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Animal models that best reproduce the clinical manifestations of human intoxication with organophosphorus compounds

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Running title: Acute and delayed clinical manifestations of OP toxicity

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Non-standard abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ADC, apparent diffusion coefficient; AMN, atropine methylnitrate; EEG, electroencephalogram; FDA, Food and Drug Administration; IC50, median inhibitory concentration; LD50, median lethal dose; PTSD, post-traumatic stress disorder; OP, organophosphorus; VEP, visual-evoked potential

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Abstract

The translational capacity of data generated in preclinical toxicological studies is contingent upon several factors, including the appropriateness of the animal model. The primary objectives of this article are: (i) to analyze the natural history of acute and delayed signs and symptoms that develop following an acute exposure of humans to organophosphorus (OP) compounds, with an emphasis on nerve agents, (ii) to identify animal models of the clinical manifestations of human exposure to OPs, and (iii) to review the mechanisms that contribute to the immediate and delayed OP neurotoxicity. As discussed here, clinical manifestations of an acute exposure of humans to OP compounds can be faithfully reproduced in rodents and non-human primates. These manifestations include an acute cholinergic crisis in addition to signs of neurotoxicity that develop long after the OP exposure, particularly chronic neurological deficits consisting of anxiety-related behavior and cognitive deficits, structural brain damage, and increased slow electroencephalographic frequencies. Because guinea pigs and non-human primates, like humans, have low levels of circulating carboxylesterases – the enzymes that metabolize and inactivate OP compounds – they stand out as appropriate animal models for studies of OP intoxication. These are critical points for the development of safe and effective therapeutic interventions against OP poisoning because approval of such therapies by the Food and Drug Administration is likely to rely on the *Animal Efficacy Rule*, which allows exclusive use of animal data as evidence of the effectiveness of a drug against pathological conditions that cannot be ethically or feasibly tested in humans.

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The discovery of the usefulness of organophosphorus (OP) compounds as pesticides in the late 1930's unfortunately led to the identification of some of the most toxic compounds synthetized by mankind, including tabun, soman, and VX, which would become collectively known as nerve agents (Tucker, 2006). These nerve agents have been stockpiled and used as weapons of mass destruction in chemical warfare and in terrorist attacks against civilians. The death toll and poor health outcomes of the alleged use of sarin against civilians in Damascus, Syria, as recently as August 2013, and the catastrophic results of the use of tabun or sarin during the Second Sino-Japanese War (1937-1945), the 1980's Iraq-Iran conflict, and the 1990's terrorist attacks in Japan are well documented (Coupland and Leins, 2005; Dolgin, 2013; Patrick et al., 2013; Romano and King, 2001; Sellstrom et al., 2013; Tucker, 2006).

The fatalities and poor health conditions resulting from acute and/or chronic exposures of humans to OP pesticides have also become a major public health concern. There are estimates that more than 3 million cases of acute OP pesticide poisoning occur per year, with more than 1 million cases being attributed to occupational exposure. Just as alarming are the conservative estimates that pesticide self-poisoning worldwide accounts for more than 250,000 deaths per year, which corresponds to about one-third of the world's suicide cases (Gunnell et al., 2007). Finally, epidemiological studies have provided compelling evidence that acute or continued exposure to sub-acute levels of OP pesticides are associated with increased risks of debilitating neurological disorders, in both adults and developing children (Dahlgren et al., 2004; Engel et al., 2011; Levin and Rodnitzky, 1976; Rosas and Eskenazi, 2008; Savage et al., 1988).

Acute signs and symptoms of OP poisoning result primarily, though not exclusively, from their common action as irreversible inhibitors of acetylcholinesterase (AChE), the enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh) (Albuquerque et al., 1985). Persistent

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stimulation of muscarinic receptors by accumulated ACh leads to a muscarinic syndrome characterized by miosis, profuse secretions, bradycardia, bronchoconstriction, hypotension, and diarrhea. Overstimulation of nicotinic receptors triggers tachycardia, and skeletal muscle fasciculation, while their subsequent desensitization contributes to muscle weakness. The broad range of CNS-related acute effects includes anxiety, restlessness, confusion, ataxia, tremors, seizures, and central cardiorespiratory paralysis (reviewed in Hurst et al., 2012; see also Yokoyama et al., 1998). Therefore, treatment of acute OP intoxication relies heavily on the use of an oxime to reactivate OP-inhibited AChE, atropine to block the over-activation of muscarinic receptors, and benzodiazepines to reduce the incidence and intensity of OP-induced convulsions and the resulting neuropathology. However, the inadequate outcomes of these treatments have been extensively scrutinized (Buckley et al., 2004). For example, AChE inhibited by some OPs, including the nerve agent soman, ages quickly and cannot be reactivated by clinically available oximes (Kassa, 2002). Some clinical trials have also revealed no clinical benefit of the use of oximes against poisoning with different pesticides, despite the fact that blood AChE was reactivated (Buckley et al., 2011). In addition, as discussed later in this article, recurrent seizures develop even after OP-induced acute seizures are controlled with benzodiazepines (Sekijima et al., 1997). Thus, there is an urgent need to develop new therapeutic strategies to treat and/or prevent the immediate and delayed toxicity of OP compounds.

