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Neurotoxicity in acute and repeated organophosphate exposure

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Abstract

The term organophosphate (OP) refers to a diverse group of chemicals that are found in hundreds of products worldwide. As pesticides, their most common use, OPs are clearly beneficial for agricultural productivity and the control of deadly vector-borne illnesses. However, as a consequence of their widespread use, OPs are now among the most common synthetic chemicals detected in the environment as well as in animal and human tissues. This is an increasing environmental concern because many OPs are highly toxic and both accidental and intentional exposures to OPs resulting in deleterious health effects have been documented for decades. Some of these deleterious health effects include a variety of long-term neurological and psychiatric disturbances including impairments in attention, memory, and other domains of cognition. Moreover, some chronic illnesses that manifest these symptoms such as Gulf War Illness and Aerotoxic Syndrome have (at least in part) been attributed to OP exposure. In addition to acute acetylcholinesterase inhibition, OPs may affect a number of additional targets that lead to oxidative stress, axonal transport deficits, neuroinflammation, and autoimmunity. Some of these targets could be exploited for therapeutic purposes. The purpose of this review is thus to: 1) describe the important uses of organophosphate (OP)-based compounds worldwide, 2) provide an overview of the various risks and toxicology associated with OP exposure, particularly long-term neurologic and psychiatric symptoms, 3) discuss mechanisms of OP toxicity beyond cholinesterase inhibition, 4) review potential therapeutic strategies to reverse the acute toxicity and long term deleterious effects of OPs.

Keywords

Pesticide; Cholinesterase inhibitor; Agriculture; Gulf War Illness; Aerotoxic Syndrome; memory

1. Introduction

The term organophosphate (OP) refers to a diverse group of chemicals that are typically derived from phosphoric, phosphonic and phosphinic acids. Prototypic OPs were first synthesized in the nineteenth century and related compounds are now found in hundreds of

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products that are used worldwide including pesticides, defoliants, fire retardants, industrial solvents, lubricants, plasticizers, fuel additives, prescription drugs, and nerve agents (see Table 1 for representative examples and reviews, Soltaninejad and Shadnia, 2014; Costa 2018). OPs are particularly common among the group of compounds generically known as pesticides, which includes insecticides, anthelmintics, fungicides, and herbicides. These compounds have contributed to improvements in agricultural productivity across the world for decades through higher crop yields and improved product quality by controlling, insects, nematodes, and plant pathogens. Moreover, they also reduce the amount of labor, machinery, and fuel used for mechanical weed control (reviewed, Fernandez-Cornejo et al., 2014). While the extensive use of OP-based pesticides is often criticized due to the risks associated with their toxicity to humans and other non-target species, an important role of OPs in agriculture is likely to continue in the near future. This is due to their high effectiveness and broad spectrum against numerous types of pests, the availability of generic OP-based products of low cost, and the decades of experience with their use (reviewed, Casida and Durkin, 2013; see also Costa, 2018). Some commonly used OPs (e.g., chlorpyrifos) have additional advantages such as a relatively short persistence in the environment after application and chemical characteristics that provide flexibility for use in multiple delivery systems (Dow AgroSciences, 2017; reviewed, Solomon et al., 2014).

In addition to optimizing agricultural productivity, the value of OPs in the control of deadly vector-borne illnesses is also clear (reviewed, Cooper and Dobson, 2007). For example, along with pyrethroids, organochlorines, and carbamates, OPs continue to play a major role in the prevention regimen against one of the world's most deadly diseases, malaria (World malaria report 2017). In at least some regions of the world OPs may offer distinct advantages over the other aforementioned insecticides. For example, in standard insecticide susceptibility testing across western Kenya it was determined that the *Anopheles gambiae* mosquito had acquired high resistance to pyrethroids and the organochlorine DDT, patchy resistance to carbamates (bendiocarb), but no resistance to the OP malathion (Wanjala et al., 2015). Notably, *Anopheles gambiae* is one of the most important vectors of malaria in sub-Saharan Africa. OP insecticides (e.g., malathion and naled) are also commonly used successfully by state and local agencies in the United States for adult mosquito control to prevent the spread of Zika, West Nile Virus, dengue, and chikungunya (Petersen et al., 2013; CDC 2017; EPA 2017). While the number of OP-based commercial products available to the public has decreased to some extent in the last several years, they continue to be available in a variety of products to combat home and garden pests (e.g., flies, roaches, ants, mosquitoes, etc.).

2. Risks associated with OP exposure

Please see Fig 1 for an illustration of several representative sources whereby humans and other non-target species may be exposed to toxic levels of OPs. Systemic absorption of OPs can occur by inhalation and after mucous membrane, dermal, conjunctival, and gastrointestinal exposure (Burillo-Putze and Xarau, 2016).

2.1 OP-Pesticides

As a consequence of their widespread use as pesticides, OPs (and their residues) are now among the most common synthetic chemicals detected in rivers, groundwater, soil, air, and plants, as well as in animal and human tissues worldwide (Schnoor, 1992; Davisson et al., 2005; Barr et al., 2011; Clune et al., 2012; Voorhees et al., 2017). This fact has become an increasing environmental concern because of the deleterious health effects of OPs that have been documented in both adults and children for decades (reviewed, Rohlman et al., 2011; Reiss et al., 2015; Worek et al., 2016; Masson and Nachon, 2017, see also section 3 below). In many places in the world, governments have had some success at reducing toxic exposures to OPs by imposing stricter regulations on their use. However, in some Eastern Mediterranean, Asiatic, and Western Pacific countries (especially those that are heavily reliant on agriculture), accidental OP exposures and suicidal ingestions remain as a persistent problem that may actually be worsening (Tsatsakis et al., 1996; Tsatsakis et al., 2008; Jin et al., 2010; Kumar et al., 2010; Costa, 2018; Abhimanyu and Madhan, 2018; reviewed, Pope, 1999; Iyer et al., 2015Gunnell et al., 2007.

2.2 OP Nerve Agents as Components of Chemical Weapons

While the risk of toxicity from pesticide exposures is considerably higher for most people in the world, the threat from intentional poisonings with OPs by rogue governments and terrorists is also an ongoing concern (reviewed, Eisenkraft and Falk, 2016). Over the last 35 years, there have been multiple (well-documented) cases where OP-nerve agents were used against military soldiers and/or civilians. For example, in the 1980s the Iraqi military attacked Iranian military soldiers (Majnoon Island) and Kurdish civilians (Halabja) with OP-based nerve agents producing casualties estimated to be as high as “tens of thousands” (Barnaby, 1988; Macilwain, 1993; O’Leary, 2002; Hawrami et al., 2004). The Tokyo Sarin attack in March of 1995 by the domestic terrorist group Aum Shinrikyo resulted in the deaths of 12 people and the emergency medical evaluation and/or treatment of more than 5,000 other individuals (Suzuki et al., 1995; Nagao et al., 1997). The Organization for the Prohibition of Chemical Weapons (OPCW) and the United Nations have now concluded that Sarin has been used against civilians in Syria on multiple occasions (Sellström et al., 2013; Guterres, 2017), and the fatal sarin poisoning in 2013 has also been verified by a network of international laboratories (John et al., 2018). The recent news reports of the use of the OP VX in the assassination of the North Korean dictator’s half-brother in Malaysia (Latiff and Chow, 2017) and the attempted assassination of a former Russian military intelligence officer and his daughter with the OP Novichok in Britain (Smout and Holden, 2018) demonstrates the continued risks of intentional OP attacks on a global scale.

