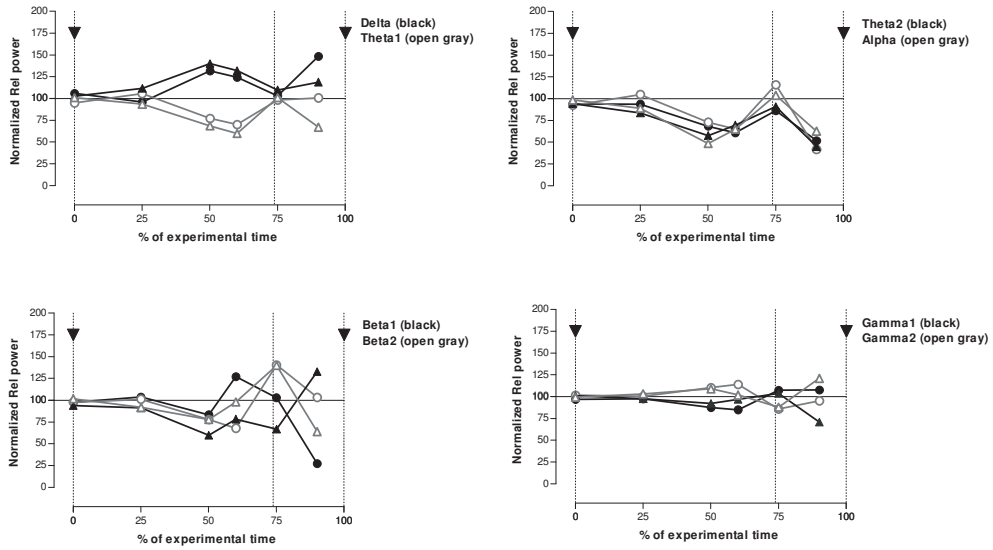

***Figure 5**. Normalized changes in relative powers of EEG bands after 500 ug/kg VX p.c. of the 2 individual animals which demonstrated seizures on their EEG at several time points during the experiment (% of experimental time, horizontal axis). Due to the small number, statistical analysis was not carried out. For clarity reasons, values of control animals were left out, see fig. 4.*

4 Discussion

The toxicokinetics of VX following skin application in the guinea pig have shown a delayed penetration through the skin (van der Schans et al. 2003), resulting in a delayed appearance of cholinergic signs (Chilcott et al. 2003; Hamilton et al. 2004). It is obvious that this delayed penetration of VX through the skin contributes to the variation with regard to onset times of clinical signs in the present study. The high VX dose used in the present study (4LD50) was chosen in order to minimize inter-individual variability. Regarding signs of poisoning, the latter goal was not completely achieved. Not all animals showed all signs. In animals showing a rapid onset of signs and a relatively short survival time for example, the chewing sign did not appear. However, looking closer at the onset time of the cholinergic signs, a less variable picture emerged. At an average of 25% of survival time, the animals showed chewing movements, shivering at 58%, salivation at 63%, tremor at 78% and respiratory distress at 85% of survival time. It is of particular interest that the deviations of these relative times belonging to the different signs are very small, indicating that this parameter is a reliable tool to predict the survival time.

The AChE activity was highly inhibited in the investigated organs of all animals. This is in line with observations from Maxwell et al. (2006), who ascribed 90% of toxicity of organophosphorous compounds to AChE inhibition. In spite of a relatively high AChE inhibition in the brain, no overt or seizure-inducing ACh levels were reached in all animals. Only 2 out of 6 animals developed seizures. The latter animals

tended to have higher levels of AChE inhibition in striate body and rest of brain. The inhibition levels in these 2 animals were approximately 90 and 95%, in striate body and brain respectively, compared to 75 and 90%, in animals that did not have seizures on their EEG. These high levels of central AChE inhibition, expected to be a requirement for seizure development, are in line with previous studies (Bueters et al. 2003; Joosen and van Helden 2007; Testylier et al. 1999). Other workers have shown in guinea pigs, using a different route of administration, that of all nerve agents tested, VX was least likely to produce seizure activity in all animals (Shih et al. 2003; Shih and McDonough 2000). Because of the failure of the microdialysis probe in one seizing animal, extracellular ACh levels could only be obtained from one of 2 seizing animals. In the latter animal, the seizures were accompanied by a high increase of extracellular ACh levels, i.e. over 10 times of basal level. From other studies it appeared that seizures did not occur until ACh levels rise over 200 times compared to basal levels and that brain AChE inhibition must have been inhibited over 65% (Tonduli et al. 1999). In a previous study, we have shown that over 75% of the AChE must be inhibited in the striate body to induce significant elevations over baseline levels of ACh, likely to induce seizures (Joosen and van Helden 2007). The other animals, 4 out of 6, showed elevations of ACh levels to approximately 5 times that of basal levels, which apparently was much too low to induce seizures. Although initiation and early expression of seizure activity are cholinergic in nature, other neurotransmitters, including glutamate and GABA can influence the maintenance of the seizures (McDonough, Jr. and Shih 1997).

All animals showed increases in extracellular levels of choline. Although choline itself has no affinity for receptors, choline dynamics are affected in several conditions of brain injury (Scremin et al. 2006). An increase of brain choline levels is found in case of cerebral trauma (Nortje and Menon 2004) and in focal and global cerebral ischemia (Scremin and Jenden 1989a; Scremin and Jenden 1989b). In these models, activation of phospholipases led to an enhanced rate of phospholipid degradation, resulting in an increasing production of free choline (Phillis and O'Regan 2003). Also in our study the net production of free choline from phospholipids may contribute to the observed increase of choline levels and may point to cerebral ischemia following VX intoxication.

Apart from increased choline levels, also some of the observed EEG changes might be the result of ischemia. Although in animals without seizures the total EEG power was not affected until the end of the experiments, significant changes of relative power in different EEG bands were observed at earlier stages in the experiment, similar to those found in animals not having seizures. Significant increases in Delta, Beta1 and Gamma2 were observed, whereas the relative power in other bands was significantly decreased. A similar significant shift to lower frequency EEG activity in the Delta band was seen within one hour following permanent occlusion of a cerebral artery in a rat model of ischemia (Cohen et al. 1994). In man, diffuse generalized slowing of the EEG is also associated with a malignant outcome of ischemia due to middle cerebral artery infarction (Burghaus et al. 2007). Additionally, short periods of circulatory arrest in man also induced increases in Delta power and more consistent decreases in Beta and Alpha bands

(Visser et al. 2001). A similar increase in relative power in the Delta band, not cholinergically mediated, was observed by Timofeeva and Gordon (2001) after oral administration of chlorpyrifos.

In case of organophosphate poisoning, it is expected that EEG changes are cholinergically mediated due to increases in ACh levels. In this respect, increases in Theta1 and Gamma2 and decreases in Theta2, Alpha, Beta1, Beta2 and Gamma1 were observed after subcutaneous administration of the muscarinic agonist oxotremorine in rats (Timofeeva and Gordon 2001). Although only slight increases in ACh were observed in animals without seizures, and not all EEG changes observed were similar to that reported by Timofeeva and Gordon, some changes in EEG bands observed in this study might be related to cholinergic effects. Particularly the increase in Gamma2 and decrease in Beta2 might be related to increased ACh levels (Joosen and van Helden 2007; Timofeeva and Gordon 2001).

Until approximately 65% of experimental time had elapsed, the EEG changes in animals that developed seizures were similar to that in animals that did not. It is not clear why some animals developed seizures and some did not. This might be related to the influence of neurotransmitters other than ACh (McDonough, Jr. and Shih 1997). One could speculate that small amounts of anaesthesia were still present while the EEG recording started, that is 14-20 hrs after surgery. However, this is highly unlikely since the half life of elimination of midazolam is about 3 hrs, that of fluanisone and fentanyl even shorter. Moreover, the latter 2 drugs are not reported to have any anti-convulsive property in case of OP poisoning. Finally, the low dose of atropine (50 µg/kg), used as premedication for surgery, is not even anticonvulsive when given immediately after OP intoxication. In conclusion, the observed EEG changes do not clearly indicate development of seizure activity, the general course of EEG changes, however, points to progressive effects of intoxication.

A major part of toxicity in case of percutaneously applied VX may be due to impact on the major organs such as the diaphragm muscle and heart. Direct effects on cardiac M2 receptors cannot be ruled out (Silveira et al. 1990). Additionally, in AChE knockout mice, which are highly sensitive to VX toxicity in spite of lacking the primary toxicity target, atropine treatment was ineffective. The latter observation implies that VX initiated the neurotoxic cascade following OP intoxication via other pathways than through cholinergic innervation (Duysen et al. 2001). The EEG effects in non-seizing animals as discussed above may be related to a decreased cerebral blood flow and ischemia due to cardiorespiratory failure. Cardiorespiratory failure was also observed in case of intoxication with VR, a structural isomer of VX. VR seems to perturb the peripheral cardiorespiratory system in a progressive way, which ultimately led to collapse of central mechanisms and death (Chang et al. 1998).

In the present study, direct central cholinergic effects, such as ACh accumulation and seizure development were not observed in most animals after percutaneous exposure to VX. Although we have only measured ACh in the striate body, it is likely to be representative for other brain regions, such as the medulla oblongata, in which the respiratory centre is located. Finding no ACh accumulation is congruent with the EEG measurements, which did not show any convulsive activity or overt cholinergic

activation in particular EEG bands. Nevertheless, we cannot completely rule out that there might have been cholinergic overactivation in the respiratory centre since the AChE activity was more inhibited in the brain stem than in the striate body in non-seizing animals. These results are similar to that of another study, in which the AChE activity in the striate body was only 40% inhibited after VX, whereas that in hippocampus and brain stem was 60% inhibited (Chang et al. 1998). At the latter levels of inhibition, no cholinergic signs were reported in that particular study, in contrast to the animals in the present study.

Taken together, our results point to a more prominent action of VX in the peripheral compartment, attributing to clinical signs and death, which is in line with results found by others (Shih and McDonough 2000). Additionally, the agent has shown much more rapid inhibition of AChE in peripheral tissues compared to that in brain tissue following VX intoxication, although the different administration route used in the latter study may have influenced the inhibition rate in different tissues (Shih et al. 2005). In contrast to red blood cell AChE, which was completely inhibited within 10 min, the whole blood ChE showed only 70% inhibition at 1 hour after injection with VX. The differential inhibition between red cells and whole blood is likely to be due to the lesser inhibition of the plasma ChE content by VX, which contributes to the inhibition of whole blood. It is apparent that most blood ChE must be inhibited before substantial inhibition of organ AChE can occur, (Sidell 1997), although it is known that most ChE-inhibitors, including nerve agents, produce differential inhibition of different enzymes and tissues. Inhibition of red blood cell AChE over 90% has shown to be associated with a severe impairment of neuromuscular transmission, whereas the latter was only slightly affected at 70-90% inhibition (Thiermann et al. 2005). At physiological pH, the VX molecule is a protonated amine, which complicates its penetration into the brain (Epstein et al. 1974; Maxwell et al. 1997). Therefore, the supposed primary toxicity in the peripheral organs may be explained by the structure of VX.

The present study has shown that percutaneous exposure to VX leads to a progressive, but predictable course of clinical signs and survival time, with overt changes in EEG. It also demonstrates that peripheral toxic effects mainly contribute to the primary cause of death, which will have implications for treatment. In conclusion, clinical signs will mainly serve as indicators for the onset and maintenance of treatment in subsequent studies.

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Chapter 4

Percutaneous exposure to the nerve agent VX: efficacy of combined atropine, obidoxime and diazepam treatment

Marloes J.A. Joosen, Marcel J. van der Schans and Herman P.M. van Helden

TNO Defence, Security and Safety, BU CBRN Protection, Rijswijk, The Netherlands

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Abstract

The nerve agent VX is most likely to enter the body via liquid contamination of the skin. After percutaneous exposure, the slow uptake into the blood, and its slow elimination result in toxic levels in plasma for a period of several hours. Consequently, this has implications for the development of toxic signs and for treatment onset. In the present study, clinical signs, toxicokinetics and effects on respiration, electroencephalogram and heart rate were investigated in hairless guinea pigs after percutaneous exposure to 500 µg/kg VX.

We found that full inhibition of AChE and partial inhibition of BuChE in blood were accompanied by the onset of clinical signs, reflected by a decline in respiratory minute volume, bronchoconstriction and a decrease in heart rate. Furthermore, we investigated the therapeutic efficacy of a single dose of atropine, obidoxime and diazepam, administered at appearance of first clinical signs, versus that of repetitive dosing of these drugs on the reappearance of signs. A single shot treatment extended the period to detrimental physiological decline and death for several hours, whereas repetitive administration remained effective as long as treatment was continued. In conclusion, percutaneous VX poisoning showed to be effectively treatable when diagnosed on time and when continued over the entire period of time during which VX, in case of ineffective decontamination, penetrates the skin.

1 Introduction

Organophosphorous compounds (OPs) are potent irreversible acetylcholinesterase (AChE) inhibitors and widely used as insecticides. The most toxic OPs might be used as chemical warfare agents. Low volatility nerve agents like VX (O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate) are likely to enter the body via the skin rather than via the respiratory route. Effective treatment of skin intoxications is difficult because of unpredictable toxicokinetics (van der Schans et al. 2003; van der Schans et al. 2008).

Previous studies demonstrated that after percutaneous exposure of anesthetized hairless guinea pigs to VX, maximum blood levels of VX were not reached until several hours after exposure, followed by a slow elimination (van der Schans et al. 2003). The variability in toxicokinetics has shown to lead to a delayed and variable onset of toxic signs (Joosen et al. 2008; Wetherell et al. 2007). In addition, effective decontamination is hampered by the unawareness of time of intoxication and skin location of exposure, causing a long duration of the toxic effects. This has important implications for military or civilian first responders with respect to triage, decontamination approach and therapeutic drug regimens (Dalton et al. 2006; Hamilton et al. 2004). The slow absorption of VX by the skin into the circulation does not parallel the short biological half lives of the current medical countermeasures, likely making a single injection treatment insufficient.

Relatively little is known about the physiological consequences of the persistent action of VX. To investigate toxicity mechanisms following percutaneous exposure to VX, we developed a hairless guinea pig model, in which toxicokinetics of VX, acetylcholinesterase and butyrylcholinesterase inhibition, and a broad range of physiological parameters can be measured. The hairless guinea pig skin closely reflects that of human skin in vitro, which justifies the use of this animal model to study toxicokinetics after VX exposure (Frasch and Barbero 2009). In this model, three characteristic target organs can be evaluated: the brain by measuring seizure activity by electroencephalogram (EEG) and acetylcholine (ACh) levels using microdialysis, the lungs by monitoring respiratory function using whole body plethysmography, and autonomic control of the heart by telemetric recording of ECG. Measuring physiological consequences is of importance as they might be independent from AChE inhibition and therefore may need separate treatment.

In the present study we investigated the toxicokinetics of percutaneously administered VX and the development of typical signs of poisoning at different physiological levels after exposure to dose levels considered relevant for human poisoning. In previous experiments, a percutaneous dose of approximately 4 LD50 (500 µg/kg), yielded a reproducible development of signs of toxicity in hairless guinea pigs (Joosen et al. 2008). The human equivalent dose, calculated by extrapolation of this dose using basal metabolic rates (BMR) or surface area corrections, is approximately 120 µg/kg, which is 1.5 times the human LDL0 (Maynard and Beswick 1992). After a thorough examination of all model parameters affected during VX poisoning, the effect of the treatment with the anticholinergic atropine, the AChE reactivator obidoxime, and the anticonvulsant diazepam, was investigated following single treatment or after repeated administration.

2 Methods

2.1 Experimental design

Five groups of hairless guinea pigs were challenged percutaneously with 500 µg/kg of VX. Levels of unbound VX and obidoxime in treated animals were determined in plasma. AChE and BuChE was determined in blood. Simultaneously, the physiological parameters heart rate and respiration were registered in these animals. The guinea pigs were either untreated, treated with atropine, obidoxime and diazepam doses at the guidance of appearance and recurrence of clinical signs or treated at first clinical signs with one single dose. Groups 1 and 2 served as control groups for the effects of percutaneous VX intoxication. Treatment efficacy was tested in groups 3, 4 and 5. The atropine dose used was 10 mg/kg, to yield a maximal anticholinergic effect in guinea pigs. The obidoxime dose (8.2 mg/kg) was corrected for its proven lower efficacy in VX reactivating potency in guinea pigs compared to humans (Worek et al. 2002). The diazepam dose used was 0.5 mg/kg, determined as ED50 value for terminating ongoing seizures independent of the atropine dose used according to the model of Shih et al. (2007). A summary of the parameters measured and treatments applied are shown in Table 1.

***Table 1**. Overview of experimental groups, number of animals per group and experimental parameters measured. Hairless guinea pigs were challenged with VX (500 µg/kg pc) and the treatment regimens applied are indicated in the table. For each group, the experimental parameters measured are indicated with X.*

Group	Number of animals	Treatment					Parameters measured per experimental group								
		Drug (mg/kg i.m.)			Nr. of injections										
		Atropine	Obidoxime	Diazepam	Single	Repetitive	Clinical signs	VX levels	AChE Activity (blood)	BuChE Activity (blood)	Obidoxime levels	Respiration	Heart rate (ECG)	EEG	ACh levels (Striatum)
1	9	0	0	0	-	-	X	X	X	X		X			
2	6	0	0	0	-	-	X						X	X	
3	6	30	24.6	1.5	X		X	X	X	X	X	X	X	X	
4	8	10	8.2	0.5		X	X	X	X	X	X	X			
5	6	10	8.2	0.5		X	X						X	X	X

To obtain EEG and ECG signals, animals were equipped with a combined set of electrodes on the skull and thorax at 5-7 days before VX exposure (groups 2, 4 and 5). For ACh determination, a microdialysis probe guide combined with an ECG/EEG electrode was attached to the skull 5-7 days before VX exposure (group 4). For analysis of VX and obidoxime levels in plasma, and cholinesterase activity in blood , animals were equipped with a carotid cannula 12- 16 hours before exposure. In these animals, respiration was measured non-invasively in a whole body plethysmograph. The procedures are described in detail below.