The public health relevance of OP toxicity to humans worldwide, the need for a comprehensive understanding of the AChE-unrelated mechanisms that contribute to the pathological conditions triggered by exposure of males and females at different ages to OPs, and the urgency to develop efficacious treatments to treat and/or prevent these conditions underscore the importance to identify optimal animal models in which the clinical manifestations of human

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OP toxicity can be replicated. In the present article, we provide a comprehensive analysis of the natural history of acute and delayed signs and symptoms that develop following exposure of humans to OP compounds, with an emphasis on nerve agents. This analysis is, then, followed by a discussion of how faithfully the clinical manifestations of human exposure to OP compounds can be reproduced in different animal models of OP intoxication and a brief review of mechanisms that contribute to the immediate and delayed OP neurotoxicity. This information placed in perspective lays the groundwork for future research aimed at developing safe and effective therapeutic interventions against the acute and delayed toxic effects induced by OP pesticides and nerve agents.

1. Clinical manifestations of human exposure to OP compounds: Acute and long-term clinical signs and symptoms

Studies of humans who had been experimentally exposed to nerve agents under "controlled" conditions provided some of the first evidence of the direct effects of nerve agents on neurological functions in humans. For example, Bowers et al. (1964) analyzed the clinical signs presented by 96 young male volunteers on active duty in the Army or the Air Force following their percutaneous exposure to a low dose of an OP compound likely to be VX. Although the volunteers did not develop overt signs of acute toxicity, they presented with a "syndrome" that was broadly referred to as a "state of altered awareness" and was characterized by difficulty in sustaining attention and slowing of intellectual and motor processes, in addition to subjective feelings of agitation, anxiety, and confusion (Bowers et al., 1964). In general, these clinical manifestations were associated with the inhibition of AChE in red blood cells and symptomatic recovery was associated with the recovery of AChE activity (Bowers et al., 1964;

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Grob and Harvey, 1953). Long-term follow-up of these individuals is not available. However, case reports of humans occupationally or intentionally exposed to nerve agents, follow-up studies of the victims of the 1995 terrorist attack with sarin in the Tokyo subway, as well as case reports and follow-up studies of humans accidently or intentionally exposed to OP pesticides, summarized below, all support the contention that persistent delayed neurological deficits develop following an initial exposure to these chemicals.

In 1969, Metcalf and Holmes reported that workers who had history of exposure to OP compounds presented years later with attention deficits, memory impairment, and difficulty in maintaining alertness that were accompanied by increased slow electroencephalographic activity in the theta range. A subsequent study compared the electroencephalograms (EEGs) recorded from industrial workers years after their confirmed accidental exposure to sarin with those recorded from control subjects (Duffy et al., 1979). In this study, visual inspection of the EEGs revealed that the exposure to sarin was associated with an increase of the slow (delta and theta) frequencies and a reduction of the alpha frequency. Spectral analysis of the EEGs also revealed a significant increase of the high-frequency activity in the beta range (12-30 Hz) years after the exposure to sarin (Duffy et al., 1979).

Long-term follow-up studies of Japanese victims of the sarin attack in Matsumoto also reported that long after the sarin exposure victims of the attack presented with significant EEG alterations, some of which were consistent with recurring electrical seizures (Sekijima et al., 1997). One of the victims was a male subject who at the time of the attack presented severe disturbance of consciousness, developed convulsions, and required assisted ventilation. At a 1-year follow-up examination, sharp wave complexes, which are consistent with epileptiform activity (Westmoreland, 1998), were present in the EEG of this victim. Two other victims were

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women who at the time of the attack presented no convulsions, needed no artificial ventilation, and had only mild disturbance of consciousness. During the 1-year follow-up examination, bursts of delta activity, which often correlate with focal brain lesions (Harmony et al., 1995), were present in their EEGs. Abnormal bursts of delta activity could still be detected in the EEG of one of the two female victims during a 2-year follow-up examination (Sekijima et al., 1997). During the 2-year follow-up examination, 14-Hz spikes were also detected in the EEG of a fourth victim, a female who at the time of the attack presented no convulsions and needed no respiratory support (Sekijima et al., 1997). Although the authors suggest that these 14-Hz spikes represented epileptic electroencephalographic changes, caution is warranted given that this patient was 16 years old at the time of the examination and 14-Hz spikes frequently appear in the EEG during adolescence (Klass and Westmoreland, 1985). These case reports clearly indicate that patients who do not develop acute convulsions in response to an acute exposure to nerve agents can, years later, present with abnormal slow delta waves that are suggestive of focal brain lesions. They also support the notion that patients who survive a severe case of nerve agent intoxication can years later present with epileptic EEG discharges.

