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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; IP_3R, 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 (AMPARs) 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[a] 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.) Blocking and reversal experiments with ATI: 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 ATI treatment (blocking): ⬆ Mortality ⬇ SE cessation ATI treatment (reversal): **[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 LCt_{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] **AEOL10150** catalytic antioxidant that scavenges multiple ROS and RNS	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.) 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[d] ratio (brain) ⬇ 3-NT[e]/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 **AEOL10150** catalytic antioxidant that scavenges multiple ROS and RNS	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-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-PAM (25 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[h] and SOD[i] ↓ MDA[i] and NO levels ↑ Bcl-2 level ↓ Bax level	Mahmoud et al. (2019) [49]
CPF[f]	**Mitoapocynin** mitochondria-targeting antioxidant	*In vitro* 10 μM CPF ± 10 or 30 μM mitoapocynin	N27 cell line	6, 12, 18, and 24 h of treatment	Mitoapocynin effects on CPF-induced cytotoxicity: ↓ DNA fragmentation ↓ ROS generation ↓ Mitochondria-derived superoxide ↓ STAT1 phosphorylation ↓ Autophagy (LC3B, beclin1)	Singh et al. (2018) [50]
		In vivo CPF (5 mg/kg/d, oral gavage) from PND 27 through PND 61 ± co-treatment of mitoapocynin (10mg/kg, oral gavage) thrice weekly	Juvenile SD rats (M and F[g])	5 weeks	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)	

">" 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-1β, TNFα, 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 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.

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].

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 β1 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 β1 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.

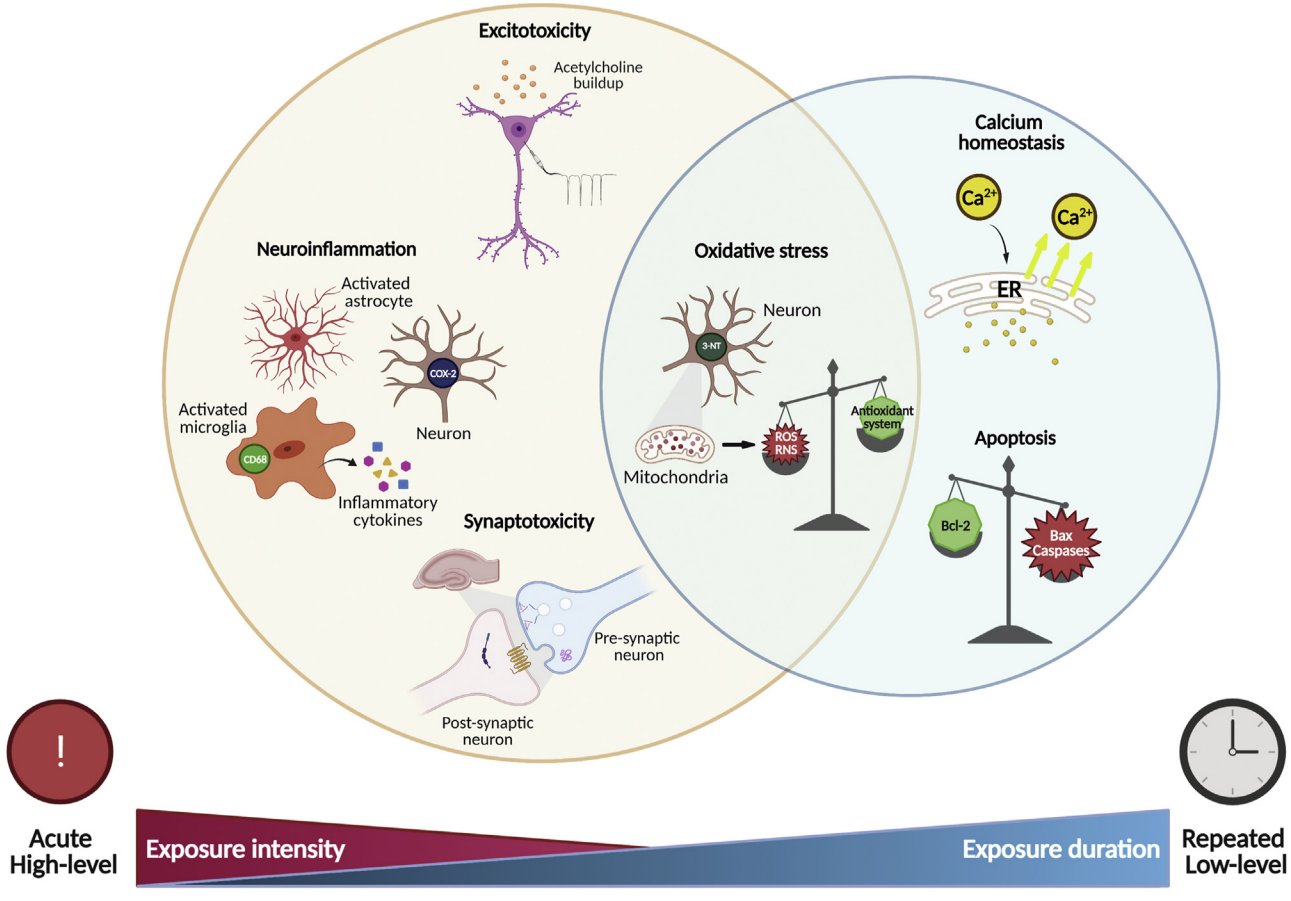
Neurotoxic mechanisms of repeated low-level OP exposures

