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Mechanisms of organophosphate neurotoxicity

Yi-Hua Tsai and Pamela J. Lein

Abstract

The canonical mechanism of organophosphate (OP) neurotoxicity is the inhibition of acetylcholinesterase (AChE). However, multiple lines of evidence suggest that mechanisms in addition to or other than AChE inhibition contribute to the neurotoxic effects associated with acute and chronic OP exposures. Characterizing the role(s) of AChE inhibition versus noncholinergic mechanisms in OP neurotoxicity remains an active area of research with significant diagnostic and therapeutic implications. Here, we review recently published studies that provide mechanistic insights regarding (1) OP-induced status epilepticus, (2) long-term neurologic consequences of acute OP exposures, and (3) neurotoxic effects associated with repeated low-level OP exposures. Key data gaps and challenges are also discussed.

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Keywords

Calcium homeostasis, Cannabinoid signaling, Glutamatergic signaling, Neuroinflammation, Oxidative stress, Synaptotoxicity.

Abbreviations

AChE, acetylcholinesterase; AMPAR, AMPA receptors; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentrations; CB1R, endocannabinoid type 1 receptors; CPF, chlorpyrifos; DFP, diisopropyl fluorophosphate; GABA_AR, GABA_A receptors; GluK1R, kainate receptors containing the GluK1 subunit; IP₃R, inositol triphosphate receptor; mAChR, muscarinic cholinergic receptor; NMDAR, NMDA receptor; OP(s), organophosphate(s); qRT-PCR, quantitative reverse transcription–polymerase chain reaction; RNS, reactive nitrogen species; ROS, reactive oxygen species; SE, status epilepticus; RyR, ryanodine receptors; TSPO, 18 kDa mitochondrial translocator protein.

Introduction

The canonical mechanism of organophosphate (OP) (Box 1) neurotoxicity is inhibition of acetylcholinesterase (AChE), which results in hyperstimulation of muscarinic cholinergic receptors (mAChRs) and

nicotinic cholinergic receptors in the peripheral and central nervous systems. Acute inhibition of AChE by ≥ 60 –70% causes ‘cholinergic crisis’, a clinical toxidrome characterized by muscle fasciculations and weakness, parasympathomimetic signs, depression of respiratory control centers in the brainstem, seizures, and death [1,2]. Clinical and experimental evidence supports AChE inhibition as the mechanism triggering acute neurotoxic effects of OPs [1,2], although AChE knockout mice exhibit signs of acute neurotoxicity similar to those observed in wild-type mice after acute OP poisoning [3]. Persistent epileptiform discharges, cognitive deficits, and anxious/depressive behavior manifest in acutely intoxicated humans [4,5] and experimental animals [1,6,7] well after AChE activity has recovered to pre-exposure levels. These observations suggest that acute and long-term effects of acute OP intoxication are mediated by mechanisms in addition to or other than AChE inhibition.

Occupational [8,9] and early-life [10] OP exposures that do not cause cholinergic crisis are also associated with neurotoxic outcomes, but there is little evidence supporting an association between AChE activity and neurobehavioral outcomes. The hypothesis that noncholinergic mechanisms contribute to the neurotoxicity of repeated low-level OP exposures is largely supported by preclinical literature [10,11]. Although the most significant and prolonged motor effects in animals are observed after OP exposures that markedly inhibit AChE activity, deficits in cognitive [12] and social [13] behavior are not as clearly correlated with AChE inhibition.

Characterization of noncholinergic mechanisms of OP neurotoxicity remains an area of active research. Here, we review selected studies published from late 2018 through early 2021 that provide mechanistic insights into (1) OP-induced status epilepticus (SE), (2) long-term neurologic consequences of acute OP exposures, and (3) neurotoxicity of repeated low-level OP exposures.

Mechanisms contributing to OP-induced SE

Seizures are generated by the initial hypercholinergic state, but are reinforced and sustained by glutamatergic activity [14]. The molecular mechanism(s) mediating the transition to SE are poorly understood. Recent electrophysiological studies of acute rat brain slices

Box 1. What are OPs?

The term “organophosphates” (OPs) refers to a group of synthetic compounds that have in common a pentavalent phosphorus bound to sulfur or oxygen via a covalent double bond. OPs were first synthesized in the early 20th century as insecticides. The discovery in the 1930s that their insecticidal activity was primarily mediated by inhibition of acetylcholinesterase (AChE), an enzyme conserved across species, including humans, led to the development during World War II of potent OP nerve agent, such as sarin, cyclosarin, soman, tabun, VR and VX, that have been weaponized for use against military and civilian targets.

Since World War II, hundreds of OP compounds have been developed for commercial applications, predominantly as insecticides, but also as plasticizers, fire retardants, and fuel additives. Despite increasing regulatory restrictions on their use in the United States and Europe, OPs remain the most commonly used group of insecticides worldwide, with particularly heavy use in developing countries because of their lower cost compared to newer insecticides. As a result, human exposure to OPs is widespread, as evidenced by data indicating that OPs are among the most commonly detected anthropogenic contaminants in human tissues.

Human and animal studies have established neurotoxicity as the primary endpoint of concern associated with OP exposures. There are several “toxic scenarios” associated with OP exposure: acute cholinergic crisis triggered by acute inhibition of AChE by more than 60–70%, long-term effects associated with acute OP intoxication, neurotoxicity associated with repeated low-level OP exposures that may inhibit AChE, but do not cause signs of cholinergic crisis.

found that paraoxon acutely enhanced the hyperpolarization-activated cation current I_h in basolateral amygdala principal neurons [15]. The M1 mAChR antagonist, VU0255035, blocked this effect, suggesting a mechanism by which cholinergic overstimulation increases glutamatergic signaling in the basolateral amygdala, a brain region critically involved in seizure initiation by OP nerve agents [14]. In support of this model, pretreatment with VU0255035 prevented the development of SE for up to 40 min in rats acutely intoxicated with paraoxon or soman [15].