2.3 OPs and Gulf War Illness

There is now a large body of published evidence indicating that approximately 25–32% of the United States (US) veterans who served in the 1990–1991 Persian Gulf War (Operation Desert Shield and Operation Desert Storm) are affected by a constellation of chronic health symptoms now known collectively as Gulf War Illness (GWI). These symptoms have not been commonly observed in US veterans of the same era who did not deploy to the Gulf region or veterans who were deployed in other areas of the world (e.g., Bosnia, Germany).

However, they have been reported in veterans from other countries who also participated in the Gulf War (United Kingdom, Canada, Australia, Denmark, and France, reviewed, Iverson et al., 2007; White et al., 2016). The symptoms of GWI are diverse and may include unexplained fatigue, headaches, respiratory problems, musculoskeletal pain, gastrointestinal distress, skin rashes, and a variety of neurological and neuropsychiatric problems including cognitive impairment (Sullivan et al., 2018).

While a variety of possible contributing factors to GWI symptoms have been discussed (heat, stress, vaccinations, smoke from oil well fires, infectious organisms), a particularly plausible explanation for the neurological-based symptoms is exposure to one or more acetylcholinesterase inhibitors (AChEIs) (see Golomb, 2008, reviewed, White et al., 2016). It has been estimated that at least 41,000 military personnel were over-exposed to insecticides that contained either carbamate or OP-based AChEIs (Winkenwerder, 2003). For general military personnel, sources of exposures included fly baits, pest strips, sprayed liquids and powders and for military pesticide applicators, pesticide fogs and prisoner delousing compounds were additional sources of exposure. In addition to OP-pesticides, exposures to OP-nerve agents may have also been a contributing factor to GWI. It is now well documented that as many as 100,000 soldiers may have been exposed to low (i.e., non-acutely toxic) levels of sarin/cyclosarin following the destruction of an Iraqi munitions storage complex at Khamisiyah, Iraq, in March 1991 (Berardocco, 1997).

It is important to note, however, that more than two decades after the Gulf War, GWI remains a controversial topic (reviewed, Nettleman, 2015; Reardon, 2015). Members of the United States Institute of Medicine (IOM) and other scientists continue to argue that there is not enough evidence implicating either the prophylactic agent pryidostigimne or pesticide use as conclusive enough to assign causality. In particular, they cite the lack of objective exposure data, the need to rely on self-reports, and a lack of consistency of symptoms across patients as barriers to establishing causality.

2.4 OPs and Aerotoxic Syndrome

For more than 20 years, there have been reports from around the world from both airline crewmembers and some passengers of sickness following exposure to toxic fumes in airplane cabins. The symptoms (now collectively referred to as “Aerotoxic Syndrome”, reviewed Winder and Balouet, 2000) are diverse and include both short and long-term effects. Examples include ear/nose/throat irritation, skin conditions, nausea and vomiting, respiratory problems, headaches, dizziness, weakness and fatigue, sensory changes and nerve pain, tremors, chemical sensitivity and cognitive impairment (see reviews, Harrison and Mackenzie Ross, 2016; Michaelis et al., 2017). Aerotoxic Syndrome is thought to occur after the cabin air of an airplane (which is “bled” in from the engines to pressurize it), is compromised due to mechanical failures, faulty seals, the overfilling of oil or hydraulic reservoirs etc. This bleed air during the so-called “fume event” can be contaminated with heated engine oil fumes that contain a variety of hazardous chemicals (e.g., phenyl-naphthylamine, toluene, xylenes and the OPs, tri-butyl phosphate and tricresyl phosphate-TCP). Of these chemicals, the tri-ortho-cresyl phosphate (ToCP) isomer of TCP (an anti-wear additive to jet engine oil), has been the most commonly implicated and studied since it

is a known neurotoxin associated with chronic OP-induced delayed polyneuropathy (OPIDP) (see Costa, 2017, see also section 3 below). It should be noted, however, that the published evidence collected thus far to support a causal connection between TCP or TOCP and the symptoms associated with aerotoxic syndrome is a subject of debate (see reviews, de Boer et al., 2015; Costa, 2017). The concentrations of these compounds in flight deck and cabin air detected thus far are very low (de Ree et al., 2014). Likewise, the levels of urine metabolites and butyrylcholinesterase adducts of these compounds in aircrews and airplane passengers, respectively, was very low (Schindler et al., 2013; Liyasova et al., 2011). Moreover, no significant inhibition of lymphocytic neurotoxic esterase (NTE) or erythrocyte AChE activity was observed in symptomatic flight crewmembers after fume events (Heutelbeck et al., 2016). In primary rat cortical neurons in culture, biochemical, morphological and electrophysiological data demonstrated that TCP isomers and mixtures induced no or only limited cytotoxicity at concentrations up to 100 μM, concentrations well above estimated systemic levels based on recent measurements of cabin air concentrations of TCPs (Duarte et al., 2017).

2.5 OPs and Incidence of Neurodegenerative Diseases

A potential role of pesticides of different classes (including OPs) in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis has long been suspected since they share common features such as the ability to induce oxidative stress, mitochondrial dysfunction, and neuronal cell loss (reviewed, Zaganas et al., 2013; Sanchez-Santed et al., 2016; Yan et al., 2016). Moreover, the notion that gene x environment interactions (e.g., epigenetic risk factors where the environmental factor is pesticide exposures) contribute to the etiology of neurodegenerative diseases is a particularly attractive hypothesis. Interestingly, some epidemiological studies have specifically linked OP exposures to the risk of Alzheimer's disease (Hayden et al., 2010). However, the epidemiological studies are unable to provide a causal connection between OP exposure and neurodegenerative illnesses and reviews of the literature point to inconsistencies and lack of strong mechanistic arguments (see Baltazar et al., 2014; reviewed, Sanchez-Santed et al., 2016). Similar limitations appear to exist in the animal literature (at least as it applies to AD). For example, Peris-Sampedro and colleagues conducted several studies with chlorpyrfos using transgenic mice that express the human abeta protein or the apoE2, apoE3 and apoE4 isoforms. In each study, chlorpyrifos was associated with cognitive dysfunction (spatial learning and memory deficits, Peris-Sampedro et al., 2014; 2015, deficits in sustained attention, Peris-Sampedro et al., 2016). However, there was no clear connection between the cognitive deficits and specific risk factors for human AD (abeta protein, apoE4 allele).