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*.

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 pro-apoptotic 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+} ($[Ca^{2+}]_i$) [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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ [53]. These findings suggested that the sustained increase in hippocampal $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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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References

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Pereira EF, Aracava Y, DeTolla Jr LJ, Beecham EJ, Basinger Jr GW, Wakayama EJ, Albuquerque EX: **Animal models that best reproduce the clinical manifestations of human intoxication with organophosphorus compounds**. *J Pharmacol Exp Therapeut* 2014, **350**:313–321.

2. Richardson JR, Fitsanakis V, Westerink RHS, Kanthasamy AG: **Neurotoxicity of pesticides**. *Acta Neuropathol* 2019, **138**: 343–362.

3. Duysen EG, Li B, Xie W, Schopfer LM, Anderson RS, Broomfield CA, Lockridge O: **Evidence for nonacetylcholinesterase targets of organophosphorus nerve agent: supersensitivity of acetylcholinesterase knockout mouse to VX lethality**. *J Pharmacol Exp Therapeut* 2001, **299**:528–535.

4. Chen Y: **Organophosphate-induced brain damage: mechanisms, neuropsychiatric and neurological consequences, and potential therapeutic strategies**. *Neurotoxicology* 2012, **33**: 391–400.

5. Figueiredo TH, Apland JP, Braga MFM, Marini AM: **Acute and long-term consequences of exposure to organophosphate nerve agents in humans**. *Epilepsia* 2018, **59**:92–99.

6. de Araujo Furtado M, Rossetti F, Chanda S, Yourick D: **Exposure to nerve agents: from status epilepticus to neuroinflammation, brain damage, neurogenesis and epilepsy**. *Neurotoxicology* 2012, **33**:1476–1490.

7. Guignet M, Lein PJ: **Neuroinflammation in organophosphate-induced neurotoxicity**. In *Role of Inflammation in environmental neurotoxicity. 3*. Edited by Aschner M, Costa LG, Academic Press; 2019:35–79.

8. Meyer-Baron M, Knapp G, Schaper M, van Thriel C: **Meta-analysis on occupational exposure to pesticides–neurobehavioral impact and dose-response relationships**. *Environ Res* 2015, **136**:234–245.

9. Rohlman DS, Anger WK, Lein PJ: **Correlating neurobehavioral performance with biomarkers of organophosphorous pesticide exposure**. *Neurotoxicology* 2011, **32**:268–276.

10. Burke RD, Todd SW, Lumsden E, Mullins RJ, Mamczarz J, Fawcett WP, Gullapalli RP, Randall WR, Pereira EFR, Albuquerque EX: **Developmental neurotoxicity of the organophosphorus insecticide chlorpyrifos: from clinical findings to preclinical models and potential mechanisms**. *J Neurochem* 2017, **142**(Suppl 2):162–177.

11. Silva MH: **Effects of low-dose chlorpyrifos on neurobehavior and potential mechanisms: a review of studies in rodents, zebrafish, and *Caenorhabditis elegans***. *Birth Defects Res* 2020, **112**:445–479.