In contrast, another group found that M1/M3 mAChR hyperactivity inhibited excitatory neurotransmission via retrograde activation of presynaptic endocannabinoid type 1 receptors (CB1Rs) [16]. Electrophysiological recordings of hippocampal Schaeffer collateral synapses revealed that paraoxon, soman, and VX depressed field excitatory postsynaptic potentials before the onset of interictal spiking. This effect was mediated by presynaptic mechanisms independent of recurrent firing or N-methyl-D-aspartate (NMDA) receptor (NMDAR) currents and was completely reversed by pharmacologic antagonism of CB1Rs or M1/M3 mAChRs, but not M2/M4 mAChRs. Based on these data and previous reports that M1/M3 mAChR agonists activated retrograde CB1R signaling in the hippocampus to inhibit

presynaptic glutamate release, the authors proposed that hyperstimulation of postsynaptic M1/M3 mAChRs triggered postsynaptic release of endocannabinoids that retrogradely diffused across the synapse to activate CB1Rs and reduce presynaptic release probability [16]. The observation that presynaptic depression occurred before interictal bursting suggested that OP suppression of presynaptic glutamate release is an early compensatory response to excessive cholinergic signaling. In support of this, pharmacologic antagonism of CB1Rs enhanced lethality in a mouse soman model [16].

The neurotransmitter receptor subtypes involved in sustaining OP-induced seizures have also been the focus of recent research (Table 1). Seizures result from imbalanced excitatory to inhibitory signaling in the brain, and prolonged seizure activity is associated with upregulated expression of all three ionotropic glutamate receptor subtypes - alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate, and NMDA - and downregulated expression of gamma aminobutyric acid (GABA)_A receptors (GABA_AR) at synapses in the hippocampus and amygdala, brain regions critically involved in OP-induced SE [14]. Downregulation of synaptic GABA_AR is posited to mediate benzodiazepine refractoriness, a characteristic feature of OP-induced SE [14,17]. Neurosteroids are positive allosteric modulators of not only synaptic but also extrasynaptic GABA_AR [17]. Post-exposure administration of neurosteroids significantly attenuated benzodiazepine-refractory seizures in rats acutely intoxicated with sarin [18] or diisopropyl fluorophosphate (DFP) [19], suggesting that activity of extrasynaptic GABA_AR influences seizure duration after acute OP intoxication.

Recent studies also support a causal role for AMPA and kainate receptor activity in sustaining OP-induced seizures. Administration of LY293558, a relatively broad-spectrum antagonist of AMPA receptors (AMPA) and kainate receptors containing the GluK1 subunit (GluK1R), 20 min after soman intoxication terminated electrographic seizures in rats and significantly repressed recurrent seizures for up to 72 h after exposure [20]. Reports that the AMPAR antagonist perampanel [19] and the dual AMPAR and NMDAR antagonist urethane [21] attenuated but did not completely suppress electrographic seizures in DFP-intoxicated rats suggest that the antiseizure activity of LY293558 involves both GluK1R and AMPAR. Co-administration of LY293558 and caramiphen, an M1 mAChR antagonist with NMDAR antagonistic properties, terminated soman-induced SE significantly faster than LY293558 alone and completely suppressed seizure recurrence for up to 72 h [20]. The authors attributed the added benefit of caramiphen to its antagonistic activity at the NMDAR. This is consistent with recent reports that ketamine and MK-801, both NMDAR antagonists, significantly mitigated OP-

Table 1 Mechanisms of OP neurotoxicity: SE (green) and long-term effects of acute (yellow) and repeated low-level (blue) exposures.

