Title

Pathogenic mechanisms of long-term neurotoxicity following repeated low-level and acute high-level organophosphate intoxication

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Pathogenic mechanisms of long-term neurotoxicity following repeated low-level and acute high-level organophosphate intoxication

By

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DISSERTATION

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Abstract

Organophosphate cholinesterase inhibitors (OPs) are a family of potent neurotoxic chemicals that includes nerve agents and pesticides. Accidental or intentional exposures to OPs account for millions of poisoning cases each year. Adverse neurobehavioral outcomes from repeated low-level and acute high-level OPs exposures have been consistently observed in epidemiological studies. Repeated exposures to levels of OPs below the threshold for acute cholinergic toxicity (such as occupational exposures) have been linked to impaired neurobehavioral performance, including memory deficits. Despite the fact that the canonical mechanism of OP neurotoxicity is inhibition of acetylcholinesterase (AChE), there is limited epidemiological or experimental evidence linking AChE inhibition to neurobehavioral outcomes caused by repeated low-level OP exposures, and the mechanism(s) that mediate the neurotoxic effects of these exposures remain speculative. For acute OP exposures, current standard of care treatment is effective in reducing mortality, but unless administered within minutes of exposure, does not effectively protect against delayed brain damage and long-term morbidity, including altered brain structure, abnormal electrographic activity and impaired cognitive behavior. The pathogenic mechanism(s) that link the acute OP intoxication to delayed and persistent neurological sequelae are not well established.

This dissertation seeks to address the functional role(s) of non-cholinergic mechanisms in OP neurotoxicity by applying two different adult rat models of OP exposures: 1) a rat model of occupational exposure to the OP pesticide chlorpyrifos (CPF); and 2) a rat model of acute intoxication with the OP diisopropylfluorophosphate (DFP). Chapter 2 demonstrates that repeated CPF exposure for 21 days caused sustained AChE inhibition, significantly impaired learning and memory, and increased biomarkers of oxidative stress and astrogliosis. Treatment with the antioxidant Trolox not only reduced expression of biomarkers of oxidative stress but also decreased astrogliosis and restored cognitive function in CPF-exposed rats. However, Trolox had no effect on CPF-induced AChE inhibition. These findings suggest a causal

relationship between oxidative stress and cognitive impairment caused by occupational CPF exposure. In chapter 3, we investigated whether acute DFP intoxication promotes the development of cellular senescence, which has been linked to behavioral deficits associated with aging and neurodegenerative disease, but not yet examined in acute OP intoxication. Our data indicated that acute intoxication with DFP upregulates expression of the senescence biomarker, p16, exclusively in neurons, with neuronal p16 expression first observed at 3 and 6 months post-DFP intoxication in hippocampus and thalamus only. The spatiotemporal profile of neuronal p16 expression was significantly correlated with FJC labeling of degenerating neurons in these same brain regions and time points. These data identify cellular senescence as a potential mechanism contributing to the chronic neurologic sequelae of acute OP intoxication. The diverse range of adverse outcomes induced by OP neurotoxicity is highly dependent on the exposure paradigm and may involve mechanisms independent of AChE inhibition. Future studies are warranted to further understand the causal roles of non-cholinergic mechanisms in long-term neurological consequences following OP exposures and to identify therapeutic windows to provide optimal neuroprotection or prevention.

The diverse range of adverse outcomes induced by OP neurotoxicity is highly dependent on the exposure paradigm and may involve mechanisms independent of AChE inhibition. Future studies are warranted to further understand the causal roles of non-cholinergic mechanisms in long-term neurological consequences following OP exposures and to identify therapeutic targets and windows to optimally preserve brain function in individuals intoxicated with OPs.

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Finally, I MADE IT!‼

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Chapter 1

Introduction

Based on a publication in Current Opinion in Toxicology (Volume 26, June 2021, Pages 49-60) with the following title and authors:

Mechanisms of organophosphate neurotoxicity

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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* non-cholinergic 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* (SE); (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.

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 = diisopropylfluorophosphate; $GABA_AR$ = $GABA_A$ receptors; GluK1R = kainate receptors containing the GluK1 subunit; IP_3R = inositol triphosphate receptor; mAChR = muscarinic cholinergic receptors; 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 = 18kDa mitochondrial translocator protein

Introduction

The canonical mechanism of OP neurotoxicity is inhibition of acetylcholinesterase (AChE), which results in hyperstimulation of muscarinic (mAChR) and nicotinic (nACR) cholinergic receptors in the peripheral and central nervous systems (**Box 1**). Acute inhibition of AChE by ≥60-70% is associated with "cholinergic crisis", a clinical toxidrome characterized by muscle fasciculations and weakness, parasympathomimetic signs, depression of respiratory control centers in the brainstem, seizures, and death (Pereira et al., 2014; Richardson et al., 2019). Acute OP intoxication annually causes an estimated 3 million life-threat (Mew et al., 2017; Pereira et al., 2014).

There are numerous reports of long-term neurologic effects in those who survive acute OP intoxication (Chen, 2012; Figueiredo et al., 2018), but a cause-effect relationship has been difficult to establish in humans (**Box 2**). This was recently addressed in a systematic review of the evidence for long-term effects in humans acutely exposed to intoxicating levels of sarin (Jett & Spriggs, 2020). 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 (Jett & Spriggs, 2020). 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 (de Araujo Furtado et al., 2012; Guignet & Lein, 2019; Pereira et al., 2014).

Clinical and experimental evidence support a role for AChE inhibition in triggering acute neurotoxic effects of OPs (Pereira et al., 2014; Richardson et al., 2019), but suggest long-term effects are mediated by mechanisms other than or in addition to AChE inhibition. Persistent epileptiform discharges, prolonged memory impairment, and anxious or depressive behavior

manifest in acutely intoxicated humans (Chen, 2012; Figueiredo et al., 2018) and experimental animals (de Araujo Furtado et al., 2012; Guignet & Lein, 2019; Pereira et al., 2014) well after AChE activity has recovered to pre-exposure levels. Further evidence includes observations that different OPs elicited varying neurotoxicity profiles at levels that cause comparable inhibition of AChE activity (Bushnell & Moser, 2006; Costa, 2018), and AChE knockout mice exhibited signs of acute neurotoxicity (including loss of motor activity, flattened posture, peripheral vasodilation, and hypothermia) similar to those observed in wildtype mice following acute OP poisoning (Duysen et al., 2001).

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 (Voorhees et al., 2017). Additionally, recent epidemiologic studies link repeated low-level OP exposures to increased risk of neurodevelopmental disorders (Munoz-Quezada et al., 2013; Rohlman et al., 2019; Sagiv et al., 2019), and neurodegenerative disease (Baltazar et al., 2014; Sanchez-Santed et al., 2016). These associations have been debated in part because of the lack of evidence of a dose-response relationship (Meyer-Baron et al., 2015; Rohlman et al., 2011). 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 (Anger et al., 2020). 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. 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 (Anger et al., 2020).

Trail Making performance was not associated with blood cholinesterase activity (Anger et al., 2020). This is consistent with earlier analyses of the epidemiologic literature that supported an association of subclinical occupational (Meyer-Baron et al., 2015; Rohlman et al., 2011) and early-life (Burke et al., 2017) OP exposures with neurotoxic outcomes, but did not find an association between blood cholinesterase activity and neurobehavioral outcomes. These findings suggest that non-cholinergic mechanisms contribute to the neurotoxicity of repeated low-level OP exposures, a hypothesis largely supported by the preclinical literature (Burke et al., 2017; Silva, 2020). While the most significant and prolonged motor effects in animals are observed following OP exposures that markedly inhibit AChE activity, deficits in cognitive (Bushnell & Moser, 2006) and social (Berg et al., 2020) behavior are not as clearly correlated with AChE inhibition.

Characterization of non-cholinergic 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 regarding: (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 (Aroniadou-Anderjaska et al., 2020). The molecular mechanism(s) mediating this transition are poorly understood. Recent electrophysiological studies of acute rat brain slices found that paraoxon acutely enhanced the hyperpolarization-activated cation current I_h in basolateral amygdala principal neurons (Miller et al., 2017). 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 following exposure to OP nerve agents

(Aroniadou-Anderjaska et al., 2020). In support of this model, pretreatment with VU0255035 significantly reduced seizure severity and prevented the development of SE for up to 40 minutes in rats acutely intoxicated with paraoxon or soman (Miller et al., 2017).

In contrast, recent studies of acute mouse hippocampal slices revealed that mAChR hyperactivity initially decreased glutamatergic neurotransmission (Brown et al., 2020; Hoffman et al., 2019). Inhibition of CA1 glutamatergic neurotransmission by acute exposure to diisopropylfluorophosphate (DFP) at concentrations that caused >75% AChE inhibition was blocked by pharmacologic antagonism of M2 mAChR (Brown et al., 2020). Another group found that mAChR hyperactivity inhibited excitatory neurotransmission via retrograde activation of presynaptic endocannabinoid type 1 receptors (CB1R) (Hoffman et al., 2019). Electrophysiological recordings of hippocampal Schaeffer collateral synapses demonstrated that paraoxon, soman and VX depressed field excitatory postsynaptic potentials prior to the onset of interictal spiking. This effect was mediated by presynaptic mechanisms independent of recurrent firing or NMDA receptor (NMDAR) currents and was completely reversed by pharmacologic antagonism of CB1R or M1/M3 mAChR, but not M2/M4 mAChR. 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 mAChR triggered postsynaptic release of endocannabinoids that retrogradely diffused across the synapse to activate CB1R and reduce presynaptic release probability (Hoffman et al., 2019). The observation that presynaptic depression occurred prior to interictal bursting suggested that OP suppression of presynaptic glutamate release is an early compensatory response to excessive cholinergic signaling. In support of this hypothesis, pharmacologic antagonism of CB1R enhanced lethality in a mouse soman model (Hoffman et al., 2019).

The neurotransmitter receptor subtypes involved in sustaining OP-induced seizures have also been the focus of recent research (**Table 1-1**). 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 (AMPA, kainate, and NMDA) and downregulated expression of $GABA_A$ receptors ($GABA_AR$) at synapses in the hippocampus and amygdala, brain regions critically involved in OP-induced SE (Aroniadou-Anderjaska et al., 2020). Downregulation of synaptic $GABA_AR$ is posited to mediate benzodiazepine refractoriness, a characteristic feature of OP-induced SE (Aroniadou-Anderjaska et al., 2020; Reddy, 2019). Neurosteroids are positive allosteric modulators of not only synaptic but also extrasynaptic $GABA_AR$ (Reddy, 2019). Post-exposure administration of neurosteroids significantly attenuated benzodiazepine-refractory seizures in rats acutely intoxicated with sarin (Lumley et al., 2019) or DFP (Dhir et al., 2020). Such observations suggest that activity of extrasynaptic $GABA_AR$ critically influences seizure duration following acute OP intoxication.

Recent studies also support a causal role for AMPA and kainate receptors in sustaining OP-induced seizures. Administration of LY293558, a relatively broad-spectrum antagonist of AMPA receptors (AMPAR) 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 hours post-exposure (Apland et al., 2018). Reports that the AMPAR antagonist perampanel (Dhir et al., 2020) and the dual AMPAR and NMDAR antagonist urethane (Rojas et al., 2020b) 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 hours (Apland et al., 2018). 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-induced SE (Niquet et al., 2019; Niquet et al., 2020); however, memantine, which also blocks NMDAR, exacerbated DFP-

induced seizures and increased mortality (Spampanato et al., 2020). Further studies are needed to identify the reason(s) for the differential effects of these drugs on OP-induced SE and to clarify the role of the NMDAR. Non-glutamatergic receptors may also be involved in sustaining OP-induced seizures. Combined midazolam and dexmedetomidine, a α2-adrenergic receptor antagonist, was superior to midazolam alone in mitigating seizure activity in rats when given 60 minutes after the initiation of SE by DFP (Spampanato et al., 2020) or soman (McCarren et al., 2018).

Whether and how the functions of these varying neurotransmitter receptor subtypes differ in a region- and/or time-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 (Olivares-Banuelos et al., 2019).

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 (Hobson et al., 2017; McDonough & Shih, 1997). However, recent studies challenge this paradigm. It was observed that antiseizure efficacy did not necessarily correlate with protection against neuronal death 24 hours post-exposure in rats acutely intoxicated with DFP. Memantine exacerbated seizure severity, but significantly reduced neuronal cell death; conversely, dexmedetomidine enhanced seizure suppression but conferred no significant neuroprotection (Spampanato et al., 2020). In another study of DFP-intoxicated rats, a subpopulation of animals was observed to exhibit minimal to no behavioral or electrographic seizures despite brain AChE inhibition comparable to that of animals with SE (Gonzalez et al., 2020). The brains of non-seizing animals exhibited significant neurodegeneration although it was delayed, less persistent, and less severe compared to

seizing animals. Micro-CT scans at 60 days post-exposure revealed extensive mineralization in the thalamus that was not significantly different between seizing and non-seizing subjects (Gonzalez et al., 2020). These data suggest that seizure-independent mechanism(s) contribute to neuropathology following acute OP intoxication.

Oxidative stress

Oxidative stress is strongly associated with excessive cholinergic and glutamatergic activity (Aroniadou-Anderjaska et al., 2020; Williamson et al., 2019), and is posited to mediate the neuropathologic consequences of OP-induced SE (Guignet & Lein, 2019; Naughton & Terry, 2018). Recent preclinical studies confirmed that acute OP intoxication upregulated brain expression of multiple oxidative stress biomarkers (**Table 1-1**) prior to the onset of long-term effects of acute OP intoxication. To probe the role of oxidative stress, structurally and mechanistically diverse antioxidants were used to reduce oxidative stress in the brain following OP-induced SE (**Table 1-1**). Administration of AEOL10150, a catalytic antioxidant that scavenges reactive oxygen and nitrogen species (ROS and RNS, respectively), within 5-15 min after rats were exposed to DFP (Liang et al., 2018) or soman (Liang et al., 2019), significantly attenuated neuroinflammation and neurodegeneration in multiple brain regions at 24 and 48 hours post-exposure. Administration of diapocynin (Putra et al., 2020a), an NADPH oxidase inhibitor, or 1400W (Putra et al., 2020b), an iNOS inhibitor, 4 hours after acute DFP intoxication significantly attenuated neuroinflammation and neurodegeneration for weeks to months. Interestingly, diapocynin reduced astrogliosis, but not microgliosis, whereas 1400W 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 in

survivors of OP-induced SE? The diapocynin and 1400W studies (Putra et al., 2020a; Putra et al., 2020b) showed that 1400W significantly suppressed epileptiform spiking for weeks; diapocynin during the first 72 hours after acute DFP intoxication, while 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 oxidative stress, neuroinflammation, neurodegeneration, and neurologic deficits following acute OP intoxication.

Neuroinflammation

As reviewed in 2019 (Guignet & Lein, 2019), a growing body of literature indicates 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 following acute DFP intoxication (Guignet et al., 2020; Putra et al., 2020a; Putra et al., 2020b; Supasai et al., 2020). Longitudinal monitoring of neuroinflammatory responses in DFP-intoxicated rats using positron emission tomography to quantify expression of the 18kDa mitochondrial translocator protein (TSPO) revealed neuroinflammation varied dynamically in a region- and time-dependent manner (Hobson et al., 2019).

TSPO is a biomarker of activated microglia and/or astrocytes (Guilarte, 2019), but its expression does not indicate whether activated microglia and astrocytes are neuroprotective or pathogenic (Guignet & Lein, 2019). A recent study began to address this question by phenotyping microglia and astrocytes in the mouse brain following DFP-induced SE (Maupu et al., 2021) using biomarkers that label microglia as pro-inflammatory (M1-like), anti-inflammatory (M2a-like) or immunoregulatory (M2b-like) (Chhor et al., 2013), and reactive astrocytes as neurotoxic (A1-like) or neuroprotective (A2-like) (Liddelow et al., 2017). These biomarkers were quantified by qRT-PCR in CD11B- (microglia/infiltrated macrophages) and GLAST (astrocytes)-

immunopositive cells isolated from whole brain at varying times after DFP-induced SE using magnetic-activated cell-sorting (Maupu et al., 2021). At 1 and 4 hours post-exposure, M1-like and A2-like markers were observed in CD11B and GLAST-positive isolated cells, respectively. At 4 and 24 hours, microglial cells transitioned from M2b-like to M2a-like. At 24 hours and 3 days, A1-like markers were increased in isolated astrocytes. While this study did not assess the functional relevance of these subtypes, the observation that these cells' phenotype shifted over time post-exposure has significant implications for understanding whether and when neuroinflammation is protective *versus* harmful following OP-induced SE.

Few studies have examined whether pharmacologic manipulation of neuroinflammation modifies long-term effects of acute OP intoxication. To date, the most compelling data come 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 (Rojas et al., 2020a). As described in detail in a recent review of these data (Rojas et al., 2020a), administration of TG6-10-1 to DFP-intoxicated rats had no effect on SE, but attenuated upregulation of inflammatory cytokines and chemokines (IL-1β, TNFα, IL-6, CCL2, CCL4) in the brain, and prevented blood-brain barrier breakdown. While TG6-10-1 did not mitigate anxiety-like behavior, it significantly improved performance in the novel object recognition task 8 to 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 neuroinflammatory mediators to specific neurologic outcomes.

Synaptotoxicity

While strongly associated with neurodegenerative disease (Marttinen et al., 2018), 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 (Farizatto et al., 2019). 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 increased β1 integrin expression was restricted to synapses. The extent of synaptic decline was 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* (Scholl et al., 2018). 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.

A potential mechanism contributing to the chronic neurologic sequelae of acute OP intoxication: *Cellular senescence*

Individuals who survive the acute cholinergic crisis triggered by exposure to high levels of OPs often suffer from long-term neuropsychiatric and cognitive deficits (reviewed in Figueiredo et al., 2018; Loh et al., 2010; Miyaki et al., 2005; Yamasue et al., 2007), which are also typical manifestations of brain aging and neurodegenerative diseases such as Alzheimer's disease (AD) and age-related dementia. While an association between acute OP intoxication and impaired cognition has been reported in humans and corroborated in preclinical models (Deshpande et al., 2014; reviewed in Figueiredo et al., 2018; Flannery et al., 2016; Guignet et al., 2020; Loh et al., 2010; Miyaki et al., 2005), the causal mechanism(s) contributing to the chronic neurodegeneration and neurobehavioral impairment remain poorly characterized.

Emerging evidence has suggested a pathogenic role of cellular senescence in aging and neurodegenerative diseases (reviewed in Baker & Petersen, 2018; reviewed in Sikora et al., 2021). More than 50 years ago, the concept of cellular senescence was first described by Dr. Hayflick and Dr. Moorhead who observed that primary human fibroblasts will enter proliferative arrest when cultured *in vitro* over extended periods (Hayflick & Moorhead, 1961). Recently, the classic definition of cellular senescence as replicative senescence has been extended beyond telomere shortening as a result of repetitive cell divisions. Researchers have proposed cellular senescence as a stress response to varying stressors including DNA damage and oxidative stress (reviewed in Baker & Petersen, 2018; reviewed in Campisi, 2013; reviewed in Walton & Andersen, 2019). Preclinical studies further confirmed that upregulated expression of multiple senescence-related biomarkers in diverse brain cells (Bhat et al., 2012; Bussian et al., 2018; Chinta et al., 2018; Jurk et al., 2012; Moreno-Blas et al., 2019; Musi et al., 2018; Vazquez-Villaseñor et al., 2020; Zhang et al., 2019). While the initial defining characteristic of cellular senescence lies on a stable growth arrest, many other phenotypic alterations associated with the senescent cells have been demonstrated and serve as important tools to understand the pathophysiological functions of senescent cells (**Figure 1-1**).

Preclinical research into a functional role for cellular senescence in disease progression has leveraged transgenic animal models and mechanistically diverse drugs called “senolytics” to target senescent cells in the brain (**Table 1-2**). A particularly impactful report in the recent published literature is the study conducted by Chinta and colleagues (Chinta et al., 2018) that investigated the role and contribution of senescent astrocytes to the pathogenesis of Parkinson’s disease (PD) triggered by the environmental neurotoxin paraquat (PQ). PQ has been identified as an environmental factor that is significantly associated with PD. This study took advantage of a transgenic mouse model, p16-3MR, that allows selective depletion of senescent cells. In p16-3MR mice, the promoter for the senescence marker p16INK4a is used to induce expression of a modified herpes simplex virus thymidine kinase (HSV-TK), which then

converts the drug ganciclovir (GCV) into a DNA chain terminator upon administration to induce apoptosis. Quantitative PCR analyses of the striatal tissues from 8-month-old transgenic p16-3MR mice injected with PQ revealed a significantly increased expression of the senescence markers p16 and one of the senescence-associated secretory phenotype (SASP) factors IL-6. Immunohistochemical analyses further showed a significant loss of nuclear lamin B1 and HMGB1 (high mobility group box 1) exclusively in GFAP-positive astrocytes in the substantia nigra pars compacta (SNpc). Importantly, the aforementioned senescent features, loss of dopaminergic neurons, and rearing behavior were rescued by treatment with GCV, which cleared senescent cells in the brains of GCV-treated p16-3MR mice. Research on cellular senescence in the brain has gained impetus, yet this is a novel concept that has not yet been investigated as a potential mechanism underlying the neurologic consequences of acute OP intoxication. Further studies are also warranted in order to uncover how environmental factors and/or endogenous stress responses can cause the induction of cellular senescence in the brain.