12. Bushnell PJ, Moser VC: **Behavioral toxicity of cholinesterase inhibitors**. In *Toxicology of organophosphate & carbamate compounds*. Edited by Gupta RC, San Diego, CA: Elsevier; 2006: 347–360.

13. Berg EL, Ching TM, Bruun DA, Rivera JK, Careaga M, Ellegood J, Lerch JP, Wohr M, Lein PJ, Silverman JL: **Translational outcomes relevant to neurodevelopmental disorders following early life exposure of rats to chlorpyrifos**. *J Neurodev Disord* 2020, **12**:40.

14. Aroniadou-Anderjaska V, Figueiredo TH, Apland JP, Braga MF: **Targeting the glutamatergic system to counteract organophosphate poisoning: a novel therapeutic strategy**. *Neurobiol Dis* 2020, **133**:104406.

15. Miller SL, Aroniadou-Anderjaska V, Pidoplichko VI, Figueiredo TH, Apland JP, Krishnan JK, Braga MF: **The M1 muscarinic receptor antagonist VU0255035 delays the development of *status epilepticus* after organophosphate exposure and prevents hyperexcitability in the basolateral amygdala**. *J Pharmacol Exp Therapeut* 2017, **360**:23–32.

16. •• Hoffman KM, Eisen MR, Chandler JK, Nelson MR, Johnson EA, McNutt PM: **Retrograde activation of CB1R by muscarinic receptors protects against central organophosphorus toxicity**. *Neuropharmacology* 2019, **155**:113–120.
Using electrophysiologic assessment of acute mouse hippocampal slices, the authors identified a novel compensatory mechanism triggered by acute OP intoxication. Specifically, they demonstrated that acute administration of the OPs paraoxon, soman or VX rapidly depressed excitatory glutamatergic signaling prior to onset of interictal spiking. This effect occurred independent of recurrent firing, and did not require NMDA recepor currents, suggesting that it was not mediated by activity-dependent calcium uptake. Pharmacologic probes demonstrated presynaptic endocanabinoid type 1 receptor (CB1R), as well as postsynaptic M1 and M3 muscarinic acetylcholine receptors were necessary for OP suppression of glutamatergic transmission. Based on these data, the authors proposed a model in which overstimulation of M1 and M3 receptors triggers release of postsynaptic endocannabinoids that retrogradely activate presynaptic CBR1 receptors to decrease presynaptic release probablilty. Administration of CB1R antagonists significantly reduced survival in mice after soman challenge, revealing an acute protective role for endogenous CB1R signaling in response to cholinergic hyperactivity. This study was also unique in showing similar effects across structurally distinct OPs.

17. Reddy DS: **Mechanism-based novel antidotes for organophosphate neurotoxicity**. *Curr Opin Toxicol* 2019, **14**:35–45.

18. Lumley L, Miller D, Muse WT, Marrero-Rosado B, de Araujo Furtado M, Stone M, McGuire J, Whalley C: **Neurosteroid and benzodiazepine combination therapy reduces status epilepticus and long-term effects of whole-body sarin exposure in rats**. *Epilepsia Open* 2019, **4**:382–396.

19. Dhir A, Bruun DA, Guignet M, Tsai YH, Gonzalez E, Calsbeek J, Vu J, Saito N, Tancredi DJ, Harvey DJ, Lein PJ, *et al.*: **Allopregnanolone and perampanel as adjuncts to midazolam for treating diisopropylfluorophosphate-induced status epilepticus in rats**. *Ann N Y Acad Sci* 2020, **1480**:183–206.

20. Apland JP, Aroniadou-Anderjaska V, Figueiredo TH, De Araujo Furtado M, Braga MFM: **Full protection against soman-induced seizures and brain damage by LY293558 and caramiphen combination treatment in adult rats**. *Neurotox Res* 2018, **34**: 511–524.

21. Rojas A, Wang J, Glover A, Dingledine R: **Urethane attenuates early neuropathology of diisopropylfluorophosphate-induced status epilepticus in rats**. *Neurobiol Dis* 2020, **140**:104863.