OP	Pharmacological probes and targets	Exposure paradigm	Model	Time	Outcomes	Citation
Soman	LY293558 AMPA and GluK1 kainate receptor antagonist Caramiphen (CRM) anti-muscarinic with NMDA receptor antagonistic properties	Soman (132 µg/kg, s.c.) ↓ 20 min Atropine sulfate (2 mg/kg, i.m.) HI-6 (125 mg/kg, i.p.) ± LY293558 (15 mg/kg, i.m.) ± CRM (50 mg/kg, i.m.)	Adult male SD rats	7 days of EEG recording	LY293558: ↓ SE duration at 24 and 72 h post-exposure LY293558 + CRM: ↓ SE duration at 24 and 72 h post-exposure *Dual therapy more effective in terminating SE than monotherapy	Apland et al. (2018) [20]
Soman	Midazolam (MDZ) GABA _A receptor PAM* that targets synaptic receptors Dexmedetomidine (DEX) α2-adrenoceptor agonist Atipamezole (ATI) α2-adrenoceptor antagonist	HI-6 (125 mg/kg, i.p.) ↓ 30 min Soman (180 µg/kg, s.c.) ↓ 1 min Atropine methyl nitrate (2 mg/kg, i.m.) ↓ 20 or 40 min after SE onset Atropine sulfate (0.45 mg/kg, i.m.) 2-PAM ^b (25 mg/kg, i.m.) MDZ (1.8 mg/kg, i.m.) ± DEX (0.1, 0.2, or 0.4 mg/kg, i.m.) <u>Blocking and reversal experiments with ATI:</u> ATI (4 mg/kg) given either 5 min before MDZ+0.4 mg/kg DEX (blocking) or 10 min after SE cessation (reversal)	Adult male SD rats	4 h of EEG recording	↑ SE termination (100% when given MDZ + 0.4 mg/kg DEX at 20 min after SE onset) ↓ Normalized spike rate and gamma power <u>ATI treatment (blocking):</u> ↑ Mortality ↓ SE cessation <u>ATI treatment (reversal):</u> [Ineffective at restoring seizure activity - SE returned in 3 out of 10 animals] ↑ Low amplitude, high frequency activity ↑ Gamma power [than MDZ+0.4 mg/kg DEX]	McCarren et al. (2018) [25]
Sarin (GB)	Diazepam (DZP) GABA _A receptor PAM that targets synaptic receptors Pregnanolone GABA _A receptor PAM that targets synaptic and extrasynaptic receptors	Sarin (vapor for 60 minutes at 3X LC ₅₀) ↓ At the onset of toxic signs Atropine sulfate (2 mg/kg, i.m.) HI-6 (93.6 mg/kg, i.m.) ↓ 30 min DZP (10 mg/kg, s.c.) ± Pregnanolone (4 mg/kg, i.v.)	Adult male SD rats	Up to 3 mo of EEG recording	↓ Seizure duration during the first 24 h [pregnanolone + DZP > DZP] ↓ (transient) delta, theta, and gamma power after treated with dual therapy	Lumley et al. (2019) [18]
DFP ^c	Diazepam (DZP) GABA _A receptor PAM that targets synaptic receptors Urethane (anesthetic): potentiates the activation of GABA and glycine receptors, and inhibits NMDA and AMPA receptors	Pyridostigmine bromide (0.1 mg/kg, s.c.) ↓ 20 min Ethylatropine bromide (20 mg/kg, s.c.) ↓ 10 min DFP (5 mg/kg s.c.) ↓ 1 h DZP (10 mg/kg, i.p.) or Urethane (0.8 g/kg, s.c.)	Adult male SD rats	24 h of EEG recording	↓ DFP-induced SE that lasts for at least 1 h [urethane > DZP] ↓ Overnight return of high power seizure activity [urethane > DZP]	Rojas et al. (2020) [21]
DFP	Midazolam (MDZ) GABA _A receptor PAM that targets synaptic receptors Phenobarbital (PHB) (anesthetic) increases GABA _A receptor activity Memantine (MEM) Blocks NMDA receptors Dexmedetomidine (DEX) α2-adrenoceptor agonist	Pyridostigmine bromide (0.026 mg/kg, i.m.) ↓ 30 min DFP (4.5 - 5.5 mg/kg s.c.) ↓ 1 min Atropine methyl nitrate (2 mg/kg, i.m.) 2-PAM (25 mg/kg, i.m.) ↓ 1 h MDZ (1.78 mg/kg, i.m.) ± PHB (10, 30, 100 mg/kg, i.p.) or MEM (18, 32, 56 mg/kg, i.p.) or DEX (0.1, 0.2, 0.4 mg/kg, i.p.)	Adult male SD rats	24 h of EEG recording	Phenobarbital: ↓ Δ in power and mean spike rate [≥ MDZ only] Memantine: ↑ Δ in power and mean spike rate [≤ MDZ only] Dexmedetomidine: ↓ Δ in power and mean spike rate [≥ MDZ only]	Spampanato et al. (2020) [24]
DFP	Midazolam (MDZ) GABA _A receptor PAM that targets synaptic receptors Allopregnanolone (ALLO) GABA _A receptor PAM that targets synaptic and extrasynaptic receptors Perampanel (PPL) AMPA receptor antagonist	DFP (4 mg/kg, s.c.) ↓ 1 min Atropine sulfate (2 mg/kg) 2-PAM (25 mg/kg) ↓ 40 min MDZ (1.8 mg/kg, i.m.) ± ALLO (6 mg/kg, i.m.) ± PPL (2 mg/kg, i.m.)	Adult male SD rats	Up to 7.5 mo of EEG recording	↓ EEG and behavioral seizures after MDZ+ALLO+PPL treatment [MDZ + ALLO + PPL >> MDZ]	Dhir et al. (2020) [19]

induced SE [22,23]; however, memantine, which also blocks NMDARs, exacerbated DFP-induced seizures and increased mortality [24]. Insufficient data are available to determine whether the differential effects of these drugs reflect differential targeting of NMDAR subunits or different pharmacological properties independent of the NMDAR. Nonglutamatergic receptors may also be involved in sustaining OP-induced seizures.

Combined midazolam and dexmedetomidine, an α₂-adrenergic receptor antagonist, was superior to midazolam alone in mitigating seizure activity in rats when given 60 min after the initiation of SE by DFP [24] or soman [25].