Many reports have suggested that hypoxia resulting from motor convulsions and respiratory distress during the acute phase of OP intoxication contribute to the neurological deficits that develop and persist long after the acute phase of intoxication subsides. For example, in 1995 Nozaki et al reported the case of a patient who presented with amnesia 15 days and six months after recovering from convulsions induced by an exposure to VX. In 1996, Hatta et al reported the case of another patient who presented with retrograde amnesia and personality changes after recovering from clonic-tonic generalized convulsions and severe episodes of dyspnea induced by sarin during the terrorist attack in Tokyo. However, case reports and follow-

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up studies of people who did not experience overt CNS signs of intoxication following an OP exposure and yet developed persistent delayed neurological deficits, as described below, support the contention that neurological impairments can develop following an acute exposure to levels of nerve agents or OP pesticides that are not sufficient to induce convulsions and/or hypoxia.

The 1995 terrorist attack with sarin in the Tokyo subway is the largest documented exposure of a civilian population to a nerve agent. Approximately 95% of the 111 victims who were admitted to hospitals and diagnosed as moderately or severely intoxicated received the conventional therapeutic interventions for OP poisoning, including atropine to block the over-activation of muscarinic receptors and pralidoxime to reactivate sarin-inhibited AChE; diazepam was also used as needed to treat victims who developed convulsions (Okumura et al., 1996). Even though the treatments effectively countered the acute signs of toxicity, victims of the sarin attack who did not present with episodes of convulsion at the time of the attack presented years later with significant memory decline (Hood, 2001; Nishiwaki et al., 2001).

Although it is well recognized that the trauma associated with the experience of a warfare attack can confound the evaluation of long-term health effects induced by the chemical used in the attack, some studies have provided evidence that post-traumatic stress disorder (PTSD) alone cannot explain changes in neurophysiological functions and disruption of the structural integrity of specific brain regions observed in victims who had been exposed to sarin in the Tokyo subway attack (Murata et al., 1997; Yamasue et al., 2007). For example, six to eight months after the sarin attack, event-related and visual-evoked potentials (P300 and VEP, respectively) were analyzed in 18 victims of the attack and 18 control subjects (Murata et al., 1997). P300 is an event-related potential associated with decision making, and VEP is an evoked potential that reflects the conduction time from the retina to the visual cortex. In victims of the attack, the

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latencies of both P300 and VEP were significantly longer than those measured in control subjects. The finding that the longer latencies of those potentials did not correlate with the victims' high PTSD scores (Murata et al., 1997) strongly indicated that sarin, rather than PTSD, was the cause of the neurophysiological alterations measured in those patients. Likewise, Nishiwaki et al. (2001) reported that first responders who were dispatched to the site of the attack in Tokyo presented three years later with memory deficits that were evident in the backward digit span test and were independent of PTSD symptoms. Finally, Yamasue et al. (2007) reported that five years after the attack victims presented with significant decrease in gray matter volume in the right insular cortex, the right temporal cortex, and the left hippocampus. In these victims, the volume of the left subinsular white matter was positively correlated with decreased serum cholinesterase levels measured after the incident, but not with the occurrence or severity of PTSD.

A case report published in 2010 corroborated the notion that exposure of humans to low levels of sarin can trigger delayed neurocognitive deficits (Loh et al., 2010). This is the report of an Army sergeant who eight months after experiencing mild signs of acute toxicity following an exposure to sarin in Iraq presented with attention deficits and impaired motor coordination in the absence of any signs or symptoms of emotional distress or mood disorders.

Persisting neurological symptoms of memory loss, decreased concentration, irritability, and personality changes have also been observed in all members of a family who were accidently exposed to the OP pesticide diazinon (Dahlgren et al., 2004). A pesticide company mistakenly sprayed the interior of the family's house with diazinon, and, soon after the exposure, all members of the family presented acute signs and symptoms of OP intoxication, including headaches, nausea, skin irritation, runny nose, and vomiting. They did not present with

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convulsion. Three months to three years later, all family members presented with cognitive deficits and mood disorders. In these cases, the persistent neurological dysfunctions following the exposure to the OP pesticide clearly developed in the absence of acute convulsions and hypoxia. Population studies have provided additional evidence that signs of emotional distress and memory deficits develop in humans long after their recovery from acute poisoning by cholinesterase inhibitors or from a hypercholinergic syndrome following acute occupational exposure to OP pesticides (Levin and Rodnitzky, 1976; Roldán-Tapia et al., 2005; Rosenstock et al., 1991; Savage et al., 1988; Steenland et al., 1994; Wesseling et al., 2002).