22. Niquet J, Lumley L, Baldwin R, Rossetti F, Schultz M, de Araujo Furtado M, Suchomelova L, Naylor D, Franco-Estrada I, Wasterlain CG: **Early polytherapy for benzodiazepine-refractory status epilepticus**. *Epilepsy Behav* 2019, **101**(Pt B): 106367.

23. Niquet J, Lumley L, Baldwin R, Rossetti F, Suchomelova L, Naylor D, Estrada IBF, Schultz M, Furtado MA, Wasterlain CG: **Rational polytherapy in the treatment of cholinergic seizures**. *Neurobiol Dis* 2020, **133**:104537.

24. • Spampanato J, Bealer SL, Smolik M, Dudek FE: **Delayed adjunctive treatment of organophosphate-induced status epilepticus in rats with phenobarbital, memantine, or dexmedetomidine**. *J Pharmacol Exp Therapeut* 2020, **375**: 59–68.

This study leveraged a rat model of acute intoxication with DFP to assess the antiseizure and neuroprotective efficacy of mechanistically diverse pharmacologic probes. EEG recordings were collected for 24 h following DFP exposure to assess seizure activity, and brains were collected at 24 h post-exposure to quantify neurodegeneration using FluoroJade B staining. The data revealed that the effect of a drug on the severity of seizure activity did not necessarily determine the drug's effect on neuronal cell death. Specifically, memantine exacerbated seizure severity, but significantly reduced neuronal cell death; conversely, dexmedetomidine enhanced seizure suppression but conferred no significant neuroprotection. These findings are among the first to challenge the paradigm that the severity of brain damage following acute OP intoxication is primarily determined by seizure activity and/or duration.

25. McCarren HS, Arbutus JA, Ardinger C, Dunn EN, Jackson CE, McDonough JH: **Dexmedetomidine stops benzodiazepine-refractory nerve agent-induced status epilepticus**. *Epilepsy Res* 2018, **141**:1–12.

26. Olivares-Banuelos TN, Martinez-Hernandez I, Hernandez-Kelly LC, Chi-Castaneda D, Vega L, Ortega A: **The neurotoxin diethyl dithiophosphate impairs glutamate transport in cultured Bergmann glia cells**. *Neurochem Int* 2019, **123**: 77–84.

27. Hobson BA, Siso S, Rowland DJ, Harvey DJ, Bruun DA, Garbow JR, Lein PJ: **Magnetic resonance imaging reveals progressive brain injury in rats acutely intoxicated with diisopropylfluorophosphate**. *Toxicol Sci* 2017, **157**:342–353.

28. McDonough Jr JH, Shih TM: **Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology**. *Neurosci Biobehav Rev* 1997, **21**:559–579.

29. •• Gonzalez EA, Rindy AC, Guignet MA, Calsbeek JJ, Bruun DA, Dhir A, Andrew P, Saito N, Rowland DJ, Harvey DJ, Rogawski MA, *et al.*: **The chemical convulsant diisopropylfluorophosphate (DFP) causes persistent neuropathology in adult male rats independent of seizure activity**. *Arch Toxicol* 2020, **94**:2149–2162.

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 demontrated 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 neurodegernation, seizure-independent mechanisms contribute to the neuropathologic consequences of acute OP intoxication. Further, since Sprague–Dawley rats are outbred, these results suggest the existence of genetic traits that confer resistance to seizures triggered by hypercholinergic signaling.

30. Williamson J, Singh T, Kapur J: **Neurobiology of organophosphate-induced seizures**. *Epilepsy Behav* 2019, **101**:106426.

31. Naughton SX, Terry Jr AV: **Neurotoxicity in acute and repeated organophosphate exposure**. *Toxicology* 2018, **408**:101–112.

32. Liang LP, Pearson-Smith JN, Huang J, McElroy P, Day BJ, Patel M: **Neuroprotective effects of AEOL10150 in a rat organophosphate model**. *Toxicol Sci* 2018, **162**:611–621.

33. Liang LP, Pearson-Smith JN, Huang J, Day BJ, Patel M: **Neuroprotective effects of a catalytic antioxidant in a rat nerve agent model**. *Redox Biol* 2019, **20**:275–284.