Whether and how the functions of these neurotransmitter receptor subtypes vary in a region- and/or time-

DFP	Midazolam (MDZ) GABA _A receptor PAM ^b	Pyridostigmine (0.1 mg/kg, i.m.) ↓ 30 min DFP (4.5 mg/kg, s.c.) ↓ 1 min Atropine methyl nitrate (2 mg/kg, i.m.) ↓ 1 min 2-PAM ^c (25 mg/kg, i.m.) ↓ 1 min, 5 min or 15 min after SE MDZ (2 mg/kg, i.m.) ± AEOL10150 (5 or 7 mg/kg s.c.) and continued at 5 mg/kg (s.c.) every 4 h until euthanasia	Adult male SD rats	24 h post- exposure	MDZ+AEOL10150 in comparison to MDZ alone: ↑ GSH: GSSG ² ratio (brain) ↓ 3-NT ² /tyrosine ratio (brain) ↑ Cysteine/cystine (plasma) ↓ TNF-α, IL-1β, IL-6, KC/GRO (hippocampus) ↓ Neurodegeneration *AEOL10150 did not protect AChE activity	Liang et al. (2018) [32]
Soman	Midazolam (MDZ) GABA _A receptor PAM	HI-6 (125 mg/kg, i.m.) ↓ 30 min Soman (154 µg/kg, s.c.) ↓ 1 min Atropine methyl nitrate (2 mg/kg, i.m.) ↓ 1 min, 5 min or 15 min after SE MDZ (2 mg/kg, i.m.) ± AEOL10150 (7 mg/kg s.c.) and continued every 4 h until euthanasia	Adult male SD rats	6, 12, 24, and 48 h post- exposure	MDZ+AEOL10150 in comparison to MDZ alone: ↑ GSH:GSSG ratio (brain) ↓ 3-NT/tyrosine ratio (brain) ↑ Cysteine/cystine (plasma) ↓ Neuroinflammation (microglial activation and proinflammatory cytokines) at 24 h post-exposure ↓ Neurodegeneration	Liang et al. (2019) [33]
DFP	Diazepam (DZP) GABA _A receptor PAM 1400W iNOS inhibitor	DFP (4 mg/kg, s.c.) ↓ 1 min Atropine sulfate (2 mg/kg, i.m.) ↓ 2 h 2-PAM (25 mg/kg, i.m.) ↓ 2 h DZP (5 mg/kg, i.m.) ↓ 2 h 1400W (20 mg/kg, i.m.) twice daily at 12 h intervals for the first 3 days	Adult male SD rats	24 h, 48 h, 7 days and 12 weeks post- exposure	1400W vs. MDZ alone: ■ Learning and memory ■ Motor function ■ Depression-like behavior ↓ Epileptiform spiking ↓ Indices of oxidative stress (brain and serum) ↓ Microgliosis and astrogliosis ↓ Proinflammatory cytokines and chemokines (hippocampus) ↓ Neurodegeneration	Putra et al. (2020) [35]

dependent manner during the evolution of OP-induced seizures are questions that warrant attention. Another research need is better understanding of the role of glia cells in the initiation and propagation of OP-induced seizures, as highlighted by a recent report suggesting that the OP metabolite, diethyl dithiophosphate, impairs glutamate transport in cultured Bergmann glia cells [26].

Mechanisms underlying the long-term outcomes of acute OP intoxication

It is generally believed that brain damage observed after acute OP intoxication is primarily caused by prolonged seizure activity [27,28]. However, it was observed that antiseizure efficacy did not necessarily correlate with protection against neuronal death 24 h after exposure in rats acutely intoxicated with DFP. Specifically, memantine exacerbated seizure severity, but significantly reduced neuronal cell death; conversely, dexmedetomidine enhanced seizure suppression but conferred no significant neuroprotection [24]. In another study of DFP-intoxicated rats, a subpopulation of animals were observed to exhibit minimal to no behavioral or electrographic seizures despite brain AChE inhibition comparable with that of animals with SE [29]. The brains of nonseizing animals exhibited significant neurodegeneration although it was delayed, less persistent, and less severe compared with seizing animals. Micro-computed tomography scans at 60 days after exposure revealed extensive mineralization in the

thalamus that was not significantly different between seizing and nonseizing subjects [29]. These observations suggest that seizure-independent mechanism(s) contribute to neuropathology after acute OP intoxication.

Oxidative stress

Oxidative stress is strongly associated with excessive cholinergic and glutamatergic activity [14,30] and is posited to mediate the neuropathologic consequences of OP-induced SE [7,31]. Recent preclinical studies confirmed that acute OP intoxication upregulated brain expression of multiple oxidative stress biomarkers (Table 1). To probe a functional role for oxidative stress, structurally and mechanistically diverse antioxidants were used to reduce oxidative stress in the brain after OP-induced SE (Table 1). Administration of AEOL10150, a catalytic antioxidant that scavenges reactive oxygen species (ROS) and reactive nitrogen species (RNS), within 5–15 min after rats were exposed to DFP [32] or soman [33] significantly attenuated neuroinflammation and neurodegeneration in multiple brain regions at 24 and 48 h after exposure. Administration of diapocynin [34], a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, or 1400W [35], an inducible nitric oxide synthase (iNOS) inhibitor, 4 h after acute DFP intoxication significantly attenuated neuroinflammation and neurodegeneration for weeks to months. Interestingly, diapocynin reduced astrogliosis, but not microgliosis, whereas 1400W