2.2 Animals

Male hairless guinea pigs [Crl:IAF(HA)BR] (375 ± 10 g), obtained from Charles River (Maastricht, The Netherlands) were used in the present study. Prior to the experiments they were housed with 2 animals per cage, and allowed to acclimate to standard conditions for two weeks. Room temperature was kept at 19-22 °C and relative humidity was maintained at 55-65% and lights were on from 7 am to 7 pm. Acidified water and standard guinea pig chow (Teklad global diet 2040, Harlan, Horst, The Netherlands) were available ad libitum. The experiments described received prior approval from the TNO Animal Ethical Committee.

2.3 Chemicals

Acetylcholinesterase, choline oxidase, acetylcholine, choline, atropine sulphate and Triton-X100 were obtained from Sigma Chemical Co. (Zwijndrecht, The Netherlands). Kathon CG (1.5% 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was provided by Rohm and Haas (Croyden, UK). LiChrosorb® NH2 was purchased from E. Merck (Amsterdam, The Netherlands). Hypnorm® (fentanyl/fluanisone) was purchased from Janssen Pharmaceutica (Beerse, Belgium) and Dormicum®(midazolam) was delivered by Roche Nederland BV (Mijdrecht, The Netherlands). Obidoxime, pralidoxime, (±) VX (O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate), the n-propyl analogue of (±) VX, O-n-propyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate and soman (O-Pinacolyl methylphosphonofluoridate) were obtained from the stocks of TNO Defence, Security and Safety and of purity ≥98% (GC). The other chemicals used were of standard purity and purchased from renowned companies. For HPLC analysis the highest purity grade was used. All solutions were prepared with water tapped from a Milli-Q system (Millipore SA, Molsheim, France).

2.4 Surgical procedures

Guinea pigs were anaesthetized with 6 ml/kg FFM-mix (1.25 mg/ml midazolam, 2.5 mg/ml fluanisone, 0.079 mg/ml fentanyl citrate) via a single i.p. injection. Premedication with 0.05 mg/kg atropine sulphate (s.c.) was administered to prevent respiratory arrest. Two stainless steel screws (A8.0 and P1.2 mm relative to bregma and 1 mm from the sagittal suture) placed at the dura mater, fixed to a plug, served as EEG electrodes. To obtain an ECG signal, two leads sutured in the superficial muscles under the skin just below the right collarbone and between the second and third rib (Lead II configuration) and were also fixed to the plug. The plug and the electrodes were fixed to the skull with dental cement. In a separate group of animals a concentric microdialysis probe guide (CMA12, CMA, Sweden) was stereotaxically (KOPF Instruments, Tujunga, CA) inserted into the ventral striatum (P1.8, L 2.8, V 8.3 mm relative to bregma and the dura mater) next to the plug. Animals received peri-operative pain medication and antibiotics by sc injections (1 ml/kg) with Rimadyl (Carprofen, 5 mg/kg) and Borgal (1:10 dilution in PBS) before and at 24 and 48 hours after surgery. Animals were allowed to recover for 5-7 days from the surgical procedure.

For toxicokinetic experiments, naive guinea pigs or guinea pigs equipped with an EEG/ECG electrode 5 days earlier, were anesthetized with isoflurane (2-4%). A cannula was installed into the carotid artery and was guided under the skin to exit the body in the neck. The cannula was filled with heparinized PBS (20 IU/ml) and closed with a plug and loosely attached to the skin. Next, the animals were allowed to recover for 12-16 hours before application of VX.

2.5 VX application, observation and treatment

After obtaining baseline data from the parameters, the guinea pigs were gently fixated by hand, and a solution of VX in Isopropylalcohol (32 mg/ml) was applied to a well-defined spot on the belly of approximately 2 cm2 at a dose of 500 µg/kg (16 µl/kg). After the fluid was absorbed(approximately after 20 seconds), the spot was covered with a stainless-steel cup, which was strapped to the skin with surgical tape and removed after one hour. Upon exposure, the guinea pigs were monitored and the onset of the following cholinergic symptoms was registered: 1) Chewing: A clear chewing-like movement of the guinea pig in which the entire head is involved as a consequence of increasing saliva production. 2) Shivering: Continuous shivering of the entire body. The animal is capable to move around. 3) Salivation: Extensive drooling and tears fluid. 4) Tremor: Extensive shivering combined with involuntary movements in which the entire body is involved. The guinea pig looks mentally dissociated from the environment and has lost control over its movements. 5) Respiratory Distress: Low respiratory rate and heavy breathing.

In groups 3-5 initial clinical signs served as indicators for treatment. The presence of two signs for a period of several minutes was chosen as an indication for treatment injection. Treatment consisted of either a combined single im injection containing atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg respectively, group 3) or repetitive treatment lower doses of these drugs (10, 8.2 and 0.5 mg/kg im respectively, groups 4 and 5). The repetitive treatments were injected at the first presence of signs as previously indicated, and repeated after clear reappearance of clinical signs, mostly within 1-2 hours. Animals received 7-10 single shot injections over a time frame of 8-13 hours. When the heart rate of guinea pigs dropped below 150 BPM the animals were humanely euthanized to minimize unnecessary suffering.

2.6 EEG/ECG acquisition and analysis

In groups 2, 4 and 5, EEG and ECG of the animals challenged with VX were registered. The signals were obtained using PhysioTel telemetry system from Data Sciences Inc. (DSI) using the ML2-11-EET-F40 transmitter body. The transmitter was attached to the plug fixed on the guinea pigs skull. A receiver board measured the signal from the transmitter, which was consolidated and stored on an IBM-compatible personal computer via a Data Exchange matrix at 250 Hz (EEG) and 500 Hz (ECG) sampling frequency. Heart rate was exported from the Data Science ART analysis program. After storage, the EEG data were converted into EDF format. Every 10 seconds power spectra were calculated using Fast Fourier Transformation (FFT).

A low cut off filter at 1.6 Hz was used to filter eye movement artefacts and high digital filter at 50 Hz was used. Relative powers of EEG bands were calculated by dividing the sum of the absolute power in the frequency range by the total power of the spectrum. The results of relative power in the Delta (0.5-3.99 Hz) band is shown as representative power band.

2.7 Microdialysis procedure for acetylcholine determination

In group 2, ACh levels in the striatum of animals exposed to VX and repetitively treated with atropine, obidoxime and diazepam was registered in combination with EEG and ECG. Five days after surgery, the guinea pigs were individually placed in a perspex cage (25 x 25 x 40 cm) with free access to food and water. The following day, experiments were started between 9 and 10 AM. The microdialysis probes used were CMA12 probes with 4 mm of exposed PAES membranes. The probes were inserted one hour before the start of the experiment. Baseline values were measured in the following hour, followed by application of VX as previously described and intra-muscular injection of treatment upon clinical signs. The microdialysis probe was perfused with a Ringer solution at 2.0 µl/min delivered by a microinjection pump (CMA 100, CMA microdialysis AB, Solna, Sweden). To obtain detectable quantities of acetylcholine in the dialysate, 100 nM neostigmine was added to the perfusate. The guinea pig was connected directly to the sample loop, allowing on-line analysis of the microdialysates. The injection valve was automatically activated every 10 min, resulting in an injection volume of 20 µl.

2.8 HPLC Instrumentation and acetylcholine analysis

Microdialysate samples were assayed for acetylcholine and choline using a HPLC system according to Damsma et al. (1987) with some modifications, as described previously (Joosen et al. 2008). Briefly, ACh was separated on a EC 125/2 Nucleosil 100-5 C18 AB analytical column (Aurora Borealis Control, Schoonebeek, The Netherlands), preloaded with 0.5% sodium lauryl sulphate, and converted into choline and hydrogen peroxide on a post-column enzyme reactor containing immobilized acetylcholinesterase (80 U) and choline oxidase (40 U). Hydrogen peroxide was electrochemically detected at + 450 mV using a VT03 flow cell with Pt work electrode (Antec Leyden BV, Hazerswoude, The Netherlands) The mobile phase consisted of a 166 mM potassium phosphate buffer (pH 8.5) containing 1 mM tetramethylammonium chloride, 0.79 mM EDTA and 250 µl/l Kathon CG. The system was run at 0.35 ml/min and temperature was maintained at 30 ^{o}C. Before the beginning and at the end of each experiment a 10 nM acetylcholine calibration standard was injected in duplicate, and if necessary corrections for reduced sensitivity during the experiment were made. Limit of quantification was 1 pmol/ml. Linear calibration curves were obtained in the range from 5 – 1000 pmol/ml ($r > 0.990$). The intra-assay coefficients of variation for 10 and 100 pmol/ml were 2.9 % and 4.1 %, respectively. Inter-assay variability could not be determined due to variable enzyme activities in the post-column reactor between days.

2.9 Toxicokinetic experiments

In experimental groups 1, 3 and 5 toxicokinetics of VX, pharmacokinetics of obidoxime and ChE activity in blood were determined upon VX exposure either with or without treatment. One hour before exposure, the cannulas were opened and clamped to have free access to blood samples without handling the animal during the measurement and the animals were placed in a whole body plethysmograph (Buxco sytems XA, USA) cage to monitor their respiration characteristics. Among other parameters, P-enhanced (Penh), an arbitrary value for bronchoconstriction, and Respiratory Minute Volume (RMV) were calculated from the signal using IOX software (EMKA technologies, France). After obtaining baseline values, VX dissolved in isopropylalcohol solution was applied on the belly of the animal as described previously (Joosen et al. 2008). Blood samples were taken at least every two hours, or every hour when clinical signs progressed rapidly. Blood samples (200 µl) were immediately processed to determine VX levels. For the obidoxime determination plasma was prepared starting with a 100 µl blood sample by centrifugation (15s, 11,000 rpm). Small blood samples (20 µl) for AChE and BuChE were drawn every hour.

2.10 Sample preparation for determination of VX

Aliquots of 20 µl blood were diluted 10 times in 1% saponin solution and frozen immediately in liquid nitrogen. These samples were used for determination of AChE and BuChE activity. Aliquots of 200 µl blood were mixed with 10 ng of O-n-propyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate as internal standard and soman (100 ng) to occupy available binding sites. Next, the sample was made alkaline with 30 µl 1M sodium hydroxide and extracted twice with two volumes of a mixture of 5% methanol in hexane. The extract was evaporated and the residue was dissolved in 100 µl n-hexane and analyzed with GC using nitrogen phosphorous detection.

2.11 Gas chromatography for VX analysis

VX levels in plasma were determined using gas chromatography as described previously (van der Schans et al. 2003). The analyses were performed on a HRGC Mega II 8560 instrument (Fisons Instruments, Milan, Italy) equipped with a nitrogen-phosphorus detector (300 °C), a split-splitless injector and an AS 800 auto sampler. The chromatographic conditions comprised a CPSil8 column length, 25 m, i.d. 0.25 mm and film thickness 1.2 µm (Varian, Middelburg, The Netherlands). Injections (8 µl) were performed using the splitless mode at an injector temperature of 260 °C. At the start, the oven temperature was maintained at 100 °C for 2 min, increased to 200 °C at a rate of 25 °C/min for 7 minutes, and finally elevated to 300 °C at a rate of 25 °C/min. The carrier gas (helium) flow was 2.5 ml/min. Detector gas flows were 35, 350, and 37 ml/min for hydrogen, air and helium, respectively.

2.12 Obidoxime analysis

Obidoxime in plasma was determined with capillary electrophoresis. Plasma samples were diluted 1:1 with a mixture of 60 U/ml heparin and internal standard (pralidoxime, 10 µg/ml). Electrophoretic analyses were performed on a P/ACE MDQ from Beckman Coulter (Fullerton, CA, USA). Running buffer consisted of 200 mM 6-aminohexanoic acid/acetate, pH 4.5. Samples were introduced by pressure injection for 5 s at 1 psi. Length of the fused silica capillary (i.d. 75 µm, PolyMicro, Phoenix, AZ, USA) was 35 cm, the effective length to the detector was 25 cm. Separation voltage was 15kV. The detection wavelength was 290 nm.

2.13 Enzyme activity

Samples were analyzed for AChE and BuChE activity using a modification of the method by Ellman et al. (1961). The assay was performed in 96 well plates, in which AChE and BuChE activity were determined simultaneously on the same plate. In short, samples were diluted in 0.8 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich B.V.). To 10 µl of diluted sample, 100 µl of 0.8 mM β-methylacetylthiocholine iodide was added, in quadruple, for determination of AChE activity. For assessment of BuChE activity in the samples, 100 µl of 0.8 mM of butyrylthiocholine was added. The delta OD per min at 412 nm at ambient temperature served as measurement for ChE activity. Guinea pig AChE does not react with butyrylthiocholine, rendering the BuChE activity determined an appropriate representation of BuChE activity. AChE activity in blood was corrected for cross reactivity of BuChE with β-methylacetylthiocholine by subtracting of 47% of the extinction of butyrylthiocholine developed by BuChE. This was calculated from separate experiments in which isolated guinea pig AChE and plasma BuChE were incubated with β-methylacetylthiocholine (Bosgra et al. 2009). AChE and BuChE activity in blood were normalized versus the baseline sample.

2.14 Data presentation and statistical analysis

All data were analyzed using one-way ANOVA or repeated measures ANOVA followed by Dunnett's post-hoc test using the VX group or baseline values as control. Results were considered significant for $p<0.05$.

For determination of continuous relationships between blood AChE and BuChE activity and occurrence of clinical signs logistic regression analysis was performed. AChE or BuChE activity of animals pc exposed to VX was coupled to presence of a sign (1) or absence of sign (0). Using binary logistic regression analysis with SPSS statistical software, the logits (l) of the probability of response for clinical signs (p) at each level of ChE activity (j) were determined. The probability of response between 0 (no response) and 1 (response) for each clinical sign was calculated from the logits of the probability p using the equation:

$$p = \frac{e^{l_j}}{1 + e^{l_j}}$$

3 Results

3.1 Toxicokinetics and clinical signs

The toxicokinetics of VX after percutaneous application was studied in hairless guinea pigs that either received no treatment, a single shot treatment or repetitive treatment (atropine 10 mg/kg; obidoxime 8.2 mg/kg and diazepam 0.5 mg/kg im per injection). During the experiment the animals were scored for clinical signs. The average onset times of these signs are shown in figure 1A. The first clinical signs appeared in a gradual fashion, starting with chewing and shivering at 15 to 220 minutes and 55 to 374 minutes, respectively. This was followed mostly by more heavy tremors at 75 to 122 minutes. In most animals this was followed by respiratory distress shown by heavy breathing. Only few animals developed convulsions. Animals died on average at 237 ±37 minutes, with a minimum of 96 minutes and a maximum of 560 minutes, indicating a large variation in the development of toxic signs.

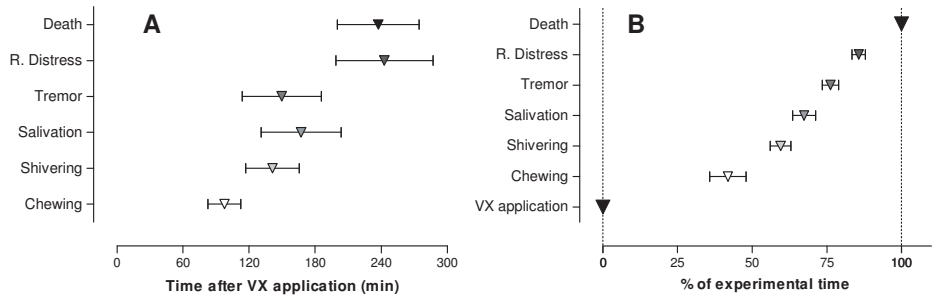

***Figure 1**. A. First appearance of cholinergic signs in individual animals (vertical axis) over experimental time (horizontal axis) between VX application (0h) and death. B. First appearance of cholinergic signs in individual animals (vertical axis) at percentages of total experimental time (horizontal axis) between VX application (0%) and death (100%). The average first appearances of signs ±SEM have been plotted (n=15).*

We scored the first appearance of the cholinergic signs versus the % of time elapsed between VX application (0%) and death (100%) (Fig. 1B). On average, the onset time of the first occurrence of each sign was predictive for the time of the occurrence of more severe signs, and signs occurred always in a similar sequence. Chewing occurs at 41±6 % of the total survival time, followed by shivering and salivation at 59±3% and 67±4% of survival time, respectively. On average, animals started showing tremor at 76±3% and the most severe symptom, respiratory distress, was only seen shortly before death at 85±2% of total time.

We measured the average concentration of VX in blood of 9 animals that were exposed percutaneously to 500 µg VX/kg (Fig. 2). In most animals, VX levels raised to more than 1 ng/ml within 1-2 hours. The maximum VX levels ranged from 0.3 to 5.5 ng/ml, which were reached after 5 hours or 90 minutes, respectively. On average, plasma levels reached maximum levels of 3-4 ng/ml at 2 hours after the intoxication. AChE was completely inhibited at that time (Fig. 2C). BuChE activity showed a similar pattern, however with a slight delay in inhibition compared to

AChE activity (Fig. 2D). In particular during the first 90 minutes after exposure a clear difference between AChE and BuChE inhibition was observed.