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 (Guignet & Lein, 2019). Identifying the mechanism(s) by which OPs cause neuroinflammation and determining the cause-effect relationship between neuroinflammation and neurotoxic outcomes remain significant data gaps that have yet to be addressed.

The literature addressing oxidative stress in repeated low-level OP exposures was also recently reviewed (Farkhondeh et al., 2020); here, we highlight recent studies that investigated cause-effect relationships between oxidative stress and neurotoxic outcomes (**Table 1-1**). Two groups (Fereidouni et al., 2019; Mahmoud et al., 2019) examined the role of oxidative stress in

apoptosis in adult rats repeatedly exposed to 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 anti-apoptotic protein Bcl-2. Co-administration of CPF and an antioxidant, either quercetin (Fereidouni et al., 2019) or N-acetylcysteine (Mahmoud et al., 2019), mitigated expression of oxidative stress biomarkers and reversed CPF effects on apoptotic protein expression. While quercetin mitigated CPF inhibition of AChE (Fereidouni et al., 2019), N-acetylcysteine did not (Mahmoud et al., 2019), suggesting that AChE inhibition is not mechanistically linked to CPF-induced apoptosis.

A significant caveat of these studies is that they did not quantify apoptotic cells or neuronal cell loss, nor did they address whether oxidative stress mediates 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 (Singh et al., 2018). 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 (Singh et al., 2018). 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 a STAT1- and mitochondrial ROS-dependent manner. *In vivo*, CPF similarly elicited STAT1 activation and oxidative stress-mediated pro-apoptotic signaling in the substantia nigra and striata, but not the cortex (Singh et al., 2018). Co-administration of mitoapocynin ameliorated these molecular responses and rescued CPF-induced motor deficits and nigrostriatal dopaminergic neurodegeneration (Singh et al., 2018).

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 other non-dopaminergic effects associate 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 (Bengtson & Bading, 2012). Repeated low-dose DFP exposure (**Table 1-1**) 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 post-exposure (Phillips & Deshpande, 2018; Phillips et al., 2019). 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 concentrations of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Phillips & Deshpande, 2018; Phillips et al., 2019). Pharmacologic block of voltage-gated Ca^{2+} ion channels, AMPA/kainate channels, or other nonspecific, gadolinium chloride-sensitive cation channel in isolated neurons, did not reduce $[Ca^{2+}]_i$. In contrast, pharmacologic antagonism of NMDAR with MK-801 produced a small but significant reduction, while ryanodine receptor (RyR) antagonism by dantrolene or inhibition of both RyR- and inositol triphosphate receptor (IP_3R)-activated Ca^{2+}-induced Ca^{2+}-release (CICR) by levetiracetam significantly decreased $[Ca^{2+}]_i$ (Phillips et al., 2019). 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 (Phillips et al., 2019). In support of this hypothesis, *in vivo* treatment with levetiracetam at 3 months post-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 (Phillips et al., 2019).

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 depressive-like and cognitive behaviors to baseline, suggesting additional mechanisms likely contribute to these phenotypes. Since levetiracetam has not only been shown to inhibit RyR- and IP_3R-activated CICR (Fukuyama et al., 2012; Nagarkatti et al., 2008), but also modulates glycine and GABA receptors and binds to SV2A protein (De Smedt et al., 2007), 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 via multiple mechanisms that vary depending on the exposure paradigm (**Figure 1-2**). 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.

Dissertation overview

Multiple lines of evidence suggest that mechanisms in addition to AChE inhibition contribute to the neurotoxic effects associated with acute and chronic OP exposures. Better understanding of the role(s) of non-cholinergic mechanisms in OP neurotoxicity remains an active area of research with significant diagnostic and therapeutic implications. The data presented in this dissertation aim to fulfill the following objectives: 1) to characterize the long-term neurologic effects following repeated OP exposure using both neuropathological and behavioral assessments; 2) to investigate the spatiotemporal progression of a specific phenotype, cellular senescence, following acute OP intoxication; and 3) to provide insights on the therapeutic potentials of targeting non-cholinergic mechanisms in alleviating OP-induced neuropathology.

Chapter 2 takes advantage of an animal model that is based on published exposure studies of agricultural workers repeatedly exposed to the OP, chlorpyrifos (CPF), over 15 - 30 days during the cotton growing season in Egypt to increase our understanding of the neurotoxic mechanisms and long-term effects induced by repeated CPF exposures. With a rat model that captures the human condition, the data presented in chapter 2 provide a robust characterization of neuropathology and neurobehavioral deficits following repeated CPF exposures. Furthermore, using the antioxidant Trolox, a vitamin E derivative, the findings identify oxidative stress as a molecular mechanism contributing to CPF-induced cognitive deficits.

While the short-term neurotoxic outcomes after acute DFP intoxication have been well-characterized by our lab (Flannery et al., 2016; Hobson et al., 2019; Li et al., 2011) and others (Deshpande et al., 2010; Kuruba et al., 2018; Rojas et al., 2018), it was only recently that studies reported that these neuropathological effects last for months post OP-intoxication (Guignet et al., 2020; Putra et al., 2020a; Supasai et al., 2020). However, identification of specific mechanism(s) driving or maintaining the neuropathogenic consequences caused by acute intoxication with DFP remains to be determined. To address this critical data gap, chapter 3 seeks to examine cellular senescence, which is implicated in neurodegenerative diseases but as yet uncharacterized following acute OP intoxication, in our rat model of DFP-induced status

epilepticus (SE). Identification of cellular senescence as a novel mechanism of OP neurotoxicity and the positive correlation between neurodegeneration and cellular senescence significantly advances the field by suggesting a novel pathogenic mechanism to be explored.

In summary, the following data chapters enhance our understanding of the neuropathology associated with both acute and chronic exposures to OPs, contributes to the body of literature describing the contribution of non-cholinergic mechanisms to OP neurotoxicity and suggests potential therapeutic targets. Collectively, these studies confirm that OPs trigger different neurotoxic mechanisms depending on the exposure paradigms and underscore the importance of understanding the functional relationships between these mechanisms and neurologic outcomes.

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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 agents, 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, and neurotoxicity associated with repeated low-level OP exposures that may inhibit AChE, but do not cause signs of cholinergic crisis.

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 (Mew et al., 2017). There are numerous reports of long-term neurologic effects in those who survive acute OP intoxication (Chen 2012; Figueiredo et al., 2018), 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 (Jett et al., 2020). 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 (Jett et al., 2020). 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 (Pereira et al., 2014; de Araujo Furtado et al., 2012; Guignet and Lein 2019).

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 (Voorhees et al., 2016). Additionally, recent epidemiologic studies link repeated low-level OP exposures to increased risk of neurodevelopmental disorders (Munoz-Quezada et al., 2013; Sagiv et al., 2018; Rohlman et al. 2019) and neurodegenerative disease (Sanchez-Santed et al., 2016; Baltazar et al., 2014]. These associations have been debated in part because of the lack of evidence of a dose-response relationship (Meyer-Baron et al., 2015; Rohlman et al., 2011). 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 (Anger et al., 2020). 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 (Anger et al., 2020). 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 (Anger et al., 2020).

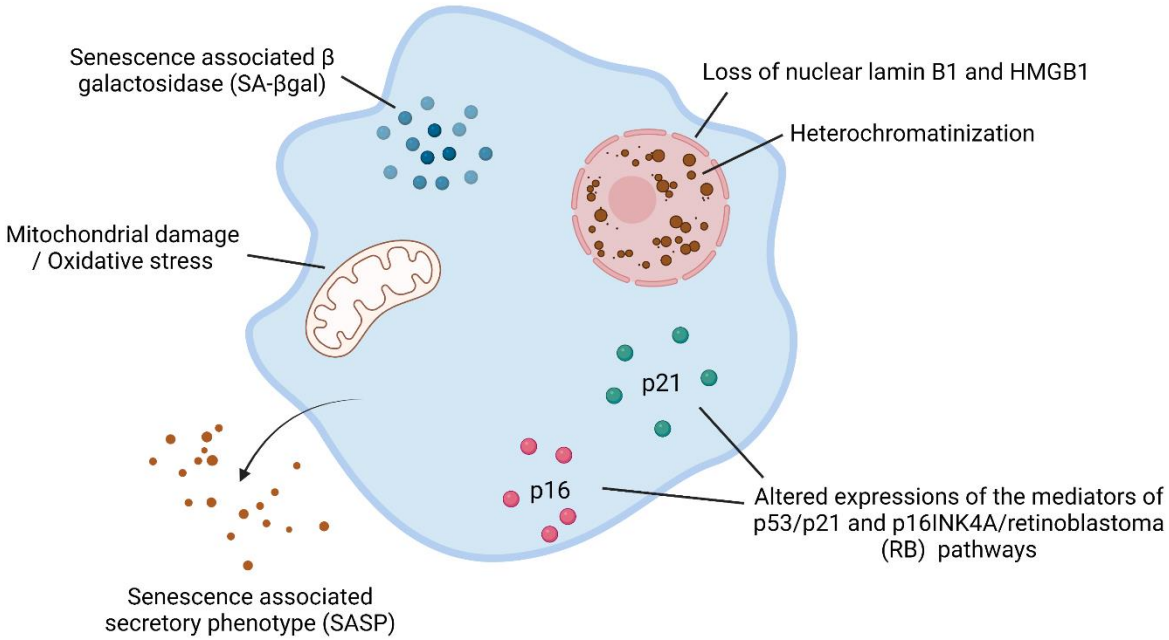

Figure 1-1. Characteristic features of senescent cells. Adapted from Baker, D. J., & Petersen, R. C. (2018). Cellular senescence in brain aging and neurodegenerative diseases: evidence and perspectives. The Journal of Clinical Investigation, 128(4), 1208-1216. doi:10.1172/JCI95145. Created with BioRender.com.

Table 1-1. Mechanisms of OP Neurotoxicity: SE (green), Long-Term Effects of Acute (yellow) and Repeated Low-Level (blue) Exposures

OP	Pharmacological probes and targets	Exposure paradigm	Model	Time point(s)	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)
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.) <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 3/10 animals]** ⬆Low amplitude, high frequency activity ⬆Gamma power [than MDZ+0.4 mg/kg DEX]	McCarren et al. (2018)

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)** 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.), 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 **[MDZ + ALLO + PPL >> MDZ]**	Dhir et al. (2020)
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 hours	Adult male SD rats	24 h post-exposure	<u>MDZ+AEOL10150 in comparison to MDZ alone</u>: ⬆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)
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 hours	Adult male SD rats	6, 12, 24, and 48 h post-exposure	<u>MDZ+AEOL10150 in comparison to MDZ alone</u>: ⬆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)

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	<u>1400W vs. MDZ alone</u>: ▬ 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)
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-hour intervals for the first 3 days	Adult male SD rats	4 and 6 weeks post-exposure	<u>Diapocynin vs. DZP alone</u>: ⬇ 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)
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	<u>LEV effects vs. DFP alone</u>: ⬇ $[Ca^{2+}]i$ of primary hippocampal neurons ⬆ 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)

CPF[f]	**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	<u>Quercetin treatment in comparison to CPF</u>: ↓Protein carbonyl contents (serum and cerebrum) ↑AChE activity (serum and cerebellum) ↑Bcl-2 protein level (brain) ↓Bax, cytochrome c, caspase-8, and caspase-9	Fereidouni et al. (2019)
CPF	**N-acetylcysteine (NAC)** antioxidant	NAC (100 mg/kg/d) ↓1 h CPF (10 mg/kg/d oral)	Adult male albino rats	28 days	<u>NAC effects vs. CPF alone:</u> ↑GSH[g] and SOD[h] ↓MDA[i] and NO levels ↑Bcl-2 level ↓Bax level	Mahmoud et al. (2019)
CPF	**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	<u>Mitoapocynin effects on CPF-induced cytotoxicity</u>: ↓DNA fragmentation ↓ROS generation ↓Mitochondria-derived superoxide ↓STAT1 phosphorylation ↓Autophagy (LC3B, beclin1)	Singh et al. (2018)
		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[j])	5 weeks	<u>Mitoapocynin effects on CPF-induced dopaminergic neurotoxicity</u>: ↓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.

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

Table 1-2. Recent evidence demonstrating the beneficial effects of targeting senescent cells in the brain

Disease implication	Genetic ablation / Senolytics	Model	Outcomes after treatment with senolytics	Citation
Alzheimer's disease	Dasatinib (5 mg/kg) + quercetin (50 mg/kg) (D+Q): one month after the first treatment, senolytic gavage continued on a biweekly basis for a total of six treatment sessions over 12 weeks	16- to 32-month-old male and female rTg4510 and rTg21221 mice on either a wild-type or *Mapt* knockout Bl6/FVB genetic background	↓the number of NFT-containing cortical neurons ↓ventricular volume and cortical brain atrophy ↓SASP gene expression (e.g. *cdkn2a*, *cdkn1a*, *Tlr4*, and *Cxcl1*) ↑cerebral blood flow ↑neuronal proteins (NeuN, PSD95, synaptophysin) ▬total tau protein levels	Musi et al. (2018)
Alzheimer's disease	1) AP20187 (2 mg/kg): twice a week beginning at weaning age (3 weeks) 2) ABT263 (navitoclax, 50 mg/kg): oral gavage starting at weaning on a regimen of five consecutive days of treatment followed by 16 days of rest	1) Till 6-month-old PS19;ATTAC male mouse 2) Till 6-month-old PS19 mice	<u>AP20187 treatment in PS19;ATTAC mice</u>: ↓gene expression of senescence markers (e.g. $p16^{INK4a}$, $p19^{Arf}$, $p21^{Cip1/Waf1}$, *Pai1*, *Il6*, *Il1b*) ↓X-gal-positive cells in the hippocampus and cortex ↓soluble tau, soluble phosphorylated tau, and insoluble phosphorylated tau ↑brain size ↑the dentate gyrus area and increased neuron density ↑cognitive function assessed by novel object recognition <u>ABT263 treatment in PS19 mice:</u> ↓gene expression of senescence markers (e.g. $p16^{INK4a}$, $p19^{Arf}$, $p21^{Cip1/Waf1}$, *Pai1*, *Il6*, *Il1b*) ↓phosphorylated tau (S202/T205) protein aggregates in the cortex and hippocampus	Bussian et al. (2018)

Alzheimer's disease	1) Short-term: Dasatinib (12 mg/kg) + quercetin (50 mg/kg) oral gavage once daily for 9 days 2) Long-term: Dasatinib (12 mg/kg) + quercetin (50 mg/kg) oral gavage once weekly for 11 weeks	1) Short-term: 7.5-month-old APP/PS1 mice 2) Long-term: beginning at 3.5-month-old female APP/PS1 AD mice	<u>Short-term treatment</u>: ➖Aβ load ⬇Aβ-plaque-associated SA-βGal activity ⬇oligodendrocyte progenitor cell marker (Olig2) and p21 ⬇Aβ-plaque-associated p16 mRNA <u>Long-term treatment</u>: ⬇hippocampal Aβ plaque load ⬇Aβ-plaque-associated SA-βGal activity in the hippocampus ⬇Aβ-plaque-associated Olig2-immunoreactive cells ⬇levels of $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus and entorhinal cortex ⬇IL-1β and TNF-α in the hippocampus and entorhinal cortex ⬆performance in the Y maze and water maze
Parkinson's disease	Ganciclovir (GCV, 25 mg/kg): i.p. injection after the second paraquat (PQ) injection and every other day for a total of 5 doses	8-month-old male and female p16-3MR mice treated with 7 mg/kg PQ at 2-day intervals for a total of 6 doses	⬇striatal $p16^{INK4a}$ and IL-6 mRNA levels as compared to PQ group ⬆nuclear lamin B1 and HMGB1 in astrocytes ⬆dopaminergic cell counts in the substantia nigra ⬆neurogenesis ⬆rearing rate
Aging	1) AP20187 (10 mg/kg): i.p. injections 2) Dasatinib (5 mg/kg) + quercetin (10 mg/kg) (D+Q): oral gavage for 3 consecutive days/week, every two weeks for the total period of 8 weeks	4 and 25-29 months of age INK-ATTAC$^{+/-}$ mice	⬇age-induced spatial memory dysfunction ⬇$p16^{Ink4a}$-positive microglia in the CA3 of the hippocampus (AP20187 treatment only) ⬆Lamin B1 and HMGB1 expression in neurons of CA3 of the hippocampus (both AP20187 and D+Q treatments) ⬇p21-positive neurons in the CA3 of the hippocampus (AP20187 treatment only) ⬇IL-1α (D+Q treatment only) and MCP-1 (AP20187 treatment only) mRNA expressions in cells of CA3 hippocampal pyramidal layer ⬇soma size of microglia in the hippocampus (both AP20187 and D+Q treatments) ⬇the number of CD3-positive cells measured in the lateral ventricle (both AP20187 and D+Q treatments)

Chapter 2

The Organophosphate Pesticide Chlorpyrifos Causes Learning and Memory Deficits via Oxidative Stress in a Rat Model of Occupational Exposure

Modified from a manuscript in preparation with the following authors:

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Abstract

Occupational exposure to organophosphate pesticides (OPs) has been associated with deficits in learning and memory; however, the mechanisms by which repeated/chronic exposures to OPs impair cognitive behavior remain speculative. Accumulating evidence suggests that oxidative stress may contribute to OP neurotoxicity associated with occupational exposures. To test this hypothesis, we used a rat model of occupational exposure to the OP chlorpyrifos (CPF) to determine whether CPF increases expression of biomarkers of oxidative stress and whether CPF-induced learning and memory deficits are blocked by antioxidant treatment. Adult male Long-Evans rats were pre-treated with a vitamin E analog Trolox (1 mg/kg/d i.p.), or corresponding vehicle (10% ethanol in 90% Neobee M-5, 1 ml/kg/d) for 5 days prior to daily exposure for 21 days to either CPF (10 mg/kg/d, s.c.) or vehicle (10% ethanol in 90% Neobee M-5, 1 ml/kg/d) in the absence or presence of concurrent Trolox treatment (1 mg/kg/d, i.p.). Repeated CPF exposure resulted in progressive inhibition of blood and brain acetylcholinesterase (AChE). Performance in the contextual fear conditioning test indicated that 21 days of CPF exposure significantly impaired learning and memory. Oxylipins (12, 13-DiHOME, 9, 10-DiHOME, and 14, 15-DiHETrE in the plasma; PGF2α in the brain) and serum and hippocampal prostaglandin E2 (PGE2) levels were elevated following repeated CPF exposure. Immunohistochemical analyses showed significantly increased expression of biomarkers of neuronal oxidative stress and astrogliosis but not neurodegeneration or microglial activation. Importantly, treatment with the antioxidant Trolox significantly ameliorated astrogliosis, mitigated biomarkers of oxidative stress, and prevented cognitive deficits associated with repeated CPF exposure but had no effect on CPF-induced AChE inhibition. Collectively, these data support the hypothesis that occupational OP exposure causes cognitive deficits via mechanisms involving oxidative stress, and suggest the therapeutic potential of using antioxidants to prevent the neurotoxic effects of repeated/chronic OP exposures.

Introduction

The use of pesticides has significantly increased and improved crop yields, yet this benefit is associated with health risks to agricultural workers. Organophosphorus (OP) pesticides are globally among the most commonly used pesticides in agriculture (Dar et al., 2020) and occupational OP exposures are frequent causes of OP poisonings especially in developing countries (Muñoz-Quezada et al., 2017). Concerns regarding health risks posed by OPs to agricultural workers and their families (Alexander et al., 2006; Anger et al., 2020; Curl et al., 2002) have resulted in increasingly restricted use and even banning of many OP pesticides in the United States and the European Union (Foong et al., 2020). Despite these regulations, OPs remain a common contaminant identified in a majority of biological samples collected from agricultural workers (Arcury et al., 2010; Arcury et al., 2016; Coronado et al., 2011; Runkle et al., 2013; Tamaro et al., 2018).

An increasing body of epidemiological evidence supports a positive correlation between occupational exposures to OP pesticides and adverse health outcomes in human populations (Anger et al., 2020; reviewed in Reiss et al., 2015; reviewed in Rohlman et al., 2011). This highlights the importance of delineating the mechanism(s) by which occupational OP exposures cause adverse neurobehavioral outcomes. Repeated exposures to OPs at a level below the threshold for acute cholinergic crisis have been associated with deficits in fine motor control and psychomotor speed, visuospatial ability, working and visual memory, and emotional control in humans and preclinical animal models (reviewed in Ross et al., 2013; reviewed in Voorhees et al., 2017). Chlorpyrifos (CPF) is one of the commonly used broad-spectrum OP pesticides not just in the United States but also around the world (Foong et al., 2020). A recently published study assessing pesticide application teams in Egypt who were primarily exposed to 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 (Anger et al., 2020). However, while epidemiological studies can identify correlations, they cannot provide conclusive

evidence of cause-effect relationships between OP exposures and neurological alterations in occupationally-exposed populations (Meyer-Baron et al., 2015; reviewed in Rohlman et al., 2011). Relevant preclinical models are required to establish causality and to investigate mechanism(s) contributing to OP-induced cognitive impairment.