DFP	Diazepam (DZP) GABA _A receptor PAM Diapocynin NADPH oxidase inhibitor	DFP (4 mg/kg, s.c.) ↓ 1 min Atropine sulfate (2 mg/kg, i.m.) ↓ 2 h DZP (5 mg/kg, i.m.) ↓ 2 h Diapocynin (300 mg/kg, oral) 6 doses at 12 h intervals for the first 3 days	Adult male SD rats	4 and 6 weeks post-exposure	Diapocynin vs. DZP alone: ↓ Motor impairment ↓ Learning and memory impairments ↓ Epileptiform spiking during the first 72 h ↓ Indices of oxidative stress at 6 weeks post-exposure (brain and serum) ↓ Activated astrogliosis ↓ Microgliosis ↓ Proinflammatory cytokines and chemokines (hippocampus) ↓ Neurodegeneration	Putra et al. (2020) [34]
DFP	Levetiracetam (LEV) inhibit both RyR- and IP3R-activated calcium-induced calcium release (CICR)	DFP (0.5 mg/kg/d s.c. once daily for 5 days) ↓ ± LEV (50 mg/kg, i.p.) twice daily for 4 days at 3 mo post-DFP exposure	Adult male SD rats	3 mo post-exposure	LEV effects vs. DFP alone: ↓ [Ca ²⁺] _i primary hippocampal neurons of DFP rats ↑ Preference for sucrose water ↓ Time immobilized in forced swim test ↑ Time spent and times of entry in the open arm of elevated plus maze ↑ Time with the novel object in novel object recognition	Philips et al. (2019) [53]
CPF	Quercetin antioxidant that scavenges ROS and RNS	CPF (13.5 mg/kg oral gavage alternate day) ± co-treatment of quercetin (50 mg/kg/d, oral gavage)	Adult male SD rats	2 mo	Quercetin treatment in comparison to CPF: ↓ Protein carbonyl contents (serum and cerebrum) ↑ AChE activity (serum and cerebellum) ↑ Bcl-2 protein level (brain) ↓ Bax, cytochrome c, caspase-8, and caspase-9 (cerebrum and cerebellum)	Fereidouni et al. (2019) [48]
CPF	N-acetylcysteine (NAC) antioxidant	NAC (100 mg/kg/d) ↓ 1 h CPF (10 mg/kg/d oral)	Adult male albino rats	28 days	NAC effects vs. CPF alone: ↑ GSH ^a and SOD ^b ↓ MDA ^c and NO levels	Mahmoud et al. (2019) [49]
CPF ^d	Mitoapocynin mitochondria-targeting antioxidant	<i>In vitro</i> 10 µM CPF ± 10 or 30 µM mitoapocynin <i>In vivo</i> CPF (5 mg/kg/d, oral gavage) from PND 27 through PND 61 ± co-treatment of mitoapocynin (10mg/kg, oral gavage) thrice weekly	N27 cell line Juvenile SD rats (M and F ^e)	6, 12, 18, and 24 h of treatment 5 weeks	Mitoapocynin effects on CPF-induced cytotoxicity: ↓ DNA fragmentation ↓ ROS generation ↓ Mitochondria-derived superoxide ↓ STAT1 phosphorylation ↓ Autophagy (LC3B, beclin1) Mitoapocynin effects on CPF-induced dopaminergic neurotoxicity: ↓ Locomotor deficits ↓ STAT1 phosphorylation (substantia nigra and striatum) ↓ Bax/Bcl-2 ratio and caspase-3 (substantia nigra and striatum) ↓ PKCδ (substantia nigra and striatum) ↓ Autophagy (LC3B) (substantia nigra and striatum) ↑ DOPAC level (substantia nigra) ↑ Tyrosine hydroxylase (TH) level (substantia nigra and striatum)	Singh et al. (2018) [50]

">" indicates more effective; "<" indicates less effective.

^a PAM = positive allosteric modulator; ^b 2-PAM = 2-pralidoxime; ^c DFP = diisopropylfluorophosphate; ^d GSSG = glutathione disulfide; ^e 3-NT = 3-nitrotyrosine; ^f CPF = chlorpyrifos; ^g M and F = male and female; ^h GSH = glutathione; ⁱ SOD = superoxide dismutase; ^j MDA = malondialdehyde.

attenuated both these responses. In all studies, mitigation of oxidative stress did not attenuate SE, ruling out the possibility that neuroprotective effects were mediated by cessation of seizure activity and suggesting that oxidative stress is not necessary for sustained seizure activity.

These studies suggest oxidative stress contributes to the neuropathologic consequences of acute OP intoxication, but is oxidative stress causally linked to neurologic deficits observed after OP-induced SE? The diapocynin and 1400W studies [34,35] showed that 1400W significantly

suppressed epileptiform spiking for weeks, diapocynin during the first 72 h after acute DFP intoxication, whereas diapocynin, but not 1400W, mitigated DFP-induced motor impairment in the rotarod assay. Neither 1400W nor diapocynin improved cognitive behavior in the Morris water maze, and 1400W had no effect on anxiety-like behavior in the forced swim test. These differential effects of mechanistically distinct antioxidants raise questions regarding mechanistic relationships between different mechanisms of oxidative stress, neuroinflammation, neurodegeneration, and neurologic deficits after acute OP intoxication.

Neuroinflammation

As reviewed in 2019 [7], a growing body of literature demonstrates that acute OP intoxication triggers a robust neuroinflammatory response. Recent studies in rodent models extended this literature by showing that astrogliosis and microgliosis persisted for months after acute DFP intoxication [34,36,37]. Longitudinal monitoring of neuroinflammatory responses in DFP-intoxicated rats using positron emission tomography to quantify expression of the 18 kDa mitochondrial translocator protein revealed neuroinflammation varied dynamically in a region- and time-dependent manner [38].