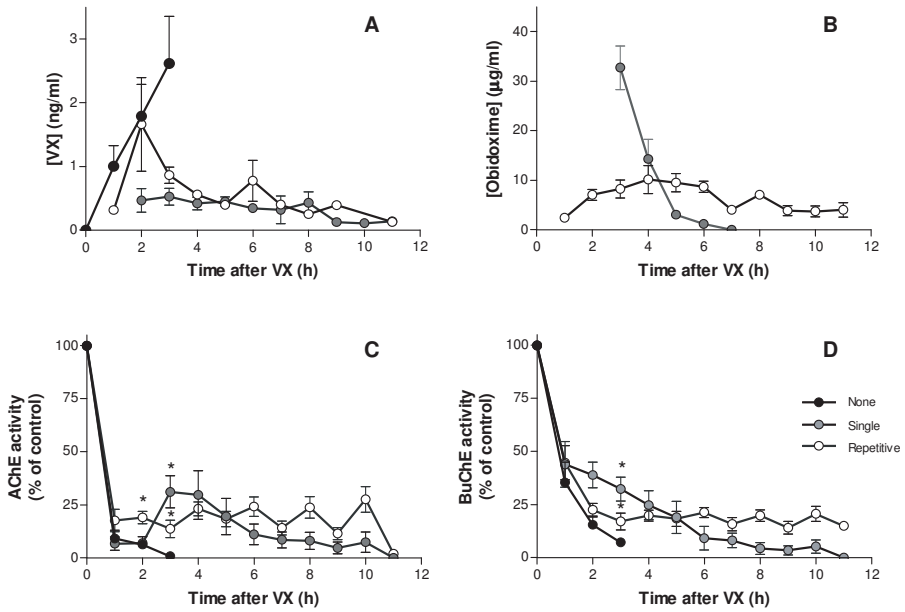

***Figure 2**. A. Toxicokinetics of VX in animals pc exposed to VX (500 µg/kg pc). B. Obidoxime plasma concentrations in animals exposed to VX (500 µg/kg pc). C. AChE and D. BuChE activity in blood of animals exposed to VX (500 µg/kg pc). Open upward triangles represent values obtained from untreated animals. Open squares represent animals treated with a single shot treatment at appearance of first signs, in general after approximately 1 hour, downward closed triangles represent animals receiving repeated injections after skin VX poisoning, in genereal every 60-90 minutes after VX poisoning. Values are represented as means ±SEM. *: p <0.05; significantly different from untreated animals, One way ANOVA followed by Dunnett's post-hoc test.*

The fraction of animals showing cholinergic clinical signs, at each level of blood ChE activity, is shown in figure 3 A-C. Analysis of the relationship between the occurrence of clinical signs and the enzyme activity of AChE and BuChE activity in blood of untreated animals by logistic regression revealed that over 80% of AChE had to be inhibited before initial clinical signs emerged (Fig. 3). The first mild signs of poisoning, chewing movements, shivering and slight tremor, generally did not occur before over 80% of AChE activity was inhibited. At that time, only 40-60% of BuChE appeared to be inhibited, resulting from a much slower inhibition rate of the BuChE activity. Full inhibition of BuChE showed to be a much better predictor of death compared to AChE inhibition. Full inhibition of AChE in blood predicted death in only 50% of the cases, whereas full BuChE inhibition was correlated with death in 80% of cases.

Animals in the group that received a single shot treatment showed similar toxicity signs. Treatment on indication of the first toxicity signs, i.e. chewing and

shivering, delayed the progression of the signs. However, they reappeared after approximately 1 hour in most animals. Three out of 6 animals died within 10-12 hours after poisoning, whereas 1 animal survived for 22 hours. Two other animals were euthanized at 10 hours because of their poor condition. Blood samples were drawn from these animals to determine AChE, BuChE, VX and obidoxime. A single shot of three auto injector equivalents led to maximum obidoxime levels of 30 µg/ml at one hour after the injection and an elimination half-life time of 1 h (Fig. 2B). Upon administration of the therapeutics, AChE and BuChE were reactivated to 40% of the control value but were completely re-inhibited within 9 hours by remaining VX (Fig. 2C,D). The VX levels in these animals ranged from 0.5 to 1 ng/ml, which was lower than in the non-treated animals (Fig. 2A).

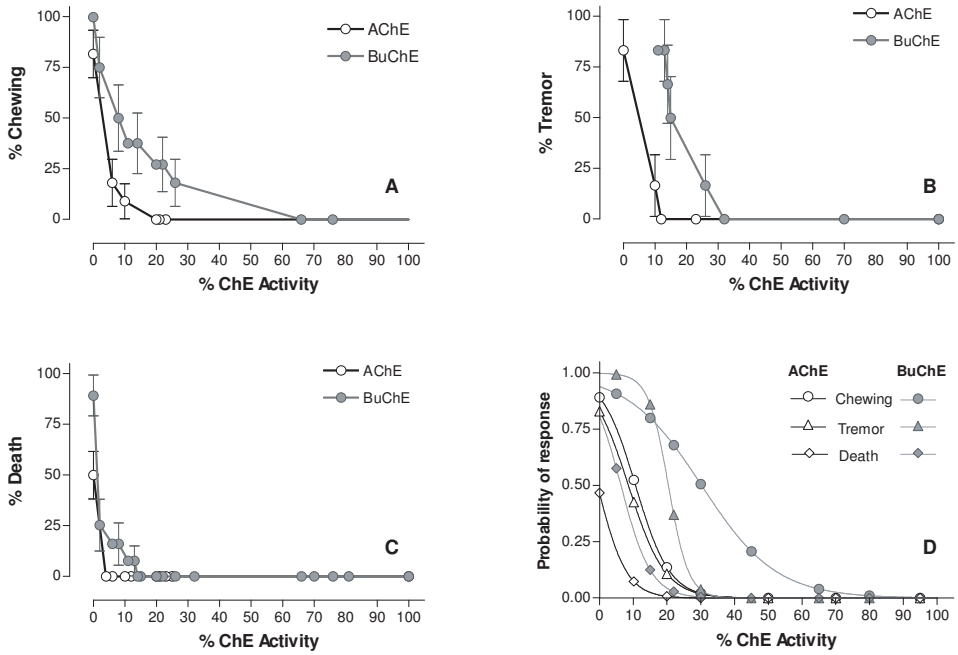

***Figure 3**. Toxicity signs in hairless guinea pigs after percutaneous exposure to 500 µg/kg of VX at a certain AChE or BuChE activity in blood. A, B and C. The percentage (mean ±SEM) of animals showing Chewing, Tremor, and Death respectively (n=9). D. The probability of response at each ChE level calculated from logistic regression analysis of the appearance of different toxicity signs following skin VX exposure in hairless guinea pigs.*

In another experimental group, treatment was given repetitively on the guidance of clinical signs. In case one injection was given after appearance of the first toxicity signs, the signs worsened again after approximately one hour. Additional treatment injections postponed further progression of signs to respiratory distress and death, but less severe signs such as shivering and tremor could not be prevented. After repetitive treatment with 6-8 auto-injector equivalents over 10-14 hours, all animals

but one survived the observational period of 24 hours. However, the animals surviving this period showed severe signs of poisoning, such as severe tremors and respiratory distress at 24 h after application of VX.

Upon repetitive injections, obidoxime levels in plasma were approximately 10 µg/ml and were stable during the period that treatment was provided (Fig. 2B). AChE and BuChE were reactivated and were steady at a level of 25% of control values during that time (Fig. 2C,D). VX levels in blood were approximately 0.8 ng/ml throughout the time of the experiment, which was again lower than in the non-treated animals (Fig. 2A). At 24 h after the application of VX, AChE and BuChE were completely inhibited, VX levels in blood were lower than 0.1 ng/ml and the obidoxime concentration in plasma was below 2 µg/ml (not shown).

3.2 Physiology

Respiration dynamics of the most representative parameters Penh (a measure for bronchoconstriction) and respiratory minute volume (RMV), were registered during the toxicokinetic experiments (Fig. 4). Bronchoconstriction showed a significant increase, together with a significant decline in RMV as the intoxication became more severe. Both the single shot treatment and repetitive treatment delayed the increase of Penh. After washout of drugs, Penh increased further (Fig. 4A). The decline in RMV was only prevented by the high dose single shot treatment (Fig. 4B). This treatment induced a short period of RMV increase, which declined again at approximately 6 hours after poisoning and 3 hours after treatment. Repetitive treatment with atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively) did not prevent a significant RMV decrease, and RMV further declined after cessation of treatment at 12 hours after poisoning.

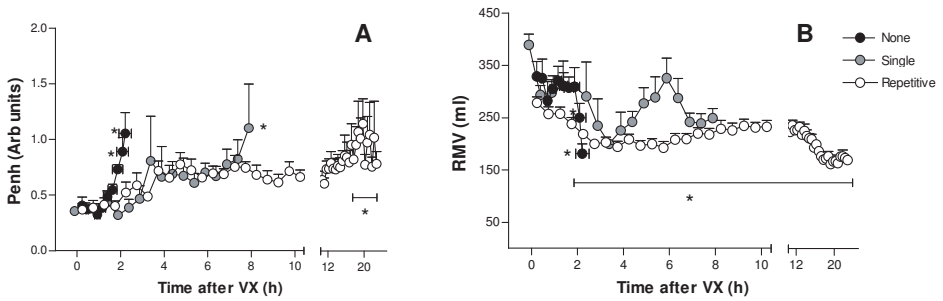

***Figure 4**. Respiration parameters after pc VX exposure (500 µg/kg). A. Averaged Penh (bronchoconstriction) after pc VX exposure. B. Averaged Respiratory minute volume. The graphs show averages ± SEM of untreated animals (black dots, n=9). Grey dots represent animals treated with one single injection of atropine, obidoxime and diazepam (10, 24.6 and 1.5 mg/kg i.m respectively,, n=6), and animals repetitively treated with injections are shown by open dots (n= 6).*: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

Animals exposed to VX (500 µg/kg pc) without treatment showed a significant decline in average heart rate at 90 minutes after poisoning (Fig. 5). A single im injection with atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg im respectively) induced an obvious delay in the heart rate decline (Fig. 5). Repeated treatment preserved the heart rate for a longer time, but after cessation of treatment the decline progressed.

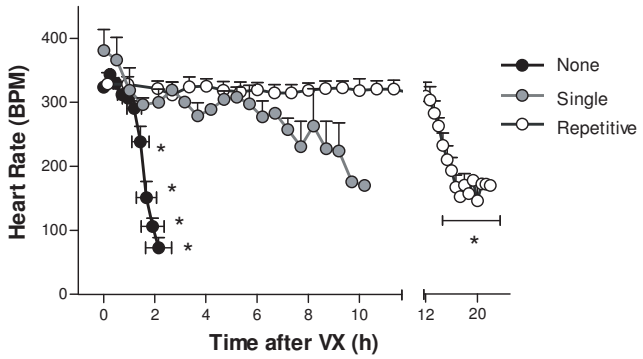

***Figure 5**. Averaged ± SEM heart rate after pc VX exposure (500 µg/kg) in untreated animals (black dots, n=6), animals treated with atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg im respectively) (grey dots, n=6), and animals repetitively treated with single injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively) (open dots, n= 6). *: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

Animals repetitively treated with im injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg respectively) eventually showed increases in extracellular ACh levels (Fig. 6). The effect of a single high dose injection on extracellular ACh levels was not tested.

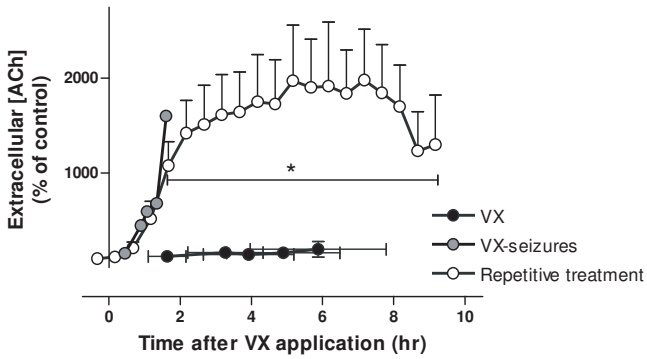

***Figure 6**. Extracellular ACh concentrations in striatum of VX exposed guinea pigs. Black dots represent untreated animals without seizures and grey dots the ACh levels of one untreated animal displaying seizures on EEG (data from Joosen et al. 2008). The open dots represent the ACh levels in the striate body of animals repetitively treated with injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively) at appearance and reappearance of clinical signs, The average time between injections was 60-90 minutes. *: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

For comparison, ACh levels of untreated animals are also presented in Figure 6. In these animals, the accumulation of ACh was not accompanied by an increase in total EEG power, and not by the appearance of seizures. The treatment applied thus prevented the occurrence of seizures induced by the increase in ACh concentration.

The EEG was also analyzed for energy shifts in power bands. The shifts in the Delta band are shown in Figure 7. The power in the Delta band of the EEG showed a clear initial increase in the first 2 hours after exposure, and then became significant. Both treatment regimens applied showed a reverse of these effects. The single shot treatment induced a shorter and steeper reversal than the repetitive treatment, and prevented statistically significant changes. The repetitive treatment induced a significant decline in the power in the delta band, which persisted for several hours.

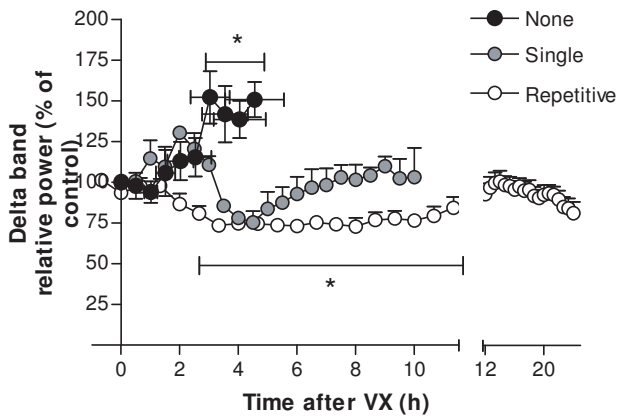

***Figure 7**. EEG effects after pc exposure to VX (500 µg/kg) in guinea pigs. The averaged normalized relative power in Delta band of the EEG is shown. Grey dots represent the animals receiving a single injection of atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg im respectively). Open dots represent the animals that received repetitive treatment with atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively). *: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

4 Discussion

The toxicokinetics of VX after percutaneous exposure to 500 µg/kg and the consequent physiological effects were investigated in hairless guinea pigs. The guinea pigs were either untreated, treated with atropine, obidoxime and diazepam doses at the guidance of appearance and recurrence of clinical signs or treated at first clinical signs with one single dose. The use of a small animal, such as the hairless guinea pig, requires the use of diluted VX to obtain a relevant level of exposure. It might be expected that both the body surface area exposed and the solvent might influence toxicokinetics. However, an effect on the toxicokinetics related to the solvent and the resulting increased surface area is not implicated (Hamilton et al. 2004). Similar to a study in the domestic swine, we found variable toxicokinetics

in untreated animals. The levels of VX in blood of animals that received treatment appeared to be lower than the VX levels in untreated animals. A possible explanation is that BuChE was also reactivated by obidoxime, thereby releasing binding sites for penetrating VX. Another possibility is that untreated animals showed to have a much lower heart rate than animals receiving treatment, implicating that the distribution and therefore the elimination rate of VX was lowered.

In line with the variability of VX toxicokinetics and AChE and BuChE inhibition, the time of onset and development of toxic signs showed a similar variability in time when left untreated. In one of our previous studies (Joosen et al. 2008), we showed that the onset time of clinical signs following percutaneous VX application showed to be a reliable predictor for the time course and presence of more severe signs and death. In this study, onset times of clinical signs were expressed as a % of the total time period between VX application and death. Using this approach in the present study, we showed again that compensation of the variable and delayed absorption through the skin by normalising the time factor, minimized inter-individual variability in the first occurrence of clinical signs (Fig. 1).

In contrast to the subcutaneous exposure route reported in the literature (Hilmas et al. 2009; Shih et al. 2007), full blown seizures were only apparent in 33% of animals skin exposed to VX. The fact that less seizures were found compared to previous work may be explained by the much slower absorbance of VX from a skin depot into the circulation than that following subcutaneous injection. The appearance of the most severe signs and death, implicates that penetration of VX into critical organs such as the brain and diaphragm will not occur until the entire scavenging pool for VX in blood is inhibited (Sidell 1997). By entering the circulation very slowly, the protonated amine in VX may be more hampering its entrance into the brain (Epstein et al. 1974; Maxwell et al. 1997), than at higher free concentrations of VX. However, at several hours after skin exposure with VX, we found substantial AChE inhibition in the brain. On the other hand, very low levels of VX have shown to lower the firing rate and amplitude of action potentials in hippocampal neurons in vitro (Rocha et al. 1999). Translated to the in vivo situation, this may cause a more generalized reduction of action potentials in the brain and a shift of EEG power to lower frequency ranges opposed to the expected increase induced by ACh accumulation. When seizures were absent, we found an increase in delta and a decrease in theta power of the EEG. Either ischemia or direct effects of VX, for example by lowering the frequency of theta power generated by hippocampal neurons, may have caused this effect (Chang et al. 1998; Cohen et al. 1994; Joosen et al. 2008).