The canonical mechanism of neurotoxicity of OPs is inhibition of acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing acetylcholine (ACh) to terminate cholinergic neurotransmission (Pope et al., 2005). Inhibition of AChE leads to an excessive accumulation of ACh at cholinergic synaptic clefts in the peripheral and central nervous systems, resulting in cholinergic overstimulation (Costa, 2018). While clinical and experimental evidence support AChE inhibition as the mechanism responsible for the acute neurotoxic effects of OPs, there is little evidence to support an association between reduced cholinesterase activity and impaired neurobehavioral outcomes following occupational exposures (Meyer-Baron et al., 2015; reviewed in Rohlman et al., 2011). Consistent with these findings, deficits in Trail Making performance that were observed in Egyptian pesticide applicators exposed to CPF were not associated with blood cholinesterase activity (Anger et al., 2020). Therefore, non-cholinergic mechanisms are hypothesized to mediate the neurotoxic outcomes observed following occupational OP exposures.

There is increasing human and experimental evidence that cellular redox homeostasis is impaired following not only acute high-level but also repeated lower-level OP exposures, suggesting oxidative stress as a potential mechanism of occupational OP neurotoxicity (reviewed in Naughton & Terry, 2018; Tsai & Lein, 2021). The brain is highly susceptible to oxidative stress (Friedman, 2011) resulting from mitochondrial dysfunction (reviewed in Farkhondeh et al., 2020; Kaur et al., 2007; Middlemore-Risher et al., 2011) and/or altered endogenous antioxidant activity (Giordano et al., 2007; Kaur et al., 2019; Mahmoud et al., 2019; Tüzmen et al., 2007). Recent preclinical studies confirmed that repeated low-level exposures to OP pesticides upregulate brain expression of multiple oxidative stress biomarkers and decrease

the antioxidant defense including glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) (reviewed in Farkhondeh et al., 2020; Fereidouni et al., 2019; Mahmoud et al., 2019; Ojha et al., 2011; Singh et al., 2018).

Recent studies have investigated cause-effect relationships between oxidative stress and neurotoxic outcomes after repeated CPF exposures in preclinical animal models. Fereidouni et al. (Fereidouni et al., 2019) and Mahmoud et al. (Mahmoud et al., 2019) specifically examined the role of oxidative stress in apoptosis in adult rats exposed repeatedly to CPF at a level that significantly inhibited AChE activity without causing cholinergic crisis. Treatment with the antioxidants quercetin (Fereidouni et al., 2019) or *N*-acetylcysteine (Mahmoud et al., 2019) attenuated CPF-induced expression of oxidative stress biomarkers and reversed CPF effects on apoptotic protein expression. In another study, Singh et al. (Singh et al., 2018) showed that a mitochondria-targeted antioxidant, mitoapocynin, ameliorated CPF-induced oxidative stress and STAT1-dependent pro-apoptotic signaling in the substantia nigra and striatum of juvenile rats. Moreover, mitoapocynin also rescued CPF-induced motor deficits and nigrostriatal dopaminergic neurodegeneration in juvenile rats chronically exposed to CPF, suggesting a role for oxidative stress in CPF-induced neurotoxicity (Singh et al., 2018). What is not yet known, however, is whether oxidative stress contributes to learning and memory deficits caused by occupational OP exposures.

The goal of this study was to determine the role of oxidative stress in neurobehavioral deficits resulting from repeated exposure to CPF at levels similar to those experienced by a well-characterized cohort of Egyptian pesticide applicators (Anger et al., 2020; Farahat et al., 2011; Farahat et al., 2010; Lein et al., 2012). Specifically, we exposed adult male Long Evans rats to CPF injected subcutaneously at 10 mg/kg/d for 21 days. This model reflected the demographics of the Egyptian occupational cohort (Lein et al., 2012) and the primary route of exposure (Fenske et al., 2012). Additionally, previous pharmacokinetic/pharmacodynamic studies (Ellison et al., 2011) demonstrated that this dosing paradigm resulted in CPF levels and

cholinesterase inhibition in the rat that approximated those observed in the Egyptian pesticide applicators (Farahat et al., 2011).

Materials and methods

Chemicals

Chlorpyrifos (CPF, C9H11Cl3NO3PS; purity 99.5%) was purchased from ChemServices, Inc. (West Chester, PA, USA). Trolox [(±)-6-Hydroxy-2,5,7,8,-tetramethylchromane-2-carboxylic acid; purity 97%] was purchased from Sigma-Aldrich (St. Louis, MO, USA). CPF and Trolox were each dissolved in 10% ethanol (Decon Laboratories, Inc., King of Prussia, PA, USA) in triglyceride of coconut oil Neobee M-5 (Spectrum Chemical Manufacturing Corporation, Gardena, CA, USA). Solutions were vortexed for 2 min followed by sonication for 1 min prior to administering to animals.

Animals

Animals were maintained in facilities fully accredited by AAALAC International, and all studies were performed with regard for alleviation of pain and suffering under protocols approved by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC protocol numbers 15646 and 20165). All animal experiments were conducted in accordance with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of laboratory animals (NIH publication No. 8023, revised 1978).

Adult male Long-Evans rats (230-280 g) were purchased from Harlan (Indianapolis, IN, USA) or Charles River (Portage, MI, USA), and upon receipt were individually housed in standard shoebox plastic cages on autoclaved corncob bedding under controlled environmental conditions (22 ± 2 °C, 40–50% humidity, 12 h light-dark cycle). Food (2018 Teklad global 18% protein rodent diet; Envigo RMS Division, Indianapolis, IN, USA) was restricted to maintain constant body weight, and water was provided ad libitum. Separate cohorts of rats were used

for behavioral and biochemical versus neuropathologic endpoints. Animals were randomly assigned to groups using a random number generator.

All animals were humanely euthanized via deep anesthesia with 4% isoflurane in medical grade oxygen and subsequent exsanguination via transcardial perfusion with cold PBS (3.6 mM Na2HPO4, 1.4 mM NaH2PO4, 150 mM NaCl, pH = 7.2) at a flow rate of 15 ml/min using a Masterflex peristaltic pump (Cole Parmer, Vernon Hills, IL, USA).

Acetylcholinesterase (AChE) activity in blood and brain

Upon euthanasia, whole blood was collected via cardiac puncture in EDTA Microtainer tubes (BD, Franklin Lakes, NJ, USA) and diluted 1:25 in 0.1 M PO4 buffer (Na2HPO4/KH2PO4, pH = 7.4) containing 0.03% (w/v) Triton. The cerebellum was microdissected from excised brains, flash frozen and stored at -80 °C until assayed for AChE activity. AChE activity was quantified using the standard Ellman's Assay (Ellman et al., 1961) with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich) and acetylthiocholine iodide (AChI; Sigma-Aldrich) as substrates. Briefly, samples were thawed on ice. Brain samples were homogenized in lysis buffer (0.1 M sodium phosphate buffer, pH 8.0 containing 1% (w/v) Triton), centrifuged at 13,400 × g, and the supernatant collected for analysis. Assays were run against blanks containing DTNB. The reaction started with the addition of AChI after equilibration with DTNB for 2-3 min. Hydrolysis of AChI was monitored by the change in absorbance at 405 nm. To inhibit pseudocholinesterase activity, 100 μM tetraisopropyl pyrophosphoramide (Sigma-Aldrich) was included in the assay. Brain AChE results were normalized to protein concentration determined using the BCA protein assay kit according to the manufacturer's directions (Pierce Chemical, Rockford, IL, USA); blood AChE activity was normalized to hemoglobin (Hgb) using a STAT-Site MHbG photometer (Stanbio Laboratory, Boerne, TX, USA).

Fear conditioning

Learning and memory were assessed using modified fear conditioning testing (Cowan & Richardson, 2018; Raybuck & Lattal, 2011). Briefly, rats were conditioned and tested in 30.5 cm× 24.1 cm× 21 cm operant chambers (Med Associates, St. Albans, VT, USA). Each conditioning chamber was equipped with an overhead closed-circuit television (CCTV) camera to record the subject during each session. Chambers were thoroughly cleaned with 70% ethanol between subjects.

On the first day of training, rats were acclimated in the testing room for 1 h prior to being placed in the operant chambers. Rats were placed in the conditioning chamber with a metal grid floor and the lights on in the presence of a vanilla scent cue (McCormick Vanilla Extract diluted 1:100 in PBS). After 2 min of silence, rats were exposed to the auditory conditioned stimulus (CS), a 75 dB white noise, for 30 s followed by delivery of a 2 s, 0.75 mA foot shock delivered through the floor grid (unconditioned stimulus or UCS). Each animal experienced three CS-UCS pairings administered 90 s apart. After the conditioning or training session, animals were placed back in their home cages and returned to the vivarium. Twenty-four hours later, the context test was administered in which animals were placed in operant chambers with the same flooring, lighting, and olfactory cue as the day before but with no white noise and no shock. Their behavior in the operant chamber was recorded for 5 min. Following completion of the context test, each subject was returned to their home cage and moved to the vivarium. Twenty-four hours after the context test, the cue stimulus test was administered. The context of the testing chamber was changed by covering the metal floor grid with a transparent Plexiglas floor insert, turning off the chamber lights, and changing the olfactory cue to a lemon scent (lemon extract diluted 1:100 in ethanol). Animals were placed in the chamber with 2 min of silence for a pre-cue stimulus test, followed by 3 min of 75 dB of white noise without the UCS foot shock for the cue stimulus test, followed by 1 min of silence for a post-cue stimulus test. Time spent freezing (defined as the absence of any movement except respiratory movement) during both the

context and cue test was recorded by CCTV and manually scored by an individual blinded to experimental groups.

Prostaglandin E_2 (PGE2) levels in blood and brain

PGE2 levels were determined using a commercial monoclonal competitive ELISA specific for PGE2 (514010, Cayman Chemical, Ann Arbor, MI, USA). Whole blood collected by cardiac puncture was dispensed into tubes containing 10 μM indomethacin (70270, Cayman Chemical) to prevent ex vivo formation of prostaglandins. Indomethacin was dissolved in 0.1 M Na_2CO_3 (Avantor J.T.Baker, Radnor, PA, USA) with gentle warming to ensure complete dissolution and then diluted with PBS (pH 7.2) to the desired concentration for the blood collection tubes. Blood samples were allowed to clot for 20-30 min and then centrifuged at 2000 x g for 10 min. Serum was aliquoted and stored at -80 °C for later PGE2 assessment. Excised brains were microdissected to obtain the cortex and hippocampus, which were washed in PBS to remove blood before being flash frozen in liquid nitrogen and stored at -80 °C. On the day of the analysis, brain tissues thawed on ice were homogenized in 5 ml buffer/1g tissue (0.1 M PO4 buffer, pH = 7.4, supplemented with 10 μM indomethacin and 1 mM EDTA) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 20 s at medium speed. Samples were centrifuged at 8000 x g for 10 minutes at 4°C. Serum and homogenates were purified using C18 cartridges (Cayman Chemical) and eluted in analytical grade ethyl acetate anhydrous (Sigma-Aldrich) plus 1% reagent grade methanol (Fisher Scientific, Hampton, NH, USA). Solvent was evaporated under a gentle stream of nitrogen, and samples were reconstituted in ELISA buffer according to the manufacturer's protocol.

Oxylipin levels in blood and brain

Free oxylipins were extracted by using solid phase extraction. Brains were homogenized with zirconia beads, 400 μl of methanol containing 0.1% butylated hydroxytoluene (BHT) and

0.1% acetic acid and 10 µl of antioxidant solution containing 0.2 mg/mL butylated hydroxytoluene (BHT), EDTA and triphenylphosphine (TPP) in water:methanol (1:1), and 10 µl of surrogate standard solution containing 2 µM of d11-11(12)-EpETrE, d11-14,15-DiHETE, d4-6-keto-PGF1α, d4-9-HODE, d4-LTB4, d4-PGE2, d4-TXB2, d6-20-HETE and d8-5-HETE in methanol. The sample was homogenized and centrifuged at 4°C at 15,871 x g for 10 min. The resultant supernatant was load onto a 60 mg Waters Oasis HLB 3cc SPE column (Waters, Milford, MA, USA), pre-rinsed with one volume of ethyl acetate and two volumes of methanol, and pre-conditioned with two volumes of SPE buffer containing 5% methanol and 0.1% acetic acid in ultrapure Millipore water. The columns were then rinsed twice with SPE buffer, before being subjected to 20 min of vacuum (≈20 psi). Oxylipins were eluted with 0.5 ml methanol and 1.5 ml ethyl acetate in 2 ml. Samples were dried under nitrogen and reconstituted in 100 µl methanol.

Oxylipins were analyzed by UPLC-MS/MS. Separation of the various oxylipins was achieved by an Agilent LC system 1290 (Agilent Corporation, Palo Alto, CA, USA), equipped with a vacuum degasser, a quaternary pump, and an autosampler. The LC was coupled to an Agilent 6460 Triple Quadrupole MS system (Agilent Corporation).

Oxylipins listed in **Table 2-1** were separated and detected by LC-MS/MS operating on dynamic multiple reaction monitoring conditions. Electrospray ionization (positive mode) was used as the ion source with the experimental parameters, as follows: Gas temperature, 300°C, Gas flow, 10 L/min; Sheath gas heather, 350 °C; Sheath gas flow, 11 ml/min; Nebulizers 35 psi; Capillary gas, 4000 V/-4000 V. UPLC separation was achieved on an Agilent Eclipse Plus C18 column (2.1 x 150 mm, 1.8 µm, Agilent Corporation) with a binary gradient consisting of solvent A (water containing 0.1 % acetic acid) and solvent B (Acetonitrile: methanol 80:15 containing 0.1 % acetic acid). The column temperature was maintained at 45°C. Electrospray ionization (negative mode) was used as the ion source with the experimental parameters, as follows: Gas

temperature, 250°C, Gas flow, 10 L/min; Sheath gas heater, 300 °C; Sheath gas flow, 11 ml/min; Nebulizers 35 psi; Capillary gas, 3500 V/-3500 V.

Histological analyses

Following euthanasia, brains were quickly excised from the skull and placed on ice. Next, brains were blocked into 2-mm thick coronal sections via a stainless-steel rat brain matrix (Kent Scientific, Torrington, CT, USA), post-fixed in 4% (w/v) paraformaldehyde (PFA; Sigma-Aldrich) in phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH = 7.2) at 4 °C for at least 24 h, and incubated in 30% (w/v) sucrose (Sigma-Aldrich) in phosphate-buffered saline (PBS, pH = 7.2) for storage at 4°C until being embedded and frozen in Tissue-Plus™ O.C.T. (Thermo Fisher Scientific, Waltham, MA, USA). Brain tissue blocks were cryosectioned into 10-µm thick coronal sections using a cryostat Microm HM550 (Thermo Fisher Scientific) onto glass slides (Thermo Fisher Scientific) and stored at -80°C until further processed.

For immunostaining, slides were dried at room temperature, rinsed with PBS, placed in 10 mM sodium citrate buffer (pH = 6.0) and heated for 30 min in a rice cooker (Black & Decker, HS2000) for antigen retrieval. Following antigen retrieval, sections were washed with PBS and blocked with 10% (v/v) normal goat serum (Vector Laboratories, Burlingame, CA, USA), 1% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS containing 0.03% (v/v) Triton X-100 (Thermo-Fisher Scientific) for 1 h at room temperature. Sections were then incubated with primary antibodies in blocking buffer overnight at 4 °C.

Primary antibodies used to assess neuropathology included rabbit anti-IBA1 (1:1000, 019-19741, AB_839504, Wako Laboratory Chemicals, Richmond, VA, USA), mouse anti-CD68 (1:200, MCA341R, AB_2291300, AbD Serotec, Hercules, CA, USA), mouse anti-GFAP (1:1000, 3670, AB_561049, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-S100β (1:300, ab52642, AB_882426, Abcam, Cambridge, UK), mouse anti-NeuN (1:1000, MAB377, AB_2298772, Millipore), and rabbit anti-nitrotyrosine (3-NT, 1:200, 06-284, AB_310089,

Millipore). The next day, sections were washed in PBS with 0.03% Triton X-100 before incubation with secondary antibodies in blocking buffer for 1 h at room temperature. The following secondary antibodies were used: for anti-IBA1, goat-anti rabbit IgG conjugated to Alexa Fluor 568 nm (1:1000, A11036, AB_10563566, Life Technologies, Carlsbad, CA, USA); for anti-CD68, goat-anti mouse IgG1(γ1) conjugated to Alexa Fluor 488 nm (1:500, A21121, AB_2535764, Invitrogen, Carlsbad, CA, USA); for anti-GFAP or anti-NeuN, goat-anti mouse IgG1 conjugated to Alexa Fluor 568 nm (1:1000, A21124, AB_2535766, Invitrogen); and for anti-S100β and anti-3-NT, goat-anti rabbit IgG conjugated to Alexa Fluor 488 nm (1:500, A11034, AB_2576217, Thermo Fisher Scientific). As a negative control, sections were incubated with blocking buffer alone rather than primary antibodies. All sections were mounted in ProLong™ Gold Antifade Mountant with DAPI (Invitrogen).

To detect degenerating neurons, Fluoro-Jade C (FJC, AG325, Millipore, Burlington, MA, USA) labeling was performed according to the manufacturer's protocol. Briefly, sections were dried at 50°C for 10-15 min, rinsed with 70% (v/v) ethanol for 2 min followed by a distilled water wash. Sections were incubated in 0.03% (w/v) potassium permanganate ($KMnO_4$; Sigma-Aldrich) in distilled water for 10 min on a shaker table followed by a 2 min distilled water wash. Next, sections were incubated in a freshly prepared solution of 0.00015% FJC in 0.1% (v/v) acetic acid (Acros Organics, Geel, Belgium) in distilled water containing 0.5 µg/ml DAPI (Invitrogen, Carlsbad, CA, USA) for 10 min, washed with distilled water, and dried at 50 °C. Dried slides were cleared by immersion in xylene (Thermo-Fisher Scientific, Waltham, MA, USA) for 1 min before sections were coverslipped with Permount mounting medium (Thermo Fisher Scientific).

Fluorescent images were acquired using the ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices, Sunnyvale, CA, USA). Images of the following brain regions were acquired (MetaXpress Version 6.2.3.733) from two serial sections from each animal based on the photographic atlas of the rat brain (Kruger et al., 1995): amygdala (Bregma

−3.6 mm to −4.2 mm), hippocampus (including the CA1, CA3, and dentate gyrus subregions; Bregma −3.6 mm to −4.2 mm), piriform and somatosensory cortex (Bregma −3.6 mm to −4.2 mm), and dorsolateral thalamus (Bregma −3.0 mm to −3.6 mm). The final image of each brain region for analysis (MetaXpress Version 6.2.3.733) was built by stitching multiple overlapping tiles that encompassed an entire brain region. Images were analyzed using ImageJ imaging software (version 1.51n, NIH, USA) to set the threshold for fluorescence intensity; cell size and circularity were used to determine the number of FJC-positive cells per unit area (mm^2). GFAP immunoreactivity was quantified using ImageJ, as previously described (Guignet et al., 2019). IBA-1/CD68 immunoreactivity was assessed using a custom-designed MATLAB script to specifically look at microglia morphology and cell number (MATLAB 2014b, The MathWorks Inc., Natick, Massachusetts). NeuN/3-NT immunoreactivity was assessed with respect to the percentage of immunopositive cells (identified by DAPI staining) with colocalized immunoreactivity via an automated solution to detect cell colocalization using convolutional neural network with in-house modification (Dou et al., 2021). A fully convolutional one-stage object detector (FCOS) was chosen to become the base model. In brief, immunopositive cells were first marked by an investigator blinded to experimental group, and then modified into training sets within the automated pipeline. The novel metric loss function, which measures the degree of colocalization, was used to provide more local information for detecting accurate colocalization.

Statistics

AChE activity and PGE2 concentrations were compared across experimental groups using one-way ANOVA as performed by Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Oxylipin measures were made from both plasma and brain tissue samples across the four experimental groups (VEH, Trolox, CPF, and CFP+Trolox) from animals at 3, 10, 15, 21 d of exposure. Linear

regression models were used for each oxylipin measure, considering exposure, day and the interaction. A best model was chosen by selecting the model (either main effects only or main effects and the interaction) with the smallest Akaike Information Criterion; all outcomes had the main effects model as the best model, so results only from this model are presented. When the F-test for group was significant, Tukey honestly significant difference (HSD) was used for post-hoc pairwise comparisons. None of the oxylipin models supported an interaction with time point so the group difference did not differ significantly across time points.

Behavioral data was analyzed using one-way ANOVA (Tukey post-hoc) or Satterthwaite-Welch t-test when Gaussian distribution and homogeneity of variance were violated.

For histological analysis, repeated measures models included exposure (VEH, Trolox, CPF, and CFP+Trolox), brain region (amygdala, CA1, CA3, cortex, dentate gyrus, piriform and somatosensory cortex, and dorsolateral thalamus), and time point (3, 10, 15, and 21 d) fit to the immunohistochemistry endpoints of GFAP (% positive area), IBA-1/CD68 (IBA-1 positive cells/mm^2), 3-NT/NeuN (% colocalization). GFAP and IBA-1/CD68 outcomes were log-transformed prior to analysis to better meet the model assumption of constant variance. Interactions between the factors were considered, and the best model was chosen using Akaike Information Criterion. Contrasts were defined to estimate differences between groups. Within a model, Benjamini-Hochberg False Discovery Rate (FDR) was used to account for multiple comparisons. Results are presented as geometric mean ratios (GMR) between groups for the log-transformed outcomes and estimated mean differences for % colocalization of 3-NT and NeuN. Point estimates of the ratios or differences and 95% confidence intervals are presented in the figures. All analyses were conducted using SAS software, version 9.4. Raw p-values are presented, but those that remained significant after an FDR correction are noted.