The 18 kDa mitochondrial translocator protein is a biomarker of activated microglia and/or astrocytes [39], but its expression does not indicate whether activated microglia and astrocytes are neuroprotective or pathogenic [7]. A recent study began to address this question by phenotyping microglia and astrocytes in the mouse brain after DFP-induced SE [40] using biomarkers that label microglia as proinflammatory (M1-like), anti-inflammatory (M2a-like), or immunoregulatory (M2b-like) [41] and reactive astrocytes as neurotoxic (A1-like) or neuroprotective (A2-like) [42]. These biomarkers were quantified by quantitative reverse transcription–polymerase chain reaction in CD11B- (microglia/infiltrated macrophages) and GLAST (astrocytes)-immunopositive cells isolated from the whole brain at varying times after DFP-induced SE using magnetic-activated cell sorting [40]. At 1 and 4 h after exposure, M1-like and A2-like markers were observed in CD11B- and GLAST-positive isolated cells, respectively. At 4 and 24 h, microglial cells transitioned from M2b-like to M2a-like. At 24 h and 3 days, A1-like markers were increased in isolated astrocytes. Although this study did not assess function, the observation that these cells' phenotype shifted over time after exposure suggests that whether neuroinflammation is protective versus harmful after OP-induced SE varies with time after exposure.

Few studies have examined whether pharmacologic manipulation of neuroinflammation modifies long-term effects of acute OP intoxication. To date, the most compelling data are from studies of TG6-10-1, a small molecular inhibitor of the prostaglandin-E2 receptor EP2, which plays a key role in neuroinflammatory responses in the brain [43]. As described in detail in a recent review of these data [43], administration of TG6-10-1 to DFP-intoxicated rats had no effect on SE, but attenuated upregulation of inflammatory cytokine and chemokines (IL-1 β , TNF α , IL-6, CCL2, CCL4) in the brain and prevented blood–brain barrier breakdown. TG6-10-1 did not mitigate anxiety-like behavior, but it significantly improved performance in the novel object recognition task 8–12 weeks after DFP-induced SE. As

Box 2. Recent data that addresses long-standing debates over human OP neurotoxicity.

Acute OP intoxication is estimated to cause 3 million life-threatening poisonings and 250,000 deaths annually across the world [55]. There are numerous reports of long-term neurologic effects in those who survive acute OP intoxication [4,5], but a cause-effect relationship has been difficult to establish in humans. This was recently addressed in a systematic review of the evidence for long-term effects in humans acutely exposed to intoxicating levels of sarin [56]. This analysis indicated that acute sarin poisoning is a neurologic hazard to humans during the first 7 days post-exposure, causing reduced cholinesterase activity and visual and ocular effects, and a suspected hazard in the subsequent weeks to years, leading to impaired learning and memory and structural changes in the brain [56]. Similar effects are documented in preclinical models of acute OP intoxication: acute cholinergic signs and seizures that transition to status epilepticus (SE), and delayed, persistent neurologic sequelae, including brain damage, cognitive dysfunction, anxiety-like behavior, and spontaneous recurrent seizures [1,6,7].

Chronic or repeated exposures to OPs at levels that do not cause cholinergic crisis are also associated with neurotoxic outcomes in humans, including cognitive deficits, depression, anxiety, and suicidal ideation [57]. Additionally, recent epidemiologic studies link repeated low-level OP exposures to increased risk of neurodevelopmental disorders [58–60] and neurodegenerative disease [61,62]. These associations have been debated in part because of the lack of evidence of a dose-response relationship [8,9]. A recently published field assessment of pesticide application teams in Egypt who were primarily exposed to a single OP, chlorpyrifos (CPF), identified a dose-related effect of CPF on performance in the Trail Making test, a behavioral test that measures processing speed, mental flexibility, and executive function [63]. Trail Making performance deficits were associated with job title, and job title was associated with varying levels of CPF exposure. Thus, pesticide applicators had the highest CPF exposures and the greatest performance deficit, while engineers had the lowest exposures and the least deficit. Control subjects who did not work in or near the fields had the lowest CPF exposures and the best Trail Making performance. Interestingly, Trail Making performance was not associated with blood cholinesterase activity [63]. Data from this and other studies met the Bradford-Hill criteria for strong evidence of a cause-effect relationship between occupational CPF exposures and neurotoxic effects in humans [63].

more studies begin to assess the cause–effect relationship between neuroinflammation and neurologic sequelae of acute OP intoxication, comparing neuroinflammatory parameters and behaviors modulated by mechanistically diverse anti-inflammatories will be important for linking specific neuroinflammatory mediators to varying neurologic outcomes.

Synaptotoxicity

Although strongly implicated in neurodegenerative disease [44], synaptotoxicity has not been widely investigated as a mechanism underlying the

neurologic consequences of acute OP intoxication. Quantitative immunocytochemical analyses of rat hippocampal slice cultures acutely exposed to paraoxon revealed progressive decline in the synaptic biomarkers synaptophysin, synapsin II, and PSD-95 in the CA1 and dentate gyrus [45]. These changes were likely not secondary to excitotoxicity because GluR1 levels were reduced over a slower timeframe, and NeuN and Nissl staining revealed no signs of neuronal damage. Declined synapsin II dendritic labeling correlated with increased staining for $\beta 1$ integrin, an adhesion molecule involved in regulating synapse maintenance and plasticity. Expression of other synaptic adhesion molecules was unchanged, and the extent of synaptic decline positively correlated with the level of $\beta 1$ integrin expression. A potential caveat of these studies is that slice cultures were obtained from postnatal day 12 rat pups, an age at which OPs do not cause seizures *in vivo* [46]. If OP-induced synaptotoxicity can be replicated in older animals that do respond to the

seizurogenic activity of OPs (postnatal day 21 and older), these findings suggest a novel mechanism to explain delayed neurologic dysfunction after acute OP intoxication.