In summary, a "cholinergic crisis" defines the acute phase of OP intoxication in humans, even though the prevalence of specific signs and symptoms is OP specific (eg, Nozaki et al., 1995). In addition, although it is true that subtle differences in the prevalence of delayed signs and symptoms of OP neurotoxicity also exist among individuals exposed to different OP pesticides and nerve agents, it appears that mood disorders, specifically anxiety and depression, and cognitive deficits, particularly attention deficits and memory impairment, are common persistent neurological conditions seen in individuals long after their exposure to these chemicals (Dahlgren et al., 2004; Hatta et al., 1996; Hood, 2001; Levin and Rodnitzky, 1976; Nishiwaki et al., 2001; Metcalf and Holmes, 1969; Nozaki et al., 1995; Roldán-Tapia et al., 2005; Rosenstock et al., 1991; Savage et al., 1988; Steenland et al., 1994; Wesseling et al., 2002). In many cases, these delayed neurological deficits have been observed in the absence of acute convulsions and/or hypoxia induced by the OP compounds. In some cases, these deficits have also been dissociated from the trauma associated with the exposure event and have been correlated with: (i) disruption of the structural integrity of different brain structures, including the hippocampus and the cingulate cortex, as seen in MRI studies of victims of the Tokyo subway terrorist attack with

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sarin (Yamasue et al., 2007), (ii) decreased regional cerebral blood flow, particularly in the occipital lobes, as seen in a single photon emission computed tomography study of subjects long after they had experienced an acute intoxication with OP pesticides (Mittal et al., 2011), and (iii) increased slow electroencephalographic activity, as reported in individuals occupationally exposed to sarin (Metcalf and Holmes, 1969). Therefore, at a minimum, translationally relevant animal models should present acute and delayed signs and symptoms of OP toxicity that recapitulate those described here and should also predict the effectiveness and safety of medical countermeasures against OP toxicity in humans.

2. Animal models of OP intoxication

Signs of acute toxicity induced by OP pesticides and nerve agents in numerous animal species ranging from small rodents such as mice, rats, and guinea pigs to large mammals, including non-human primates, closely resemble those presented by humans exposed to these compounds. Following exposure of different animal species to high doses of nerve agents, a cholinergic crisis develops and is characterized by chewing and gnawing, profuse secretions, diarrhea, muscle fasciculation, restlessness, tremors, motor convulsions, and respiratory distress that can lead to death (Albuquerque et al., 2006; Deshpande et al., 1986; Despain et al., 2007; Kawabuchi et al., 1988; Maxwell et al., 2006). The rank order of lethal potencies (LD50s) of the nerve agents is invariably the same among the different species, with the LD50s increasing from VX to soman to sarin (Bajgar, 1992; Fawcett et al., 2009; Koplovitz and Stewart, 1994; Lenz et al., 2005; Maxwell et al., 2006). In addition, the LD50s of different nerve agents correlate well with their IC50s to inhibit *in vitro* AChE activity in brain extracts from rats and guinea pigs (Fawcett et al., 2009; Sivam et al., 1984), supporting the contention that, as in humans, AChE

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inhibition is an important determinant of the acute toxicity of nerve agents in animal models of acute OP intoxication.

Studies from various laboratories further confirmed that, similar to humans (Nozaki et al., 1995), different animal species present signs of acute toxicity that are nerve agent dependent. For example, guinea pigs challenged with 0.8xLD50 VX, soman, or sarin present facial twitches, chewing, slight hyperlocomotion, and head tremors (Fawcett et al., 2009). With increasing doses of soman or sarin, guinea pigs present progressively with forelimb clonus, increased secretions, muscle fasciculation, rearing, strong grinding, gnashing or clenching of the teeth (bruxism), unremitting tonic-clonic convulsions, and severe respiratory distress. In contrast, even at a dose as high as 1.2xLD50, VX does not induce recurrent or unremitting motor convulsions in guinea pigs. Motor convulsions induced by 1.2xLD50 VX are brief and show spontaneous termination (Fawcett et al., 2009). In earlier studies, McDonough and collaborators also reported that electrical seizures are usually detected in rats and guinea pigs following a subcutaneous injection of ≥ 2xLD50 soman, sarin, tabun, cyclosarin, VX, or VR, with the incidence of these seizures being higher with soman, sarin, tabun, cyclosarin, and VR than with VX (Shih and McDonough, 1999; Shih et al., 2003). They further reported that: (i) onset of electrical seizures is slowest with VX than with the other nerve agents and (ii) doses of anticonvulsants needed to control seizures induced by VX or sarin are significantly lower than those needed to control seizures induced by the other nerve agents.