Because multiple cells were analyzed per animal in order to determine morphology ratios of the microglial population, repeated measures models with an animal-specific random intercept were used to assess the association between experimental group (VEH, Trolox, CPF, and

CFP+Trolox), brain region (amygdala, CA1, CA3, cortex, dentate gyrus, piriform and somatosensory cortex, and dorsolateral thalamus), and time point (3, 10, 15, and 21 d) and the ratio of process length to cell body for microglia morphology ratios. The ratio of process length to cell body was natural log-transformed prior to analysis to better meet the model assumption of constant variance. Interactions between the factors were considered, and the best model was chosen using Akaike Information Criterion. Contrasts were defined to estimate differences between treatment groups, by brain region and days post exposure. Due to the large number of contrasts, FDR was used to account for multiple comparisons; only those results that remained significant after FDR correction are presented. Results are presented as GMR between treatment groups. Point estimates of the GMR and 95% confidence intervals are presented in the text. A confidence interval that does not include one suggests a difference between the groups. All analyses were conducted using SAS software, version 9.4.

Results

Acetylcholinesterase (AChE) inhibition, learning and memory deficits, and increased serum PGE2 level in a rodent model of occupational CFP exposure

We initially validated our modified rat model of occupational CPF exposure (Voorhees et al., 2019), which is based on an Egyptian cohort of pesticide applicators (Anger et al., 2020) to confirm that repeated CPF exposure inhibited AChE and impaired learning and memory. In this modified model, adult male Longs-Evans rats were exposed to CPF at 10 mg/kg/d daily for 21 days (**Figure 2-1A**). The dose was chosen based on CPF PK/PD analyses in the Longs-Evans rat (Ellison et al., 2011) following a 21-day exposure that mimicked exposures documented in Egyptian pesticide applicators working in the cotton fields (Farahat et al., 2011). AChE activity was measured in the blood at multiple time points throughout the 21-day exposure period and in the cerebellum after 21 days of exposure. As shown in Figure. 2-1B, AChE activity was

significantly inhibited relative to vehicle controls after 4 days of CPF exposure and remained suppressed throughout the remainder of the exposure period. There were no statistically significant differences in blood AChE activity at 4, 11, 18, and 21 days of CPF exposure (**Figure 2-1B**). Similarly, AChE activity in cerebellar tissue was significantly inhibited at the end of the 21-day exposure period (**Figure 2-1B**). As an initial measure of oxidative stress, PGE2 levels in the serum were quantified after 7 d of CPF exposure (**Figure 2-1B**). At this time point, the concentration of PGE2 in serum of CPF-exposed rats was significantly elevated compared to that of VEH controls (**Figure 2-1B**).

To assess the impacts of repeated CPF exposure on learning and memory, VEH and CPF animals were assessed using contextual and cue fear conditioning at the end of the exposure period (**Figure 2-1A**). Animals were initially trained to associate the unconditioned stimulus (foot shock) with the conditioned stimulus (auditory cue). CPF animals exhibited impaired performance in both the context and cue tests relative to VEH controls (**Figure 2-1C**). Specifically, CPF animals spent significantly less time freezing relative to VEH animals during context test (VEH: 44.08 ± 7.031%; CFP: 16.84 ± 3.643%, t-test, $p = 0.0326$) and during cue test (VEH: 63.04 ± 8.704%; CFP: 34.78 ± 3.889%, t-test, $p = 0.0119$).

Repeated CPF exposure increased expression of biomarkers of oxidative stress in the blood and brain and this effect was prevented by co-exposure to the antioxidant, Trolox

We next determined whether CPF increased expression of biomarkers of oxidative stress in the blood and brain, and if so, whether this effect could be blocked by co-exposure to the antioxidant, Trolox (**Figure 2-2**). PGE2 levels in sera, cortices, and hippocampi were measured by ELISA at the end of the 21-day exposure period (**Figure 2-3A**). The concentration of PGE2 in sera and cortices after a 21-day exposure to CPF was not significantly different from VEH controls. In contrast, hippocampal concentrations of PGE2 after 21 days of exposure were significantly higher in CPF animals relative to VEH animals (one-way ANOVA with *post-hoc*

Tukey test, $p < 0.05$). Co-exposure to Trolox significantly reduced PGE2 levels in the hippocampus of CPF animals compared to CPF animals who were not administered Trolox. Hippocampal levels of PGE-2 in the CPF+Trolox animals were not significantly different from that measured in VEH controls, and Trolox alone did not alter PGE levels in any of the tissues we evaluated.

Among the 81 oxylipins measured in plasma (**Table 2-1**), levels of 12, 13-DiHOME ($p<0.001$), 14, 15-DiHETE ($p=0.006$), and 9, 10-DiHOME ($p=0.001$) differed significantly between experimental groups but did not differ significantly across time points (3, 10, 15, 21 days) (**Figure 2-3B**). CPF animals had significantly higher levels of 12, 13-DiHOME (mean=13.5, 95% CI: (1.7, 25.2)) and 14, 15-DiHETrE (mean=0.2, 95% CI: (0.03, 0.3)) than VEH animals. Similarly, CPF animals had significantly higher plasma levels of 12, 13-DiHOME (mean = 17.4, 95% CI: (5.8, 29.0)) and 9,10-DiHOME (mean = 3.0, 95% CI: (0.8, 5.3)) than Trolox animals. Levels of these oxylipins were not significantly altered by exposure to Trolox alone, and co-exposure to Trolox did not block CPF-induced elevations in 12-13-DiHOME or 14,15-DiHETrE.

Among all the oxylipins measured in brain tissue (**Table 2-1**), 13-HODE ($p=0.047$), PGF2a ($p<0.001$), and 8-HETE ($p=0.04$) differed significantly between experimental groups but did not vary across time points (3, 10, 15, 21 days) (**Figure 2-3B**). 13-HODE levels were elevated in Trolox animals compared to VEH animals (mean = 5.9, 95% CI: (0.1, 11.6)), while CPF animals exhibited higher levels of PGF2α compared to VEH animals (mean = 7.4, 95% CI: (1.6, 13.1)) and Trolox animals (mean=7.6, 95% CI: (1.9, 13.4)). Animals exposed to CPF+Trolox had significantly higher PGF2a levels than VEH (mean = 6.9, 95% CI: (1.1, 12.6)) or Trolox animals (mean = 7.1, 95% CI: (1.4, 12.9)). Co-exposure to CPF+Trolox significantly increased 8-HETE levels relative to VEH (mean = 0.6, 95% CI: (0.03, 1.2)), but did not reduce elevated levels of PGF2a in CPF animals.

3-Nitrotyrosine (3-NT), the result of the reaction of tyrosine residues with reactive nitrogen

species, such as peroxynitrite anion, is a well-established biomarker of oxidative damage (Ahsan, 2013). To corroborate with biochemical analyses of levels of PGE2 and oxylipins, we assess global levels of neuronal oxidative stress in brain regions including not only the hippocampus but also the amygdala, piriform and somatosensory cortex, and dorsolateral thalamus. While both neurons and non-neuronal cells could exhibit signs of oxidative stress, we found that it is exclusively neurons that co-express the oxidative stress marker 3-NT in the brain after repeated CPF intoxication, as indicated by co-localization of 3-NT and NeuN immunoreactivity (**Figure 2-4A**). Differences between experimental groups did not vary by brain region or time point (3, 10, 15, 21 days). Therefore, comparisons are presented as an overall estimate of group differences (**Figure 2-4B**). After FDR correction, there was a significant increase in the percent colocalization of NeuN-immunopositive cells that expressed 3-NT in CPF animals compared to VEH animals (on average 20.8 percentage points higher, $\beta = 20.8$, SE = 1.9, $p < 0.001$). Conversely, Trolox ($\beta = -15.4$, SE = 1.5, $p < 0.001$) and CPF+Trolox ($\beta = -16.4$, SE = 1.8, $p < 0.001$) groups had significantly decreased 3-NT/NeuN percent colocalization compared to the CPF group.

CPF causes astrogliosis but not microgliosis or neurodegeneration

Astrogliosis, microgliosis, microglial activation and neurodegeneration were evaluated in sections from the same brains. Brain regions examined include the amygdala, CA1, CA3, dentate gyrus, piriform and somatosensory cortex, and dorsolateral thalamus. Astrogliosis was assessed by GFAP and S100β immunohistochemistry (**Figure 2-5A**) (Eng & Ghirnikar, 1994; Raponi et al., 2007) and differences between groups presented as the geometric mean ratio (GMR) (**Figure 2-5B**). CPF elevated the % area immunopositive for GFAP compared to VEH regardless of days of exposure: 3 d (GMR = 1.6, $p < 0.001$, 95% CI= (1.4,1.8)), 10 d (GMR = 1.5, $p < 0.001$, 95% CI = (1.4,1.6)), 15 d (GMR = 1.6, $p < 0.001$, 95% CI = (1.4, 1.7)) and 21 d (GMR = 1.5, $p < 0.001$, 95% CI = (1.4, 1.7)). The % area of GFAP immunoreactivity was also

elevated in the CPF+Trolox group compared to VEH but only at 3 d (GMR = 1.1, $p < 0.001$, 95% CI = (1.07,1.21)) and 15 d (GMR = 1.2, $p < 0.001$, 95% CI = (1.1, 1.3)). However, the CPF+Trolox group consistently had a significantly lower % area immunopositive for GFAP than the CPF group across all time points examined. All noted differences remained significant after FDR correction. In contrast, CPF had minimal effect on the % area of S100β immunoreactivity in the seven brain regions across all time points examined (**Figure 2-S1**).

Microgliosis and microglial activation were measured as the cell counts of IBA-1-immunopositive cells (Ito et al., 1998) and determined by the co-expression of CD68 (Holness & Simmons, 1993) and microglial morphology, respectively (**Figure 2-6**). As shown in representative photomicrographs in **Figure 2-6A**, there was a lack of CD68 immunoreactivity across experimental groups at all time points examined. There were also no significant differences in IBA-1 immunopositive cell counts between groups and across brain regions at all four time points (**Fig. 2-6B**). Although CPF+Trolox co-exposure resulted in a higher count of IBA-1 positive cells than VEH, the overall effect of exposure was not significant ($p = 0.13$).

To further examine the effects of repeated CPF exposures on microglia activation, microglia morphology was assessed in the amygdala, CA1, CA3, dentate gyrus, piriform cortex, and thalamus (**Figure 2-S2A**) using a custom-designed MATLAB script to automatically determine the ratio of process length to the area of cell body in IBA-1 immunopositive cells. Microglia have a ramified cell shape with many thin processes extending from their soma while they are at resting state. These thin processes become condensed when microglia are activated, giving them an amoeboid-like appearance (Kreutzberg, 1996). Therefore, methods for morphological assessment of microglia have been developed and used as an indicator for microglial activation (Heindl et al., 2018; Kozlowski & Weimer, 2012). Significant differences in morphology were found at 3 and 21 d of exposure. At 3 d of exposure, relative to VEH animals, Trolox animals had morphology ratios that were 55% lower (GMR=0.45, 95% CI=0.35- 0.57, $p<.001$) and CPF animals had ratios 46% lower (GMR=0.54, 95% CI=0.42-0.71, $p<0.001$) in the CA3. At 21 d,

microglia morphology ratios in the amygdala were approximately 35% lower in CPF animals (GMR=0.65, 95% CI=0.51-0.83, p<.001) or CPF+Trolox animals (GMR=0.64, 95% CI=0.51-0.81, p<.001) compared to VEH (**Figure 2-S2B**).

Neurodegeneration, assessed by FJC staining, was found to be negligible regardless of brain region, experimental group, or time of treatment (**Figure 2-S3**).

Co-administration of the antioxidant Trolox prevented CPF induced neurobehavioral deficits but not AChE inhibition

Learning and memory function was assessed by contextual and cue fear conditioning at the end of the 21-day exposure period (**Figure 2-2**). In contrast to our initial experiments (Figure 2-1), there were no significant differences in the percentage time immobilized among the CPF, VEH, Trolox, and CPF+Trolox during the context test; however, the CPF animals displayed a significant decrease in time spent freezing during the cue test when compared to VEH, Trolox or CPF+Trolox animals (one-way ANOVA with *post-hoc* Tukey test, p = 0.0052) (**Figure 2-7A**). Co-exposure to Trolox significantly blocked the CPF-induced impairment in the cue test, and the time CPF+Trolox animals spent freezing did not differ significantly from that of VEH animals (one-way ANOVA with *post-hoc* Tukey test, p = 0.0267) (**Figure 2-7A**).

We next evaluated whether the protective effects of Trolox on behavior in CPF-exposed animals was related to protection of AChE activity. AChE activity in the blood and cerebellum of Trolox animals was comparable to that of VEH animals at all time points, while blood and brain AChE activity was significantly inhibited to a similar extent in both CPF and CPF+Trolox animals (**Figure 2-7B**).

Discussion

The association between occupational exposures to OPs and deficits in neurobehavioral performance is supported by numerous epidemiological studies (Anger et al., 2020; reviewed in

Meyer-Baron et al., 2015; reviewed in Rohlman et al., 2011; reviewed in Ross et al., 2013). Among the various potential molecular targets and non-cholinergic mechanisms proposed to mediate OP-induced neurobehavioral deficits following occupational exposures, oxidative stress has been of interest because biomarkers of oxidative stress have been found to be upregulated by chronic OP exposure in humans and preclinical animal studies (Ranjbar et al., 2002; reviewed in Soltaninejad & Abdollahi, 2009; reviewed in Voorhees et al., 2017). Additionally, clinical observations of neurological diseases with neurobehavioral and neuropsychiatric symptoms that are similar to those associated with occupational OP exposures provide indirect support for a mechanistic role of oxidative stress in occupational OP neurotoxicity (Black et al., 2015; reviewed in Fedoce et al., 2018; reviewed in Montine et al., 2005; reviewed in Wang et al., 2014).

In order to better understand the mechanisms associated with neurotoxicity of repeated occupational OP exposures, preclinical animal models using exposure paradigms that can be rigorously controlled have demonstrated impaired behavioral outcomes in learning and memory function (reviewed in Voorhees et al., 2017). For example, impaired performance in the Morris water maze was observed in rats exposed to CPF at 18 and 25 mg/kg/d for 14 days when tested 24 hours following the last injection (Terry et al., 2003). Using the same CPF exposure paradigm we used in this study, a recent report showed that early-life repeated CPF exposure exacerbates not only the pathological formation of cortical and hippocampal vacuoles and neuronal cell loss in TgF344-AD rats but also behavioral deficits in learning and memory (Voorhees et al., 2019).

In the present study, we used a CPF exposure paradigm that was based on a well-characterized occupational cohort in Egypt in which agricultural workers were dermally exposed to CPF for up to 21 consecutive days during the cotton growing season (Anger et al., 2020; Fenske et al., 2012; Lein et al., 2012). We confirmed that this translationally-relevant exposure resulted in impaired memory in adult male Long Evans rats, evident as significant deficits in the

context and cue fear conditioning tasks. While the context test is predominantly hippocampal-dependent, the cue test relies on both hippocampal and amygdalar function. Therefore, this observation suggests that repeated CPF intoxication impairs memory function that involves these two brain regions. These data corroborate epidemiologic observations indicating a link between occupational CPF exposure and cognitive impairment (Farahat et al., 2010; reviewed in Voorhees et al., 2017) and confirm observations of other experimental models of occupational OP exposures (Hussein et al., 2018; Terry et al., 2012; reviewed in Voorhees et al., 2017).

An extensive literature implicates mechanisms other than or in addition to AChE inhibition in chronic OP neurotoxicity (reviewed in Naughton & Terry, 2018; reviewed in Rohlman et al., 2011; Tsai & Lein, 2021). Based on literature demonstrating that OPs, including CPF, alter multiple enzymatic/non-enzymatic markers of the oxidant/antioxidant system and increase expression of biomarkers of oxidative stress (Akpa et al., 2021; Albasher et al., 2020; Fereidouni et al., 2019; Mahmoud et al., 2019), it is posited that oxidative stress contributes to the neurotoxic outcomes observed following occupational OP exposures. We confirmed that the CPF exposure paradigm we used similarly increased biomarkers of oxidative stress. The brain is susceptible to oxidative stress in part because of its high composition of lipids (Pearson & Patel, 2016). Oxylipins, which are oxidized products of polyunsaturated fatty acids (PUFAs), such as arachidonic acid and linoleic acid, can be metabolized through different enzymatic pathways, including cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) to generate lipid signaling mediators that play a role in responses to oxidative stress responses (Yang et al., 2011). To the best of our knowledge, this is the first study characterizing the temporal changes of lipid mediators in the rat brain after repeated exposures to CPF. In the present study, we screened over 80 different oxylipins and found significant changes in 6 lipid mediators (12, 13-DiHOME, 9, 10-DiHOME, and 14, 15-DiHETrE in the plasma; 13-HODE, PGF2α, and 8-HETE in the brain). CPF significantly increased soluble epoxide hydrolase (sEH) metabolites in the plasma while COX metabolites were increased in the brain. We also found a

transient increase in serum PGE2 level after 7 days of CPF exposure while brain PGE2 levels were significantly increased in the hippocampus, but not cortex, after 21 days of CPF exposure. Additionally, 3-NT immunoreactivity was detected in multiple brain regions following repeated CPF intoxication and this biomarker of oxidative stress was observed to be colocalized with the neuronal biomarker NeuN, suggesting that CPF specifically elicited oxidative stress in hippocampal neurons.

Our data extend previous studies demonstrating that CPF upregulated lipid peroxidation, increased levels of reactive oxygen and nitrogen species, and decreased antioxidant enzymes in the brain following weeks of exposure in animal models (Albasher et al., 2020; Hussein et al., 2018; Mahmoud et al., 2019). However, these previous studies have not evaluated the spatiotemporal relationship between these biomarkers of oxidative stress and brain regions implicated in mediating the neurobehavioral functions altered by occupational OP exposure. In contrast, our data demonstrated that oxylipins, PGE2 levels and neuronal 3-NT expression were increased in a brain region, the hippocampus, critically involved in performance in the context and cue fear conditioning tasks (Curzon et al., 2009). These findings provide further support for the hypothesis that oxidative stress contributes to the impaired cognitive function observed following repeated CPF exposures.

Evidence of a causal relationship between CPF-induced oxidative stress and CPF effects on cognitive function was obtained in our studies to determine whether Trolox blocked the CPF-induced neurobehavioral deficits. Trolox is a water-soluble vitamin E derivative which that protects against oxidative stress by scavenging free radicals (Davies et al., 1988). Trolox treatment significantly improved performance in CPF-exposed animals, specifically in the cue test. While significant deficits in the cue text were observed in both cohorts of animals we exposed to CPF, significant deficits in the context test were observed only in the first cohort of CPF-exposed animals that did not include a Trolox treatment group Interestingly, while CPF animals from the second cohort showed a trend towards decreased performance in the context

test, they exhibited a wider range of variation in the freezing time during the context test compared to animals of the first cohort. This may suggest that CPF effects on the hippocampus, which is involved in the context test, might be variable between these two cohorts of animals.

Consistent with the hypothesis that oxidative stress mediates the neurobehavioral deficits of occupational OP exposures, Trolox significantly inhibited CPF induction of hippocampal PGE2 levels and significantly reduced neuronal 3-NT expression in the hippocampus of CPF-exposed animals. In contrast, Trolox did not mitigate CPF effects on oxylipins. One caveat regarding the brain oxylipin data is that oxylipin concentrations are potentially overestimated because we did not use head-focused microwave fixation, which is the recommended fixation method to prevent the effects of post-mortem ischemia on oxylipins (Hennebelle et al., 2019). Therefore, these results should be interpreted with caution and future lipidomic experiments should take the fixation method into account to reduce potential artifacts.

Reactive oxygen and nitrogen species are important signaling molecules in innate and adaptive immune responses that can activate microglia and astrocytes (reviewed in Hsieh & Yang, 2013; reviewed in Mittal et al., 2013). In turn, activated microglia and astrocytes release not only pro-inflammatory mediators but also toxic free radicals, which further amplify oxidative stress. Given the close interplay between oxidative stress and neuroinflammation, there is growing interest in the temporal and functional relationships between these pathways in the context of OP intoxication (reviewed in Guignet & Lein, 2019; reviewed in Naughton & Terry, 2018). For example, *in vitro* studies demonstrated that the NLRP3 inflammasome is induced by CPF in a mitochondrial reactive oxygen species (mROS)-dependent manner in human HaCat cells (Jang et al., 2015). In addition, antioxidant treatment was reported to attenuate oxidative stress and neuroinflammation following repeated low-level OP exposures (Abolaji et al., 2017; Mohammadzadeh et al., 2018).