3. Long Term Neuropsychiatric Effects of Acute and Repeated Exposures to OPs

The acute toxicity of OPs in humans has been associated with a host of central nervous system, cardiovascular, respiratory, gastrointestinal, sensory, and motor manifestations (see reviews, Bardin, 1994; Collombet, 2011; Pereira et al., 2014). Depending on the exposure level, some of the acute effects of OPs including excessive secretions, cardiorespiratory

depression, and seizures can be life threatening. However, acute exposures to OPs can also lead to a variety of long-term neurological and psychiatric consequences in survivors. These consequences can include motor impairments, psychotic episodes, depressed mood, as well as deficits in signal detection and information processing, sustained attention, memory, sequencing and problem solving, abstraction, and cognitive flexibility (Savage et al., 1988; Rosenstock et al., 1991; Steenland et al., 1994; Dassanayake et al., 2007, Pereira et al., 2014). Chronic exposure to Ops can also be associated with neurologic and psychiatric abnormalities including motor dysfunction (extrapyramidal symptoms) deficits in eye-hand coordination and reaction time, anxiety, depression, psychotic symptoms, deficits in attention, information processing, and learning and memory (Amr et al., 1997; Salvi et al., 2003; Stephens et al., 1995; and reviewed Singh and Sharma, 2000). These symptoms are collectively known as chronic OP-induced neuropsychiatric disorders (COPIND).

In addition to the consequences of acute and chronic OP poisoning described above, a variety of long-term effects of repeated exposures to levels of OPs below the threshold for acute “cholinergic” toxicity have been documented. This type of exposure is relatively common in agricultural workers and pesticide sprayers and it has been associated with persistent alterations in psychomotor speed, executive function, visuospatial ability, working and visual memory (see Ross et al., 2013). Many of the symptoms described above for GWI and Aerotoxic Syndrome may also be result of OP exposures that were below the threshold for symptoms of acute toxicity.

Prospective animal studies support the clinical and epidemiological observations described above and indicate that chronic (or repeated) exposures to OPs at levels that are not associated with acute toxicity can result in a variety of neurobehavioral symptoms, particularly cognitive deficits (reviewed, Terry, 2012).

Several epidemiological studies have also found associations between prenatal or early postnatal exposure to OPs and behavioral abnormalities, particularly deficits in attention, learning and memory (see reviews, Eskenazi and Castorina, 1999; Eaton et al., 2008; Muñoz-Quezada et al., 2013; Gonzalez-Alzaga et al., 2014; Reiss et al., 2015). Here it is important to note that exposure to OPs, as revealed in biological monitoring studies in children, was elevated in inner cities and farming communities, but at levels below those causing significant AChE inhibition (reviewed in Costa, 2017). There is also considerable evidence from animal studies to suggest that both prenatal and early postnatal exposure to OPs can result in a variety of protracted cognitive impairments (Levin et al., 2001; reviewed, Slotkin 2004; Slotkin 2005; Timofeeva et al., 2008). Moreover, these impairments can become more pronounced as the exposed subject ages (Levin et al., 2010). These adverse developmental effects of OPs, however, with few exceptions, were only observed at levels causing significant AChE inhibition (reviewed Eaton et al., 2008; Timofeeva and Levin, 2010).

4. Mechanisms of the Long Term Neuropsychiatric Effects of Acute and Repeated Exposures to OPs

4.1 Acetylcholinesterase Inhibition

In the case of acute OP poisoning, the mechanism of both the acute symptoms of OP toxicity and the long-term neurological consequences has been attributed to the inhibition of acetylcholinesterase (AChE). This inhibition leads to marked elevations in synaptic acetylcholine levels which in turn lead to excessive stimulation of both muscarinic and nicotinic acetylcholine receptors (reviewed Ecobichon, 2001; Pereira et al., 2014). However, it is important to note that COPIND can occur without antecedent cholinergic symptoms and it does not necessarily appear to be dependent on acetylcholinesterase inhibition (Brown and Brix, 1998; reviewed, Ray and Richards, 2001 and Singh and Sharma, 2000).

While the inhibition of acetylcholinesterase undoubtedly plays a key role in both the acute and long-term toxicology of OPs, there is nearly twenty years of experimental evidence to support the argument that this mechanism cannot alone account for the wide range of symptoms that have been reported (reviewed, Pope, 1999; Duysen, et al., 2001; Terry, 2012; Burke et al., 2017). OPs are known to affect literally hundreds of enzymes, receptors, and other proteins and it is becoming increasingly evident that some of these targets may be especially relevant to the long-term CNS effects of chronic OP exposure and developmental neurotoxicity. Consequently, interactions with these targets should be considered as part of a cumulative risk assessment of OPs (reviewed, Costa, 2018). In Table 2, we have provided a referenced (representative) list of potential non-acetylcholinesterase targets of OPs and their known or theoretical functions. In Fig 2, we have illustrated potential consequences of OP interactions with some of these targets (i.e., axonal transport deficits, oxidative stress, neuroinflammation, and autoimmunity). In the text below, we have provided an overview of the effects of OPs beyond acetylcholinesterase inhibition, which includes interactions with particular targets as well as physiological processes that these targets are known to influence. It is also important to note that acetylcholinesterase inhibition can occur in the absence of acute OP toxicity, but not vice versa; therefore the non-acetylcholinesterase targets discussed could be affected in both the case of acute-high level exposure as well as repeated lower level exposure, however, in the acute setting the non-acetylcholinesterase related physiologic effects might be difficult to distinguish. While each of these putative (noncholinergic) mechanisms of toxicity are discussed separately, it is unlikely that they are mutually exclusive and they may converge to induce deleterious effects.