The absence of profound central effects after percutaneous VX exposure in the majority of animals prompted us to put focus on physiological peripheral effects such as respiration and heart rate in addition to toxicokinetics and EEG. A significant life threatening decrease in heart rate and respiratory minute volume in combination with bronchoconstriction developed at approximately 60-70 % of survival time, while AChE and BuChE in blood were inhibited for 90% and 80%, respectively. These physiological changes paralleled the alterations found by EEG. In contrast, obvious less severe clinical signs, such as chewing occurred as early as

at 40% of survival time, at lower levels of BuChE and AChE inhibition of over 80 and 60%, respectively. These findings showed that mild clinical signs are good predictors for the toxicological status, and that measurement of blood cholinesterase is a proper indicator for initial severity of poisoning and treatment efficacy in case of skin exposure involving VX.

The treatment employed improved the clinical outcome on all parameters as long as treatment was continued on worsening of clinical signs. Discontinuation of treatment led to worsening of signs of poisoning after some time, resulting from VX slowly diffusing from the skin into the blood. Treatment with an intramuscular bolus of atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg i.m. respectively)at first signs of poisoning led to a delay of progression of life-threatening signs and prolonged survival time. This increase in treatment efficacy was probably for a great deal due to reactivation of AChE and only to some extent BuChE activity. Although critical physiological parameters were mainly preserved, the animals receiving either single or repetitive treatment kept showing mild to moderate clinical signs such as shivering and slight tremor, rendering the animals most likely incapable of performing any tasks. A single high dose treatment broadened the time window needed to receive additional intensive medical care, which was prolonged when the repetitive treatment scenario was employed.

Continuous treatment with single injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg i.m. respectively) led to a substantial level of approximately 10 µg/ml of obidoxime in blood, which continuously reactivated a small amount of AChE and a very small fraction of BuChE. The single shot high treatment, leading to 3 times higher levels of obidoxime, induced a larger initial reactivating effect, after which the reactivated enzymes gradually were re-inhibited within the following hours by continuing VX release from the skin, again leading to toxicologically relevant levels.

In addition to oxime treatment, atropine served as a major component for treatment of peripheral signs. First of all, atropine is effective in counteracting salivation and respiratory deterioration and prevents vagally induced bradycardia by blocking mAChRs. Clinical findings suggest that atropine demand strongly depends on the level of AChE inhibition. In patients showing less than 90% of AChE inhibition, only very low doses of atropine were necessary (less than 10 µg/kg/h), whereas in patients showing over 90% of RBC AChE inhibition, very high levels of atropine were needed to treat mAChR mediated cholinergic transmission (Thiermann et al. 2009).

Changes in relative power in frequency bands of the EEG, probably induced by a severe impairment of respiration and decline in heart rate, were also reversed by repetitive treatment. In these treated animals, there was a huge accumulation of ACh in the brain, pointing to the entrance of VX into the brain and insufficient restoration of AChE activity to prevent ACh accumulation. Similar accumulation of ACh levels caused by OP poisoning in general, leads to development of seizures (Joosen et al. 2008; Joosen et al. 2009; Lallement et al. 1992). The treatment with the combination of atropine and diazepam probably prevented the development of seizures in spite of high levels of ACh in these animals. In addition, the toxicological process induced by skin applied VX is relatively slow, enabling synaptic adaptation

of the brain to the excess of ACh. The present findings imply that in spite of the initial absence of seizures in percutaneous VX exposure, diazepam or an alternative anticonvulsant is a necessity in the treatment of VX poisoning through the skin.

In conclusion, parameters such as EEG, heart rate and respiration showed to be less sensitive than typical cholinergic clinical signs for determining the toxicological status of the animal, but showed to be major and objective read-out parameters for treatment efficacy. The use of clinical signs as early indicator for starting and continuation of treatment with atropine, obidoxime and diazepam showed to be highly effective in preventing life-threatening effects on heart rate and respiration in the guinea pig. Percutaneous VX exposure appeared to be effectively treatable when diagnosed on time and if an appropriate timing of treatment is employed. Moreover treatment needs to be continued over the period of time during which all VX, that cannot longer be removed by skin decontamination, has entered the body and has been eliminated.

Although extrapolation of results from small animal models to man is difficult due to dose calculations, we predict that our findings in the guinea pig with clear consequences for treatment in this model, will hold predictive value for the human situation.

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Chapter 5

Long-term cognitive deficits accompanied by reduced neurogenesis after soman poisoning

Marloes J.A. Joosen[1], Edwin Jousma[2], Tom M. van den Boom[1], Willem C. Kuijpers[1], August B. Smit[3], Paul J. Lucassen[2] and Herman P.M. van Helden[1]

1: TNO Defence, Security and Safety, BU CBRN Protection, Rijswijk, The Netherlands
2: SILS Center for Neuroscience, University of Amsterdam, The Netherlands
3: Center for Neurogenomics and Cognitive Research, VU University, Amsterdam, The Netherlands

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Abstract

To date, treatment of organophosphate (OP) poisoning shows several shortcomings, and OP-victims might suffer from lasting cognitive deficits and sleep-wake disturbances. In the present study, long-term effects of soman poisoning on learning ability, memory and neurogenesis were investigated in rats, treated with the anticholinergic atropine and the oxime HI-6 for reactivation of soman-inhibited acetylcholinesterase. We also investigated whether sub-chronic treatment with the reported neurogenesis enhancer olanzapine would stimulate neurogenesis and possibly normalize the anticipated long-term deleterious effects of soman intoxication. Animals were treated with HI-6 (125 mg/kg i.p.), followed after 30 minutes by soman (200 µg/kg s.c.) and atropine sulphate (16 mg/kg i.m.) 1 minute thereafter. Soman poisoning led to an elevation of extracellular acetylcholine levels to 1500% over baseline values as assessed by striatal microdialysis. Brain acetylcholinesterase was inhibited over 95%. This was accompanied by short recurrent seizures lasting for 40 minutes. Osmotic minipumps releasing olanzapine (7.5 mg/kg/day) or vehicle were subcutaneously implanted 24 hours post-intoxication. After drug delivery for 4 weeks, newborn cells were BrdU labeled. Learning and memory performance were assessed 8 weeks after soman poisoning, followed by analysis of surviving newborn cells (BrdU) and neurogenesis (doublecortin, DCX). Eight weeks after soman-intoxication a significantly impaired learning ability was found that was paralleled by significantly lower numbers of DCX-positive cells but no changes in the number of BrdU-labeled cells. Apparently, the present Olanzapine regime was ineffective. We conclude that soman poisoning has long lasting effects on learning ability, a finding that was accompanied by impaired neurogenesis. Although we confirm a correlation between impaired neurogenesis and cognitive deficits, establishing the true causal relationship between these processes in OP exposed animals awaits future research.

1 Introduction

Organophosphates (OPs) like pesticides and chemical warfare agents, irreversibly inhibit acetylcholinesterase (AChE), which leads to accumulation of acetylcholine (ACh) and overstimulation in both central and peripheral synapses. This overstimulation results in epileptiform activity, which may cause respiratory failure and death. The currently available treatment (atropine, oxime, diazepam) of OP-poisoning is insufficient in that victims still suffer from persisting adverse effects on cognitive processes, mood, and sleep-wake rhythm (Dawson 1994; Lallement et al. 1998; McDonough 2002; Shih and McDonough, Jr. 1997; van Helden and Bueters 1999; Willems et al. 1993). These effects are supposed to be due to progressive neurodegeneration following the epileptiform activity (Carpentier et al. 2000; Kadar et al. 1995; Lallement et al. 1998). Brain areas involved in the OP-induced neurodegeneration are the hippocampus, amygdala, striatum and prefrontal cortex (Baille et al. 2005). The drastic effects of OP poisoning urge for research to prevent or stop neurodegeneration and to stimulate brain repair.

Over the past decade, it has become apparent that in particular parts of the adult brain new neurons are still formed, a process called neurogenesis (Eriksson et al. 1998; Gross 2000; Kuhn et al. 1996; Schinder and Gage 2004). In 2 main regions, i.e. the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus, the newborn cells undergo extensive migration before they become mature neurons (Schinder and Gage 2004). Adult hippocampal neurogenesis has been found in various species including humans (Boekhoorn et al. 2006; Eriksson et al. 1998) and is heavily regulated. It has furthermore been implicated in hippocampal learning and memory (Dobrossy et al. 2003; Dupret et al. 2007). Counterintuitively, neurogenesis is also increased in responses to damage (Emsley et al. 2005) and pathological conditions like seizures or ischemia are powerful stimuli of neurogenesis in the sub-granular zone (SGZ) of rodents shortly after injury (Collombet et al. 2005; Parent 2007; Sharp et al. 2002; Wiltrout et al. 2007). On the other hand, after prolonged traumatic brain injury, decreases in hippocampal neurogenesis occur, which is associated to decrements in cognition (Rola et al. 2006). Damage to the cholinergic circuitry projecting to the hippocampus has been shown to suppress neurogenesis (Cooper-Kuhn et al. 2004; Van der Borght et al. 2005). Therefore, both brain injury as well as cholinergic impairments following OP-poisoning could possibly interfere with neurogenesis and might therefore be involved in the cognitive deficits observed.

Chronic treatment of rats with several different classes of antidepressants has been shown to increase dentate neurogenesis. Also other studies indicate that antidepressants may act through promoting cell survival, structural plasticity and neurogenesis (Malberg et al. 2000), also in related brain areas (Kodama et al. 2004; Wang et al. 2004).

The notion that OP-induced seizures due to persistent AChE inhibition produce neuronal loss and persisting decrements in cognition, prompted us to investigate whether drug-induced neurogenesis can be used to counteract OP-induced effects. In the present study, soman-poisoned rats were treated with HI-6 and atropine. ACh levels, AChE inhibition and the occurrence of seizures was measured in these animals. Long-term effects of the intoxication and of the neurogenesis enhancer olanzapine were investigated at the behavioral and cellular level. Because neurogenesis is

most abundant in the hippocampus, which is a preferred site for soman induced damage, acquisition and memory retrieval was tested in a hippocampus specific water maze paradigm 8 weeks after soman exposure. Neurogenesis was measured by immunohistochemistry for doublecortin (DCX) and BrdU.

2 Methods

2.1 Animals

Male Sprague Dawley rats, body weight 160-180 g at arrival, were obtained from Harlan, Horst, The Netherlands. Until surgery, the animals were housed in pairs and allowed to get accustomed to standard conditions for at least one week. Temperature was kept at 19-22 ^{o}C, relative humidity was maintained at 55-65% and lights were on from 7 a.m. to 7 p.m. Acidified water and standard rodent chow (Teklad Global Diet, Harlan, Horst, The Netherlands) were available ad libitum. After the first surgery, animals were individually housed under standard conditions. The experiments described received prior approval from the Ethical Committee on Animal Experimentation of TNO.

2.2 Chemicals

Acetylcholinesterase, choline oxidase, acetylcholine, choline, atropine sulphate and Triton-X100 were obtained from Sigma Chemical Co. (Zwijndrecht, The Netherlands). Kathon CG (1.5% 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was provided by Rohm and Haas (Croyden, UK). LiChrosorb® NH_2 was purchased from E. Merck (Amsterdam, The Netherlands). Hypnorm® (fentanyl/fluanisone) was purchased from Janssen Pharmaceutica (Beerse, Belgium) and Dormicum® (midazolam) was delivered by Roche Nederland BV (Mijdrecht, The Netherlands). Soman (pinacolyl methylphosphonofluoridate) was obtained from the stocks of TNO Defence, Security and Safety and of purity ≥98% (GC). Olanzapine was kindly provided by Eli Lilly (Greenfield, IN, USA).The other chemicals used were of standard purity and purchased from renowned companies. For HPLC analysis the highest purity grade was used. All solutions were prepared with water tapped from a Milli-Q system (Millipore SA, Molsheim, France).

2.3 Experimental design

The experiment consisted of two parts (Fig. 1). In the first part of the study (Experiment A, groups 1 and 2, see Table 1), a rat model was established according to Shih et al. (1991). Extracellular ACh levels, EEG effects and AChE levels in blood and brain were determined upon intoxication with soman. To ensure survival, the animals were treated i.p. with 125 mg/kg HI-6, followed by soman 200 µg/kg s.c. 30 min later. One minute after soman, the animals were injected i.m. with atropine sulphate (16 mg/kg). Clinical signs were registered and ACh levels and EEG were assessed during 4 hours after soman treatment. AChE activity was measured at the end of the 4-hour period.

***Table 1**. Group sizes and individual treatment protocols before and after intoxication.*

Group size (n)	Pretreatment T= -30 min HI-6 *(mg/kg i.p.)*	Intoxication T=0 soman *(µg/kg s.c.)*	Treatment T= 1min atropine sulphate *(mg/kg i.m.)*	Subchronic treatment (28 days) Olanzapine *(mg/kg/day)*
Experiment A				
1 (5)	125	200	16	none
2 (4)	125	0	16	none
Experiment B				
3 (3)	125	200	16	0
4 (6)	125	200	16	7.5
5 (3)	125	0	16	0
6 (4)	125	0	16	7.5

The second part of the study was designed to establish long-term effects of the latter soman-intoxication as well as those of subchronic treatment with the anti-depressant olanzapine (groups 3-6, see Table 1). The experimental timeline is shown in figure 1B. To this end, male sprague dawley rats were equipped with EEG electrodes and intoxicated by the aforementioned regimen or vehicle one week after surgery. A four week-lasting treatment with olanzapine started one day later, directly followed by BrdU labeling over 6 days (200 mg/kg i.p.). Another three weeks later, the animals were trained for 5 days in the Morris Water Maze. After 2 days without training, spatial memory was tested, immediately followed by preparation of the brains of the animals by means of perfusion fixation to determine histopathological effects and changes in neurogenesis.

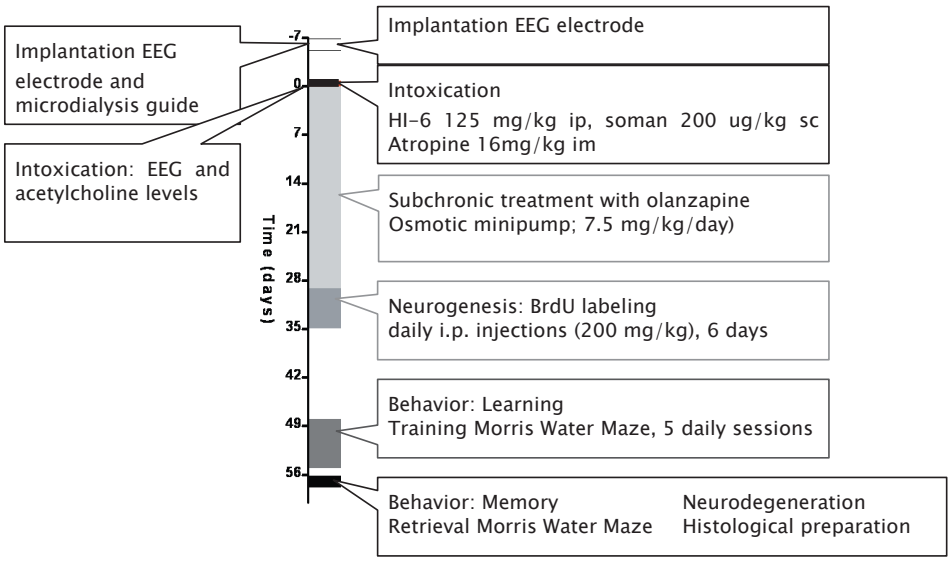

***Figure 1**. Experimental timeline (vertical axis) of the two separate experiments performed. The microdialysis experiment (A) for determination of acute effects is shown at the left side of the picture. The experimental timeline for determination of long-term effects is shown at the right side (B). Day 0 at the vertical axis represents the day of soman intoxication. The different experimental stages are defined by the colored blocks and flags. See methods section for experimental details.*

2.4 Surgical procedures

Male Sprague Dawley rats were anaesthetized with 2.7 ml/kg FFM-mix (2.5 mg/ml fluanisone, 0.079 mg/ml fentanyl citrate and 1.25 mg/ml midazolam,) via a single i.p. injection after premedication with 0.05 mg/kg atropine sulphate to prevent respiratory arrest and 5 mg/kg Rimadyl® (both s.c.) as analgesia. A guide for a CMA10 microdialysis probe (CMA, Sweden) was stereotactically (KOPF Instruments, Tujunga, CA) inserted into the ventral striatum (A 0.5, L 3.0, V 3.0 mm) relative to bregma and the dura mater). Additionally, two stainless steel screws (A3.0 and P3.0 mm relative to bregma and 3 mm from the sagittal suture) placed at the dura mater, fixed to a plug, served as EEG electrodes. The microdialysis guide and the electrodes were fixed to the skull with dental cement. A heating pad was used to maintain body temperature. Post-surgery analgesia with Rimadyl® (5 mg/kg s.c.) continued for another 2 days.

For the long-term experiments the rats were subjected to similar surgery in which stainless steel screws were placed at the dura mater and fixed with dental cement, but no microdialysis guide was placed.