Here, we applied two commonly used biomarkers of astrocytes, GFAP and S100β, to capture a broader pool of activated astrocytes (reviewed in Jurga et al., 2021). GFAP, an

intermediate filament structural protein, primarily labels astrocytic processes and therefore is a commonly used biomarker for assessing astrocyte morphology associated with reactive astrogliosis (reviewed in Jurga et al., 2021). S100β is a cytosolic Ca^{2+}-binding protein, that labels cell bodies of small astrocytes with less extended branching (reviewed in Jurga et al., 2021). However, compared to the increasingly clearer proinflammatory role of extracellular S100β (reviewed in Michetti et al., 2019; Villarreal et al., 2014), data on the role of intracellular S100β has yet reached a consensus. We observed a significant increase in GFAP immunoreactivity in CPF-exposed animals when compared to VEH controls while the percent area of S100β immunoreactivity was not significantly altered by CPF exposure except in the cortex (21 days) and thalamus (10 and 15 days). To gain insight regarding the toxicological significance of the differential effects of CPF on GFAP vs. S100β, future studies might focus on profiling the spatiotemporal expression of these two biomarkers within groups as there have been studies suggesting the colocalization profile of astrocyte biomarkers may change depending on the degree of the injury (Gil-Martínez et al., 2018; Himeda et al., 2006). In contrast to increased astrogliosis noted in CPF-exposed animals, repeated CPF exposure had no effect on multiple measures of microglial activation. These findings suggest that CPF did not trigger a general neuroinflammatory responses but rather targeted astrocytes selectively.

Oxidative stress in the brain is tightly associated with the homeostasis of astrocytes, and astrocytes can play both beneficial and detrimental roles in response to oxidative stress (reviewed in Chen et al., 2020). A recent in vitro study using primary cortical astrocytes showed that astrocytes survived high levels of oxidative stress induced by high concentrations of the herbicide Paraquat developed oxidative stress tolerance against subsequent high-concentration exposure. These astrocytes continued to offer protection to neighboring neurons from intense oxidative toxicity in neuron/astrocyte co-culture, suggesting that stress-reactive astrocytes could provide neuroprotective efficacy and might not be necessarily neurotoxic (Bhatia et al., 2019). Conversely, it has also been shown that persistent overactivation of astrocytes can contribute to

oxidative stress via impaired glutamate transport, mitochondrial dysfunction, and a feed-forward loop that exacerbates inflammatory responses (reviewed in Chen et al., 2020; reviewed in Guignet & Lein, 2019). Given that oxidative stress and neuroinflammation are dynamic, co-dependent responses, the mechanistic relationships between oxidative stress and neuroinflammation, and their relative contributions to neurologic outcomes following occupational OP exposure remain outstanding research questions.

Our observation that increased GFAP immunoreactivity and 3-NT immunoreactivity overlapped spatially and temporally in the brain of CPF-exposed animals makes it difficult to determine whether oxidative stress triggers neuroinflammation or vice versa. However, CPF-induced astrogliosis as determined by GFAP immunoreactivity was found to be significantly attenuated by Trolox. This suggests that oxidative stress is likely playing a role in triggering and/or maintaining astrogliosis. Substantially increased expression of GFAP has been reported in varying neurological diseases, including neurodegeneration and neuropsychiatric diseases (Dossi et al., 2018; Hol & Pekny, 2015; Kim et al., 2018). Future studies are needed to further understand the mechanistic role of astrogliosis in behavioral deficits resulting from occupational OP exposures.

Oxidative stress and neuroinflammation have been suggested to contribute to neurodegeneration (reviewed in Fischer & Maier, 2015; reviewed in Hsieh & Yang, 2013), and early neuroinflammatory responses and oxidative stress correspond with neurodegeneration in preclinical models of acute OP intoxication (Liang et al., 2019; Liang et al., 2018; Rojas et al., 2015). However, unlike models of acute OP intoxication, we did not observe neurodegeneration in our preclinical model of occupational CPF exposure, as indicated by the absence of FJC staining. This suggests that following occupational exposures to OPs, neurodegeneration is neither a cause nor a consequence of oxidative stress and neuroinflammation.

AChE inhibition is the canonical mechanism of action of acute OP neurotoxicity (Costa, 2018). However, its role in mediating neurotoxic outcomes associated with occupational OP

exposures remains controversial. While occupational exposures can result in significant AChE inhibition in both humans (Farahat et al., 2011; reviewed in Rohlman et al., 2011) and animals (Ellison et al., 2011; reviewed in Voorhees et al., 2017), this is not associated with cholinergic crisis, and it has not been strongly associated with behavioral deficits (Anger et al., 2020; reviewed in Rohlman et al., 2011). In light of these observations, it has been proposed that prolonged OP exposure can lead to development of tolerance to sustained AChE inhibition due to down-regulation of muscarinic and nicotinic acetylcholine receptors (Bushnell et al., 1993; Costa et al., 1982). Additionally, it is widely speculated that noncholinergic mechanisms that contribute to chronic OP neurotoxicity, such as oxidative stress, might be independent of AChE inhibition (Pope, 1999; Rohlman et al., 2011). For example, *in vitro* studies demonstrated that CPF-induced cytotoxicity and oxidative stress are antagonized by antioxidant treatments including phenyl-*N*-tert butylnitrone (PBN), catalase (CAT), and glutathione monoethyl ester (GSHEE) but not by the cholinergic receptor antagonists mecamylamine and atropine (Giordano et al., 2007). In another study of adult mice exposed to malathion at 30 mg/kg for 15 days, exposed animals exhibited impaired spatial memory (assessed by object location task) and a significant reduction in the activity of hippocampal mitochondrial complex I while whole hippocampal AChE activity remained at levels comparable to control animals (dos Santos et al., 2016). These findings support the hypothesis that AChE inhibition and oxidative stress occur independently following repeated OP exposures.

A previous report that used the same CPF dosing paradigm employed in the current study (Ellison et al., 2011) characterized the time course of CPF-induced blood and brain AChE inhibition. Average blood and brain AChE activity in animals exposed to CPF at 10 mg/kg/d (s.c.) were found to decline with increasing duration of CPF exposure, with blood AChE activity decreased to 67% of control on day 2 and 33% on day 10, and brain values ecreased to 67% of control on day 4 and 28% on day 10. Consistent with this study (Ellison et al., 2011), we also observed inhibition of blood AChE after 4 days of exposure that was sustained throughout the

remainder of the 21-day exposure and significant inhibition of brain AChE at the end of the exposure period. Of importance, our results demonstrated that Trolox did not influence the sustained AChE inhibition caused by repeated CPF exposure, further supporting the hypothesis that oxidative stress and cholinergic dysfunction occur independently of each other in experimental animal models of occupational CPF exposure.

In conclusion, our study demonstrated that in a rat model of occupational CPF exposures based on human exposure data collected from Egyptian pesticide applicators occupationally exposed to CPF (Anger et al., 2020; reviewed in Meyer-Baron et al., 2015; reviewed in Rohlman et al., 2011; reviewed in Ross et al., 2013), neurobehavioral changes coincided with elevated levels of oxidative stress and neuroinflammation. Treatment with the antioxidant Trolox significantly mitigated cognitive deficits, hippocampal PGE2 level, neuronal oxidative stress, and astrogliosis associated with repeated CPF exposure but had no effect on CPF-induced AChE inhibition. These findings are among the first evidence that CPF effects on oxidative stress are causally linked to impaired cognitive behavior. These observations suggest the possibility that diets high in vitamin E or other antioxidants may provide neuroprotection to exposed human populations, and conversely that individuals with low antioxidant capabilities are more susceptible to the neurotoxic effects of repeated CPF exposures. Whether these findings extend to other OP pesticides remains a critical question to be addressed.

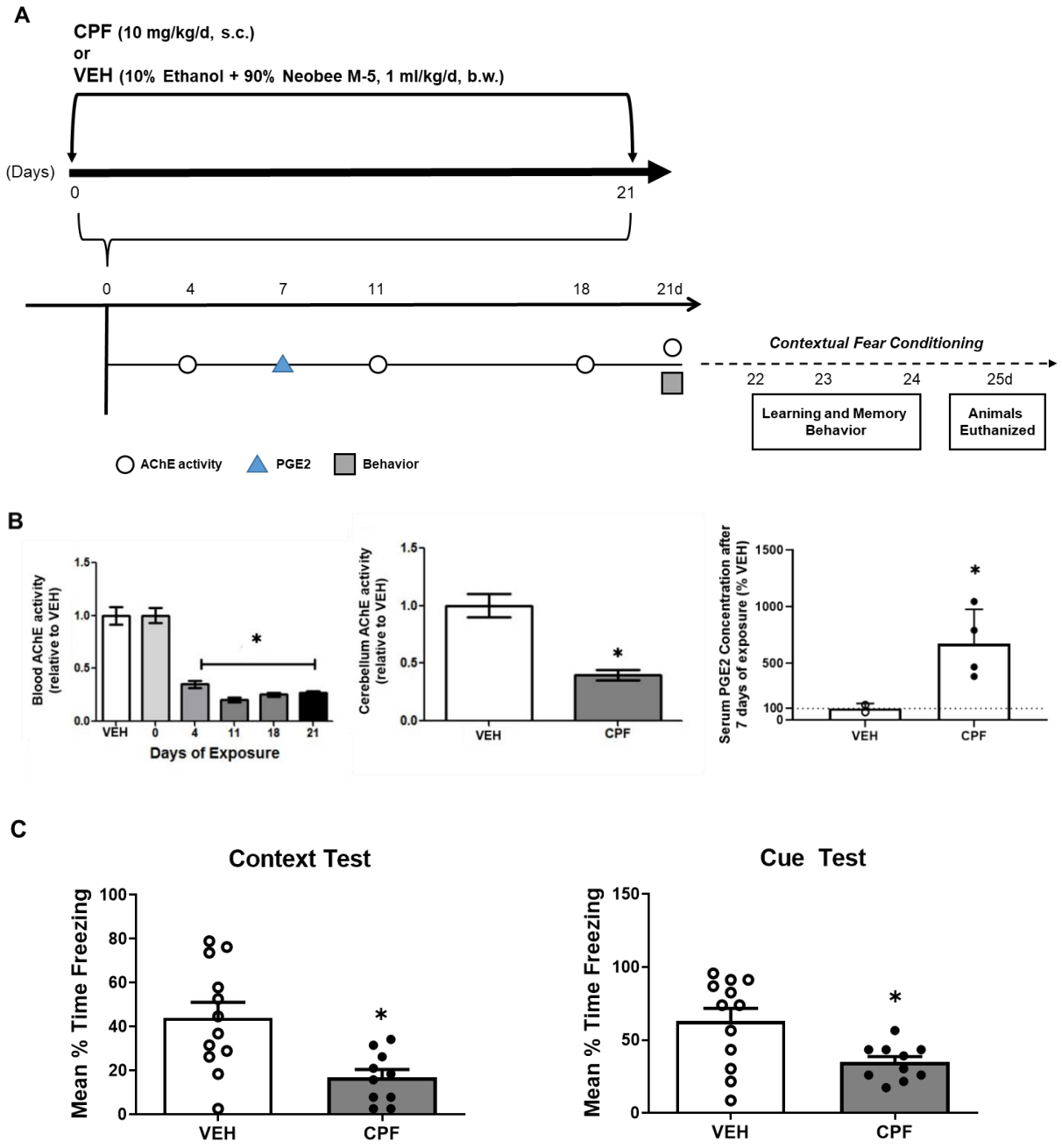


Figure 2-1. Effects of repeated CPF exposures on acetylcholinesterase (AChE) activity and fear conditioning. ***(A)*** Adult male Long-Evans rats were administered CPF (10 mg/kg/d, s.c.) or an equivalent volume (1 ml/kg/d) of VEH (10% ethanol in 90% Neobee M-5) daily for 21 days. Blood was collected at varying times throughout the exposure period. Upon conclusion of behavioral testing, the cerebellum was collected for AChE analyses. ***(B)*** AChE activity in the blood and cerebellum. Data are presented as the mean ± SEM (N = 8-12 for blood; N = 4-5 for

cerebellum). * Significantly different from VEH at $p < 0.05$ as determined by one-way ANOVA with post-hoc Tukey's test for blood AChE activity and by Student's t-test for brain AChE activity. PGE2 levels in the serum of VEH and CPF animals after 7 days of CPF exposure, expressed as relative percentage of the mean VEH values. Data presented as the mean ± SEM with individual data points per animal (N = 2 for VEH, and N = 4 for CPF). * Significantly different from VEH at $p < 0.5$ as determined by unpaired t test. ***(C)*** Performance in the context and cue fear conditioning tests. Data are presented as the mean ± SEM with individual data points per animal (N = 12 for VEH, and N = 10 for CPF). *Significantly different from VEH at $p < 0.05$ as determined using the Satterthwaite-Welch t-test.

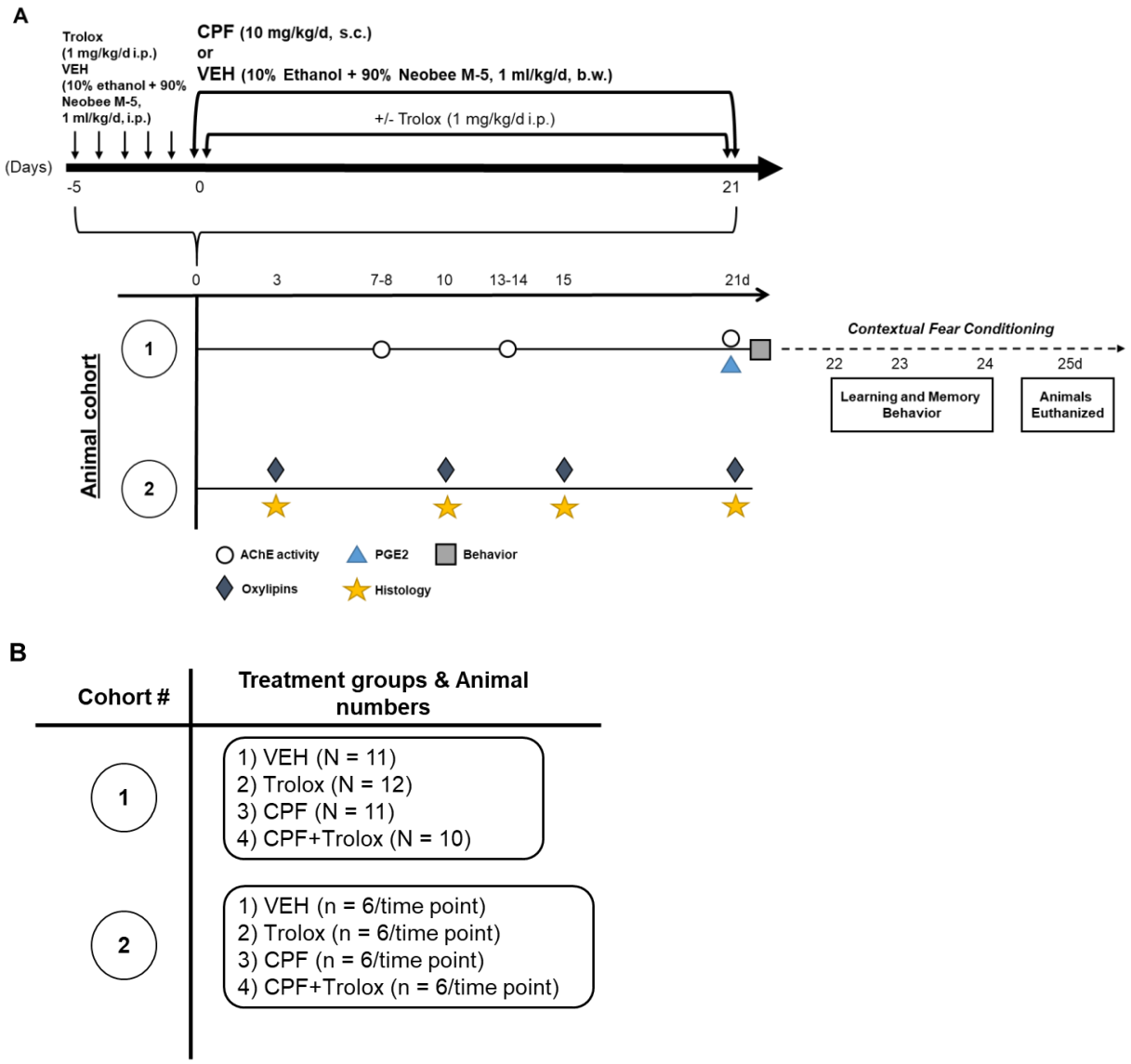


Figure 2-2. Schematic illustrating the experimental design and dosing paradigm for studies with the antioxidant Trolox. ***(A)*** Adult male Long-Evans rats were administered chlorpyrifos (CPF, 10 mg/kg/d, s.c.) or an equal volume (1 ml/kg/d) of vehicle (VEH, 10% ethanol in 90% Neobee M-5, 1 ml/kg/d, s.c.) for 21 days. Within the CPF and VEH groups, animals were randomly assigned to receive VEH or Trolox (1 mg/kg/d, i.p.) starting 5 days prior to CPF administration and continuing until the conclusion of the CPF exposure. Two independent cohorts of animals were set up for collecting various endpoints. ***(B)*** Sample sizes in each cohort.

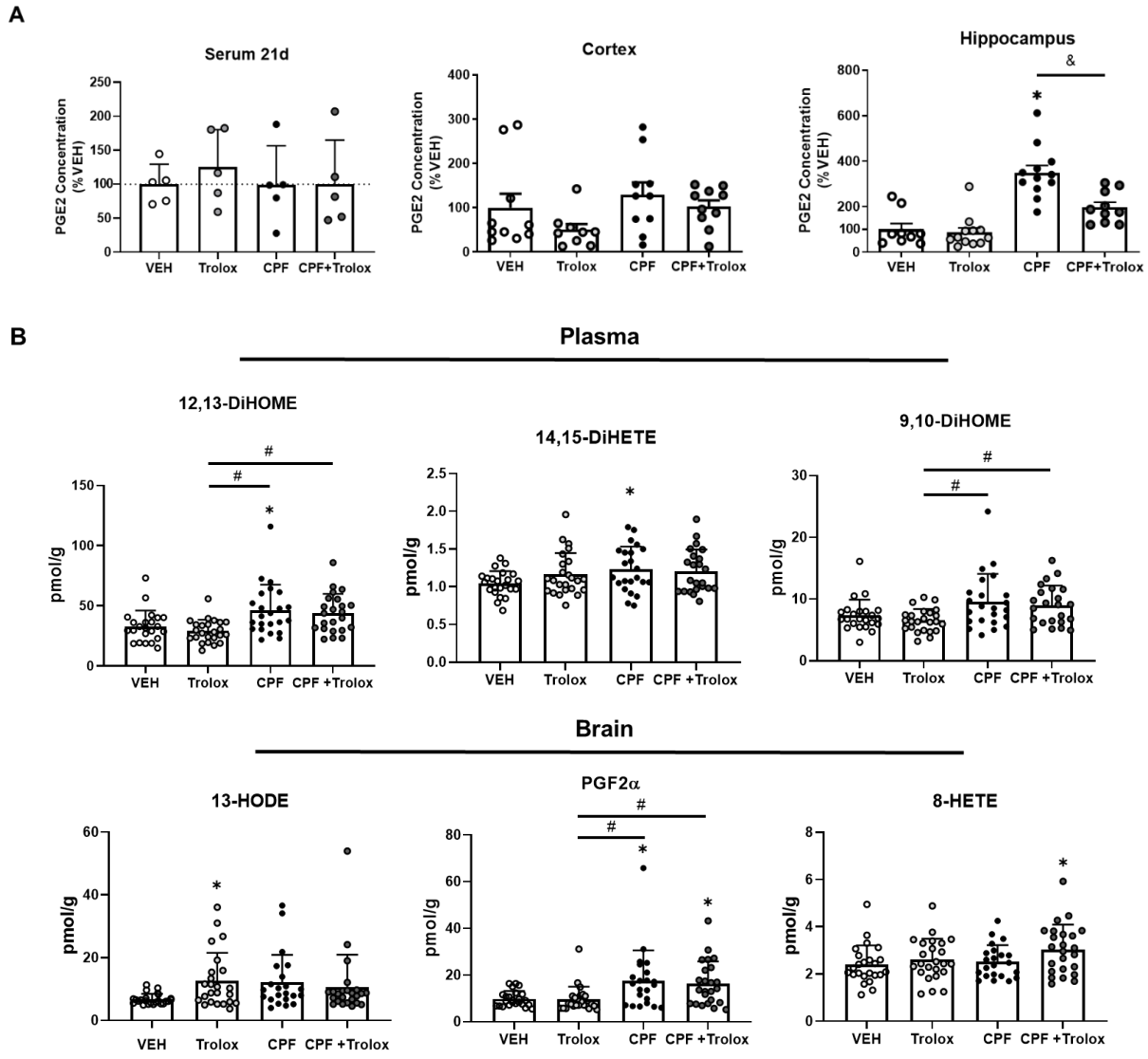


Figure 2-3. CPF significantly increases soluble biomarkers of oxidative stress in the blood and brain. ***(A)*** PGE2 levels in the serum, cortex, and hippocampus at the end of the 21-day exposure period are expressed as relative percentages of the mean value in vehicle control animals. Data presented as the mean ± SEM with individual data points per animal (individual dots represent individual animals; N = 5 for serum; N = 9-11 for cortex; n = 10-12 for hippocampus). * Significantly different ($p < 0.05$) from the VEH group; & significantly different ($p < 0.05$) from the CPF group as determined by one-way ANOVA with post-hoc Tukey's test. ***(B)*** Oxylipins measured in plasma and whole brain homogenates after 3, 10, 15 and 21 days of

exposure. There were no time by group interactions, so the group difference applies to all exposure times. Data presented as the mean ± SEM with individual data points per animal (N = 24 animals per group combined across time points). * Significantly different ($p < 0.05$) from the VEH group; # significantly different ($p < 0.05$) from the Trolox group as determined by one-way ANOVA with post-hoc Tukey's test.