A recent study conducted with guinea pigs treated with atropine methylnitrate (AMN) prior to their challenge with 1.0xLD50 sarin or VX further revealed that only among sarin-exposed animals was lethality proportional to the percentage of animals that developed electrical seizures (O'Donnell et al., 2011). In that study, approximately 70% of all guinea pigs injected

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with 1.0xLD50 sarin developed electrical seizures. Nearly 50% of sarin-challenged animals that developed electrical seizures did not survive the first 24-h post-challenge, whereas all animals that did not develop seizures survived the sarin challenge (O'Donnell et al., 2011). In contrast, approximately 40% of the AMN-treated animals developed seizures when challenged with 1.0xLD50 VX and all AMN-treated guinea pigs survived the VX challenge regardless of whether or not they developed seizures (O'Donnell et al., 2011). Because unremitting convulsions induced by soman and sarin contribute to their lethal potency, timely treatment of soman- or sarin-challenged animals with benzodiazepines significantly improves their survival and increases the effectiveness of the standard therapy consisting of the muscarinic antagonist atropine and such AChE reactivators as pralidoxime in reducing mortality induced by these nerve agents (Lallement et al., 1997, 1998, 1999; McDonough et al., 1999, 2000). This is in agreement with the well-reported increased risk of death associated with *status epilepticus* in humans (Ferlisi and Hocker, 2013; Jette and Trevathan, 2014).

A single exposure of different animal species to high levels of soman, sarin, or VX causes profound neuropathology, with significant neuronal loss in various brain regions, including the hippocampus, amygdala, piriform cortex, and thalamus (Kadar et al., 1995; Lemercier et al., 1983; McLeod et al., 1984; Petras, 1994; Shih et al., 2003). The finding that the severity of the neuropathology induced by the nerve agents correlates well with the duration and severity of convulsions led to the suggestion that early management of nerve agent-induced convulsions would be sufficient to reduce the neuropathology and the accompanying neurological deficits (Hayward et al., 1990; Lallement et al., 1993, 1994, 1997, 1998; Martin et al., 1985; McDonough et al., 2000; McDonough and Shih, 1997; Raveh et al., 2002; Shih et al., 2003). However, spontaneous recurrent motor convulsions and electrical seizures in addition to

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neurodegeneration have been observed in rats long after acute seizures following an exposure to nerve agents were terminated by benzodiazepines (de Araujo Furtado et al., 2010). Spontaneous recurrent motor convulsions and/or electrical seizures have also been reported in soman-exposed rats and non-human primates long after the acute toxicity of the nerve agent subsided (Despain et al., 2007; Romano et al., 2001). Therefore, treatment with anticonvulsants, although essential to control the acute tonic-clonic convulsions and improve survivability among animals exposed to high levels of nerve agents, is not sufficient to counter the development of delayed neurological deficits. In this respect, it is noteworthy that neurodegeneration and the resulting neurological dysfunctions observed in soman-intoxicated rodents can be significantly reduced by therapeutic interventions that, although unable to control the seizures, effectively decrease glutamate excitotoxicity (Filliat et al., 1999).

Neurological dysfunctions and structural brain damage that resemble those presented by humans exposed to these agents have been successfully reproduced in rats, guinea pigs, and non-human primates exposed to these agents. And, of major significance, in many studies these neurological deficits have been detected at doses of nerve agents that are not sufficient to induce overt signs of acute toxicity in the animals. This is a crucial point given that, as described in the previous section, follow-up clinical studies revealed neurological deficits in human subjects who were occupationally or intentionally exposed to levels of nerve agents or OP pesticides that were not sufficient to trigger overt signs of acute toxicity.

Anxiety-related behavior, a clinical manifestation of OP exposure in humans, develops and lasts long after a single exposure of rats and guinea pigs to sarin or soman. Several studies revealed that rats and guinea pigs presented with anxiety related-behavior when tested in the open field and in the elevated T-maze days to months after an acute exposure to a low dose of

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soman (Baille et al., 2001; Mamczarz et al., 2010; Sirkka et al., 1990). In addition, Bajgar et al. (2004) reported that one month after the exposure to 0.7xLCt50 sarin female guinea pigs presented increased locomotor activity and exacerbated stereotypic behaviors that are suggestive of anxiety (Choi et al., 2013).

Cognitive deficits have also been observed in animals exposed to nerve agents. One study reported that, following a single inhalation exposure to a level of sarin sufficiently low to trigger no or only mild signs of cholinergic hyperstimulation, rats showed significant impairment in spatial discrimination in the Y-Maze (Kassa et al., 2002). Other studies reported that rats and mice that developed severe signs of acute toxicity, including convulsions, following a single subcutaneous exposure to 1.0-1.2xLD50 soman showed immediate and delayed cognitive impairments in the Morris water maze (Filliat et al., 1999; Raveh et al., 2002). Of particular interest is a report that in asymptomatic, soman-challenged mice cognitive deficits could be detected at three months, but not one month following the challenge (Filliat et al., 2007). Guinea pigs, too, develop cognitive deficits long after their exposure to soman (Mamczarz et al., 2011; Pereira et al., 2012). Three months after surviving the challenge with 1.0xLD50 soman, prepubertal female guinea pigs present spatial learning impairment in the Morris water maze (Mamczarz et al., 2011) and adult male guinea pigs present both spatial learning and memory retention deficits (Pereira et al., 2012).