34. • Putra M, Gage M, Sharma S, Gardner C, Gasser G, Anantharam V, Thippeswamy T: **Diapocynin, an NADPH oxidase inhibitor, counteracts diisopropylfluorophosphate-induced long-term neurotoxicity in the rat model**. *Ann N Y Acad Sci* 2020, **1479**:75–93.

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.

35. • Putra M, Sharma S, Gage M, Gasser G, Hinojo-Perez A, Olson A, Gregory-Flores A, Puttachary S, Wang C, Anantharam V, Thippeswamy T: **Inducible nitric oxide synthase inhibitor, 1400W, mitigates DFP-induced long-term neurotoxicity in the rat model**. *Neurobiol Dis* 2020, **133**:104443.

In this study, the authors used the same model as in the previous Putra et al., 2020 reference [34] to investigate the effects of the iNOS inhibitor, 1400W, on long-term effects of acute DFP intoxication. Unlike diapocynin, 1400W significantly attenuated not only DFP-induced astrogliosis but also microgliosis, and reduced neurodegeneration in the CA3 region of the hippocampus (other brain regions were not analyzed). Also in contrast to diapocynin, 1400W suppressed epileptiform spiking and spontaneous recurrent seizures for up to 12 weeks post-exposure, but had no effect on motor dysfunction in the rotarod or horizontal bar test. 1400W also did not alter DFP effects on learning and memory in the Morris water maze task or anxiety-like behavior in the forced swim test. While these findings are similar to those of the diapocynin study in that they demonstrated a cause-effect relationship between oxidative stress and a subset of long-term effects of acute DFP intoxication, in contrast to diapocynin, 1400W protected against long-term electrographic abnormalities but not motor dysfunction. These results suggest that different oxidative stress mechanisms differentially influence long-term effects of acute OP intoxication.

36. Guignet M, Dhakal K, Flannery BM, Hobson BA, Zolkowska D, Dhir A, Bruun DA, Li S, Wahab A, Harvey DJ, Silverman JL, *et al.*: **Persistent behavior deficits, neuroinflammation, and oxidative stress in a rat model of acute organophosphate intoxication**. *Neurobiol Dis* 2020, **133**:104431.

37. Supasai S, Gonzalez EA, Rowland DJ, Hobson B, Bruun DA, Guignet MA, Soares S, Singh V, Wulff H, Saito N, Harvey DJ, *et al.*: **Acute administration of diazepam or midazolam minimally alters long-term neuropathological effects in the rat brain following acute intoxication with diisopropylfluorophosphate**. *Eur J Pharmacol* 2020, **886**:173538.

38. Hobson BA, Rowland DJ, Siso S, Guignet MA, Harmany ZT, Bandara SB, Saito N, Harvey DJ, Bruun DA, Garbow JR, Chaudhari AJ, *et al.*: **TSPO PET using [18f]PBR111 reveals persistent neuroinflammation following acute diisopropylfluorophosphate intoxication in the rat**. *Toxicol Sci* 2019, **170**: 330–344.

39. Guilarte TR: **TSPO in diverse CNS pathologies and psychiatric disease: a critical review and a way forward**. *Pharmacol Ther* 2019, **194**:44–58.

40. •• Maupu C, Enderlin J, Igert A, Oger M, Auvin S, Hassan-Abdi R, Soussi-Yanicostas N, Brazzolotto X, Nachon F, Dal Bo G, Dupuis N: **Diisopropylfluorophosphate-induced status epilepticus drives complex glial cell phenotypes in adult male mice**. *Neurobiol Dis* 2021, **152**:105276.

Numerous studies have demonstrated that acute OP intoxication triggers robust activation of microglia and astrocytes; however, this is the first study to date to analyze the phenotype of these cells during the first 3 days after DFP-induced SE. Using qRT-PCR to quantify biomarkers that label microglia as pro-inflammatory (M1-like), anti-inflammatory (M2a-like) or immunoregulatory (M2b-like) [40], and reactive astrocytes as neurotoxic (A1-like) or neuroprotective (A2-like) in microglia and astroytes isolated from the whole brain, the authors demontrated that the phenotypes of these cells shift over time post-exposure, which has significant implications for understanding whether and when neuroinflammation is protective versus harmful following OP-induced SE.

41. Chhor V, Le Charpentier T, Lebon S, Ore MV, Celador IL, Josserand J, Degos V, Jacotot E, Hagberg H, Savman K, Mallard C, *et al.*: **Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro**. *Brain Behav Immun* 2013, **32**:70–85.

42. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Munch AE, Chung WS, Peterson TC, Wilton DK, *et al.*: **Neurotoxic reactive astrocytes are induced by activated microglia**. *Nature* 2017, **541**:481–487.

43. •• Rojas A, Ganesh T, Wang W, Wang J, Dingledine R: **A rat model of organophosphate-induced status epilepticus and the beneficial effects of EP2 receptor inhibition**. *Neurobiol Dis* 2020, **133**:104399.

This is a review of data collected by the Dingledine lab using a small molecular inhibitor of the prostaglandin-E2 receptor, EP2, to demonstrate a cause-effect relationship between EP2 activation and neuroinflammation, hippocampal neurodegeneration, blood–brain barrier leakage, and cognitive deficits following acute DFP intoxication. Since EP2 is critically involved in promoting neuroinflammatory effects in the brain, these studies are among the first to provide data supporting a causal role for neurinflammation in the long-term effects of acute OP intoxication.

44. Marttinen M, Takalo M, Natunen T, Wittrahm R, Gabbouj S, Kemppainen S, Leinonen V, Tanila H, Haapasalo A, Hiltunen M: **Molecular mechanisms of synaptotoxicity and neuroinflammation in Alzheimer's disease**. *Front Neurosci* 2018, **12**:963.

45. • Farizatto KLG, Almeida MF, Long RT, Bahr BA: **Early synaptic alterations and selective adhesion signaling in hippocampal dendritic zones following organophosphate exposure**. *Sci Rep* 2019, **9**:6532.

While synaptotoxicity is strongly assicated with neurodegenerative disease, this is among the few studies to investigate synaptotoxicity in the context of acute OP intoxication. Using hippocampal slice cultures, the authors demonstrated that acute paraoxon exposure progressively reduces pre- and postsynaptic biomarkers as determined by quantitative immunocytochemistry. These effects occurred prior to GluR1 decline and in the absence of signs of neurodegeneration. Decreased expression of synaptic biomarkers correlated with increased levels of the β1 integrin subunit at the synapse. If confirmed *in vivo*, these observations suggest a novel mechanism underlying the delayed neurologic dysfunction after acute OP intoxication.

46. Scholl EA, Miller-Smith SM, Bealer SL, Lehmkuhle MJ, Ekstrand JJ, Dudek FE, McDonough JH: **Age-dependent behaviors, seizure severity and neuronal damage in response to nerve agents or the organophosphate DFP in immature and adult rats**. *Neurotoxicology* 2018, **66**:10–21.

47. Farkhondeh T, Mehrpour O, Forouzanfar F, Roshanravan B, Samarghandian S: **Oxidative stress and mitochondrial dysfunction in organophosphate pesticide-induced neurotoxicity and its amelioration: a review**. *Environ Sci Pollut Res Int* 2020, **27**:24799–24814.

48. Fereidouni S, Kumar RR, Chadha VD, Dhawan DK: **Quercetin plays protective role in oxidative induced apoptotic events during chronic chlorpyrifos exposure to rats**. *J Biochem Mol Toxicol* 2019, **33**, e22341.

49. Mahmoud SM, Abdel Moneim AE, Qayed MM, El-Yamany NA: **Potential role of N-acetylcysteine on chlorpyrifos-induced neurotoxicity in rats**. *Environ Sci Pollut Res Int* 2019, **26**:20731–20741.

50. •• Singh N, Lawana V, Luo J, Phong P, Abdalla A, Palanisamy B, Rokad D, Sarkar S, Jin H, Anantharam V, Kanthasamy AG, *et al.*: **Organophosphate pesticide chlorpyrifos impairs STAT1 signaling to induce dopaminergic neurotoxicity: implications for mitochondria mediated oxidative stress signaling events**. *Neurobiol Dis* 2018, **117**:82–113.

Using molecular genetic and pharmacologic approaches, this study demonstrated an essential role of STAT1 in CPF-induced dopaminergic neurodegeneration and mitochondria-mediated oxidative stress *in vitro* and *in vivo*. The authors also demonstrated the efficacy of mitoapocynin (a mitochondria-targeting antioxidant) in protecting against CPF-induced dopaminergic apoptosis via inhibition of STAT1-dependent ROS generation and proapoptotic signaling events. This study is among the first to causally link oxidative stress to neurotoxic outcomes following repeated low-dose OP exposures.