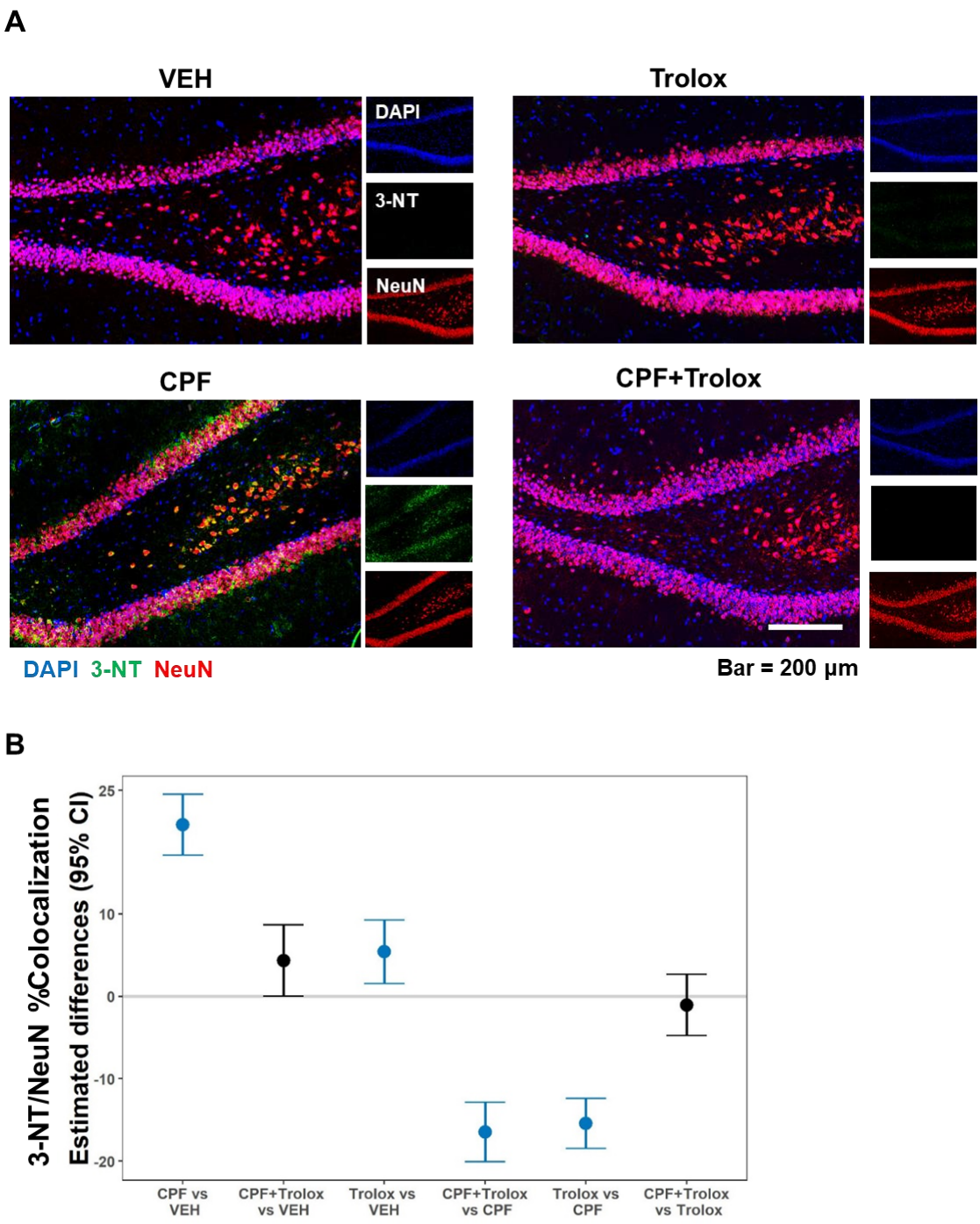


Figure 2-4. CPF significantly increases neuronal oxidative stress. ***(A)*** Representative photomicrographs of the dentate gyrus after 21 days of exposure to VEH or CPF (10 mg/kg/d, s.c.) in the absence or presence of Trolox (1 mg/kgk/d, i.p.). Brain sections were labeled with DAPI (blue) to identify cell nuclei and immunostained for 3-NT (green) and NeuN (red) to

specifically identify neuronal 3-NT expression. Scale bar = 200 μm. ***(B)*** The differences between groups did not vary by brain region (amygdala, CA1 and CA3 of the hippocampus, dentate gyrus of the hippocampus, piriform cortex, somatosensory cortex, thalamus) or time point (3, 10, 15, or 21 days) so an overall estimate of group differences based on all of the available data is presented. The estimated difference in the percentage of NeuN immunopositive cells immunoreactive for 3-NT between two groups with 95% confidence intervals (N = 24 animals per group combined across time points). Confidence intervals that do not encompass 0 (light grey line) suggest a significant difference between the two groups being compared with raw p values < 0.05. Confidence intervals colored blue survived false discovery rate (FDR) correction for multiple comparisons.

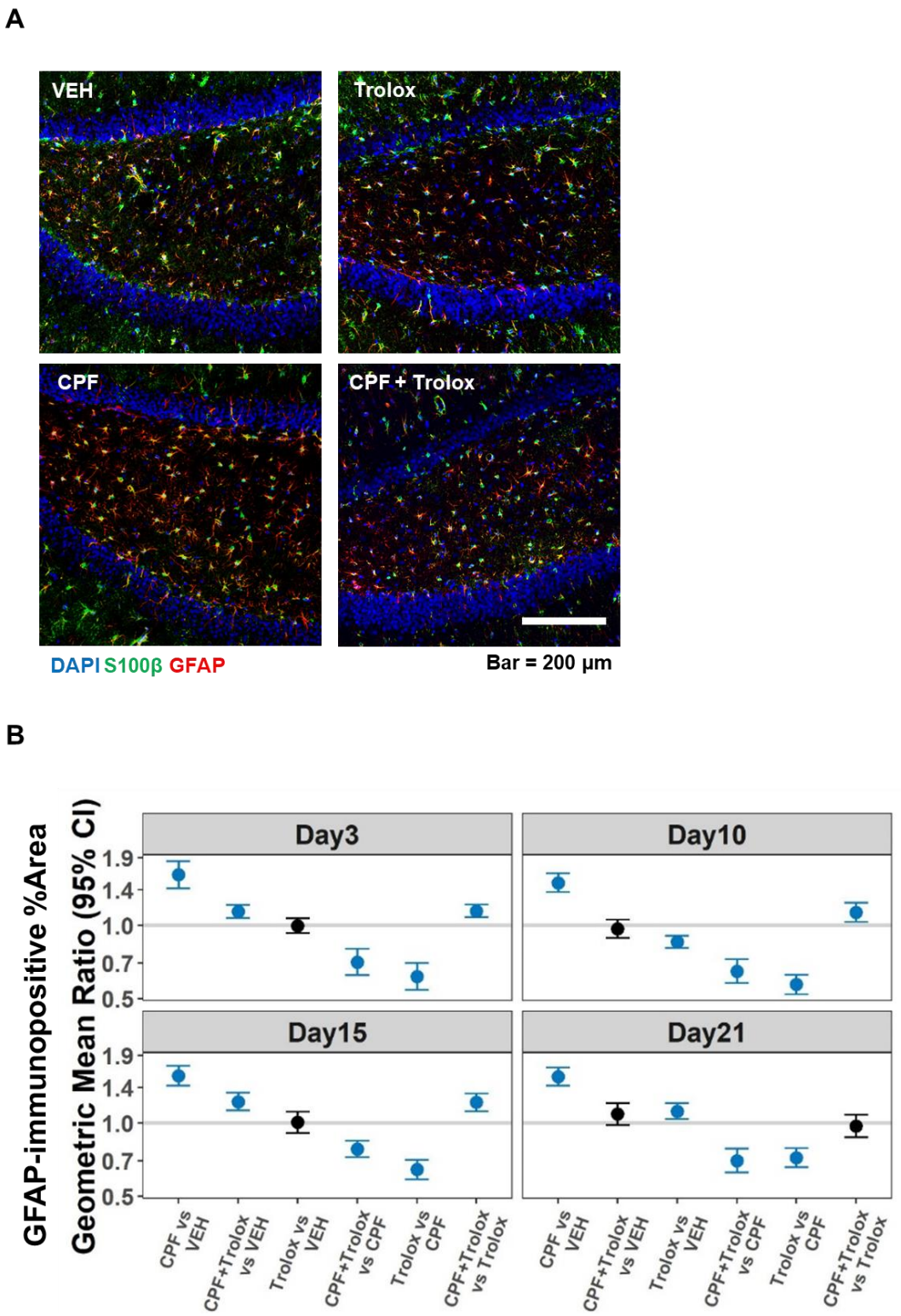


Figure 2-5. Repeated CPF exposure triggered persistent reactive astrogliosis in multiple brain regions. ***(A)*** Representative photomicrographs of GFAP and S100β immunoreactivity in

the dentate gyrus of the hippocampus after 21 days of exposure to VEH or CPF (10 mg/kg/d, s.c.) in the absence or presence of Trolox (1 mg/kgk/d, i.p.). Brain sections were labeled with DAPI (blue) to identify cell nuclei and immunostained for S100β (green) and GFAP (red) to identify astrocytes. Scale bar = 200 μm. ***(B)*** The % area immunopositive for GFAP varied between groups as a function of exposure duration so data for each time point are presented separately. Results are presented as the geometric mean ratio (GMR) and 95% confidence interval (N = 6 / group). Confidence intervals that do not include 1 indicate a significant difference between groups with raw p values < 0.05. Confidence intervals colored blue identify significant differences that survived the FDR correction for multiple comparisons. No significant differences between groups were identified for the % area immunopositive for S100β (see Figure 2-S2 in the supplemental materials).

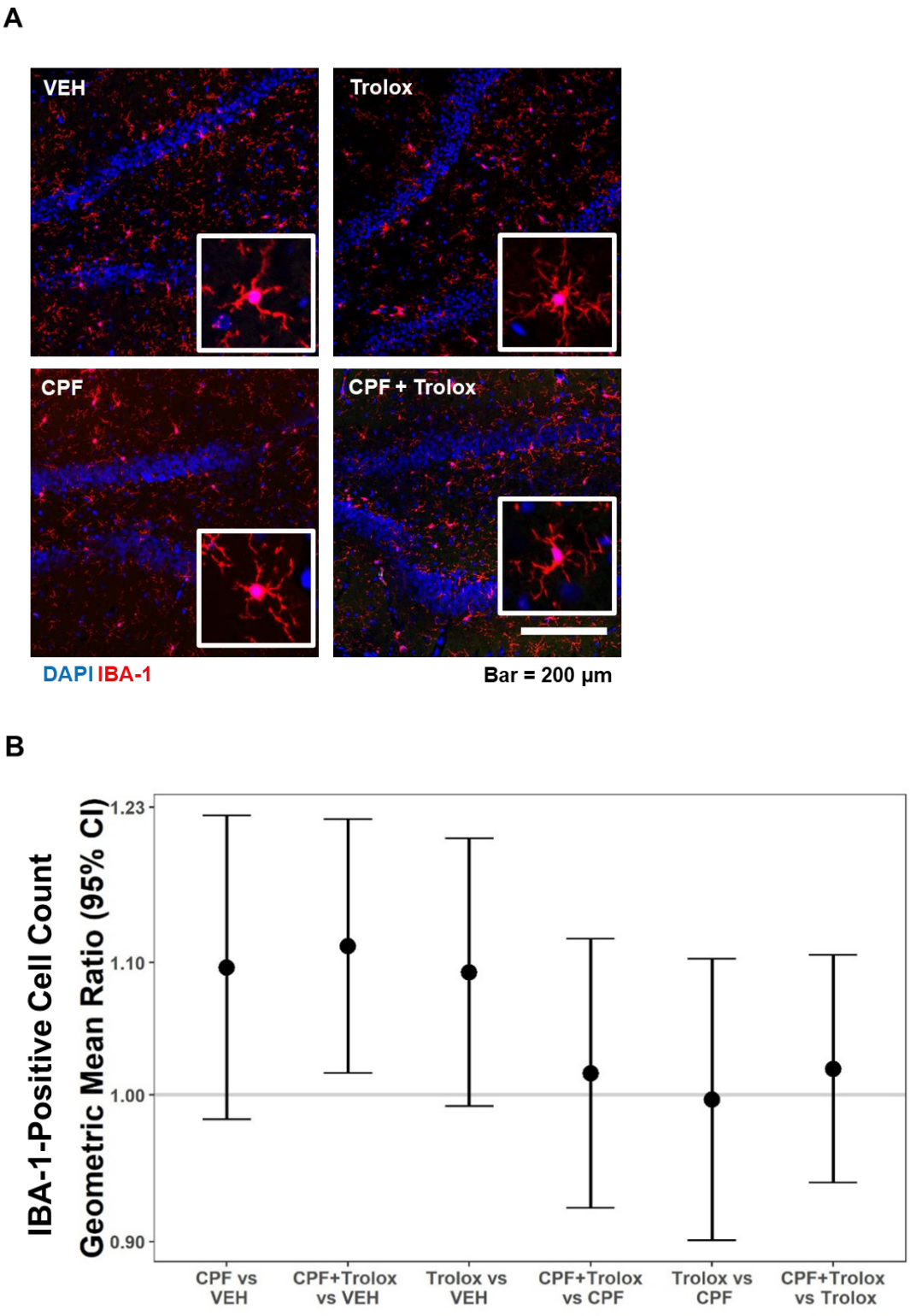


Figure 2-6. Repeated CPF exposure did not trigger microgliosis. ***(A)*** Representative photomicrographs of the dentate gyrus of the hippocampus after 21 days of exposure to VEH or

CPF (10 mg/kg/d, s.c.) in the absence or presence of Trolox (1 mg/kgk/d, i.p.). Brain sections were labeled with DAPI (blue) to identify nuclei and immunostained for IBA-1 (red) to identify microglia and CD68 (green) to identify phagocytic microglia. Scale bar = 200 μm. ***(B)*** Negligible CD68 immunoreactivity was detected in all samples, so microgliosis was quantified as the total number of IBA-1+ cells per unit area in the brain regions of interest. Group differences in IBA-1 positive cell count did not differ by brain region or by day and so are presented as an overall estimate of the difference. Results are presented as the geometric mean ratio (GMR) with 95% confidence interval (N = 24 animals per group combined across time points). Confidence intervals that do not include 1 indicate a significant difference between groups at $p < 0.05$

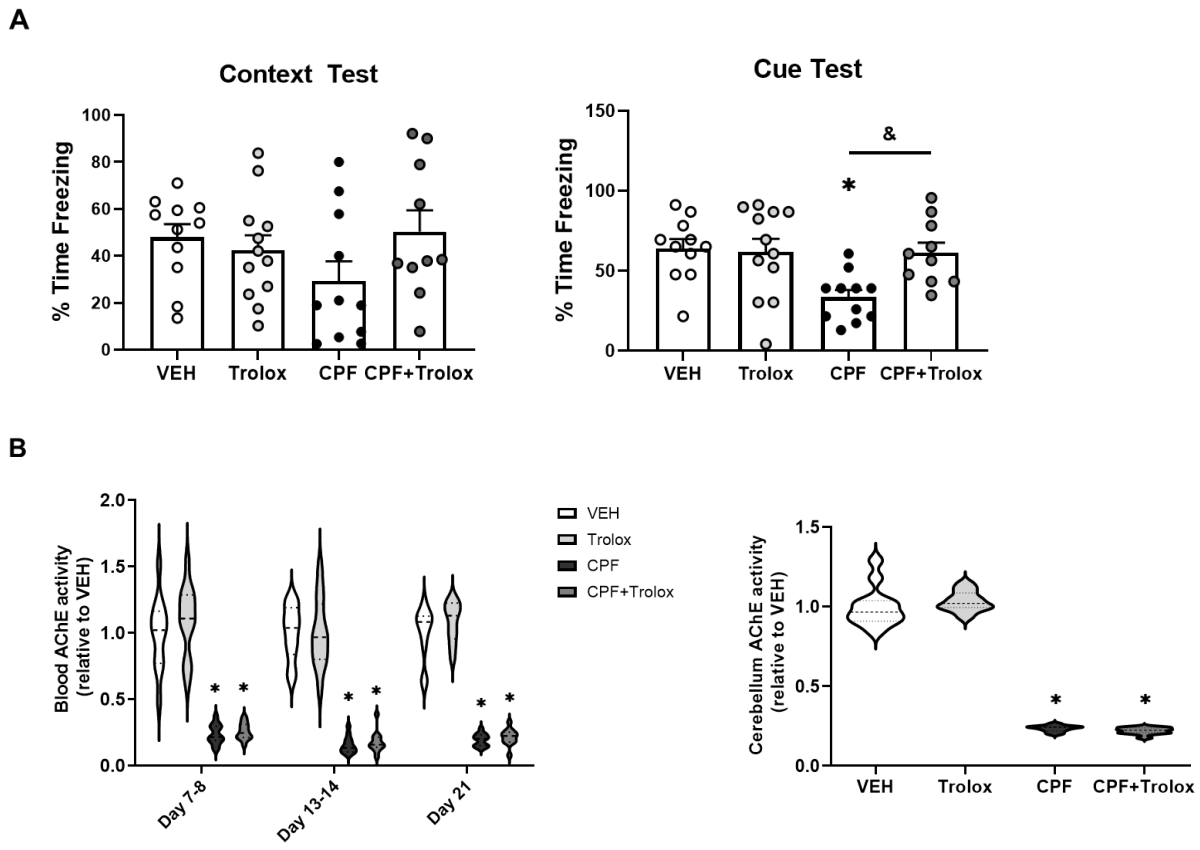
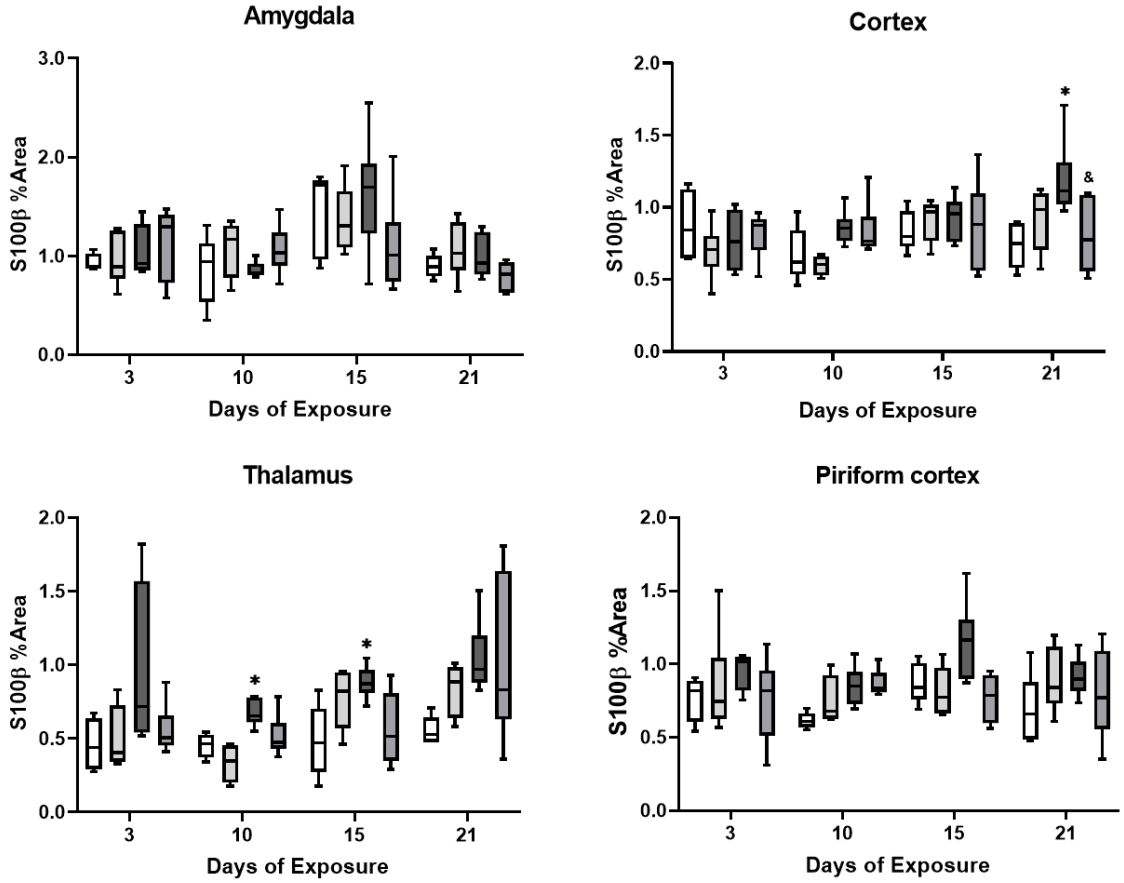


Figure 2-7. Treatment with the antioxidant Trolox prevents CPF-induced neurobehavioral deficits but not AChE inhibition. ***(A)*** At the conclusion of the 21-day exposure period, contextual and cue fear conditioning were assessed as a measure of learning and memory. Data presented as the mean ± SEM with individual data points corresponding to individual animals (N = 10-12 / group). * Significantly different ($p < 0.05$) from the VEH group; & significantly different ($p < 0.05$) from the CPF group as determined by one-way ANOVA with post-hoc Tukey test. ***(B)*** AChE activity was measured in blood collected at different times throughout the exposure period and in the cerebellum after 21 days of exposure. Data are presented as the mean ± SEM (N = 5-6 / group). AChE activity was measured using the Ellman assay; blood AChE activity was normalized to hemoglobin levels in the sample; brain AChE activity was normalized to total protein. *Significantly different ($p < 0.05$) from the VEH group as determined by one-way ANOVA with post-hoc Tukey test.