Twenty-four hours after intoxication, rats were subcutaneously implanted for 4 weeks with Alzet® osmotic minipumps (Model 2ML4, Durect, Charles River, The Netherlands) with a delivery rate of 0.25 µl/hr. They were implanted under isoflurane anaesthesia (2-3%) in a subcutaneous pocket on the back of the rat. The dose of olanzapine was aimed at 7.5 mg/kg/day per rat, a dose which has been shown to lead to optimal plasma concentrations (Kapur et al., 2003, Turrone et al., 2005). To this end, olanzapine was dissolved in Milli-Q, acidified with 1% glacial acetic acid in a concentration of 37.5 mg/ml. After 4 weeks, the pumps were explanted under isoflurane anaesthesia, and checked for any residual fluid, which was maximally 200 µl, pointing to an adequate delivery. The content of olanzapine and degradation products due to hydrolysis in the residual fluid was confirmed by electrospray MS/MS. The estimated concentration of olanzapine in the residual fluid was 28 mg/ml, resulting in an estimated dose of 5 mg/kg/hr at the end of the study.

Pump removal was immediately followed by injection of the first BrdU dose (200 mg/kg i.p.)(Heine et al., 2004). BrdU was dissolved at 20 mg/ml in PBS containing 50 mM NaOH. In view of the longterm followup and to minimize variation, BrdU injections were repeated for another 5 days, yielding a total of 6 BrdU injections.

2.4 Microdialysis procedure

Microdialysate samples were assayed for acetylcholine and choline using a HPLC system previously described by Damsma et al. (1987) with some modifications. The system consisted of a LC-10AD VP pump (Shimadzu, Den Bosch, The Netherlands), a pulse damper (SSI, Alltech, Breda, The Netherlands), a refillable guard column (silica pellicular packing material, Alltech, Breda, The Netherlands), an EC 125/2 Nucleosil 100-5 C18 AB analytical column (Aurora Borealis Control, Schoonebeek, The Netherlands), preloaded with 0.5% sodium lauryl sulfate, an enzyme reactor, an electrically actuated injector (VALCO VICI AG, Schenkon, Switzerland) with a 20 µl sample-loop, an DECADE potentiostate equipped with a VT03 flow cell with Pt work electrode (Antec Leyden BV, Hazerswoude, The Netherlands) and a chromatography

data acquisition system (Azur, Gynkotek, Germering, Germany). The post-column enzyme reactor contained immobilized acetylcholinesterase (80 U), which converted acetylcholine to choline, and choline oxidase (40 U), which converted choline to hydrogen peroxide that was electrochemically detected at + 450 mV. The mobile phase consisted of a 166 mM potassium phosphate buffer (pH 8.5) containing 1 mM tetramethylammonium chloride, 0.79 mM EDTA and 250 µl/l Kathon CG. The system was run at 0.35 ml/min and temperature was maintained at 30 °C. Limit of detection was 1 pmol/ml. Linear calibration curves were obtained in the range from 5 – 1000 pmol/ml ($r > 0.990$). The intra-assay coefficients of variation for 10 and 100 pmol/ml were 2.9 % and 4.1 %, respectively. Inter-assay variability could not be determined due to variable enzyme activities in the post-column reactor between days.

All experiments started between 9 and 10 AM. At the beginning and end of each experiment, a 10 nM acetylcholine/ choline calibration standard was injected in duplicate, and if necessary corrections for reduced sensitivity of the enzyme reactor during the experiment were made. The microdialysis probe was perfused with a Ringer solution at 2.0 µl/min delivered by a microinjection pump (CMA 100, CMA microdialysis AB, Solna, Sweden). To obtain detectable quantities of acetylcholine in the dialysate, 100 nM neostigmine was added to the perfusate. The rat was connected directly to the sample loop, allowing on-line analysis of the microdialysates. The injection valve was automatically activated every 10 min, resulting in an injection volume of 20 µl.

In the experiments for determination of acetylcholine accumulation in the striatum, the animals were allowed to recover for a week from surgery. During this period, the rats were housed in their home cages with 2 animals per cage. One day before soman exposure, they were individually placed in a perspex cage (25 x 25 x 40 cm) with free access to food and water. The following day, EEG measurements were started and a microdialysis probe was slowly lowered into the guide cannula, and allowed to stabilize for one hour, after which they received HI-6, soman and atropine as previously described. The measurement continued for 4 hours, after which the animals were euthanized by decapitation, allowing collection of blood and brain tissue for AChE determination. The hemisphere containing the microdialysis probe was fixed in 4% formaldehyde to verify probe placement.

2.5 Acetylcholinesterase activity

Brain parts (hippocampus and striatum) were homogenized (900 rpm, 10 % w/v homogenate) in ice-cold TENT buffer, which consisted of 50 mM Tris, 5 mM EDTA, 1 M NaCl and 1% v/v Triton X-100, pH 7.4. The homogenates were centrifuged at 12000 g in an eppendorf centrifuge at 4 °C and supernatants were immediately frozen in liquid nitrogen and stored at -20 °C until analysis of enzyme activity, within one month. Heparinized blood aliquots were 1:10 (v/v) diluted in 1% saponin and immediately frozen in liquid nitrogen.

Samples were analyzed for AChE activity using a modification of the method by Ellman et al. (1961). Shortly, after appropriate dilution, 10 µl samples were incubated with 0.8 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich B.V.) and 0.8 mM acetylthiocholine iodide. Absorbance at 415 nm was measured directly

after the addition of the acetylthiocholine substrate and 40 minutes later. The increase in absorbance per µl homogenate per min at ambient temperature served as measurement for AChE activity.

2.6 EEG acquisition and analysis

EEG was obtained using PhysioTel telemetry system from Data Sciences Inc. (DSI) using the TA11ETA-F40 transmitter body. The transmitter was attached to the plug fixed on the skull of the rat. A receiver board consolidated and stored the signal from the transmitter on an IBM-compatible personal computer via a Data Exchange matrix at 100 Hz sampling frequency. After storage, the data were converted into European Data Format (EDF) and seizure duration in the 180 minute evaluation period was determined by visual inspection of the EEG trace. Seizures were defined as high-frequency spiking, at least 3 times elevating form baseline. Animals were awake during the measurement.

2.7 Behavior: Morris water maze

To investigate behavioral deficits following soman intoxication, rats were tested for learning ability and memory formation in the Morris water maze 8 weeks after poisoning. The diameter of the container was 2 m, with an invisible Plexiglas platform, placed 2 cm below the water surface. Water temperature was 21 oC, and figures attached to the wall served as visual spatial clues. The swimming pool was divided into 4 imaginary quadrants, of which the one with the platform was designated as goal quadrant. All movements of the rats were registered by a video camera, and later on digitized and analyzed with EthoVision tracking software.

The Morris water maze test was divided into two phases. The first phase consisted of a training period, in which the animals had to learn the position of the platform in the bath. The training period lasted for 5 sessions, one session per day. Prior to each session, the rat was placed on the platform for 30 seconds, after which the first trial was started. In each session the rat was tested 4 times (trials), in which the animal was placed in each quadrant in a random order (similar for all rats). The trial lasted until the animal had reached the platform or after 90 seconds of swimming. The time taken to reach the platform was defined as escape latency. Regardless of reaching the platform, the rat was allowed to stay on the platform for 30 seconds to establish a cognitive map. Acquisition performance was expressed cumulatively (Gallistel et al., 2004) and the training curves were fitted using a mathematical model (Y=Ymax*(1-exp(-K*x)) using GraphPad Prism Software. Statistical differences between the training curves (Ymax) of experimental groups were tested using one-way ANOVA followed by Dunnet's post-hoc test, using the saline-vehicle group as control group.

On the 3rd day after the last training session, the spatial retention of the animals was measured during a retrieval or probe trial, in which the animals had to swim for 90 seconds without a platform and the time spend in each of the pool quadrants was recorded. The crossing between two adjacent quadrants served as starting point.

2.8 Histology

2.8.1 Histological preparation

Directly after completing the retrieval trial, i.e. between 12:00 and 14:00 h the animals were sacrificed for histological studies. Under deep sodium pentobarbital (Nembutal) anaesthesia the animals were transcardially perfused with 4% freshly prepared paraformaldehyde solution, after washing out blood with heparinized saline. After perfusion, the fixed animals were stored at 4 °C, and the brains were carefully removed form the skull 24 hrs later. The brains were stored at 4 °C in dissector buffer (PB) with 0.01% sodium azide, cryo-protected with a two-step 15% to 30% sucrose solution in PB, directly followed by freezing and serial coronal sectioning on a sliding microtome into 40 µm thick slices and collected in PB with azide.

2.8.2 Histopathology and immunohistochemistry

Slices were mounted on Superfrost®Plus glasses (Menzel-Glâser) and Nissl-stained with 0.25% cresyl-violet, for histological orientation.

To detect BrdU labeling, free-floating slices (40µm) were rinsed extensively with 0.1M phosphate buffer (PB) pH 7.4. Endogenous peroxidase activity was blocked, using a 0.5% hydrogen-peroxide/PB solution. To denature DNA, slices were incubated for 2 hours in a 50% formamide/2xSSC solution at 65 °C, followed by a 30 minute incubation in 2N HCl at 37°C. Slices were then incubated for 10 minutes in 0.1M boric acid pH 8.5. Murine anti-BrdU (Roche Diagnostics, The Netherlands) was used as a primary antibody diluted 1:1000 in diluens (0.1M PB/0.1% Bovum Serum Albumine (BSA) /0.5% Triton X-100/ 1% Normal Goat Serum (NGS) for 1 hour at room temperature (RT) and then overnight (ON) at 4°C. Subsequently, sections were intermittently rinsed with PB and incubated with biotynilated sheep-anti mouse IgG second antibody (GE Healthcare UK Limited 1:200). Amplification was performed with avidin-biotin (ABC-kit, Vectastain, Brunschwig Chemie, Amsterdam) 1:1000 for 2 hours. Labeled cells were visualized with 0,05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in 0.05M Tris buffer. Sections were mounted on Superfrost®Plus glasses (Menzel-Glâser), counterstained with haematoxyline acc. to Ehrlich, rinsed with aqua dest, dehydrated through a graded series of alcohol and xylene and coverslipped with Entallan (Merck).

DCX-expressing neurons were visualized as described in Oomen et al. (2007b) and Lucassen et al. (2008). In short, sections were rinsed with 0.05M Tris buffer and 0.9% saline pH 7.6 (TBS). After blocking endogenous peroxidase activity (0.5% hydrogen peroxidase) and a-specific binding sites (2% milk powder, Campina Elk), free floating sections were incubated with polyclonal DCX primary antibody (1:1600, Goat Polyclonal IgG, SantaCruz®) in supermix (0.25% gelatine/0.1% Triton X-100/ TBS) for 1 hour at RT and ON at 4 oC. With intermittent rinses in 0.05 M TBS, sections were subsequently incubated with biotinylated donkey anti-goat (Jackson, 1:500) second antibody in supermix for 2 h at RT. Signal amplification was carried out for 2 h at RT with ABC complex (1:800). Labeled cells were visualized with 0.05% DAB and 0.01% hydrogen peroxide in 0.05M Tris buffer. Slices were then further processed for quantification.

2.8.3 Quantification and stereology

Stereological quantification of the number of DCX-positive cells was performed unilaterally by systematic random sampling in every 10th section using the StereoInvestigator System (Microbrightfield, Germany) on a Zeiss Axiophot microscope with a 1000x magnification and the optical fractionator probe in the software (Oomen et al., 2007a). Cells were counted using a 60µm (X) by 60µm (Y) counting frame, a dissector height (Z) of 14µm and upper and lower guard zones of 3µm, in a sampling grid of 100x100 µm. Cells were only considered if their cell bodies were located within the counting frame or intersected by its inclusion edges (top and right edge). DCX cell-numbers were assessed using the following formula: Nobj = 1/ssf×1/asf×1/tsf×∑ Q (ssf = section sampling fraction, asf = area sampling fraction, tsf = thickness sampling fraction and ∑ Q is the sum of all objects in the dissector probe).

Due to restrictions of the automated sampling process (clustering of cells and the relative low number of BrdU positive cells) BrdU cells were counted manually on a Zeiss microscope at 200x magnification in every 10th hippocampal section and multiplied by 10 to estimate the total number of BrdU positive cells. Cells were assessed per hippocampal subregions of the hilus, sub-granular zone (SGZ) and granular cell layer (GCL), in a stereological approach over the entire rostrocaudal extent of the hippocampus. The SGZ was defined as a two-to- three cell thick layer along the inner border between the GCL and the hilus.

2.8.4 Morphology assessment

In the DCX-stained sections, the proliferation stage of the DCX-positive cells was assessed according to Plumpe et al. (2006). Groups of cells in the DG were assigned to a category of maturity dendritic morphology, A through F. Category A represents cells with no dendrites at all, B short plump processes, both categories representing cells in the proliferation stage. The intermittent stage is characterized by category C, cells with medium process, and category D, in which the dendrites of the cells reach the molecular layer. The final and post-mitotic stage of newborn neurons is represented by category E, cells with one strong dendrite branching in the molecular layer and category F, to which cells with delicate branching in the granule cell layer were assigned.

2.9 Data analysis

Data are represented as means +/- SEM. Statistical analysis was performed using one-way ANOVA and followed by Dunnet's Post-hoc test or two-way ANOVA followed by Bonferoni's post-hoc test whenever appropriate. Data were considered significantly different for p-values < 0.05.

3 Results

3.1 Experiment A: acute effects

Soman poisoning with a dose of approximately 2LD50 (200 µg/kg s.c.) led to a very rapid and excessive increase of extracellular ACh levels (1500%) in the brain, in spite of pretreatment with a high dose of HI-6 (125 mg/kg i.p.) and post-intoxication treatment with atropine sulphate (16 mg/kg i.m.) (Fig. 2A). The highest level of ACh was reached in about 20 minutes after poisoning, after which a decline to baseline levels occurred in the following 20 minutes. In saline treated animals, no effects on ACh levels were observed. Recurrent short-lasting seizure periods accompanied the excessive ACh levels after soman intoxication, which lasted until ACh levels had returned to baseline at approximately 40 minutes in most animals (Fig. 2B). It must be noted that only one of the animals showed continuous seizures, whereas seizures had stopped within 40 minutes in all other animals. During this period, animals also exhibited typical cholinergic signs, shown as heavy chewing-like movements which rapidly evolved into convulsive activity represented by intense jerking of the body. The AChE activity in the striatum and hippocampus, measured at 4 hours after the intoxication was inhibited >95%, whereas the AChE activity in blood was inhibited to approximately 40% of control values (Fig. 2C). All animals survived the 4-hour period.

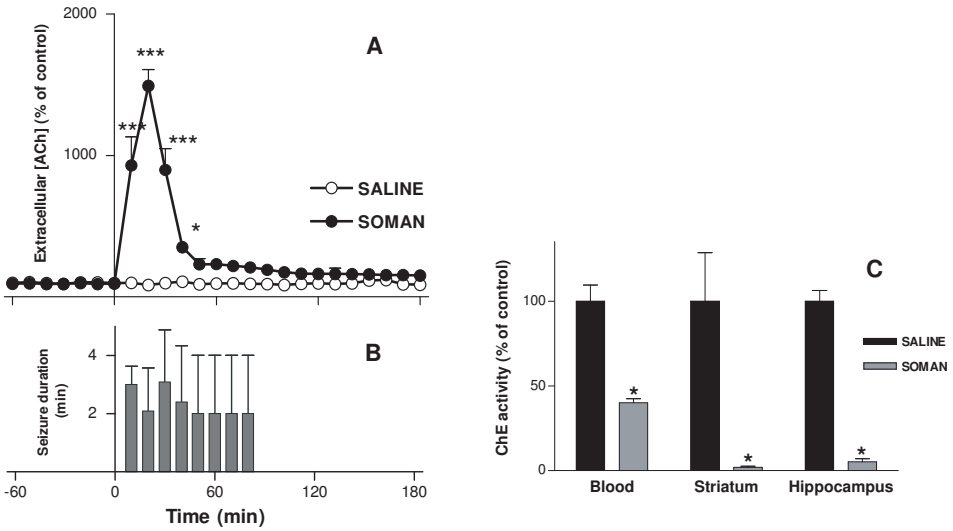

Figure 2. *A. Extracellular ACh levels in the striatum of rats before and after soman poisoning in saline (n=4, open dots)- or soman-treated animals (n=5, closed dots). (soman: 200 µg/kg s.c., t=0, pretreatment with 125 mg/kg HI-6 i.p. at t= -30 min; treatment with 16 mg/kg AS i.m. at t=1 min) are shown. Significant (*, p<0.05, two-way-ANOVA followed by Bonferoni's post-hoc test) elevation of ACh levels is present at 10-40 minutes after soman intoxication. B. Seizure duration in minutes (mean +/- SEM) of the soman-intoxicated animals synchronized with the 10-min periods of the ACh sampling. C. Cholinesterase activity at 4 hours after poisoning in blood, striatum and hippocampus. Soman poisoning induced significant inhibition in both blood and brain (* p<0.05, two-way-ANOVA followed by Bonferoni's post-hoc test).*

3.2 Experiment B: long term effects

A dose of 200 µg/kg soman (s.c.) administered 30 minutes after a pretreatment with HI-6 (125 mg/kg i.p.) and post-treatment with atropine sulphate (16 mg/kg i.m.) led to overt cholinergic signs and a survival rate of 67% at 24 hours. The cholinergic signs appeared within 3 minutes after soman injection. The convulsive activity was accompanied by short-lasting full-blown seizure periods on the EEG, for approximately 30 minutes (Fig. 3B). No differences between animals that were treated later with vehicle or olanzapine were present, ensuring a similar starting-point after soman for both groups. After this period, the animals returned to a hyperreactive state without convulsions.