4.2 Axonal Transport Deficits

Axonal transport is a fundamental process within neurons, whereby various organelles, membrane bound vesicles, and additional cargoes are transported along microtubules between the cell body and distal tip of the axon. The process is essential to neuronal development and axonal outgrowth as well as the maintenance and function of fully developed neurons (reviewed, Maday et al., 2014). Early indicators that OPs might impair axonal transport were evident in a report where relatively high doses of OPs (i.e., phenylphosphonothioate esters and TOCP) known to be associated with OPIDN impaired fast anterograde axonal transport in a rat optic nerve preparation (Reichert and Abou-Donia,

1980). Later studies in our laboratories (Terry et al., 2003; Terry et al., 2007) indicated that both anterograde and retrograde transport of vesicles in the sciatic nerves (*ex vivo*) were impaired in rats repeatedly exposed to chlorpyrifos, an OP not associated with OPIDN except at doses well above the LD50 (reviewed, Richardson, 1995). Importantly, the chlorpyrifos doses in these studies were below the threshold for acute toxicity and the deficits in axonal transport were persistent (detected for up to 14 days after the last chlorpyrifos injection). In more recent studies in our laboratory, using time-lapse imaging techniques in primary neuronal culture, we have observed impairments in the axonal transport of mitochondria (Middlemore-Risher et al., 2011) as well as membrane bound organelles (MBOs) (Gao et al., 2016) in axons associated with both chlorpyrifos and its metabolite CPF-oxon. Similar impairments in vitro were observed with the nerve agent diisopropylfluorophosphate (DFP) (Gao et al., 2016). In all of these in vitro studies, the impairments in axonal transport could be detected at concentrations that did not inhibit acetylcholinesterase activity and they were not blocked by cholinergic receptor antagonists. These data indicate that OPs with considerably different chemical structures (alkylphosphate versus the oxon metabolite of a phosphorothioate) can exhibit quite similar profiles of fast, anterograde axonal transport impairments at very low concentrations. Importantly, we have also observed persistent deficits in axonal transport in vivo in living rats exposed to chlorpyrifos or DFP using manganese-enhanced magnetic resonance imaging (MEMRI) (Hernandez et al., 2015; Naughton et al., 2018).

Other indications of OP-induced dysfunction in axons are evident in studies in vitro demonstrating decreased axonal outgrowth after exposure to chlorpyrifos or chlorpyrifos-oxon (CPO) (Howard et al., 2005; Yang et al., 2008). Chlorpyrifos-oxon has also been shown to disrupt axonal growth as well as motor behavior in zebrafish (Yang et al., 2011).

The mechanisms of the OP-related deficits in axonal transport noted above are unclear, but several hypotheses have been developed. There is now a significant amount of evidence that OPs may alter the function of motor proteins such as kinesin and/or components of the neuronal cytoskeleton (e.g., microtubules) that are important for axonal transport. In vitro evidence that OPs might interact with kinesin to negatively affect kinesin-driven axonal transport is evident in microtubule motility experiments where an increase in the number of locomoting microtubules that detached from kinesin-coated glass when kinesin was preincubated with the OPs chlorpyrifos, chlorpyrifos-oxon, or DFP was observed (Gearhart et al., 2007). These data suggested that OPs modify kinesin and weaken the kinesin-microtubule interactions that facilitate anterograde axonal transport. This hypothesis was supported by a later mass spectrometry study where (using the biotin-tagged OP agent, FP-biotin) OP binding to tyrosine in the human kinesin 3C motor domain was demonstrated (Grigoryan et al., 2009)

There is also evidence that OPs impair tubulin polymerization and other factors that can affect microtubule assembly and stability, which in turn leads to impairments of axonal transport. For example in spectrophotometric studies, Prendergast et al., 2007 demonstrated that chlorpyrifos-oxon inhibited the polymerization of tubulin, and, moreover, utilizing organotypic slice cultures of rodent brain and histological methods that chlorpyrifos-oxon caused a marked decrease in the concentration of microtubule associated protein-2 (MAP-2).

Interestingly, multiple OPs (soman, sarin, DFP, CPO, FP-Biotin) have been shown to covalently bind to tyrosine residues on tubulin, an effect that may explain the disruptions in tubulin polymerization (Grigoryan et al., 2008; Jiang et al., 2010).

Other studies suggest that OPs can trigger changes in the post-translational modifications of molecules that are important for axonal transport. As an example, DFP was recently shown to decrease tubulin acetylation to impair the axonal movements of mitochondria in both cultured rodent and human derived neurons. The effect of DFP was exacerbated by corticosterone or cortisol, in rat and human neurons, respectively (to mimic stress), and these negative effects were attenuated by tubacin, a drug that inhibits HDAC6, the tubulin deacetylase (Rao et al., 2017). Moreover, our recent study indicated that chronic exposure to chlorpyrifos leads to a significant and persistent inhibition of tubulin acetylation in the brain of rats (Yang et al., 2018).

Finally, decreases in retrograde axonal transport related proteins dynein and dynactin have been observed in the spinal cord and cerebral cortex of hens after exposure to acutely toxic doses of TOCP (Song et al., 2012).

4.3 Oxidative Stress and Neuroinflammation

It is well accepted that OPs, particularly at high concentrations, can increase oxidative stress and DNA damage to cells, (reviewed, Soltaninejad and Abdollahi, 2009; Pearson and Patel, 2016; Vanova et al., 2018). In addition, chlorpyrifos, can induce lipid peroxidation in the developing rat brain at concentrations that only cause mild signs of systemic toxicity (Slotkin, 2005). Chronic low-level exposure to dichlorvos in adult rats has been shown to induce apoptotic neurodegeneration by raising mitochondrial Ca^{++} levels, impairing the activity of mitochondrial complexes I, III and IV, and increasing oxidative stress (Kaur et al., 2007). The OP-exposure regimen in this study was also associated with an increase in lipid peroxidation and decreases in the mitochondrial antioxidants glutathione and superoxide dismutase. Moreover, long-term exposure by rabbits to the OPs, diazinon or propoxur (at doses not associated with overt signs of acute toxicity) resulted in oxidative stress, histopathological lesions, and genotoxic effects in the liver and kidneys (Tsitsimpikou et al., 2013). Low-level repeated exposure to chlorpyrifos has also been shown to induce inflammatory responses in cultured astrocytes (i.e., increases in IL-6, GFAP, and p-ERK1/2) (Mense et al., 2006). Acute and chronic exposures to multiple OPs have also been shown to trigger neuroinflammation via microglia activation and increases in proinflammatory cytokines in mice (reviewed, Banks and Lein, 2012; Viviani et al., 2014), as well as in 3-dimensional brain cell cultures (Monnet-Tschudi et al., 2007). In a mouse model developed to mimic Gulf War Illness (GWI), acute exposure to DFP was found to produce a strong inflammatory response across multiple brain regions. Increased levels of mRNA for the inflammatory markers TNF-α, IL6, CCL2, IL-1β, LIF and OSM were reported and interestingly, these effects were potentiated by pretreatment with the stress related hormone corticosterone (O'Callaghan et al., 2015). A follow-up (GWI-related) study in rats later demonstrated that acute DFP exposure increased the expression of a number of cytokine-related genes associated with inflammation (IL-6, IL1β, CCL2 etc.) in a manner that was exacerbated by corticosterone pre-treatment. Using a novel high-order diffusion magnetic

resonance imaging (MRI) technique, alterations in micro-scale diffusivity in the frontal cortex and hippocampus were found to accompany the alterations in cytokine mRNA expression (Koo et al., 2018). The authors noted that the association of these diffusion changes with neuroinflammatory cytokine expression indicates the potential for GWI-relevant exposures to result in connectivity changes in the brain.