In an *in vivo* magnetic resonance imaging (MRI) study, analyses of T2-weighted images obtained from prepubertal female guinea pigs that survived the challenge with 1.0xLD50 soman demonstrated significant and long-lasting damage to the structural integrity of brain regions known to play a central role in cognitive processing and in control of emotional behaviors (Gullapalli et al., 2010). Segmentation of the brain images obtained from all soman-exposed

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guinea pigs before and seven days after the injection of the nerve agent revealed a significant increase in ventricular CSF that was suggestive of brain atrophy. In animals that developed severe signs of intoxication in response to 1.0xLD50 soman, there was a significant, long-lasting increase of T2 values in the piriform cortex, amygdala, and thalamus in addition to a transient increase of T2 values in the hippocampus. A significant amount of cell lysis may have accounted for the regional increase in T2 values, given that loss of neurogranin mRNA signal (a neuronal marker) and large numbers of Fluoro-Jade-B-positive neurons were observed in the brains of the animals that were severely intoxicated with soman.

Using diffusion-weighted MRI to assess brain damage in mice exposed to a convulsive dose of soman (172 μg/kg), Testylier et al (2007) demonstrated that three hours following the soman exposure there were changes in apparent diffusion coefficient (ADC) that were also compatible with edema in different brain regions. In agreement with the findings obtained from soman-exposed guinea pigs, the MRI and histopathological studies carried out in mice exposed to soman led to the conclusion that the cerebral edema induced by soman is primarily intracellular and cytotoxic.

Bhagat et al (2001; 2005) used T2-weighted imaging and diffusion weighted imaging to evaluate the evolution of brain pathology following exposure of adult male Sprague Dawley rats to a convulsive dose of soman (180-200 μg/kg; 1.8-2.0xLD50). The authors observed a significant 23% decrease in ADC at 12 h after soman exposure in the hippocampus and thalamus. In these rats, ADC returned to normal values in seven days. They also observed at 12 h post-soman exposure a significant decrease in T2 values, which returned to near normal values at 24 h. Although these studies lasted no longer than seven days, the acute ADC changes seen in the hippocampus and thalamus of rats that survived the exposure to soman were

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compatible with those seen in the follow-up studies of the victims of the Tokyo terrorist attack with sarin and were suggestive of disruption of the integrity of white matter.

Studies from numerous laboratories have also shown that the relative contribution of delta waves to the EEG power spectrum increases significantly following an exposure of rats, mice, guinea pigs, and non-human primates to convulsing doses of soman (Carpentier et al., 2001; McDonough et al., 1998). This increase in the slow delta EEG frequency resembles that described in the previous section for humans exposed to sarin and other OP compounds and appears to be a predicting factor of the neuropathology that eventually develops in the brain of these animals (Carpentier et al., 2001; McDonough et al., 1998). Also, as reported for workers who had been occupationally exposed to sarin (Duffy et al., 1979), an increase in beta activity was detected in the EEG of non-human primates one year after their exposure to sarin (Burchfiel and Duffy, 1982; Burchfiel et al., 1976).

In summary, it is clear that signs of acute OP intoxication in rats, mice, guinea pigs, and non-human primates closely resemble the clinical manifestations of acute OP intoxication in humans. Evidence also exists that delayed neurological deficits, particularly anxiety-related behavior and cognitive deficits, structural brain damage, and functional EEG alterations (specifically an increase of the slow EEG frequencies) seen in humans long after a single exposure to nerve agents can be reproduced in rats, mice, guinea pigs, and non-human primates exposed to these agents. The question, then, arises as to whether any one animal model is better than the others.

Undoubtedly, non-human primates, particularly macaques, hold a number of advantages over small mammals as animal models in biomedical research. The genetic, anatomic, physiological, behavioral, and metabolic similarities between humans and non-human primates

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make the non-human primates the most appropriate animal model of a variety of human conditions, including OP intoxication (Anderson, 2008; Lallement et al., 1998; Lenz et al., 2005; Sun et al., 2008). It is particularly noteworthy that, because the anatomy and physiology of the non-human primate brain are very close to those of humans, neurobehavioral tasks normally used to assess cognitive functions in humans (eg, delayed-matching-to-sample task) have been successfully adapted for non-human primates, and, thereby, increased the translational relevance of non-human primates in research (Buccafusco, 2008). Specifically as it relates to the field of OP toxicity, the sensitivity of non-human primates to different OPs closely resembles that of humans. For example, the intravenous LD50s of sarin in Rhesus monkeys and humans are 15 μg/kg and 14 μg/kg, respectively (Miller et al., 2004; Woodard et al., 1994). However, taking into consideration the principles of humane experimental research, less sentient species are needed, particularly, for proof-of-concept studies. In this respect, guinea pigs have stood out as an appropriate small animal model of OP intoxication. Specifically, the sensitivity of guinea pigs to OP compounds closely resembles that of humans. For example, the intravenous LD50 of sarin in guinea pigs (24 μg/kg) is only 1.7-fold higher than that reported for humans (Miller et al., 2004; Spruit et al., 2000). Furthermore, many anatomical features of the human brain are closely related to those in the guinea pig brain, making the guinea pigs a robust small, non-primate model in which to study multidimensional neurobehaviors of direct relevance to humans (see Lee et al., 2014 and references therein).