A


Amygdala
S100β %Area
3.0
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15
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Days of Exposure
Cortex
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*
&
3
10
15
21
Days of Exposure
Thalamus
S100β %Area
2.0
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Days of Exposure
Piriform cortex
S100β %Area
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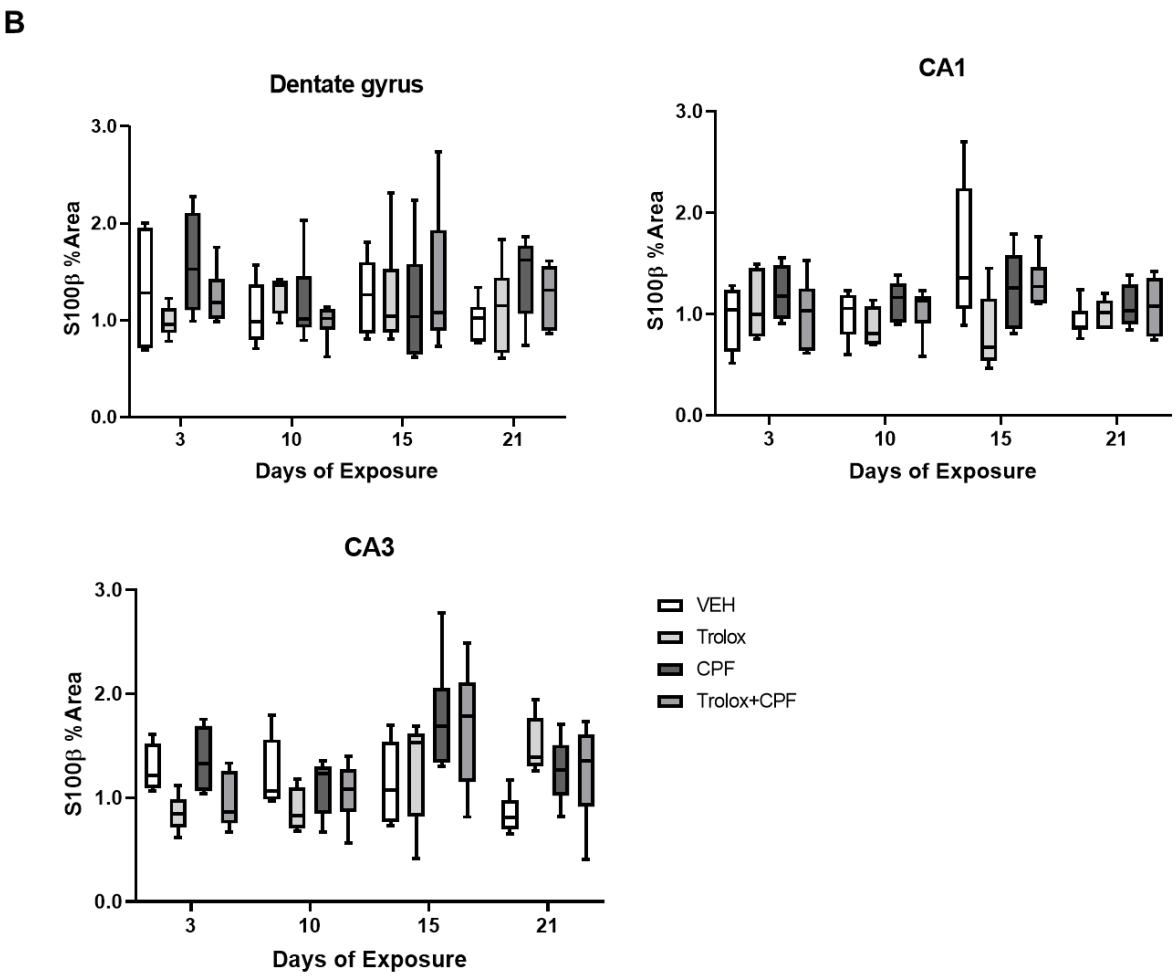

Figure 2-S1. Repeated CPF exposure had no effect on the % area of S100β immunoreactivity in multiple brain regions. Edges of each box refer to the 25th and 75th percentiles of the raw data; and the horizontal line within the box refers to the median value; the whiskers represent the minimum and maximum values (N = 6 / group). * Significantly different ($p < 0.05$) from the VEH group; & significantly different ($p < 0.05$) from the CPF group as determined by one-way ANOVA with post-hoc Tukey test.

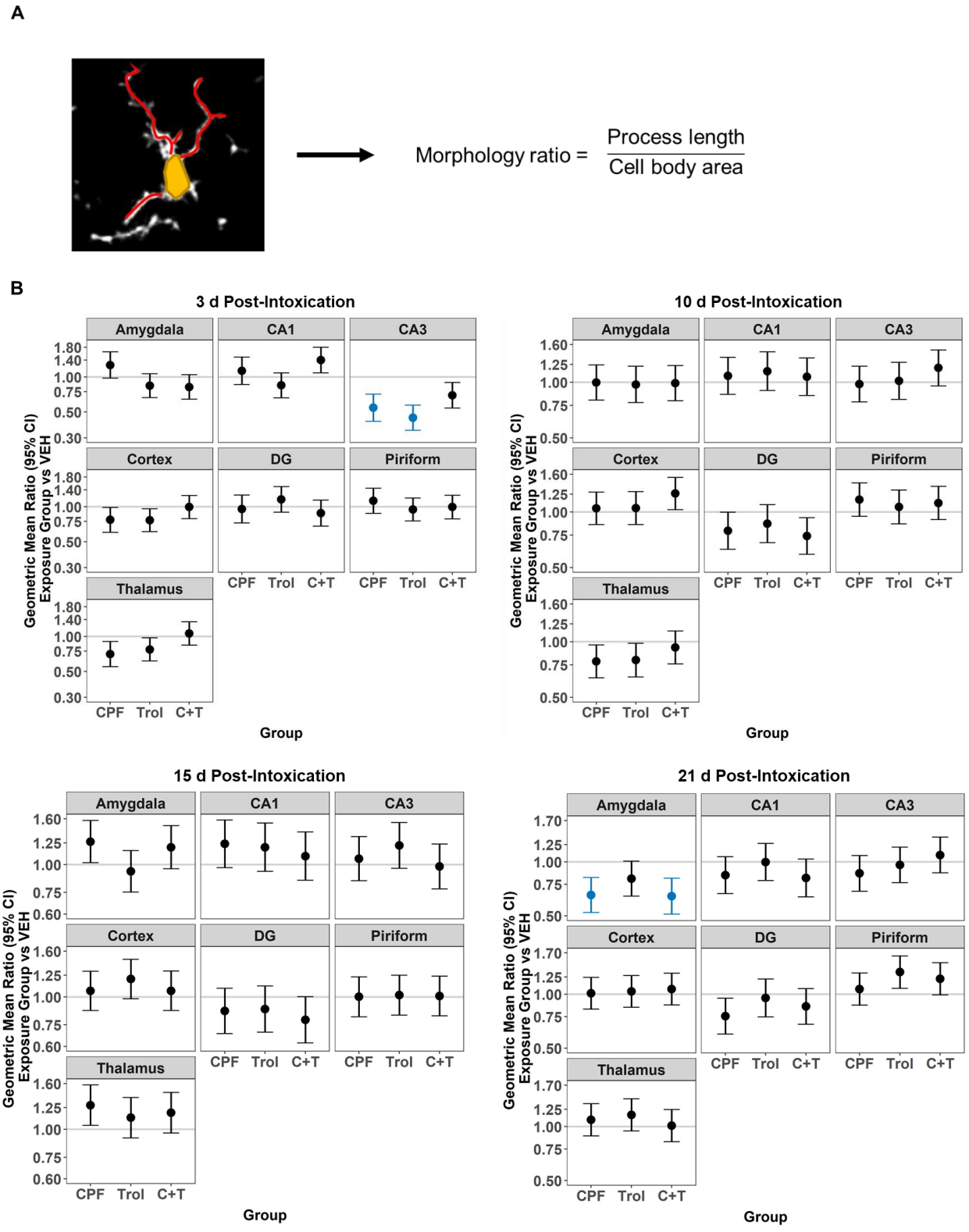

Figure 2-S2. Repeated CPF intoxication did not trigger microglial activation as indicated by morphological criteria. ***(A)*** Schematic representing the morphological criteria used to

assess microglia activation. Morphology ratios are calculated as the process length (highlighted by red lines) divided by the cell body area (highlighted in yellow). ***(B)*** Microglia morphology was analyzed in seven brain regions (amygdala, CA1 and CA3 of the hippocampus, dentate gyrus of the hippocampus, piriform cortex, somatosensory cortex, and thalamus) after 3, 10, 15, or 21 days of repeated CPF or VEH exposure. Data presented as the geometric mean ratio (GMR) with 95% confidence interval (N = 6 / group). Confidence intervals that do not include 1.00 indicate a significant difference between the two groups being compared at $p < 0.05$; confidence intervals colored blue indicate significant differences that survived FDR correction. CPF = CPF only; Trol = Trolox only; C+T = CPF+Trolox.

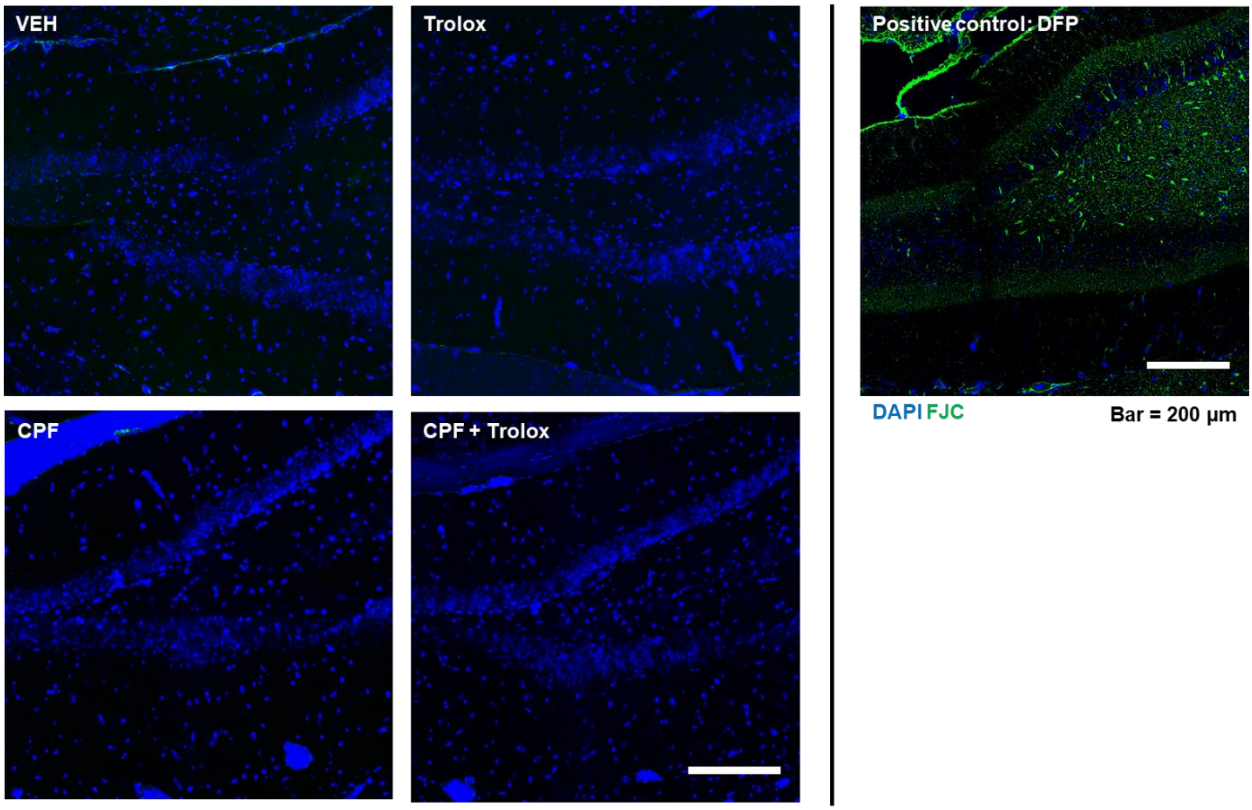

Fig. 2-S3. Repeated CPF exposure does not cause neurodegeneration as indicated by FluorJade-C (FJC) staining in the amygdala, CA1 and CA3 of the hippocampus, dentate gyrus of the hippocampus, piriform cortex, somatosensory cortex, thalamus after 3, 10, 15, or 21 days of repeated CPF exposure, even in the absence of Trolox. Representative photomicrographs illustrating the lack of FJC staining in the dentate gyrus of the hippocampus after 21 d of exposure to VEH or CPF (10 mg/kg/d, s.c.) in the absence or presence of Trolox (1 mg/kgk/d, i.p.). As a positive control, brain sections from adult male rats acutely intoxicated with the OP diisopropylfluorophosphate (DFP) showing positive FJC staining. Brain sections were labeled with DAPI (blue) to identify cell nuclei and FJC (green) to identify degenerating neurons. Scale bar = 200 µm.

Table 2-1. List of oxylipins analyzed in the plasma and the brain (abbreviations, names, and precursor fatty acid).

Abbreviation	Compound name	Precursor
d11-11(12)-EpETrE	d-11-11(12)-epoxyeicosatrienoic acid	AA
d11-14,15-DiHETrE	d11-14,15-dihydroxyeicosatrienoic acid	AA
d4-6-keto-PGF1a	d4-6-keto-prostaglandin F1 alpha	AA
d4-9-HODE	d4-9-hydroxyoctadecadienoic acid	LA
d4-LTB4	d4-Leukotriene B4	AA
d4-PGE2	d4-Prostaglandin E2	AA
d4-TXB2	d4-Tromboxane B2	AA
d6-20-HETE	d6- 20-hydroxyeicosatetraenoic acid	AA
d8-5-HETE	d8- 5-hydroxyeicosatetraenoic acid	AA
12(13)-EpOME	12(13)-epoxyoctadecamonoenoic acid	LA
13-HODE	13-hydroxyoctadecadienoic acid	LA
13-oxo-ODE	13-oxo-octadecadienoic acid	LA
9-HODE	9-hydroxyoctadecadienoic acid	LA
9-oxo-ODE	9-oxo-octadecadienoic acid	LA
9,10,13-TriHOME	9,10,13-trihydroxyoctadecamonoenoic acid	LA
9,12,13-TriHOME	9,12,13-trihydroxyoctadecamonoenoic acid	LA
9(10)-EpOME	9(10)-epoxyoctadecamonoenoic acid	LA
20-HETE	20-hydroxyeicosatetraenoic acid	AA
11-HETE	11-hydroxyeicosatetraenoic acid	AA
11,12-DiHETrE	11,12-dihydroxyeicosatrienoic acid	AA
11(12)-EpETrE	11(12)-epoxyeicosatrienoic acid	AA
12-HETE	12-hydroxyeicosatetraenoic acid	AA
12-oxo-ETE	12-oxo-eicosatetraenoic acid	AA
14,15-DiHETrE	14,15-dihydroxyeicosatrienoic acid	AA
14(15)-EpETE	14(15)-epoxyeicosatrienoic acid	AA
14(15)-EpETrE	14(15)-epoxyeicosatrienoic acid	AA
15-deoxy-PGJ2	15-deoxy-Prostaglandin J2	AA

15-HETE	15-hydroxyeicosatetraenoic acid	AA
15-oxo-ETE	15-oxo-eicosatetraenoic acid	AA
20-COOH-LTB4	20-COOH- Leukotriene B4	AA
20-OH-LTB4	20-OH-Leukotriene B4	AA
5-HETE	5-hydroxyeicosatetraenoic acid	AA
5-oxo-ETE	5-oxo-eicosatetraenoic acid	AA
5,6-DiHETrE	5,6-dihydroxyeicosatrienoic acid	AA
5(6)-EpETrE	5(6)-epoxyeicosatrienoic acid	AA
6-keto-PGF1a	6-keto-prostaglandin F1 alpha	AA
6-trans-LTB4	6-trans-leukotriene B4	AA
8-HETE	8-hydroxyeicosatetraenoic acid	AA
8,9-DiHETrE	8,9-dihydroxyeicosatrienoic acid	AA
8(9)-EpETrE	8(9)-epoxyeicosatrienoic acid	AA
9-HETE	9-hydroxyeicosatetraenoic acid	AA
LTB4	Leukotriene B4	AA
LTC4	Leukotriene C4	AA
LTD4	Leukotriene D4	AA
LTE4	Leukotriene E4	AA
LXA4	Lipoxin A4	AA
PGB2	Prostaglandin B2	AA
PGD2	Prostaglandin D2	AA
PGE2	Prostaglandin E2	AA
PGF2α	Prostaglandin F2 alpha	AA
PGJ2	Prostaglandin J2	AA
TXB2	Tromboxane B2	AA
12,13-DiHOME	12,13-dihydroxyoctadecamonoenoic acid	ALA
13-HOTrE	13- hydroxyoctadecatrienoic acid	ALA
9-HOTrE	9- hydroxyoctadecatrienoic acid	ALA
9,10-DiHOME	9,10-dihydroxyoctadecamonoenoic acid	ALA

PGD1	Prostaglandin D1	DGLA
PGE1	Prostaglandin E1	DGLA
10(11)-EpDPE	10(11)-epoxydocosapentaenoic acid	DHA
13(14)-EpDPE	13(14)-epoxydocosapentaenoic acid	DHA
16(17)-EpDPE	16(17)-epoxydocosapentaenoic acid	DHA
17-HDoHE	17- hydroxydocosahexaenoic acid	DHA
19(20)-EpDPE	19(20)-epoxydocosapentaenoic acid	DHA
7(8)-EpDPE	7(8)-epoxydocosapentaenoic acid	DHA
11(12)-EpETE	11(12)-epoxyeicosateteaenoic acid	EPA
12-HEPE	12-hydroxyeicosapentaenoic acid	EPA
14,15-DiHETE	14,15-dihydroxyeicosatetraenoic acid	EPA
15-HEPE	15-hydroxyeicosapentaenoic acid	EPA
17,18-DiHETE	17,18-dihydroxyeicosatetraenoic acid	EPA
17(18)-EpETE	17(18)-epoxyeicosateteaenoic acid	EPA
5-HEPE	5-hydroxyeicosapentaenoic acid	EPA
5,15-DiHETE	5,15-dihydroxyeicosatetraenoic acid	EPA
5,6-DiHETE	5,6-dihydroxyeicosatetraenoic acid	EPA
8-HEPE	8-hydroxyeicosapentaenoic acid	EPA
8,15-DiHETE	8,15-dihydroxyeicosatetraenoic acid	EPA
8(9)-EpETE	8(9)-epoxyeicosateteaenoic acid	EPA
PGD3	Prostaglandin D3	EPA
PGE3	Prostaglandin E3	EPA
Resolvin E1	Resolvin E1	EPA
LTB3	Leukotriene B3	ETA
15(S)-HETrE	15(S)-hydroxyeicosatrienoic acid	GLA

AA: Arachidonic acid; LA: Linoleic acid; ALA: α-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; EPA: eicosapentaenoic acid; GLA: gamma-linolenic acid

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Chapter 3

Acute Intoxication with Diisopropylfluorophosphate Promotes Cellular Senescence in the Adult Rat Brain

Modified from a manuscript in preparation with the following authors:

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Abstract

Acute intoxication with high levels of organophosphate (OP) cholinesterase inhibitors can cause cholinergic crisis, which is associated with acute, life-threatening parasympathomimetic symptoms, respiratory depression and seizures that can rapidly progress to *status epilepticus*. Clinical and experimental data demonstrate that individuals who survive the acute neurotoxic effects often develop significant chronic morbidity, including behavioral deficits. The pathogenic mechanism(s) that link acute OP intoxication to chronic neurological deficits remain speculative. Cellular senescence has been linked to behavioral deficits associated with aging and neurodegenerative disease, but whether acute OP intoxication causes cellular senescence has not been investigated. Here, we test this hypothesis in a rat model of acute intoxication with the OP diisopropylfluorophosphate (DFP). Adult male Sprague-Dawley rats were administered DFP (4 mg/kg, s.c.); control animals were administered an equal volume (300 µl) of sterile phosphate-buffered saline (PBS; s.c.). Both groups were subsequently injected with atropine sulfate (2 mg/kg, i.m.) and pralidoxime (25 mg/kg, i.m.). DFP injection triggered seizure activity within minutes that rapidly progressed to *status epilepticus*, as determined using behavioral seizure criteria. Brains were collected from animals at 7 days, 28 days, 3 months, and 6 months post-exposure for immunohistochemical analyses of cellular senescence in the amygdala, the somatosensory and piriform cortex, the hippocampus, and the thalamus. Cellular senescence is specifically assessed via a well-established biomarker of cellular senescence, p16, which is a cyclin-dependent kinase inhibitor (CDKI). Significantly increased p16 immunoreactivity was observed in the hippocampus and thalamus, but not other brain regions, at 3 and 6 months post-DFP exposure. Experiments to determine whether p16 immunoreactivity colocalized with NeuN, GFAP, IBA1 or CD31, biomarkers of neurons, astrocytes, microglia and endothelial cells, respectively, revealed that p16 expression in the brain of DFP animals is neuron-specific. The spatial distribution of p16-immunopositive cells overlapped that of degenerating neurons identified by Fluoro-Jade C staining, and the co-occurrence of these two biomarkers was

positively correlated. This study implicates cellular senescence as a novel pathogenic mechanism underlying the chronic neurological deficits observed in humans and experimental animals who survive OP-induced cholinergic crisis.