Some studies suggest that the increases in inflammation observed after OP exposure may be directly related to changes in oxidative stress. For example, chlorpyrifos exposure can lead to the induction of the NLRP3 inflammasome in a manner that is reversible by the blockade of mitochondrial reactive oxygen species (mROS). Stimulation of mROS by chlorpyrifos is thought to be first step in a process by which mROS-induced inflammation can lead to pyrotosis and apoptosis in human keratinocyte HaCat cell lines (Jang et al., 2015). More recent experiments in rats demonstrated that increased levels of oxidative stress markers (GSH, GSGG, 3-NT/Tyrosine) in the brain after acute DFP exposure were accompanied by increases in inflammatory cytokines (TNF-α, IL1-β, IL-6, KC/GRO) (Liang et al, 2018). Interestingly, DFP induced changes in both oxidative stress and inflammation could be attenuated by treatment with a catalytic antioxidant. Similar findings in which antioxidant treatment was able to attenuate an increase in inflammatory markers elicited by exposure to the OP malathion have also been reported (Mohammadzade et al., 2018). Furthermore, the co-occurrence of oxidative stress and inflammation has been reported in rats exposed to chlorpyrifos, an effect that could be reduced by a drug with known antioxidant properties (Abolaji et al., 2017). Please see further discussion to support the relationship between neuroinflammation and oxidative stress in the “Potential Therapeutic Strategies” section below.

4.4 Autoimmunity

There is a small, but evolving body of literature to suggest that OP exposures may lead to autoimmune responses in some individuals, effects that might offer one explanation for why OP exposures can lead to chronic (in some cases lifelong) symptoms. Examples come from studies designed to identify peripheral biomarkers of neurotoxicity in veterans with GWI and farmers exposed to pesticides who developed neurological symptoms or evidence of neurological damage (Abou-Donia et al., 2017; El Rahman et al., 2018). In the Abou-Donia et al. study, the serum of 20 veterans with GWI and 10 non-veteran symptomatic (low back pain) controls were screened for the presence of autoantibodies to central nervous system-specific proteins. In the El Rahman et al. 2018 study, serum samples from 50 farmers chronically exposed to different mixtures of pesticides (including a variety of OPs) and 25 non-exposed controls were analyzed. In each study, higher levels of autoantibody reactivity against the following proteins were detected in both the veterans with GWI and the pesticide exposed farmers (compared to controls): neurofilament triplet proteins (NFP), tubulin, microtubule associated tau proteins (Tau), microtubule associated protein-2 (MAP-2), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP), and calcium-calmodulin kinase II (CaMKII). The findings of these two studies were similar to a previous study (Abou-Donia et al., 2013) of flight crewmembers who experienced symptoms now referred to as “Aerotoxic Syndrome” and who reported being exposed to cabin air emissions which may have contained OPs. Collectively, while the

three human studies described here were relatively small and retrospective, they support the argument that OP exposure may lead to the generation of autoantibodies that target proteins, especially those known to play important roles in both the structure and function of neurons including myelination and axonal transport.

4.5 Gene Expression

Recent advances in gene sequencing have made it possible to simultaneously evaluate the expression of hundreds of different genes in a single study (reviewed, Wang et al., 2009). Due to the ability of OPs to modify a number of different targets, such studies may be of particular utility in understanding the vast array of OP-induced toxic effects. Alterations in the expression of hundreds of different genes in the rat hippocampus have been recently reported via microarray and RNA-seq analysis after repeated chlorpyrifos exposure (Lee et al., 2016). In particular, alterations in six mRNAs were found to be common among multiple transcriptomics analyses (Bdnf, Cort, Crhbp, Nptx2, Npy and Pnoc). The genes affected by chlorpyrifos exposure are involved in the expression of neurotrophins and secreted neuropeptides. Alterations in the expression and function of the aforementioned neurotrophins and neuropeptides have been associated with a range of cognitive and psychiatric disorders (Lee et al., 2016) and thus could offer one explanation for the cognitive and psychiatric disturbances in OP-exposed individuals. Furthermore, a recent study demonstrated that chronic, low level exposure to the OP diazinon in adult rats induces changes in the expression of genes associated with psychological disorders and cell-to-cell signaling specifically involving glutamatergic and monoaminergic neurotransmission (Savy et al., 2018).

In vitro changes in gene expression in response to both acute and long-term exposure to OPs have also been reported. For example, multiple changes in the expression profiles of genes related to apoptosis and necrosis were observed after 24hrs of acute, or 14 days of repeated, exposures to chlorpyrifos in SN56 cells (Moyano et al., 2017). Given that the SN56 cell line is derived from cholinergic septal neurons, it is believed that the reported changes in cell death related pathways may underlie cholinergic neurodegeneration and may be responsible for previously reported deficits in learning and memory after chlorpyrifos exposure.

5. Potential Therapeutic Strategies

5.1 Antioxidants

Given the aforementioned perturbations in mitochondrial function and related oxidative stress, the use of antioxidants to treat OP related dysfunction has become a growing field of study. In support of this premise, the mitochondria-targeted antioxidant MitoQ attenuated dichlorvos-induced ROS production, it increased MnSOD activity and glutathione levels, and it decreased lipid peroxidation, protein and DNA oxidation in rats. MitoQ also suppressed dichlorvos-related DNA fragmentation, cytochrome c release and caspase-3 activity and it attenuated mitochondrial swelling, loss of cristae and chromatin condensation (Wani et al., 2011). More recently, the antioxidant carotenoid compound crocin was shown to be protective against parkinsonian-like motor deficits resulting from chronic exposure to the OP malathion in rats (Mohammadzadeh et al., 2018). In addition, the catalytic

antioxidant AEOL10150 was shown to reduce markers for oxidative stress, inflammation, and cell death after administration of an acute, seizure-inducing, dose of DFP in rats. The neuroprotective nature and blood-brain-barrier permeability of AEOL10150 makes it a particularly strong candidate as a potential therapeutic (Liang et al., 2018). Further evidence for the benefit of antioxidants in treating OP toxicity has been provided by studies in rats using nanoparticles to deliver coenzyme Q10 (CoQ10). Eftekhari and colleagues demonstrated that nanoparticle based delivery of CoQ10 is effective in protecting against dichlorvos induced hepatotoxicity. Here, the authors demonstrated protection against mitochondrial dysfunction and the generation of reactive oxygen species with increased bioavailability over traditional CoQ10 (Eftekhari et al., 2018).