Most studies of the acute and delayed toxicity induced by an exposure to different levels of OP compounds have been carried out in rats and mice. The relatively small size, short generation time, and accelerated lifespan of rats and mice help to keep space and time required to perform a study manageable. In addition, most behavioral tests have been developed and

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validated in rats and mice, antibodies against thousands of proteins are commercially available for both species, and manipulation of mouse and rat DNA has proven to be feasible, allowing scientists to explore how specific genes function in health and disease (Broverhof et al., 2011; see also Lee et al., 2014 and references therein). However, the sensitivity of rats and mice to the acute toxicity of OP compounds is reportedly lower than that of humans. For example, the intravenous LD50s of sarin in rats and mice (45-63 µg/kg and 83 µg/kg, respectively) are approximately 3- to 6-fold higher than the intravenous LD50 of sarin in humans (14 µg/kg; John et al., 2009; Miller et al., 2004). The species-specific sensitivity to the toxic effects of OP compounds has been correlated with the activity of circulating carboxylesterases – the liver enzymes that catalyze the metabolism and inactivation of OP compounds. In fact, Maxwell and colleagues reported that inhibition of carboxylesterases by cresylbenzodioxaphosphorin oxide eliminates the species-related differences in the LD50 of soman (Maxwell et al., 1987).

The effectiveness of medical therapies against OP intoxication in humans is also species specific and can be more reliably predicted in guinea pigs and non-human primates than in mice and rats. For example, pralidoxime, a reactivator of OP-bound AChE, confers significant protection against soman intoxication in rats and mice, but not in guinea pigs and non-human primates (Inns and Leadbeater, 1983). Likewise, some clinical trials have reported the poor effectiveness of pralidoxime to treat acute poisoning of humans with OP compounds (Buckley et al., 2011). Thus, to standardize testing of antidotes (particularly pre-treatments) against OP intoxication, initial studies are generally carried out in rodents, including guinea pigs, and results are subsequently confirmed in non-human primates (Inns and Leadbeater, 1983; Maxwell et al., 1987).

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An ever growing number of epidemiological studies have provided evidence that *in-utero* exposure to OP pesticides is associated with increased prevalence of attention deficit hyperactive disorder, pervasive developmental disorder, and reduced IQ among children (reviewed in Rosas and Eskenazi, 2008). A recent MRI study also revealed significant structural abnormalities in the brain of children prenatally exposed to OP pesticide chlorpyrifos (Rauh et al., 2012). Thus, the availability of small animal models of OP-induced developmental toxicity is needed to prioritize and standardize safe and efficient medical therapies to treat even the most sensitive sector of the population in the event of a terrorist attack involving deployment of nerve agents or OP pesticides.

Although prenatal and/or early neonatal exposure of rats and mice to OP pesticides has been shown to compromise their brain development and cause neurocognitive deficits later in life that resemble those seen in humans exposed *in utero* to these compounds (Levin et al., 2001, 2002; Turgeman et al., 2011), striking differences exist between the CNS development of mice or rats and that of humans making it difficult to extrapolate sensitive gestational periods from rats and mice to humans. In short-gestation species such as the rat and mouse, the majority of CNS growth spurt occurs postnatally, generally within the first three weeks after birth. In long-gestation species, including guinea pigs, non-human primates, and humans, this growth spurt starts mid-gestation in the uterus, peaks during the last third part of pregnancy, and ends shortly after birth (Byrnes et al., 2001; Dobbing and Sands, 1970, 1973). Thus, the guinea pig emerges as a more appropriate rodent model system than rats and mice to study the deleterious effects of developmental exposure to OP pesticides and nerve agents.

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3. Molecular mechanisms that contribute to the acute and delayed toxicity of OP compounds

It is generally accepted that irreversible inhibition of AChE is the primary mechanism underlying the acute toxicity of OP pesticides and nerve agents. However, the fact that the prevalence of the various signs of acute toxicity varies among different OP compounds in humans and in animal models strongly suggests that direct interactions of these compounds with other molecular targets contribute to their acute toxicity. The most direct evidence that AChE is only one of the proteins targeted by the OP compounds came from studies of mice with a null mutation in the gene encoding AChE; injection of the OP compound diisopropylfluorophosphate or VX in these mice triggers a cholinergic syndrome identical to that seen in wild-type mice (Duysen et al., 2001; Xie et al., 2000).