51. Bengtson CP, Bading H: **Nuclear calcium signaling**. *Adv Exp Med Biol* 2012, **970**:377–405.

52. • Phillips KF, Deshpande LS: **Chronic neurological morbidities and elevated hippocampal calcium levels in a DFP-based rat model of Gulf War Illness**. *Mil Med* 2018, **183**:552–555.

Repeated exposures to DFP (0.5 mg/kg/d over 5 days) resulted in significant neuronal damage in the hippocampus associated with depressive signs and cognitive deficits in adult male rats at 3 and 6 months post-exposure. Ca^{2+} imaging studies of hippocampal neurons acutely isolated 3 or 6 months post-exposure revealed that DFP exposure was associated with a significantly greater percentage of neurons with elevated intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Levetiracetam, which blocks RyR and IP_3R, reversed these *in vivo* and *in vitro* effects, suggesting that dysregulation of internal calcium stores in hippocampal neurons contributes to the long effects of repeated low-level OP exposures.

53. Phillips KF, Santos E, Blair RE, Deshpande LS: **Targeting intracellular calcium stores alleviates neurological morbidities in a DFP-based rat model of Gulf War Illness**. *Toxicol Sci* 2019, **169**:567–578.

54. De Smedt T, Raedt R, Vonck K, Boon P: **Levetiracetam: the profile of a novel anticonvulsant drug-Part I: preclinical data**. *CNS Drug Rev* 2007, **13**:43–56.

55. Mew EJ, Padmanathan P, Konradsen F, Eddleston M, Chang SS, Phillips MR, Gunnell D: **The global burden of fatal self-poisoning with pesticides 2006-15: systematic review**. *J Affect Disord* 2017, **219**:93–104.

56. •• Jett DA, Sibrizzi CA, Blain RB, Hartman PA, Lein PJ, Taylor KW, Rooney AA: **A National Toxicology Program systematic review of the evidence for long-term effects after acute exposure to sarin nerve agent**. *Crit Rev Toxicol* 2020, **50**:474–490.

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.

57. Voorhees JR, Rohlman DS, Lein PJ, Pieper AA: **Neurotoxicity in preclinical models of occupational exposure to organophosphorus compounds**. *Front Neurosci* 2016, **10**:590.

58. Munoz-Quezada MT, Lucero BA, Barr DB, Steenland K, Levy K, Ryan PB, Iglesias V, Alvarado S, Concha C, Rojas E, Vega C: **Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: a systematic review**. *Neurotoxicology* 2013, **39**:158–168.

59. Sagiv SK, Harris MH, Gunier RB, Kogut KR, Harley KG, Deardorff J, Bradman A, Holland N, Eskenazi B: **Prenatal organophosphate pesticide exposure and traits related to autism spectrum disorders in a population living in proximity to agriculture**. *Environ Health Perspect* 2018, **126**, 047012.

60. Rohlman DS, Ismail A, Bonner MR, Abdel Rasoul G, Hendy O, Ortega Dickey L, Wang K, Olson JR: **Occupational pesticide exposure and symptoms of attention deficit hyperactivity disorder in adolescent pesticide applicators in Egypt**. *Neurotoxicology* 2019, **74**:1–6.

61. Sanchez-Santed F, Colomina MT, Hernandez E: **Organophosphate pesticide exposure and neurodegeneration**. *Cortex* 2016, **74**:417–426.

62. Baltazar MT, Dinis-Oliveira RJ, de Lourdes Bastos M, Tsatsakis AM, Duarte JA, Carvalho F: **Pesticide exposures as etiological factors of Parkinson's disease and other neurodegenerative disease – a mechanistic approach**. *Toxicol Lett* 2014, **230**:85–103.

63. •• Anger WK, Farahat FM, Lein PJ, Lasarev MR, Olson JR, Farahat TM, Rohlman DS: **Magnitude of behavioral deficits varies with job-related chlorpyrifos exposure levels among Egyptian pesticide workers**. *Neurotoxicology* 2020, **77**:216–230.
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.