Many compounds extracted from plants of the *Zingiberaceae* (ginger) family are also well known for their medicinal value. Curcumin is one such compound, and is an extract from *Curcuma longa* (Turmeric). Curcumin pretreatment in SH-SY5Y and IMR-32 cells in vitro was shown to reduce oxidative stress caused by a synergistic interaction between amyloid beta and the commonly used OPs monocrotophos or chlorpyrifos. The positive effects of curcumin in this study were reported to depend on activation the Nrf2-antioxidant response element-signaling pathway (Sarkar et al., 2017). Due to its activity in both antioxidant and DNA repair settings, curcumin may thus make an effective prophylactic treatment against OP exposure (Sarkar et al., 2017). Other compounds of the *Zingiberaceae* family may also be effective therapeutics against OP toxicity by acting in a multifunctional manner. Due to the wide variety of targets affected by OPs, compounds with the ability to target both multiple molecular pathways as well as multiple organs may be of particular utility. 6-Gingerol-rich fraction (6-GRF) from *Zingiber officinale*(Garden Ginger) was recently shown to be protective against chlorpyrifos-induced oxidative stress (H2O2 and MDA) and disruption of antioxidant related proteins (catalase, GST, GSH, SOD, GPx) in rats. Moreover, 6-GRF reduced CPF-related increases in inflammatory (NO, MPO and TNF-α) and apoptotic (caspase-3) markers (Abolaji et al., 2017).

5.2 Axonal Transport: Microtubule and Signaling Kinase Targeted Therapies

Increasing microtubule stability may serve as another potential strategy for reversing OP induced deficits in axonal transport and cognition. While no data are currently available regarding OP exposures and microtubule-target therapeutic agents, studies in other disease contexts may be relevant. For example, the microtubule stabilizing compound epitholone D has previously shown efficacy in reducing axonal dysfunction as well as improving axonal transport and cognitive performance in a transgenic mouse model for Alzheimer's disease (Zhang et al., 2012; Brunden et al., 2010). Tubulin acetylation may be another potential target for reversing OP induced deficits in axonal transport and microtubule dysfunction. It was recently shown that chronic exposure to CPF can lead to decreased acetylation of α-tubulin at Lys-40 (Yang et al., 2018) in rats. Similarly, Rao et al demonstrated reductions in tubulin acetylation after DFP exposure in both cultured rat and human (stem cell derived) neurons. Moreover, the effect of DFP was reversed by treatment with the HDAC6 inhibitor tubacin (Rao et al., 2017).

In addition to microtubule-associated proteins and post-translational modifications, a number of different kinases can also act as regulators of axonal transport. A recent review identified 10 different kinases that could regulate various aspects of axonal transport (JNK, Cdk5, GSKIIIβ, ERK1/2, PKA etc.), including the phosphorylation of motors, adapter proteins, and cargoes (reviewed, Gibbs et al., 2015). Accordingly, some of these kinases could be targeted by new therapeutic agents to improve axonal transport.

5.3 Additional Strategies against Acute OP Toxicity

The current standard of care for treating acute poisoning with OPs includes airway support (intubation), the muscarinic antagonist atropine, the AChE reactivating agent pralidoxime (2-PAM), and benzodiazepines (e.g., diazepam) to control seizures (reviewed, Kats and Brooks 2017). Unfortunately, the mortality rate with this regimen is relatively high and the efficacy of some components of this regimen (especially 2-PAM) has been challenged (reviewed, Eddleston & Chowdhury, 2016). Accordingly, there remains a need for more effective therapeutic regimens and several relatively recent studies have identified new (potential) approaches. For example, blockade of intracellular Ca2+ release following OP induced status epilepticus has shown to be an effective strategy for neuroprotection against OP induced toxicity. As an example, the compounds dantrolene and carisbamate reduced hippocampal cell death resulting from a seizure inducing exposure to the OP paraoxon in rats via the inhibition of a post injury Ca2+ plateau (Deshpande et al., 2016). N-acetylcysteine (NAC) is an antioxidant compound used in the treatment of acetaminophen overdose, which may also be a useful adjunct to the traditional standard of care after acute OP exposure. In a randomized, controlled, parallel-group clinical trial, intravenous NAC was shown to reduce the amount of atropine required for symptomatic relief after acute organophosphate poisoning (El-Ebiary et al., 2016). Recently it has also been shown that the essential omega-3 polyunsaturated fatty acid alpha-linolenic acid can rescue soman induced behavior deficits in rats and to reduce rates of corresponding cell death in multiple brain regions (Pan et al., 2015). Catalytic bioscavengers represent another potential route for protecting against OP toxicity, especially in a prophylactic context. Bacterial derived enzymes with phosphotriesterase (PTE) are highly efficient in the degradation of OPs, but present certain immunogenic concerns. Similar, human derived, enzymes such as paraoxonase-1(PON1) represent additional possibilities with less immunogenic concerns. In particular, the PON1 mutant IIG1 may offer prophylactic protection against a broad range of OPs (reviewed, Iyer et al., 2015). The use of butyrylcholinesterase as a bioscavenger to remove OPs from circulation, thus preventing their toxic effects is another potential avenue for therapeutic intervention (see review, Pope and Brimijoin, 2018). Equine serum derived BChE delivered via a gel coated nanocapsule has been shown to bind the OP paraoxon in a non-immunogenic fashion in rats (Zhang et al., 2016).

Supplemental antidotes to acute OP toxicity, such as magnesium, clonidine and sodium bicarbonate, have also been evaluated in small clinical trials; however, the results to date have been equivocal. Other, potential antidotes such as nicotinic receptor antagonists, beta-adrenergic agonists, and lipid emulsions (to bind and sequester lipophilic OPs, see review Eisenkraft and Falk, 2016) are currently being evaluated in animal models and in small pilot clinical trials (reviewed, Eddleston & Chowdhury, 2016).

6. Conclusion

With the widespread use of OPs worldwide to control pests as well as to prevent vector borne illnesses comes the environmental risks to humans and other non-target organisms. This risk is exacerbated by the tendency of rogue governments and terrorists to employ OPs against individuals they consider enemies which can include both military personnel and civilians. It is essential that novel treatments be developed to combat both the acute toxicity of OPs as well as their long-term deleterious health effects, since there are currently no ideal therapeutic approaches available. In order for these new treatments to be developed it is imperative that a better understanding of the mechanisms that contribute to the toxicity be of OPs be elucidated. In addition to the well-known inhibitory effects of OPs on acetylcholinesterase, there is an evolving literature to suggest that OPs affect a number of additional targets that lead to oxidative stress, axonal transport deficits, neuroinflammation, and autoimmunity. There is significant (and growing) evidence that some of these targets could exploited for therapeutic purposes.