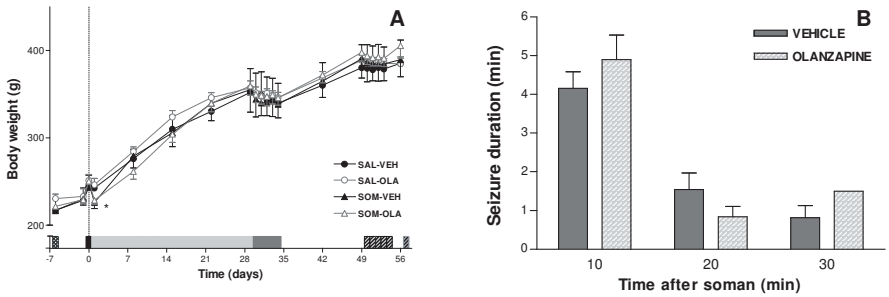

***Figure 3**. A. Average relative body weight of animals at different time points during the experiment, for timeline see figure 1 (mean +/- SEM). B. The average duration of full-blown seizures in soman-poisoned animals (200 µg/kg s.c., pretreatment with 125 mg/kg HI-6 i.p. at t= -30 min; treatment with 16 mg/kg AS i.m. at t=1 min. The seizure periods are divided into 10-min periods after intoxication. Note that there are no differences in the seizure durations of animals that were later treated with olanzapine or vehicle, ensuring a similar basal condition.*

Histological evaluation of the brain 24 hours after intoxication in a separate group of animals showed hardly any signs of gross morphological alterations or obvious indications for neuropathology. The body weight of this group of animals showed a significant decline of approximately 10% at 24 hours after soman poisoning, which was significantly lower than that of saline-treated animals (Fig. 3A). Within one week the soman-intoxicated animals had reached body weights comparable to that of saline-treated animals, irrespective of subchronic treatment with olanzapine.

3.2.1 Morris Water Maze

Acquisition in the Morris Water Maze was measured in the 8th week after soman intoxication. The cumulative latency per trial is demonstrated in figure 4A indicating that learning ability of soman-intoxicated animals was affected. The fitted maximum cumulative latency is significantly higher for soman-intoxicated animals compared to saline-treated animals ($p<0.05$, ANOVA followed by Dunnett's post-hoc test, saline-vehicle as control group). Subchronic olanzapine had no effect on learning ability in saline-treated animas, but it tended to affect the learning ability further in soman-intoxicated animals, as Ymax of the soman-olanzapine group was significantly higher than that of the soman-vehicle group ($p<0.05$, ANOVA followed by Dunnett's post-hoc test, soman vehicle as control group).

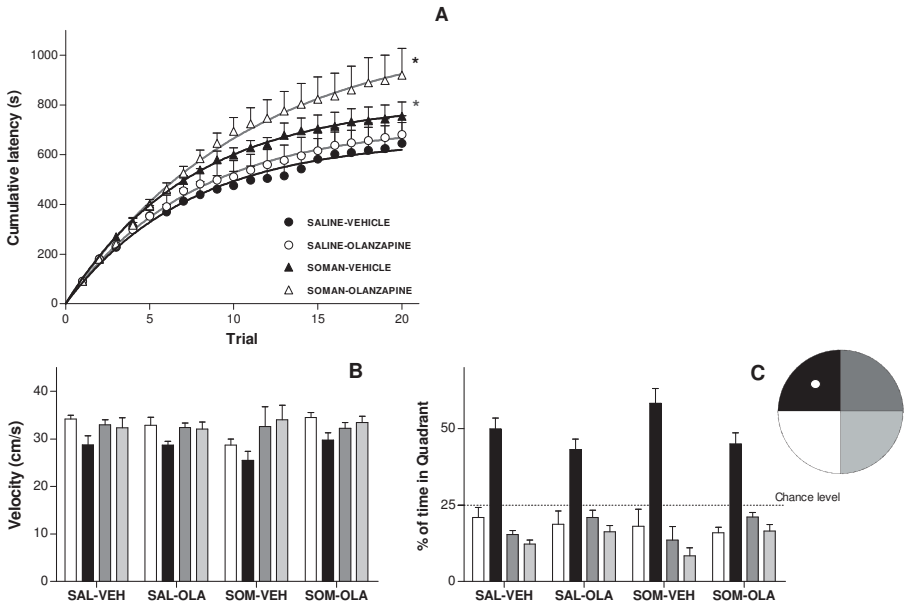

***Figure 4**. Performance in Morris water maze. A. Average cumulative seconds to reach the platform (mean +/- SEM). The curves are fitted using a mathematical model (Y=Ymax*(1-exp(-K*x)) using GraphPad Prism Software and checked for statistical differences. Soman-intoxicated animals used significantly more time to find the platform irrespective of subchronic treatment with olanzapine or its vehicle than saline-injected animals (Ymax, one-way ANOVA followed by Dunnett's post-hoc test vs saline-vehicle). B. Swim speed and C. Time spent in the maze quadrants during a retrieval trial in the Morris water maze. The colors represent the coloured maze qudrants, of which the balck one is the goal quadrant. No significant differences were observed between groups regarding swim speed or time spent in the different maze quadrants (two-way ANOVA).*

Swim speed and time spent in the target quadrant during the probe trial in the different groups of animals was unaffected (Two way ANOVA, $p>0.05$, Fig. 4B and 4C). The animals subchronically treated with olanzapine, showed a trend towards a shorter time spent in the goal quadrant, whereas soman-vehicle-treated animals showed a trend towards an increased time spent in the goal quadrant. This is probably caused by a lower swimming speed in the goal quadrant of the soman-vehicle treated animals than in other quadrants of the pool.

3.3.2 Histology and immunohistochemistry

Nine weeks after soman intoxication no signs of overt cell loss or obvious neuropathology were observed in any of the experimental groups. Subchronic olanzapine treatment did not affect the total number of surviving BrdU-labelled cells, nor their number in the different subregions of the DG (Fig. 5A and 5B).

Soman-poisoned animals showed a significantly lower number of DCX-labelled young neurons in the DG of the hippocampus ($p<0.05$, ANOVA followed by Dunnett's post-hoc test, saline vehicle as control group, Fig. 5C). Subchronic treatment with olanzapine did not affect the number of DCX-positive cells in either saline-treated or soman-intoxicated animals (Fig. 5C).

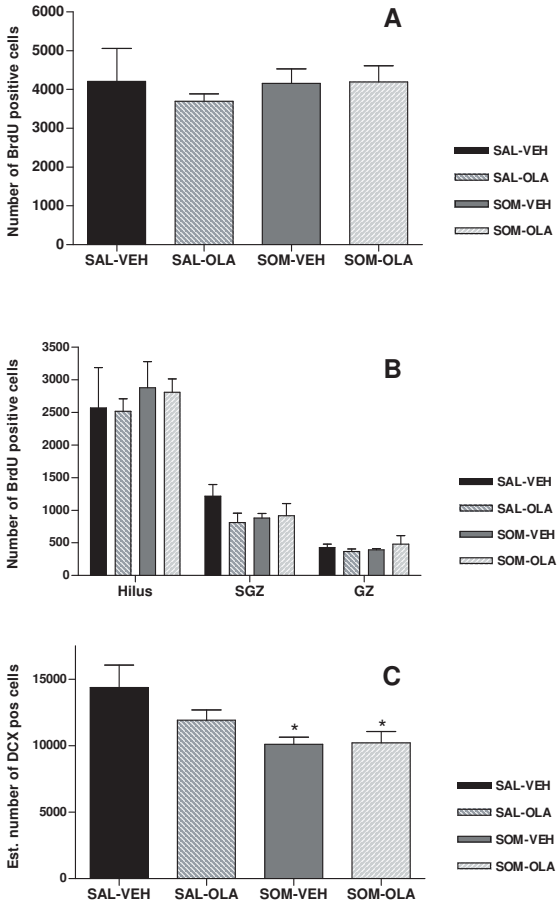

***Figure 5**. A. Estimated numbers of BrdU-positive cells in DG of rats in the different treatment groups. B. The number of BrdU positive cells in different brain areas. No significant differences between groups were observed in either brain area. C. Estimated numbers of DCX-positive cells in different treatment groups determined by stereological quantification. The number of DCX- positive cells in both soman-intoxicated groups is significantly lower than that of saline-treated animals (one-way ANOVA followed by Dunnet's post-hoc test, $p<0.05$ compared to saline-vehicle exposed animals).*

Assessment of the morphology of DCX-labelled cells showed no differences between the groups in the different developmental stages of individual DCX-positive neurons. In all groups, over 70% of the cells were found to be in a mature post-mitotic stage (E-F), as was clear from their strong dendritic branching into the molecular and granular layer.

4 Discussion

A major objective of the present study was to investigate long-term effects of soman-poisoning on cognition. In spite of life-saving treatment with HI-6 and atropine, soman intoxication led to an excessive ACh accumulation in the striatum which lasted approximately 40 minutes. During this period, there was some recurrent seizure activity, which mostly remained limited to the primary stage of accumulation. Without the above treatment, animals generally die during or after this primary stage of ACh accumulation (Bueters et al. 2002; van Helden et al. 1998). Although we did not measure other neurotransmitters, our results are in agreement with data reported by Shih and McDonough (1997) showing a cholinergic phase that lasted about 40 min, which is followed by a glutamatergic phase characterized by recurrent full-blown seizures leading to excitatory amino acid-mediated neuronal death. However, in the present study a clear recurrent seizure pattern in a glutamatergic phase did not occur, because of the therapeutically effective doses of HI-6 and atropine sulphate used. Similar anticonvulsive effects, even with lower doses of atropine are reported by Shih et al. (1999). The absence of recurrent seizures is in line with our histological data obtained at 24 hours after soman intoxication, indicating that hardly, if any, signs of neurodegeneration were present.

After the primary cholinergic stage, ACh levels returned to control values indicating a negative feedback on ACh release. The striatum was chosen as representative brain area for ACh microdialysis, as this brain area is more sensitive to ACh accumulation during AChE inhibition than the hippocampus (Joosen and van Helden 2007). As AChE inhibition levels do not considerably differ between brain areas upon soman poisoning (Shih et al. 2005), which we also found in the present study regarding the striatal and hippocampal AChE activity, significant ACh accumulation in other brain areas, such as the hippocampus, was most likely present. Four hours after intoxication, brain AChE activity was inhibited over 95%, and in blood to approximately 40% of basal AChE activity, which can be explained by the fact that unlike HI-6, soman easily passes the blood brain barrier. As such, HI-6 cannot properly reactivate the inhibited AChE (Kassa and Bajgar 1998).

Other studies reporting poisoning with other anti-cholinesterase agents have shown that recovery of inhibited brain AChE activity is rather slow. On average the recovery of AChE activity in different brain regions is about 20% per 10 days (Abou-Donia et al. 2002; Kobayashi et al. 2007). We therefore predicted that at the start of our Morris water maze training, i.e., 50 days post poisoning, AChE would have recovered to baseline values in all brain regions. Nevertheless, we observed a decrement in learning ability as soman-poisoned and oxime/atropine-treated animals needed more time to learn to find the platform than control animals. The latter finding is consistent with data showing that the current treatments of OP poisoning, that consist of reactivation of the organophosphate-inhibited enzyme with oximes, anticholinergic treatment with atropine and the anti-epileptic drug diazepam, does not provide complete protection of the CNS and may result in long-term neuronal deficits (Anderson et al. 1997; Dawson 1994; Shih et al. 1999; van Helden and Bueters 1999).

Other investigators have shown impaired cognition 3 months after soman poisoning without neurodegeneration at short term (Filliat et al. 2007). In our study, the impaired learning ability in the soman-poisoned animals was accompanied by significantly fewer newborn DCX+ neurons compared to saline treated animals. Various studies have implicated levels of neurogenesis in structural plasticity and hippocampal learning (Gould et al. 1999a; Imayoshi et al. 2008; Kempermann 2008; Leuner et al. 2004; Shors et al. 2001). As shown by Dupret et al. (Dupret et al. 2007; 2008) and Dobrossy et al. (2003), spatial learning regulates neurogenesis in the DG of the hippocampus. Our DCX-staining identifies newborn neurons from 4-14 days of age, and represents new cells from approximately one week before the start of training until the end of the retrieval trial. Since before the start of the training, no differences were observed in the number of BrdU labeled cells in the DG of the hippocampus, the survival phase of the new born cells does not appear to be affected but rather indicates a shift in differentiation towards less new neurons. Although it is tempting to speculate that the significantly fewer DCX-+ cells in soman-exposed rats may causally relate to the different learning ability, this requires further investigation. The changes in cognition and neurogenesis might e.g. also relate to the cholinergic dysfunctioning, as described by Mohapel et al. (2005), who have shown that neurogenesis and spatial memory were both impaired after lesion of the forebrain cholinergic input.

A second objective of the present study was to investigate whether subchronic treatment with olanzapine would stimulate hippocampal neurogenesis and could counteract any cognitive deficits. With the present experimental design, we could not detect an effect of olanzapine on the number of BrdU-labeled, and hence on the survival of the newly born cells. Although other studies have shown stimulating effects of subchronic treatment of olanzapine on neurogenesis (Kodama et al. 2004; Wakade et al. 2002; Wang et al. 2004), some discrepancies exist with the present study. First of all, the first BrdU injection was administered after termination of the olanzapine treatment, which implies that at the time of BrdU incorporation into DNA, olanzapine was absent. Although olanzapine is likely to affect survival and neuronal survival anyway, early stimulatory effects on the proliferating progenitor population are less likely. In the above mentioned previous studies, BrdU was injected during olanzapine treatment, which suggests that olanzapine should be available during the entire process of neurogenesis including the early proliferation phase. The aim of the present study, however, was to stimulate the entire process of neurogenesis with olanzapine. Another explanation could be that proliferation enhancement by olanzapine has remained undetected because the newborn neurons did not survive until the time of sacrifice. The latter possibility seems less probable since in other studies 89% of the newborn neurons in the DG promoted by olanzapine were NeuN double-positive while these cells were reported to survive for at least 28 days (Kodama et al. 2004), after which it is unlikely that they will die (Dayer et al. 2003; Gould et al. 1999b).

A third question in the present study was whether olanzapine had effects on learning ability. As discussed, the lack of effect of olanzapine treatment on neurogenesis may parallel the absence of improved learning and memory ability

compared to animals not treated with olanzapine. Moreover, both for learning ability as well as for memory performance, olanzapine even has a tendency to decrease learning and memory ability. This might be explained by reported effects of olanzapine on cholinergic pathways, such as inhibition of choline acetyltransferase (Terry, Jr. et al. 2002; Terry, Jr. and Mahadik 2007), and anti-muscarinic actions (Chew et al. 2006). In soman-poisoned rats, the cholinergic pathway has already been affected, which is why an even more pronounced adverse effect of olanzapine on learning behavior in soman-poisoned animals could occur. Also, cholinergic pathways are involved in the regulation of neurogenesis and of learning and memory (Cooper-Kuhn et al. 2004; Mohapel et al. 2005).

In conclusion, the present study showed that, in spite of life-saving treatment with HI-6 and atropine, an acute soman-intoxication led to excessive central acetylcholine accumulation and a lasting impairment of learning ability which was accompanied by a decline in neurogenesis. In the protocol used, subchronic olanzapine treatment neither enhanced the process of neurogenesis in the hippocampus, nor prevented the cognitive deficit. The causal relationship between impaired neurogenesis and cognitive deficits in OP exposed animals should be target of future research.

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Chapter 6

General Discussion

Although the probability of nerve agents being used for military purposes has diminished considerably, use by non-state actors on military or civilian targets is a realistic scenario, which has a high impact on society. Consequently, exposures to OPs are most likely to be unanticipated. Given this, the present medical countermeasures should be efficacious and not reliant on pretreatment. The risk of human exposure to OP insecticides mainly arises from agricultural use in developing countries leading regularly incidents, or are even used in suicide attempts; estimated at approximately 3 million exposures and 200 thousand fatalities each year (Eddleston et al. 2005; Eyer 2003). Relevant animal models allowing a more integrated analysis of sequelae of the toxicological impact of different nerve agents are required to enable advances in medical countermeasures, which is one of the main goals of this thesis. Furthermore, the efficacy of current treatment protocols and possible improvements were investigated in the models developed. In addition, long term-effects of OP-exposure and possible interventions were investigated. Due to the similarity in chemical structure and in mechanism of action, insights acquired by studying nerve agents can also be used to improve treatment of intoxications with OP insecticides, the latter of which represents a worldwide problem.

1 Improved animal models to study nerve agent poisoning

The differences in chemical structure of nerve agents result in different risk profiles for exposure, distribution, and toxic effects. Volatile agents such as soman, sarin and tabun, will most likely enter the body via inhalation leading to a rapid development of signs. Although inhalation exposure would be the most relevant route for these agents, experimental approaches are difficult and were therefore replaced by the subcutaneous exposure route in the present studies. In contrast, for low volatile agents, such as VX and most OP insecticides, exposure via the skin is more likely and the persistence of these agents in the circulation results in a delayed and continuous development of clinical signs. An important requirement of animal models is that they allow assessment of rapid diagnosis, differential toxicological profiles and cognitive impairments to enable adaptations of the emergency protocols presently in use.