Neurotoxic mechanisms of repeated low-level OP exposures

Neuroinflammation and oxidative stress

We recently reviewed the evidence demonstrating that repeated low-level OP exposures triggered neuroinflammation [7]. Identifying the mechanism(s) by which OPs cause neuroinflammation and determining the cause–effect relationship between neuroinflammation and neurotoxic outcomes remain as significant data gaps that have yet to be addressed.

The literature addressing oxidative stress in repeated low-level OP exposures was also recently reviewed [47]; here, we highlight recent studies that investigated cause–effect relationships between oxidative stress and neurotoxic outcomes (Table 1). Two groups [48,49]

Figure 1

Schematic summarizing mechanisms implicated in mediating the neurotoxic effects of OPs. Mechanisms postulated in mediating the long-term neurologic consequences of acute, high-level OP exposures are depicted on the left; mechanisms implicated in the neurotoxicity associated with repeated low-level OP exposures are shown on the right. Oxidative stress resulting from an imbalance between the production of pro-oxidants (reactive oxygen/nitrogen species; ROS/RNS) and the antioxidant capacity of the system are implicated in both acute OP poisoning and chronic OP neurotoxicity. This image was created with [BioRender.com](https://www.biorender.com).

examined the role of oxidative stress in apoptosis in adult rats repeatedly exposed to chlorpyrifos (CPF) at levels that did not cause cholinergic crisis but significantly inhibited brain AChE activity by the end of the exposure period. Both studies found that CPF increased expression of oxidative stress biomarkers coincident with upregulated expression of caspases and the proapoptotic protein Bax and reduced expression of the antiapoptotic protein Bcl-2. Co-administration of CPF and an antioxidant, either quercetin [48] or N-acetylcysteine [49], mitigated expression of oxidative stress biomarkers and reversed CPF effects on apoptotic protein expression. Although quercetin mitigated CPF inhibition of AChE [48], N-acetylcysteine did not [49], suggesting that AChE inhibition is not mechanistically linked to CPF-induced apoptosis.

A significant caveat of these studies is that they neither quantified neuronal cell loss nor determined whether oxidative stress mediated behavioral deficits associated with repeated CPF exposures. These questions were, however, addressed by a third group that investigated the relationship of mitochondria-dependent oxidative stress to dopaminergic cell death and locomotor deficits in juvenile rats chronically exposed to CPF [50]. Initial mechanistic studies using the N27 immortalized murine mesencephalic dopaminergic cell line showed that CPF promoted apoptosis via STAT1-dependent signaling, which triggered mitochondrial dysfunction and ROS generation in part via enhanced proteolytic cleavage of protein kinase C delta [50]. CPF also enhanced autophagy via STAT1-dependent ROS generation. Mitoapocynin, a mitochondrially targeted antioxidant, protected against CPF-induced dopaminergic cell death via improved clearance of autophagic vacuoles in an STAT1- and mitochondrial ROS-dependent manner. *In vivo*, CPF similarly elicited STAT1 activation and oxidative stress-mediated proapoptotic signaling in the substantia nigra and striatum, but not the cortex [50]. Co-administration of mitoapocynin ameliorated these molecular effects and rescued CPF-induced motor deficits and nigrostriatal dopaminergic neurodegeneration [50].

These findings support a role for oxidative stress in mediating dopaminergic neurotoxicity associated with chronic CPF exposure but raise numerous questions: How does CPF activate STAT1, and what is the biological explanation for the regional specificity of STAT1-dependent apoptosis? Do OPs other than CPF similarly trigger dopaminergic cell death via oxidative stress? Does oxidative stress mediate nondopaminergic effects associated with chronic OP neurotoxicity?

Calcium dysregulation

The role of Ca^{2+} -dependent signaling in cognitive behavior and mood is well documented, and Ca^{2+} dysregulation is observed in many neurologic disorders [51]. Repeated low-dose DFP exposure (Table 1) was recently

shown to cause significant neuronal damage in the hippocampal region associated with depressive signs and cognitive deficits in adult male rats at 3 and 6 months after exposure [52,53]. Ca^{2+} imaging studies of hippocampal neurons acutely isolated 3 or 6 months after exposure revealed that DFP was associated with a significantly greater percentage of neurons with elevated concentrations of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) [52,53]. Pharmacologic block of voltage-gated Ca^{2+} ion channels, AMPA/kainate channels, or other nonspecific, gadolinium chloride-sensitive cation channel did not reduce $[\text{Ca}^{2+}]_i$. In contrast, pharmacologic antagonism of NMDARs with MK-801 produced a small but significant reduction, whereas ryanodine receptor (RyR) antagonism by dantrolene or combined block of the RyR and inositol triphosphate receptor with levetiracetam significantly decreased $[\text{Ca}^{2+}]_i$ [53]. These findings suggested that the sustained increase in hippocampal $[\text{Ca}^{2+}]_i$ originated from persistent release of Ca^{2+} from intracellular stores, a possibility supported by western blot data demonstrating DFP reduced levels of the RyR stabilizing protein calstabin2 [53]. In support of this hypothesis, *in vivo* treatment with levetiracetam at 3 months after DFP exposure mitigated depression-like behavior in the sucrose preference test, elevated plus maze, and forced swim test and improved learning and memory behavior in the novel object recognition task [53].