Among the non-AChE molecular targets for OP compounds are the nicotinic and muscarinic receptors (Albuquerque et al., 1985; Bomser and Casida, 2001; Jett et al., 1991; Silveira et al., 1990). Direct interactions of OP compounds with nicotinic receptors at the neuromuscular junction lead to receptor desensitization that can contribute to the muscle weakness and paralysis seen in the acute phase of OP poisoning (reviewed in Albuquerque et al., 1987). In addition, direct interactions of the nerve agents sarin and soman with presynaptic muscarinic receptors in the hippocampus reduce GABAergic transmission onto CA1 pyramidal neurons (Chebabo et al., 1999; Santos et al., 2003), an action that can certainly contribute to the occurrence of seizures in subjects intoxicated with the nerve agents. AChE-independent reduction of neuronal M2 muscarinic receptor function by OP pesticides also potentiates bronchoconstriction induced by stimulation of the vagus in guinea pigs (Lein and Fryer, 2005), an action that can weigh in the respiratory distress induced by these compounds.

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The initial irreversible inhibition of AChE by OP compounds can trigger mechanisms that eventually lead to the neurotoxicity that develops either in the absence of an overt acute phase of intoxication or long after the acute phase of OP poisoning has resolved. Accumulated ACh resulting from OP-induced AChE inhibition has been linked to activation of a number of signal transduction mechanisms (Bodjarian et al., 1992; Savolainen and Hirvonen, 1992) that can contribute to time-dependent up- or down-regulation of expression of genes that code for proteins involved in sustaining cell viability. In fact, toxicogenomic studies have reported that expression of genes linked to neuroprotective and neurodegenerative pathways is altered time dependently following an injection of rats with either a sub-convulsive or a convulsive dose of sarin or soman (Damodaran et al., 2006; RamaRao et al., 2011; Spradling et al., 2011). In this respect, it is noteworthy that the frequency of sister chromatid exchanges was found to be significantly higher in human lymphocytes from victims of the 1995 sarin attack in Japan than in control subjects at two time points: 2-3 months and 3 years after the attack (Li et al., 2004). The increased frequency of sister chromatid exchanges in sarin-exposed humans who did not present with overt signs of acute toxicity is consistent with the notion that the nerve agent per se, in the absence of convulsions and/or hypoxia, can have a significant impact on gene expression.

Direct interactions of OPs with molecular targets other than AChE have also been proposed to play a key role in the development of delayed OP neurotoxicity. For example, OP compounds can decrease neuronal viability by disrupting the axonal transport of nutrients from the cell body to the axon terminals of neurons (Grigoryan et al., 2008; Prendergast et al., 2007). Cellular mechanisms underlying the OP-induced axonal transport impairment may include direct covalent binding of OP compounds to such structural proteins as tubulin, kinesin, and dynein (reviewed in Androutsopoulos et al., 2013; Terry, 2012). In fact, the ability of chlorpyrifos-oxon

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to inhibit the polymerization of tubulin has been associated with neurodegeneration in the hippocampus (Prendergast et al., 2007). Additional mechanisms that have been proposed to contribute to the delayed toxicity of OP compounds include exacerbated oxidative stress, imbalanced intracellular Ca^{2+} homeostasis, increased signaling mediated by inflammatory mediators such as interleukins and cytokines, changes in cellular signaling mediated by neurotrophin receptors and protein kinases, and mitochondrial disruption (reviewed in Androustsopoulos et al., 2013; Banks and Lein, 2012; Terry, 2012). Elucidating the cause-consequence relationship between the various cellular and molecular mechanisms known to be affected by specific OP compounds and the clinical manifestations induced by an acute, sub-acute, and/or chronic exposure to these compounds is a critical step towards the design and development of effective therapeutic interventions to treat OP toxicity.

The Food and Drug Administration (FDA) uses the *Animal Efficacy Rule* to approve medical treatments to reduce and/or prevent serious and/or life-threatening conditions caused by exposure to a permanently disabling toxic agent, such as OP pesticides and nerve agents, where human efficacy trials are not feasible or ethical. Based on this rule, the FDA can rely exclusively on animal studies to provide substantial evidence of drug effectiveness if: (i) the mechanisms of action of the toxicant are reasonably well understood; (ii) the effects of the toxicants and the effectiveness of the treatments can be demonstrated in animal species expected to react with a response predictive for humans; (iii) the animal study endpoints are clearly related to the desired benefits in humans; and (iv) data on the pharmacokinetics and pharmacodynamics of the treatments in animals and humans can allow selection of an effective dose for humans. In fact, it was on the basis of the *Animal Efficacy Rule* that in 2003 the FDA approved pyridostigmine

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bromide as a pre-treatment to prevent the toxicity of soman in military personnel under the threat of exposure to this agent (Aebersold, 2012).

Recognizing the limitations of available therapies against OP poisoning, we need to seek more effective medical countermeasures to treat and/or prevent the acute and delayed neurotoxicity of nerve agents and OP pesticides. Therefore, identification of animal models of the immediate and delayed clinical manifestations of an acute exposure of humans to OP compounds and their mechanisms of action is crucial for studies aimed at the discovery of effective medical countermeasures to treat and/or prevent those conditions.

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Authorship Contributions

All authors contributed equally to the writing of the manuscript.

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Footnotes

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