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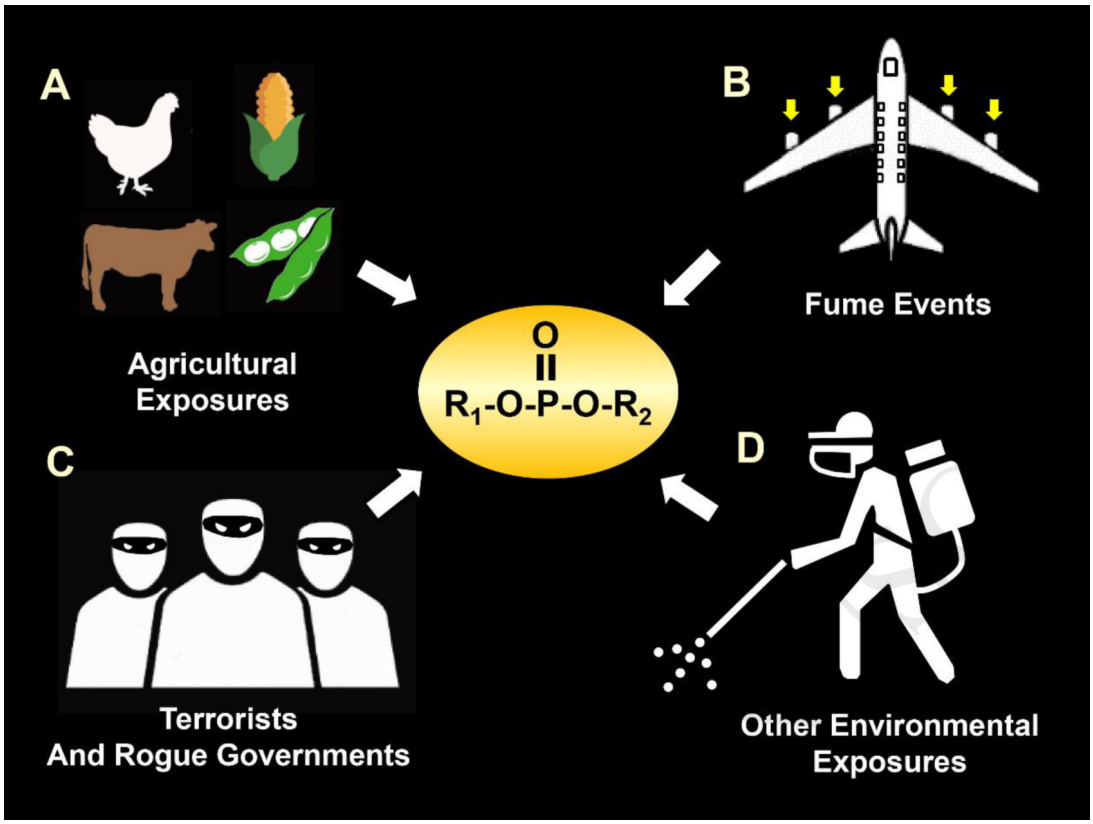


Fig 1.

Illustration of several representative sources of toxic exposures to OPs by humans and other non-target species. A. OP exposures in the agricultural setting, which may come from insecticides, anthelmintics, fungicides, and herbicides. B. OP exposures from terrorist attacks and chemical warfare assaults by rogue governments. C. Exposures as a result of "fume events" where the cabin air of an airplane can be contaminated with heated engine oil fumes that contain OPs. D. Exposures from insecticides used by public health officials to combat vector borne illnesses.

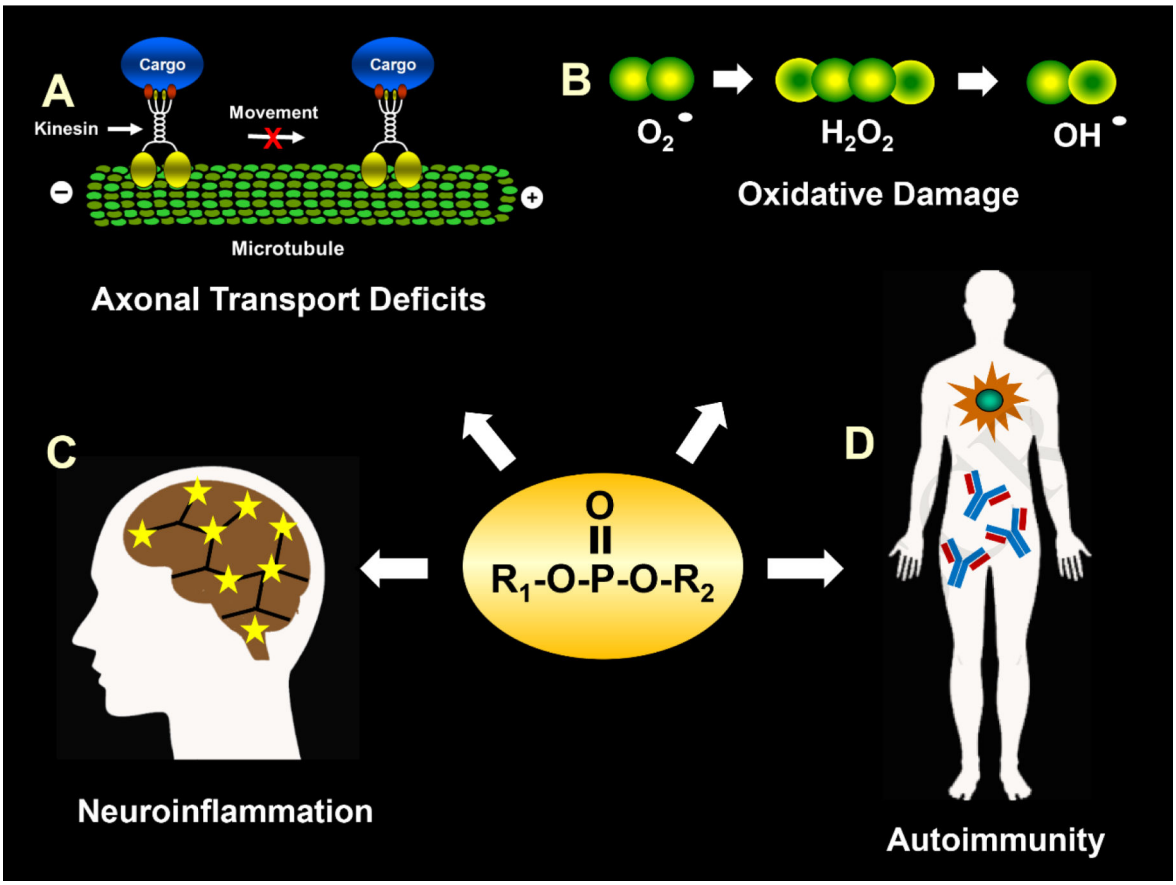
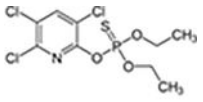
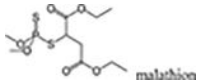
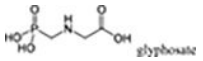
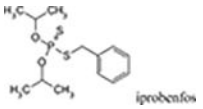
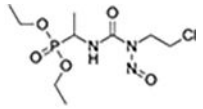
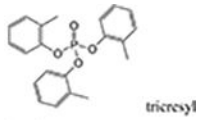
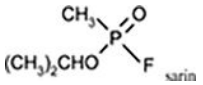


Fig 2.