1.1 Rapid diagnosis

A crucial point in treatment of OP poisoning is the timing of treatment, which is highly dependent on the ability to diagnose exposure as early as possible. To this end, animal models were developed in which toxicological effects of nerve agents could be measured at physiological and biochemical level upon different risk and exposure scenarios. Animals were either subcutaneously exposed to soman or percutaneously to VX. Biochemical verification of exposure is best accomplished by determination of AChE activity. Direct measurement of tissue AChE activity is technically not possible; the inhibition levels of both cholinesterases in blood can be regarded as measures of exposure. Blood AChE activity closely reflects the AChE activity at the neuromuscular junction (Thiermann et al. 2009). In the experiments described in chapter 2 and 4, the type of nerve agent and exposure route showed to affect the predictive validity of AChE inhibition for the appearance of a clinical sign. The rapid evolvement of clinical signs after soman exposure was associated with a very rapid full inhibition of blood and probably also brain AChE, thereby lowering the use of AChE screening as a predictor for the toxicological status of the animal. In contrast, the slower profile of blood AChE inhibition exhibited after pc exposure to VX, showed to be more applicable for screening. Both after sc soman and pc VX, full inhibition of blood AChE was not always associated with death. This implies that in addition to clinical signs, monitoring AChE activity is reliable to establish the toxicological status in scenarios of slowly progressing poisoning or to follow up recovery, whereas development of clinical signs is the most reliable in case of a rapid inhalation exposure to a nerve agent.

1.2 Differential toxicological profiles

Although both soman and VX act via a similar mechanism, that is inhibition of acetylcholinesterase, physiology was affected quite differently. As expected, toxic signs upon sc exposure to soman developed very rapidly and seizures were always present, as discussed in Chapter 2. Progression of poisoning was accompanied by a dramatic increase in respiratory minute volume, while heart rate remained practically unaffected in animals surviving the experimental period of 90 minutes. In contrast, after pc exposure to VX, animals showed a delay in development of clinical

cholinergic signs compared to subcutaneous soman exposure. In addition, only a few animals showed seizures on their EEG (Joosen et al. 2008; van der Schans et al. 2003). Most animals suffered from life threatening respiration problems such as bronchoconstriction and decreased minute volume, and a severely lowered heart rate. The differential toxicological profiles have implications for timing and type of treatment.

An overview of the generic and differential patterns upon exposure to both nerve agents is shown in figure 1. In particular the difference in seizure development in animals exposed to either sc soman or pc VX is striking. A likely explanation is the difference in distribution of both agents due to their chemical structures on the one hand, and the exposure route on the other hand. Both VX and soman consist of mixtures of stereo-isomers. In contrast to the stereo-isomers of VX, the isomers of soman show very different profiles of persistence in the circulation and toxicities, resulting from their selective binding to AChE (van der Schans et al. 2008). Since the type and number of binding sites for soman and VX are assumed to be the same, the higher persistence of VX, charged at physiological pH, in the circulation increases the chance for VX to encounter binding sites before entering the brain compared to soman. The results presented in this thesis indicate that soman acts predominantly in the CNS, whereas VX is more confined to the peripheral compartment, indicating that different treatment approaches are required.

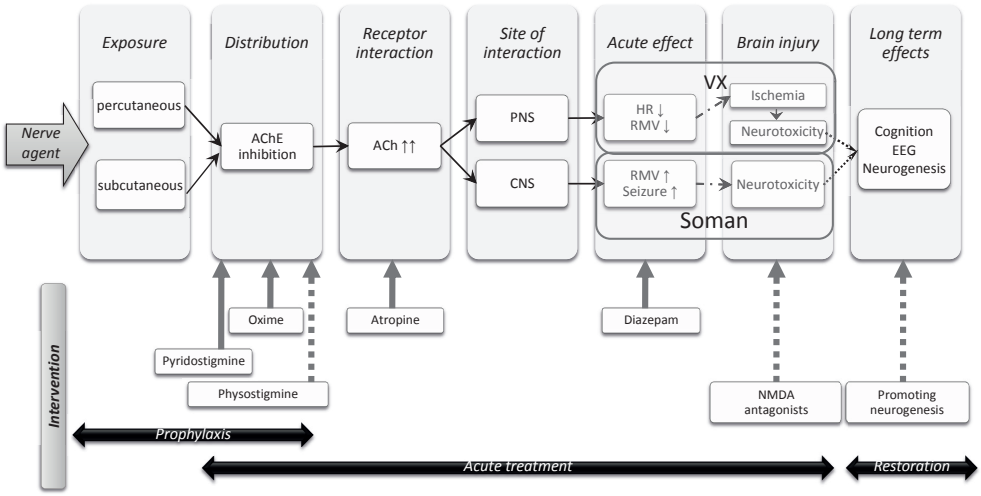

***Figure 1**. Overview of differential nerve agent effects and intervention strategies. The subcutaneous route is an approximation of the inhalation route regarding toxicokinetics and effects. Grey arrows indicate current countermeasures in use. Dotted grey arrows indicate novel countermeasures.*

Apart from different toxicokinetics, different effects independent from AChE inhibition and determined mostly in vitro, might contribute to differential seizure development of both agents. Very low levels of VX have shown to lower the firing rate and amplitude of action potentials in hippocampal neurons in vitro (Rocha et al. 1999). Translated to the in vivo situation, this may cause a more

generalized reduction of action potentials in the brain and a shift of EEG power to lower frequency ranges by VX opposed to the expected increase induced by ACh accumulation. In contrast to VX, soman has shown to induce internalization of GABAA receptors in cultured hippocampal neurons (Wang et al. DRDC Canada, personal communication), which probably contributes to the resistance of soman-induced seizures to benzodiazepines.

The differential effects on heart rate and respiratory control are more difficult to explain. Cholinergic overstimulation due to AChE inhibition is expected to result in a dramatic decrease in heart rate, which was present in animals pc exposed to VX. In addition, these animals suffered from suppression of respiration, probably resulting from muscle paralysis and stimulation of the vagal nerve. In animals exposed to sc soman, heart rate remained normal, but the development of seizures was accompanied by an increase in respiratory minute volume. This might be explained by stimulation of the respiratory centers in the brain. In addition, bronchorrhea and bronchoconstriction mediate lower pO2 levels in blood, thereby stimulating chemoreceptors in the respiratory centers (Wiener and Hoffman 2004). These results indicate that in case of pc exposure to VX treatment should be aimed at restoring these vital parameters, whereas soman exposure requires a higher protection against seizure development.

1.3 Cognitive impairment

Long term effects on cognition after nerve agent poisoning are connected with brain injury resulting from excitotoxicity following seizure initiation by excessive ACh accumulation. A rat model was used to evaluate such effects in Chapter 5. However, the results obtained in the rat model imply that cognition deficits can be present, even when soman-induced seizures were only short-lasting (Joosen et al. 2009). We have not investigated long-term effects after pc exposure to VX. It might be anticipated that the decrease in RMV and heart rate result in ischemia (Joosen et al. 2008). Ischemia induces brain injury, which is associated with cognitive decline (Bendel et al. 2005). The results imply that apart from diminishing seizure activity, stabilization of the cholinergic pathway, that regulates neurogenesis in the hippocampus, and prevention of ischemic injury following pc exposure to VX might be targets for treatment of long-term cognition deficits (Fig. 1).

2 Advances in treatment protocols

2.1 Physostigmine efficacy: importance of timing

To obtain a high efficacy, it is recommended to initiate treatment after exposure to a nerve agent as early as possible, within one minute after exposure. However, a more practical time point for starting treatment is the appearance of first clinical signs. To date, a combination of pretreatment and therapy is required to provide the most optimal protection against nerve agent poisoning (Aas 2003). The risk of asymmetric and unpredictable terrorist use, targeting not only at armed forces but also at civilian population, makes a therapy necessary which is less reliant on pretreatment. A proof of principle study in guinea pigs showed that a combination

of scopolamine, HI-6 and physostigmine administered immediately after poisoning was effective in protecting animals from the lethal effects of tabun, sarin, soman and VX (Wetherell et al. 2006). An alternative to physostigmine could be galantamine, which has additional neuroprotective properties (Pereira et al. 2009).

In Chapter 2, the added therapeutic value of physostigmine was investigated in a soman-exposed guinea pig model, either in the presence of obidoxime and/or atropine or in the presence of a single drug. Single physostigmine treatment showed an U-shape efficacy, with low efficacy at the lowest dose, high efficacy at the middle dose and no efficacy or aggravating toxic signs of soman at the highest dose of physostigmine. In combination with obidoxime and atropine, results were similar, however, the middle physostigmine dose even showed a higher ability in preventing spreading and continuation of seizures compared to single treatment with physostigmine. Delay of treatment with physostigmine to appearance of first clinical signs at 10 minutes after poisoning, without addition of atropine, showed to be dangerous and aggravated signs at a dose that was highly effective when administered at one minute after exposure. This demonstrated that physostigmine is effective by competing with soman for binding sites.

Although there is room for inclusion of a carbamate in the immediate treatment regimen against nerve agent toxicity, it should be employed with care. An antimuscarinergic agent is required to prevent unwanted effects if physostigmine is applied at time points when AChE is fully inhibited. In addition, the type of nerve agent and the oxime to be used is of great importance. The combination HI6, scopolamine and physostigmine showed to be ineffective against tabun poisoning (Wetherell et al. 2006). HI6 is unable to reactivate tabun-inhibited AChE, implying that the efficacy also depends on the efficacy of the oxime. In case of soman poisoning, obidoxime is a bad reactivator, but probably sufficient to provide a pool of AChE subsequently protected by physostigmine (Chapter 2). In general, soman-inhibited AChE 'ages' very rapidly, making the enzyme refractory to treatment with the currently available oximes (Maxwell et al. 2006). This implies that in particular after soman exposure carbamate treatment should be applied as soon as possible.

2.2 Percutaneous VX exposure: need for continuous treatment

It is anticipated that the current treatment regimen in case of percutaneous exposure to VX will provide insufficient protection. The slow penetration of VX after pc exposure and its high persistence in the circulation, are not aligned with pharmacokinetics of drugs administered in case of exposure to a nerve agent (Joosen et al. 2008; van der Schans et al. 2003). The present regimen, consisting of administration of 3 autoinjector doses at once used at first signs showed to postpone severe toxicity. The efficacy of the treatment was improved when the timing of the antidotes was adjusted to the appearance of clinical signs (Chapter 4). This is in line with results obtained from case studies from patients exposed to OP insecticides, who required intravenous treatment with oximes up to one week to reactivate AChE, which was correlated to restoration of neuromuscular function (Thiermann et al. 2009).

The high incidence of the absence of seizures initially indicated that anticonvulsive treatment by diazepam would not be necessary in a pc VX exposure scenario

(Chapter 3). However, microdialysis results showed high levels of ACh accumulation in the brain, indicating that VX entered the brain in sufficient amounts to cause dangerously high levels of ACh (Chapter 4). While in animals left untreated after pc exposure to VX, the decline in heart rate most likely prevents full distribution of VX into the brain, the preservation of heart rate by atropine and obidoxime probably enabled a more continuous distribution of VX into the brain (Chapter 4). Because obidoxime only enters the brain in very limited amounts, thereby insufficiently reactivating inhibited AChE, addition of atropine and diazepam are necessary to prevent the development of seizures in spite of high amounts of ACh.

Treatment of pc VX exposure with a carbamate, such as physostigmine, combined with an effective reactivator might be an advisable approach to investigate further. The slow entrance of VX into the circulation provides a suitable time window for protection of AChE by physostigmine.

2.3 Impairment of cognition: treatment options

Regarding long-term effects, animals showed a decline in neurogenesis and cognition at 8 weeks after exposure to soman, in spite of effective and life saving treatment with the oxime HI-6 and atropine. As seizures were only present for a short time, not inducing observable brain injury, the cognitive deficits may not have resulted from excitoxicity, but probably were due to an imbalance in cholinergic input into the dentate gyrus. In the present thesis, it was aimed to enhance neurogenesis by means of subchronic antipsychotic treatment with olanzapine (Chapter 5). This drug, however, was not able to induce neurogenesis at 8 weeks after poisoning and was, probably therefore, not effective in restoring cognitive deficits. However, other pharmacological agents might be able to increase neurogenesis in such a way that they could improve cognition. To this end, it is recommended to test growth factors (Collombet et al. 2005) and anticholinesterases such as physostigmine or galantamine when cognition deficits show up (Kotani et al. 2008). In addition, physical exercise might help to restore the balance in neurogenesis in the DG of the hippocampus (Van Praag et al. 1999).

3 Conclusion

The animal models developed allow an integrative assessment of toxicology and treatment efficacy. The toxicological profiles of sc soman exposure and pc VX exposure showed to be very different and accordingly demand a very different treatment approach. In particular, timing of treatment is an important issue in both cases. Soman exposure requires rapid treatment, directly aimed at restoring AChE activity and terminating seizure activity, whereas pc VX exposure requires repeated treatment on appearance of clinical signs until all nerve agent has entered the circulation and has been hydrolyzed. The results from the VX experiments are useful to the clinical practice of OP insectide poisoning, because of their similar persistence in the body. Another important conclusion of the present study is that an effective life-saving treatment at short term, may not be sufficient to prevent long term cognition deficits. This implies that a more thorough understanding of the cause of such deficits is needed to design improved treatment strategies. In this

thesis, impaired neurogenesis is proposed as a cause of cognition deficits, which could be a starting point for treatment.

Extrapolation of the results to humans remains difficult. Sensitivity for OPs and antimuscarinergics, oxime-induced reactivation and 'aging' of OP-inhibited AChE markedly differs between species. Whereas the guinea pig is a suitable model to investigate the impact of AChE protection, it is less suitable for the assessment of subtle cognition impairments, due the lack of proper tests; the rat model is more suitable to assess impact on cognition. To provide the best translational results, it is recommended to include both models in screening of treatment efficacy.

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Summary

Towards improved models for treatment of organophosphate poisoning

Organophosphates (OPs) are highly toxic compounds. They represent a large class of pesticides used worldwide, and (un)intentional poisoning is recognized as a global problem. The research towards more effective insecticides led to the discovery of nerve agents. Although the Chemical Weapons Convention, an agreement to ban the use, proliferation and stockpile of OP nerve agents, is ratified by most nations this cannot prevent the threat of such compounds being used by terrorists. Therefore research into mechanism of OP action and into clinical countermeasures when poisoning occurs is necessary.

Exposure to an OP leads to inhibition of Acetylcholinesterase activity and to an excessive buildup of Acetylcholine (ACh) in cholinergic nerve terminals. The overstimulation of muscles and nerve cells by ACh leads to muscular paralysis, excessive mucus secretion and to epileptiform brain activity. In case of exposure to high levels of OP, these symptoms can lead to death. The current medical countermeasures for OP poisoning are effective to a reasonable extent; yet, there is room for improvement. In order to be able to improve medical countermeasures a thorough understanding of the routes of entry, fate within the body and the mechanism of action is needed.

This thesis focussed on nerve agents, which can be divided into different classes based on their chemical structure. The first type of these chemical warfare agents, such as soman, sarin and tabun, are highly volatile and upon dispersion these agents will most likely enter the body via inhalation. In contrast, other types of nerve agents, such as VX, have a much lower volatility, making exposure via the skin most likely. In addition, their chemical structures encompass a nitrogen atom, charging the molecule in a physiological environment, which hampers their entrance into brain. These differential properties of chemical agents result in different risk profiles with regard to exposure, distribution, and toxic effects. The differential toxicology might urge for adaptations in the emergency protocols presently in use. Due to the similarity in chemical structure and mechanism of action, the insight acquired by studying nerve agents can also be used to improve treatment of intoxications with OP-pesticides.

In order to enable improvement of medical management, research in this thesis focussed on optimizing animal models to further investigate the mechanism of OP toxicity and treatment efficacy. Two types of OPs were used: soman, representing a highly toxic volatile OP, entering the body via inhalation and producing toxic signs very quickly, and VX, representing a much less volatile OP entering the body via the skin and demonstrating a clinical delay of toxic signs and long persistence. In particular treatment efficacy was tested.

In Chapter 2, an integrative guinea pig model was established to evaluate biochemical and vital physiological parameters following soman poisoning. Then, the efficacy of a current treatment regimen consisting of obidoxime and atropine was tested at 1 minute after exposure, as well as after appearance of first clinical signs after exposure to different doses of soman. This regimen appeared to be hardly effective; it provided low efficacy following challenge with a high dose of soman, and also when the treatment was postponed. Addition of the carbamate physostigmine to this treatment appeared to improve the outcome dramatically when administered at 1 minute after poisoning. However, when physostigmine

treatment was delayed to 10 minutes after poisoning, its efficacy showed to be redundant or even harmful.

The VX-exposed hairless guinea pig model is presented in Chapter 3. Exposure of VX via the skin was shown to be unpredictive and variable in terms of toxicokinetics. After a lag period following skin application, VX levels in blood continue to rise for several hours. The physiological consequences of accumulating central ACh levels, clinical signs and AChE activity were investigated. It was shown that the highly variable development of clinical signs upon exposure was predictive for the clinical outcome. Only few animals exposed to VX developed seizures, while most of them suffered from EEG signs that pointed to ischemia.

In Chapter 4 the VX model was extended to investigate toxicokinetics in freely moving animals combined with physiology, e.g. respiration, EEG and heart rate effects. Exposure to VX led to an increase in bronchoconstriction and a dramatic decline in heart rate, which was in line with the signs of ischemia on the EEG pointed out in Chapter 3. After full establishment of all parameters, a treatment protocol consisting of a one-shot injection consisting of 3 auto-injector equivalents of atropine, obidoxime and diazepam was tested. Initially, this treatment effectively postponed clinical signs of skin VX poisoning, but clinical signs reappeared after some time, progressing in a similar way as in untreated animals. Repetitive treatment on reappearance of signs, however, showed to be effective as long as treatment was continued. These findings imply that in case of skin exposure with VX, treatment should be continued until all VX has been eliminated from the body.