These findings support a mechanistic link between calcium dysregulation and behavioral effects of repeated low-dose DFP. However, levetiracetam did not restore $[\text{Ca}^{2+}]_i$ or behaviors to baseline, suggesting additional mechanisms likely contribute to these phenotypes. Because levetiracetam not only blocks RyR and inositol triphosphate receptor activity, but also modulates glycine and GABA receptors and binds to SV2A protein [54], it will be important to determine whether levetiracetam reversed the underlying molecular changes responsible for elevated $[\text{Ca}^{2+}]_i$ in DFP neurons and how DFP reduced calstabin2 expression.

Conclusions

OPs cause neurotoxicity (Box 2) via multiple mechanisms that vary depending on the exposure paradigm (Figure 1). Recent data implicate oxidative stress in both acute high-level and repeated low-level OP exposure. However, significant questions remain regarding functional relationships between oxidative stress, neuroinflammation, and neurodegeneration, the mechanism(s) by which OPs trigger these processes, and their contribution(s) to neurologic outcomes. Untangling these relationships is complicated by the dynamic nature of these processes that vary in a time- and region-dependent manner. There is also a critical need for determining whether neurotoxic mechanisms generalize across OPs. Answering these questions is critical for developing diagnostic biomarkers to identify OP-

intoxicated individuals at greatest risk for neurologic outcomes and for determining therapeutic targets and windows that provide optimal neuroprotection from acute or chronic OP neurotoxicity.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yi-Hua Tsai: Conceptualization, Investigation, Writing - original draft, Visualization. **Pamela J. Lein:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Several preclinical studies of acute OP intoxication have noted that a small percentage of animals intoxicated with OP nerve agents or pesticides do not exhibit electrographic or behavioral seizures. Using a Sprague–Dawley rat model of acute DFP intoxication, this study demonstrated that non-seizing animals had comparable levels of AChE inhibition in the brain as seizing animals, indicating that the lack of seizure activity was not due to technical issues related to DFP administration. Non-seizing animals also had significant neurodegeneration as evidenced by FluoroJade C staining of multiple brain regions and by microCT analyses of mineralization in the thalamus. While FluoroJadeC staining in the brain of non-seizing animals was delayed in onset, less severe, and resolved more quickly than FluoroJade C staining in seizing animals, the extent of thalamic mineralization was comparable in seizing and non-seizing animals. These data provide evidence that while seizure severity and/or duration influence the severity of neurodegeneration, seizure-independent mechanisms contribute to the neuropathologic consequences of acute OP

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Using a rat model of acute DFP intoxication, the authors demonstrated that administration of the NADPH oxidase inhibitor, diapocynin, 2 h following termination of behavioral seizures with diazepam, partially rescued DFP-induced motor impairment on rotarod and horizontal bar test but had no effect on learning and memory in the Morris water maze. Diapocynin significantly reduced DFP-induced upregulation of oxidative stress biomarkers, proinflammatory cytokines, and reactive astrogliosis, but not microgliosis. Further, diapocynin significantly attenuated neurodegeneration in the piriform cortex, CA1 and dentate gyrus, but not the CA3 region, as determined by FluoroJade B staining of NeuN immunopositive cells. These results are among the first to provide evidence that oxidative stress is causally linked to at least a subset of long-term neuropathologic and behavioral consequences of acute DFP intoxication, and specifically suggest that oxidative stress contributes to motor dysfunction.

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- While immediate effects of acute OP intoxication are established, whether effects persist after initial signs have subsided is debated. To address this controversy, the National Toxicology Program (NTP) conducted a systematic review to evaluate the evidence for long-term neurological effects following acute exposure to sarin. The literature search and screening process identified 32 data sets within the 34 human studies and 47 data sets within the 51 animal studies (from 6837 potentially relevant references) that met the objective and the inclusion criteria. Four main health effect categories of neurological response were identified as having sufficient data to reach hazard conclusions: (1) cholinesterase levels; (2) visual and ocular effects; (3) effects on learning, memory, and intelligence; and (4) morphology and histopathology in nervous system tissues. NTP concluded that acute sarin exposure is known to be a neurological hazard to humans in the period following exposure up to 7 days and suspected to be a hazard week to years after exposure, given a lower level of evidence in later time periods. These findings are significant because they are the first to establish that acute OP intoxication causes long-term effects in humans.
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This study is the first to demonstrate a dose-related effect of occupational OP exposure on human neurobehavior. Pesticide application

teams in Egypt, which represented a unique exposure cohort because they were primarily exposed to a single OP, chlorpyrifos (CPF), were recruited into a field assessment. Trail Making A and the more challenging Trail Making B tests were administered to 54 engineers (who supervise the pesticide application process, usually from the side of the field), 59 technicians (who guide the pesticide applicators in the field), 31 applicators (who mix and apply pesticides using knapsack sprayers), and 150 controls (who did not work in the fields) at two different times during the OP application season as well as immediately after applications had ended and 1.5 months later. On the same days neurobehavioral tests were conducted, urine and blood were collected to measure urinary levels of 3,5,6-trichloro-2-pyridinol (TCPy), a specific metabolite of CPF, and cholinesterase activity, respectively. Urinary TCPy levels confirmed a pattern of higher to lower CPF exposures from applicators to technicians to engineers, and these were all greater than urinary metabolite levels in controls. A consistent relationship between job title and performance speed on the behavioral task was observed: Controls had the best (fastest) performance on Trail Making A and B tests throughout the application season, and applicators had significantly slower performance than engineers on Trail Making tests. However, individual urinary TCPy and blood cholinesterase activity did not predict individual performance. These results established that chronic occupational exposure to chlorpyrifos is neurotoxic. Further, these findings suggested that the classic biomarkers of recent CPF exposure are not predictive of chronic exposure effects.