Illustration of several representative physiological processes that may be affected by OPs to result in long-term deleterious health effects. A. OPs may impair axonal transport by altering the function of motor proteins such as kinesin and/or components of the neuronal cytoskeleton (e.g., microtubules). B. OPs can increase free radical formation and oxidative stress, which can lead to mitochondrial dysfunction and DNA damage to cells. C. OPs can lead to microglia activation and an increase in proinflammatory cytokines, which in turn may lead to neuroinflammation. D. OP exposure may lead to the generation of autoantibodies that target multiple proteins known to play important roles in both the structure and function of neurons including myelination and axonal transport.

Table 1.

Commonly used Organophosphates in the United States and Worldwide and Nerve Agents (representative examples)

Application	Representative Examples	Representative Chemical Structure
Agricultural Insecticides	chlorpyrifos, malathion, diazinon	 chlorpyrifos
Vector-Born Illness Prevention	malathion, fenitrothrion, pirimiphos-methyl, naled	 malathion
Herbicides	glyphosate, glufosinate	 glyphosate
Fungicides	iprobenfos, edifenphos	 iprobenfos
Pharmaceutical Agents	echothiophate, fosinopril, fotemustine	 fotemustine
Flame Retardants	tris (1, 3 dichloro-2-propyl) phosphate - TDCPP, tris (2chloroethyl) phosphate – TCEP tricresyl phosphate (TCP)	 tricresyl phosphate
Highly Toxic Nerve Agents	sarin, soman, tabun, VX	 sarin

References: EPA 2017, CDC 2017; Costa 2018; Killeen et al., 2017

Table 2.

Non-Cholinesterase Targets of OPs and Downstream Effects

Target	Description/Function	Reference(s)
Neuroinflammation	Cytokines mediate a variety of inflammatory processes (IL-6, IL-1β, CCL2).	Koo et al., 2018, Mohammadzadeh et al., 2018
Oxidative Stress	Process by which reactive oxygen species are generated in excess or improperly cleared to cause widespread cell damage.	Eftekhari et al., 2018, Abolaji et al., 2017
Gene Expression	Changes in gene expression can occur throughout all biological systems in virtually all cell types. Pathological changes in gene expression have been associated with a wide array of disorders.	Moyano et al., 2017, Savy et al., 2018, Lee et al., 2016
Autoimmunity	Formation of autoantibodies that target proteins, especially those known to play important roles in the structure and function of neurons including myelination and axonal transport (e.g., MBP, NFP, Tau, MAP-2)	Abou-Donia et al., 2013; AbouDonia et al., 2017; El-Rahman et al., 2018
Mitochondria	Organelle responsible for ATP production. Changes in transport and morphology can affect function. Also associated with the release of apoptotic factors.	Middlemore-Risher et al 2011, Eftekhari et al., 2018
Gut Microbiome	Large group of bacteria found in the gut existing in symbiosis with the host. Implicated in a wide range of functions and downstream effects ranging from immune function to cognitive development.	Gao et al., 2017
DNA	DNA Damage (i.e. fragmentation, single and double strand breaks) is associated with carcinogenic and reproductive toxicity	Li et al., 2015
Dynactin	Modulates the binding of cargoes to molecular motor proteins (dynein and kinesin-2)	Song et al., 2012
Dynein	Motor protein responsible for retrograde axonal transport.	Song et al., 2012
Tubulin	Globular proteins that polymerize into microtubules (i.e., α- and β-tubulin assemblies which serve structural roles in the cytoskeleton, support intracellular transport, mitosis, etc.).	Jiang et al., 2010
Transferrin	Plasma glycoprotein that regulates iron levels.	Grigoryan et al., 2009
Kinesin	Motor protein responsible for anterograde axonal transport in neurons as well as other cellular functions such as mitosis, meiosis, etc.	Grigoryan et al., 2009
ATP Synthase	Enzyme responsible for ATP synthesis in mitochondria.	Grigoryan et al., 2009
Nerve Growth Factor (NGF) and its receptors TrkA and P-75^{NTR}	NGF-Neurotrophin associated with survival, growth, and proliferation in neurons. TrkA-high affinity NGF receptor responsible for mediating neurotrophic effects of NGF. P-75^{NTR}-low affinity neurotrophin receptor, which mediates apoptosis and cell death.	Terry et al., 2011
Choline acetyltransferase (ChAt)	Enzyme important for the synthesis of acetylcholine.	Terry et al., 2007, 2011
α7-nicotinic acetylcholine receptors (nAChRs)	Acetylcholine receptor with known role in learning and memory.	Terry et al., 2007, 2011
Butyrylcholinesterase	Serine hydrolase which nonspecifically hydrolyzes choline-based esters.	Li et al., 2007
Monoacylglycerol lipase	Responsible for 2-AG hydrolysis in endocannabinoid inactivation.	Quistad et al, 2006, Reviewed Casida et al.,2008
Serine Hydrolase KIAA 1363	Regulates lipid metabolism, involved with the invasiveness of tumors.	Nomura et al., 2006, Reviewed Casida et al.,2008
Albumin	Globular protein responsible for transport	Peeples et al., 2005
Cannabinoid CB1 receptors	G protein-coupled receptors, which mediate endocannabinoid signaling.	Quistad et al., 2002

Target	Description/Function	Reference(s)
Fatty acid amide hydrolase	Serine hydrolase enzyme that catabolizes a class of bioactive lipids called the fatty acid amides, traditionally associated with endocannabinoid catabolism.	Quistad et al., 2001
M_2 muscarinic acetylcholine receptors	Second messenger coupled autoreceptor, modulates acetylcholine signaling.	Bomser and Casida 2001 *
Acylpeptide hydrolase	Serine protease enzyme that catalyzes the removal of N-acylated amino acids from acetylated peptides, this plays a role in the coordinated protein-degradation	Richards et al., 2000
Adenylyl cyclase	Enzyme that is required for the conversion of ATP to cyclic AMP/ Important in the G protein signaling cascade	Huff et al., 1994; Song et al., 1997; Auman et al., 2000
Neuropathy target esterase	Phospholipase enzyme with known role in phospholipid metabolism, neurite outgrowth and process elongation during neuronal differentiation.	Lush et al., 1998
Papain	Lysozomal cysteine protease.	Chaiken and Smith, 1969

* OP-phosphorylation of M2 receptors was initially reported by Bomser and Casida 2001 (using chlorpyrifos oxon), but was not observed in experiments where paraoxon or a biotinlabeled fluorophosphonate were evaluated (Proskocil et al., 2010)