Long-term behavioural deficits of OP-poisoning and a possible intervention by drug-induced neurogenesis were investigated in a soman-poisoned rat model in Chapter 5. The rats were injected with a seizure-inducing dose of soman, combined with life-saving treatment consisting of atropine and HI-6. All animals suffered from seizures for a short period of time, which was accompanied by a huge increase of ACh levels in the brain. Although brain damage was very limited at 24 hours after poisoning, rats showed an impaired learning curve in a spatial memory task at 8 weeks after exposure. This was accompanied by a decrease in neurogenesis in the hippocampus, which could not be restored by the antipsychotic Olanzapine, which was reported to enhance neurogenesis. Although Olanzapine was ineffective in enhancing neurogenesis and normalizing behaviour, influencing neurogenesis by drug or other means might be an attractive approach in the treatment of long-term cognitive deficits after OP poisoning.

Overall, the results described in this thesis led to improved insights in the treatment of exposure to the agents soman and VX. The animal models developed allow an integrative assessment of toxicology and treatment efficacy. The sensitivity for these OPs and anti-muscarinic receptor compounds, the oxime-induced reactivation and ‘aging’ of OP-inhibited acetylcholinesterase markedly differs between species, complicating the extrapolation of these results to man. Whereas the guinea pig model is most suitable to investigate drugs for acetylcholinesterase protection, it is less suitable for the assessment of subtle cognition impairments, due to the lack of proper tests; the rat model is more suitable to assess impact of drugs on cognition. Thus, to provide the best translational results, it is recommended to include both the guinea pig and rat model in screening treatment efficacy. In the

rat model it was shown that an effective life-saving treatment at short term might not be sufficient to prevent long-term cognition deficits. This implies that a better understanding of the cause of such deficits is needed in order to design improved treatment strategies. It is assumed that impaired neurogenesis attributes to cognition deficits, which might be a starting point for research into future treatment of OP poisoning.

The toxicological profiles of soman and VX exposure in the guinea pig model showed to be very different and accordingly demand a completely different treatment approach. In particular the timing of treatment is an important factor to enhance efficacy in case of exposure to both VX and soman. Whereas soman exposure requires fast treatment, aimed at immediate recovery of acetylcholinesterase activity and terminating seizure activity, VX exposure requires repeated treatment on (re)appearance of clinical signs until all nerve agent has entered the circulation and has been hydrolysed. The results from the VX experiments are useful to the clinical treatment of OP insectide poisoning, because of similarity in persistence in the body.

Samenvatting

Verbetering van modellen voor organofosfaat vergiftiging

Organofosfaatverbindingen (OPs) behoren tot een klasse van hoogtoxische stoffen die wereldwijd gebruikt worden als pesticiden en insecticiden. Onderzoek naar meer effectieve verbindingen leidde tot de ontdekking van voor de mens zeer giftige verbindingen, zenuwgassen. Ondertekening van de Chemische Wapen Conventie, een verbod op het gebruik, proliferatie en opslag van zenuwgassen door de meeste naties, bleek onvoldoende om misbruik van dergelijke verbindingen door terroristen te voorkomen.

Blootstelling aan een OP veroorzaakt irreversibele remming van de activiteit van acetylcholinesterase (AChE), een belangrijk enzym in het centraal zenuwstelsel dat zorgt voor de afbraak van de neurotransmitter Acetylcholine (ACh). De remming van de afbraak van ACh leidt tot overstimulatie van spieren en zenuwcellen met als gevolg spierverlamming, overmatige afscheiding van slijm en epileptische activiteit. In geval van blootstelling aan hoge doseringen OP, kunnen deze symptomen leiden tot de dood. De huidige medische tegenmaatregelen zijn redelijk effectief, maar er is behoefte aan en ruimte voor verbetering. Om dit te kunnen bewerkstelligen, is het noodzakelijk een zeer goed begrip te hebben van het werkingsmechanisme, de besmettingsroutes en het gedrag van de stoffen in het lichaam.

Het onderzoek in dit proefschrift was gericht op het gedrag van zenuwgassen met als doel de behandelingsprotocollen te verbeteren. Zenuwgassen kunnen op basis van hun chemische structuur onderscheiden worden in twee klassen. Tot de eerste klasse zenuwgassen behoren relatief vluchtige verbindingen, zoals soman, tabun en sarin met als meest waarschijnlijke besmettingsroute het ademhalingsstelsel. Via deze route treden symptomen van vergiftiging snel op. Tot de tweede klasse zenuwgassen, behoren juist nauwelijks vluchtige verbindingen, zoals VX, waardoor een vloeistofbesmetting via de huid het meest waarschijnlijk is. Deze route van besmetting gaat gepaard met een vertraging in het optreden van symptomen, die ook lang aanhouden. Daarnaast bevatten de zenuwgassen in deze klasse in hun chemische structuur een stikstofatoom waardoor het molecuul in het lichaam geladen wordt en minder gemakkelijk de hersenen bereikt. Het verschil in eigenschappen tussen deze agentia resulteert in verschillende risicoprofielen met betrekking tot blootstelling, verspreiding en toxische effecten. Het verschil in toxicologisch profiel kan vragen om aanpassingen van de eerste hulp protocollen zoals die nu gehanteerd worden. Vanwege de gelijkenis in chemische structuur en werkingsmechanisme, kunnen de inzichten verworven bij het bestuderen van zenuwgassen ook gebruikt worden om de behandeling bij besmetting met OP pesticiden te verbeteren.

In Hoofdstuk 2 werd een model in de cavia ontworpen, waarin de effecten van verschillende doseringen soman op biochemische en vitale fysiologische functies konden worden onderzocht. In dit model werd de effectiviteit van een behandelprotocol bestaande uit obidoxime en atropine, toegediend 1 minuut na soman, of na het optreden van vergiftigingsverschijnselen. Deze behandeling was nauwelijks effectief in geval van besmetting met een hoge dosis en bij het uitstellen van de behandeling. Toevoeging van het carbamaat fysostigmine aan deze behandeling 1 minuut (direct) na soman zorgde voor een zeer hoge effectiviteit. Wanneer de behandeling met fysostigmine echter werd uitgesteld tot na het optreden van vergiftigingsverschijnselen bleek de effectiviteit volledig afwezig en in

enkele gevallen zelfs schadelijk te zijn.

Een model voor blootstelling aan VX via de huid in de haarloze cavia is beschreven in Hoofdstuk 3. Blootstelling aan VX via de huid leidde tot een onvoorspelbaar verloop van het optreden van verschijnselen van vergiftiging. De effecten van VX op het EEG, ACh concentraties in de hersenen, klinische verschijnselen en AChE activiteit werden onderzocht. Ondanks de onvoorspelbaarheid van het verloop van de klinische verschijnselen, bleken deze in individuele dieren zeer voorspelbaar te zijn voor het klinische verloop ten gevolge van de blootstelling. Slechts enkele dieren vertoonden epileptische activiteit en hoge levels ACh, terwijl de meeste dieren verschijnselen vertoonden wijzend op zuurstofgebrek.

In hoofdstuk 4 werd dit model uitgebreid om in vrij bewegende dieren de toxicokinetiek van VX te meten, gecombineerd met het meten van de ademhaling, hartslag en EEG. Blootstelling van de huid aan VX leidde tot benauwdheid en een afname in hartslag, waardoor de effecten ten gevolge van ischemie gevonden in Hoofdstuk 3 verklaard konden worden. Na het in kaart brengen van de toxische effecten, werd een behandelprotocol getest waarbij 1 injectie werd gegeven, waarvan de inhoud gelijkwaardig was aan 3 autoinjectorequivalenten atropine, obidoxime en diazepam. Deze behandeling bleek ernstige verschijnselen van toxiciteit enige tijd uit te stellen, maar na enige tijd kwamen de symptomen terug. Herhaalde behandeling op geleide van terugkerende symptomen bleek effectief te zijn, zolang de behandeling werd voortgezet. Dit wijst erop dat in geval van besmetting met VX via de huid, de behandeling moet worden voortgezet zo lang er VX uit de huid in het lichaam komt.

Gedragseffecten op lange termijn ten gevolge van besmetting met soman en mogelijke interventie met neurogenese bevorderende farmaca werden onderzocht in een ratmodel in Hoofdstuk 5. De ratten werden blootgesteld aan dosis soman, in combinatie met een levensreddende therapie met atropine en het oxim HI-6, waardoor er kortdurend epileptische aciviteit ontstond, gepaard gaande met zeer hoge concentraties ACh in de hersenen. Alhoewel er nauwelijks sprake was van hersenschade op 24 uur na blootstelling, vertoonden de blootgestelde ratten slechter leervermogen in een gedragstest gebaseerd op ruimtelijk oriëntatievermogen 8 weken na blootstelling. De mate van neurogenese in de hippocampus was significant lager in deze dieren. Het antipsychotium Olanzapine, dat volgens de literatuur neurogenese zou bevorderen, deed dat in onze studie niet, en zorgde ook niet voor verbeteringen in het gedrag. Het bevorderen van neurogenese door middel van bijvoorbeeld farmaca , zou een aantrekkelijke benadering zijn om lange termijn effecten ten gevolge van Op blootstelling te behandelen.

De resultaten beschreven in dit proefschrift hebben geleid tot meer inzicht in de effecten van besmetting met de zenuwgassen soman en VX en de behandeling hiervan. De ontwikkelde diermodellen maken het mogelijk zowel toxische effecten van de zenuwgassen en de effectiviteit van behandelingen integratief te benaderen.

Tussen verschillende species bestaat een verschillende gevoeligheid voor deze OPs en antimuscarinerge stoffen en reactivatie van OP-geremd AChE door oximen, waardoor de extrapolatie van deze resultaten naar mensen bemoeilijkt wordt.

Terwijl het caviamodel erg geschikt is voor het onderzoeken van de effectiviteit van farmaca met betrekking to AChE reactivatie, is dit model door gebrek aan gevoelige gedragstesten minder geschikt voor het evalueren van subtiele cognitieve defecten. Het rattenmodel is meer geschikt voor dit soort onderzoek. Daarom wordt het aanbevolen om beide modellen te gebruiken om te komen tot zo goed mogelijk extrapoleerbare resultaten.

In het rattenmodel werd aangetoond dat een effectieve levensreddende behandeling mogelijk niet genoeg is om lange termijn effecten op cognitie te voorkomen. Dit wijst erop dat meer begrip van de oorzaak van dergelijke effecten noodzakelijk is om verbeterde behandelstrategieën te kunnen ontwerpen. De aantasting van het neurogeneseproces in de rat zou kunnen bijdragen aan de effecten op cognitie veroorzaakt doorblootstelling aan soman en hiermee een startpunt kunnen zijn voor een nieuwe behandeling met neurogenese bevorderende middelen.

De toxicologische profielen van subcutane besmetting met soman en besmetting via de huid met VX bleken erg verschillend te zijn, waardoor beide scenario's vragen om verschillende behandelingsstrategieën. Met name de timing van behandeling vereist de aandacht. Terwijl soman besmetting vraagt om zeer snelle behandeling, gericht op bescherming van AChE activiteit en het zo snel mogelijk stoppen van epileptische activiteit, vereist huidbesmetting met VX een herhaalde behandeling op geleide van symptomen, totdat al het VX opgenomen in de huid in de circulatie is gekomen en is gemetaboliseerd. Met name de resultaten uit de VX experimenten zijn van waarde voor de klinische behandeling van OP pesticide blootstelling, vanwege de gelijkenis in chemische structuur en vergelijkbare persistentie in het lichaam.

Abbreviations

2-PAM	-	Pralidoxime
5-HT	-	Serotonin
A	-	Anterior
ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
AChET	-	Acetylcholinesterase tetramer
Am	-	Amygdala
ANOVA	-	Analysis of variance
AUC	-	Area under curve
BMR	-	Basal metabolic rate
BPM	-	Beats per minute
BrdU	-	Bromodeoxyuridine
BSA	-	Bovine serum albumin
BuChE	-	Butyrylcholinesterase
CA3	-	Hippocampal
Ch	-	Choline
CNS	-	Central nervous system
CWA	-	Chemical warfare agent
CWC	-	Chemical Weapons Convention
cys	-	Cysteine
DAB	-	3,3′-diaminobenzidine tetrahydrochloride
DAM	-	Diacetylmonoxime
DCX	-	Doublecortin
DG	-	Dentate gyrus
EAA	-	Excitatory amino acid
EC	-	Entorhinal Cortex
ECG	-	Electrocardiogram
EDF	-	European data format
EEG	-	Electroencephalogram
FFT	-	Fast Fourier Transformation
GABA	-	Gamma-aminobutyric acid
GCL	-	Granular layer of the dentate gyrus
Glu	-	Glutamic acid
H	-	Hilus
HI-6	-	[(E)-[1-[(4-carbamoylpyridin-1-ium-1-yl)methoxymethyl]pyridin-2-ylidene]methyl]-oxoazanium dichloride
Hip	-	Hippocampus
His	-	Histidine
HPLC	-	High performance liquid chromatography
HR	-	Heart rate
Ht	-	Hypothalamus

im	-	Intramuscular
ip	-	Intraperitoneal
L	-	Lateral
LD50	-	Median lethal dose
LDL0	-	Lowest Dose causing lethality
mAChR	-	Muscarinic Acetylcholine receptor
MANOVA	-	Multivariate analysis of variance
MINA	-	Monoisonitrosoacetone
ML	-	Molecular layer of the dentate gyrus
MO	-	Medulla oblongata
MQ	-	Milli Q
MS	-	Medial septum
nAChR	-	Nicotinic Acetylcholine receptor
Nb	-	Nucleus Basalis
NMDA	-	N-methyl-D-aspartic acid
NOS	-	Nitric Oxide Synthase
NPC	-	Neuronal progenitor cell
OP	-	Organophosphate
OPCW	-	Organisation for the Prohibition of Chemical Weapons
P	-	Posterior
PB	-	Phosphate buffer
PC	-	Piriform cortex
pc	-	Percutaneous
Penh	-	Arbitrary value for bronchoconstriction
PM	-	Pontomesenphalon
PNS	-	Peripheral nervous system
PP	-	Perforant path
PrC	-	Perirhinal Cortex
PTSD	-	Post-traumatic Stress Disorder
RMV	-	Respiratory minute volume
RSDL	-	Reactive Skin Decontamination Lotion
sc	-	Subcutaneous
SEM	-	Standard error of mean
Ser	-	Serine
SGZ	-	Subgranular zone
STR	-	Striatum
SVZ	-	Subventricular zone
Th	-	Thalamus
V	-	Ventral
VX	-	(O-ethyl-S-2-diisopropylaminoethyl-methylphosphonothioate

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Curriculum Vitae

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Marloes (Maria Johanna Adriana) Joosen was born in Bergen op Zoom, The Netherlands in 1977. After finishing high school (grammar school) at the Gymnasium Juvenaat H. Hart in Bergen op Zoom in 1995, she started studying Medical Biology at the Hogeschool West-Brabant in Etten-Leur, The Netherlands. Main subjects were histology, microbiology and zoology. Her final internship was followed under supervision of Dr. Herman van Helden at the research group Pharmacology at the TNO Prins Maurits Laboratory (TNO-PML) in Rijswijk, The Netherlands, during which she evaluated a model for dementia using intracerebroventricular administration of an oxygen radical generator in the lateral ventricle of the rat. During the internship she started working at TNO-PML as a technician in the Pharmacology group of Dr. P.L.B. Bruijnzeel. During this period, she participated in research on the development of animal models for asthma and chronic bronchitis. In addition, she investigated therapeutic effects of exogenous surfactants in the treatment of lung injury induced by toxic gasses such as phosgene and PFIB. In 2003 her research efforts shifted towards improvement of medical countermeasures against nerve agents by application of adenosine A1 agonists and improvement of pretreatment regimens in the research group Medical Countermeasures led by Dr. J.P. Langenberg. After publication of several papers, she started a PhD project regarding the improvement of medical countermeasures against nerve agents in 2007, of which the results are described in this thesis. The PhD project was carried out at TNO Defence, Security and Safety (formerly TNO-PML) in the Business Unit CBRN Protection under supervision of Dr. Herman van Helden at TNO and Prof. Guus Smit from the Centre for Neurogenomics and Cognitive Research at the VU University, Amsterdam, the Netherlands. She participated in the Onderzoeksschool Neurowetenschappen Amsterdam (ONWA). Next to the PhD project, she participated in several research projects, amongst others the improved design of oximes to enhance blood brain barrier penetration, a Parkinson's model, the toxicokinetics of Botulinum Toxin and the characterization of biomarkers for the development of stress related disorders. From 2010 she continues her career at TNO Defence, Security and Safety as scientist and project manager.

Published papers

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T.J.H. Bueters, M.J.A. Joosen, H.P.M. van Helden, A.P. IJzerman, M. Danhof. "Adenosine A_1 receptor agonist N^6-cyclopentyladenosine affects the inactivation of acetylcholinesterase in blood and brain by sarin." J Pharmacol Exp Ther. 2003 Mar;304(3):1307-13

Book Chapters

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M.J.A. Joosen, A.B. Smit, H.P.M. van Helden. "Organophosphate poisoning: Short-term therapeutic effects of physostigmine and obidoxime." Book chapter in: The Neurochemical Consequences of Organophosphate Poisoning in the CNS, 2010: 135-147. Eds. B.A. Weissman and L. Raveh