Introduction

Organophosphate cholinesterase inhibitors (OPs) are a family of potent neurotoxic chemicals that includes nerve agents and pesticides. OP poisoning as a consequence of accidental or intentional (suicidal) exposures, war, and terrorism is a significant global public health issue (Eddleston & Phillips, 2004; Haley, 2018; UN, 2017; Vogel, 2013). It is estimated that acute OP intoxication annually causes 3 million life-threatening poisoning cases and 250,000 deaths worldwide (Mew et al., 2017; Pereira et al., 2014).

The canonical mechanism of acute OP neurotoxicity is acetylcholinesterase (AChE) inhibition, which results in hyperstimulation of muscarinic (mAChR) and nicotinic (nAChR) cholinergic receptors in the peripheral and central nervous systems due to excessive accumulation of acetylcholine (ACh) (Pope et al., 2005). When AChE activity is acutely inhibited by ≥ 60-70%, this triggers a clinical toxidrome called “cholinergic crisis” which includes seizures that rapidly progress to *status epilepticus* (SE) (Pereira et al., 2014; Richardson et al., 2019). Current medical countermeasures for OPs are effective in reducing mortality, but unless administered within minutes following exposure, do not effectively terminate OP-induced seizures or protect against delayed brain damage and long-term morbidity (Chen, 2012; Eddleston et al., 2008; Jett & Spriggs, 2020). There are reports of long-term neurologic effects such as neuropsychiatric and cognitive deficits in those who survive acute OP intoxication (Chen, 2012; reviewed in Figueiredo et al., 2018; reviewed in Jett et al., 2020). This sobering fact coupled with observations that OPs remain significant public health concerns, underscores the importance of better understanding the pathogenic mechanisms underlying these outcomes in order to identify novel, mechanistically based neuroprotective therapies.

Impaired cognitive function has been reported as an adverse neurological outcome in humans who survived acute OP exposures (reviewed in Figueiredo et al., 2018; reviewed in Jett et al., 2020; Loh et al., 2010; Miyaki et al., 2005; reviewed in Vale & Lotti, 2015; Yamasue et al., 2007). For example, long-term neurological alterations later in life, including structural brain

damage, decreased gray matter volume in certain brain regions (Yamasue et al., 2007), and deficits in cognitive function (Miyaki et al., 2005), have been reported in survivors of the Tokyo subway sarin attack in 1995. Deficits in cognitive function have been corroborated in preclinical models of acute OP intoxication (de Araujo Furtado et al., 2012; Deshpande et al., 2014; Flannery et al., 2016; Guignet et al., 2020; Pereira et al., 2014). For instance, in adult rats acutely intoxicated with the OP, diisopropylfluorophosphate (DFP), memory impairment was observed at 1-month post-exposure as assessed using the Morris water maze (Brewer et al., 2013), at 2 months post-exposure as evaluated by Pavlovian fear conditioning (Guignet et al., 2020), and at 3 months post-exposure using novel object recognition (Rojas et al., 2016).

Extensive experimental and epidemiological data implicate non-cholinergic mechanisms in a diverse range of neurotoxic outcomes (reviewed in Naughton & Terry, 2018; Tsai & Lein, 2021). In the context of the chronic neurotoxicity associated with acute OP intoxication, several non-cholinergic mechanisms are widely posited, including neuroinflammation (reviewed in Andrew & Lein, 2021; reviewed in Guignet & Lein, 2019) and oxidative stress (reviewed in Pearson & Patel, 2016; reviewed in Vanova et al., 2018). The spatiotemporal patterns of biomarkers of neuroinflammation and oxidative stress have recently been characterized in the rat model of acute DFP intoxication (Flannery et al., 2016; Guignet et al., 2020; Hobson et al., 2019; Supasai et al., 2020). However, few studies to date have determined whether experimental manipulation of neuroinflammation or oxidative stress ameliorate long-term effects of acute OP intoxication (Putra et al., 2020a; Putra et al., 2020b; Rojas et al., 2020).

Emerging evidence links cellular senescence to neurodegenerative disease and age-related cognitive impairment (reviewed in Baker & Petersen, 2018; reviewed in Kritsilis et al., 2018). Cellular senescence, which was originally described as the finite capability of cell replication before experiencing stable growth arrest (Hayflick & Moorhead, 1961), is now thought to be a stress response triggered by a variety of intrinsic and extrinsic insults, such as oncogenic activation, oxidative and genotoxic stress, or mitochondrial dysfunction (reviewed in

Kuilman et al., 2010; reviewed in McHugh & Gil, 2017). Cellular senescence can be beneficial or deleterious (reviewed in Burton & Krizhanovsky, 2014). For example, induction of cellular senescence can act to irreversibly halt proliferation of cells that at risk for malignant transformation (reviewed in Campisi, 2013); conversely, persistent senescent cells in aged tissues are thought to contribute to age-related pathologies (Baker et al., 2016; reviewed in van Deursen, 2014). Distinguishing characteristics of senescent cells include upregulated gene and protein expression of $p16^{INK4a}$ and p21 (which are cyclin-dependent kinase inhibitors, CDKIs), chromatin reorganization, loss of nuclear lamin B1, adoption of a pro-inflammatory phenotype known as the senescence-associated secretory phenotype (SASP), and increased β-galactosidase (SA-βgal) activity (reviewed in Baker & Petersen, 2018; reviewed in Childs et al., 2017). While the role of cellular senescence in brain aging and neurodegenerative disease has yet to be fully elucidated, various cell types of the central nervous system have been found to display characteristics of senescence in the context of Alzheimer's and Parkinson's disease (Arendt et al., 1998; Bhat et al., 2012; Chinta et al., 2018; Musi et al., 2018; Riessland et al., 2019).

Reports that repeated exposure to Paraquat in a preclinical model of Parkinson's disease promotes senescence of astrocytes suggest that neurotoxic chemicals can promote the development of cellular senescence in the brain (Chinta et al., 2018). Here, we test the hypothesis that acute DFP intoxication promotes cellular senescence in the brain by assessing biomarkers of cellular senescence during the 6 months following DFP-induced SE.

Materials and methods

Animals

Animals were maintained in facilities fully accredited by AAALAC International, and all studies were performed with regard to alleviation of pain and suffering under protocols approved by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC

protocol number 20165). All animal experiments were conducted in accordance with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of laboratory animals. Adult male Sprague Dawley rats (8 ± 1 weeks old, 225 - 250 g) were purchased from Charles River Laboratories (Hollister, CA), and upon receipt were individually housed in standard plastic shoebox cages on autoclaved corncob bedding under controlled environmental conditions (22 ± 2 °C, 40–50% humidity, 12 h light-dark cycle). Food and water were provided *ad libitum*, and rats were allowed to acclimate for at least 7 d before experimentation.

DFP exposure paradigm and behavioral seizure monitoring

DFP was purchased from Sigma-Aldrich (St. Louis, MO, USA), and stocks were confirmed to have a purity of 90 ± 7% as determined by ^{1}H-, ^{13}C, ^{19}F and ^{31}P NMR methods (Gao et al., 2016). DFP aliquots were stored at - 80°C to ensure chemical stability for over a year (Heiss et al., 2016). On the day of dosing, DFP was prepared in ice-cold sterile phosphate-buffered saline (PBS, 3.6 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 150 mM NaCl; pH 7.2) and administered at 4 mg/kg (s.c.). Control animals were administered a comparable volume (300 µl) of ice-cold sterile PBS. Rats (n = 4 – 6 animals per group) were randomly divided into DFP or vehicle control (VEH) groups using a random number generator. Both DFP and VEH groups received a combined intramuscular (i.m.) injection of atropine sulfate (2 mg/kg, purity ≥ 97%, Sigma-Aldrich) and 2-pralidoxime (2-PAM, 25 mg/kg, purity ≥ 99%, Sigma-Aldrich) in sterile saline (0.9% NaCl) in the inner thigh of the hind leg 1 min following DFP/VEH administration. This post-exposure treatment has been shown to significantly increase survival by reducing peripheral cholinergic symptoms associated with acute OP intoxication (Pessah et al., 2016). This exposure paradigm is illustrated in Figure 3-1.

All animals were continuously monitored for seizure behavior for 4 h post-exposure. Seizure behavior was scored every 5 minutes for the first 2 hours and then every 20 minutes for the next 2 hours using a modified Racine scale as previously described (Deshpande et al.,

2010). Briefly, seizure behavior was scored as: 0) no signs of symptoms; 1) salivation, lacrimation, urination, defecation (SLUD); 2) muscle fasciculations, tremors; 3) forelimb clonus; 4) rearing of torso, hindlimb clonus; and 5) rearing and falling, and loss of righting reflex. DFP-intoxicated animals with consecutive seizure scores ≥ 3 have previously been shown to be in SE as determined using electroencephalographic criteria (Deshpande et al., 2010). The DFP rats included in this study had consecutive seizure scores of ≥ 3 during 40 minutes after DFP injection. At the end of the 4 h exposure period, DFP animals were administered 10 ml of 5% dextrose in 0.9% isotonic saline (Baxter International, Deerfield, IL, USA) to supplement fluids lost before being returned to their home cages. Soft chow was provided for the following 3 to 5 days following DFP intoxication until the animals resumed normal consumption of water and solid food.

Histochemistry and immunohistochemistry

At 7d, 28 d, 3 months and 6 months post-exposure, animals were humanely euthanized with 4% isoflurane in medical grade oxygen and subsequently perfused transcardially with PBS at a flow rate of 15 ml/min using a Masterflex peristaltic pump (Cole Parmer, Vernon Hills, IL, USA). Following euthanasia, brains were quickly excised from the skull and placed on ice. The brain was bisected and the right hemisphere of the brain was sliced into 2-mm thick coronal sections that were postfixed in 4% (w/v) paraformaldehyde (PFA; Sigma-Aldrich) in phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH = 7.2) at 4 °C overnight. Sections were then switched to 30% (w/v) sucrose (Sigma-Aldrich) in PBS for storage at 4°C until being embedded and frozen in Tissue-Plus™ O.C.T. (Thermo Fisher Scientific, Waltham, MA, USA). Brain tissue blocks were cryosectioned into 10-µm thick coronal sections and stored at -80°C.

To detect degenerating neurons, Fluoro-Jade C (FJC, AG325, Millipore, Burlington, MA, USA) labeling was performed according to the manufacturer's protocol. Briefly, after drying at 50°C for 10-15 min, sections were rinsed with 70% (v/v) ethanol for 2 min followed by a distilled

water wash. Sections were incubated in 0.03% (w/v) potassium permanganate ($KMnO_4$; Sigma-Aldrich) in distilled water for 10 min on a shaker table followed by a 2 min distilled water wash. Next, sections were incubated in a freshly prepared solution of 0.00015% FJC in 0.1% (v/v) acetic acid (Acros Organics, Geel, Belgium) in distilled water containing 0.5 µg/ml DAPI (Invitrogen, Carlsbad, CA, USA) for 10 min. Sections were then washed with distilled water, and dried at 50°C. Dried slides were cleared by immersion in chemical grade xylene (Thermo-Fisher Scientific, Waltham, MA, USA) for 1 min before being coverslipped in Permount mounting medium (Thermo-Fisher Scientific).

For immunostaining, slides were air dried at room temperature before being rinsed with PBS. Antigen retrieval was performed by incubating the slides in 10 mM sodium citrate buffer (pH = 6.0) and heated for 30 min in a rice cooker (Black & Decker, HS2000). Following antigen retrieval, sections were washed 3 times with PBS before being blocked in 5% (w/v) bovine serum albumin (Sigma-Aldrich) and 10% (v/v) normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS plus 0.03% (v/v) Triton X-100 (Thermo-Fisher Scientific) for 1.5 hour at room temperature. Sections were then incubated with primary antibodies in blocking buffer overnight at 4 °C. Primary antibodies used to assess neuropathology included mouse anti $p16^{INK4a}$ (1:500, MA5-17142, RRID AB_2538613, Thermo-Fisher Scientific), rabbit anti-IBA1 (1:1000, 019-19741, RRID AB_839504, Wako Laboratory Chemicals, Richmond, VA, USA), guinea pig anti-GFAP (1:500, 173 004, RRID AB_10641162, Synaptic Systems, Göttingen, Germany), rabbit anti-S100β (1:300, ab52642, RRID AB_882426, Abcam, Cambridge, UK), rabbit anti-NeuN (1:500, PA5-37407, RRID AB_2554049, Thermo Fisher Scientific), rabbit anti-CD31/PECAM-1 (1:250, NB100-2284, RRID AB_10002513, Novus Biologicals, Littleton, CO, USA), and anti-β-amyloid, 1-16 (1:1000, 803001, RRID AB_2564653, BioLegend, San Diego, CA, USA). The next day, sections were washed 3 times in PBS with 0.03% (v/v) Triton X-100 before incubation with secondary antibodies in blocking buffer for 1 h at room temperature. The following secondary antibodies were used: for anti-$p16^{INK4a}$, goat anti-mouse IgG1 Alexa Fluor

conjugated to 488 nm (1:500, A21121, RRID AB_2535764, Life Technologies, Carlsbad, CA, USA) or goat anti-mouse IgG1 conjugated to Alexa Fluor 568 nm (1:500, A21124, RRID AB_2535766, Life Technologies); for anti-IBA1, goat-anti rabbit IgG conjugated to Alexa Fluor 568 nm (1:1000, A11036, RRID AB_10563566, Life Technologies); for anti-GFAP, goat anti-guinea pig IgG conjugated to Alexa Fluor 647 nm (1:1000, A21450, RRID AB_2735091, Thermo-Fisher Scientific); for anti-NeuN, goat anti-rabbit IgG (H+L) conjugated to Alexa Fluor 488 (1:500, A11034, RRID AB_2576217, Life Technologies); for anti-CD31, goat anti-rabbit IgG (H+L) conjugated to Alexa Fluor 647 nm (1:1000, A21245, RRID AB_2535813, Life Technologies); and for anti-β-amyloid, 1-16, goat anti-mouse IgG1 conjugated to Alexa Fluor 568 nm (1:1000, A21124, RRID AB_2535766, Life Technologies). Negative controls in which sections were reacted with secondary antibodies only were run with each batch. All slides were mounted in ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, Waltham, MA, USA).

For dual immunohistochemistry and FJC labeling, sections were processed for immunohistochemistry first as described above, and then processed for FJC labeling with the following modifications: immersion in 0.015% $KMnO_4$ for 1 min followed by incubation in 0.0001% solution of FJC solution for 10 min. The sections were rinsed with distilled water and coverslipped in Permount mounting medium.

High content imaging and image analysis

Fluorescent images were acquired using the ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices, Sunnyvale, CA, USA). Images of p16 immunoreactivity in the following brain regions were acquired from two serial sections from each animal (n = 4 - 6 animals per group): amygdala, hippocampus (CA1, CA3, dentate gyrus), piriform and somatosensory cortex, and dorsolateral thalamus. For FJC labeling, images were acquired from two serial sections from each animal (n = 4 - 6 animals per group) in the hippocampus (CA1, CA3, dentate gyrus) and the dorsolateral thalamus. The final image of each

brain region for analysis was built by stitching multiple overlapping tiles that encompassed an entire brain region (MetaXpress Version 6.2.3.733). ImageJ imaging software (version 1.51n, NIH, USA) was applied to set the threshold for fluorescence intensity; cell size and circularity were used to determine the number of FJC-positive cells per unit area (mm^2). p16/NeuN immunoreactivity was assessed using a custom-designed module (MetaXpress Version 6.2.3.733) with respect to the percentage of NeuN-immunopositive cells (identified by DAPI staining) with colocalized p16 immunoreactivity (**Supplemental method**).

Statistics

FJC data were analyzed using two-way or mixed-effects ANOVA as performed by Prism 9. 1. 2 (GraphPad Software, La Jolla, CA, USA). For the histological analysis of neuronal expression of the senescence marker p16, mixed-effects models (including animal-specific random effects) were fit to assess differences between exposure groups. Primary factors of interest included exposure (DFP, VEH), region (CA3 and hilus of the hippocampus and the thalamus), and time post-exposure (7 days, 28 days, 3 months and 6 months). Interactions between the factors (exposure, region, and time point) were considered and the best model was chosen using Akaike Information Criterion. To better meet the assumptions of the model, the outcome was transformed using the natural logarithm after shifting all values by 1 to enable analyses of animals with no colocalization. Contrasts for group differences by time point and region were constructed and tested using a Wald test. Further contrasts were constructed between time points by region within each exposure group to assess the temporal differences in p16/NeuN colocalization. The Benjamini-Hochberg false discovery rate (FDR) was used to account for multiple comparisons across contrasts. Results are presented as geometric mean ratios (GMR) between exposure groups or between time points within the DFP group. Point estimates of the ratios and 95% confidence intervals are presented in the figures. When the confidence interval for the GMR includes 1, there is no statistical evidence of a difference

between groups; similarly, when the confidence interval for the differences includes 0, there is no statistical evidence of a difference between groups. All analyses were performed using SAS software, version 9.4 and alpha was set at 0.05; all reported significant results remained so after the FDR procedure. Correlations between the number of cells expressing p16 and FJC-positive degenerating neurons were analyzed by Spearman's correlation coefficient by Prism 9. 1. 2 (GraphPad Software).

Results

p16 immunoreactivity is detected in multiple brain regions at 3-months post-DFP intoxication

We determined whether there is evidence of cellular senescence in the brain following acute DFP intoxication. p16 is a well-established biomarker of cellular senescence that is associated with aging-related pathologies (Baker et al., 2011). In the brain regions examined (amygdala, hippocampus (including CA1, CA3, hilus), somatosensory and piriform cortex, and thalamus), significant expression of p16-immunopositive cells were found in the hilus and CA3 of the hippocampus and thalamus of the DFP animals at 3 months post-intoxication (**Figure 3-2**). There was negligible p16 expression in the CA1 of the hippocampus of DFP animals, the amygdala or cortical regions of DFP animals or in any brain region of VEH at 3 months-post-exposure.

Localization of p16 immunoreactivity to specific brain cell types in the acute DFP rat model

To identify cell types that expressed p16 in the DFP brain at 3 months post-exposure, brain sections were co-labeled for p16 and cell type-specific markers, including NeuN for neurons (Mullen et al., 1992), GFAP and S100β for astrocytes (Eng & Ghirnikar, 1994; Raponi et al., 2007), IBA-1 for microglia/monocytes (Ito et al., 1998), and CD31 for endothelial cells (Mantovani & Dejana, 1998). As shown in representative photomicrographs of the CA3 region of hippocampus of DFP-exposed animals at 3 months post-intoxication (**Figure 3-3**), p16

immunoreactivity colocalized with NeuN immunoreactivity but not with the other cell type-specific biomarkers, suggesting that acute DFP intoxication specifically induced cellular senescence in neurons. Similar results were observed in the hilus of the hippocampus and the thalamus (data not shown).

Temporal profile of p16 expression following acute DFP intoxication

We next examined the time course and persistence of cellular senescence in the brain following DFP-induced SE by comparing p16 expression of DFP-intoxicated animals versus VEH controls at 7 days, 28 days, 3 months and 6 months post-intoxication. As illustrated in representative photomicrographs of the dentate gyrus of the hippocampus (**Figure 3-4A**), relative to time- and region-matched samples from VEH controls, significant DFP-induced p16 immunoreactivity was first evident at 3 months post-exposure. Consistent with data shown in Figure 3-3, at this time point, p16 immunoreactivity co-localized with NeuN immunoreactivity. Significant neuronal senescence persisted in the hilus and CA3 of the hippocampus and thalamus of DFP animals at 6 months post-intoxication (**Figure 3-4B**). Quantification of neuronal senescence confirmed significantly increased neuronal expression of p16 in the hippocampus and thalamus of DFP animals when compared to VEH controls at 3 and 6 months post-exposure (**Figure 3-4C**; $p < 0.001$, GMR > 5.6). The difference between DFP and VEH animals in the percentage of NeuN immunopositive cells co-labeled for p16 varied by brain region and time point ($p = 0.007$), suggesting region-specifc temporal profile of neuronal senescence in DFP animals. There were no significant differences in colocalization between 3 months and 6 months in the three regions examined in VEH animals (CA3: GMR = 0.8, 95% CI = 0.3-2.3, $p = 0.7$; hilus: GMR = 0.4, 95% CI = 0.2-1.1, $p = 0.08$; thalamus: GMR = 1.4, 95% CI = 0.5-3.7, $p = 0.5$). Within the DFP group, the percent colocalization at 6 months in the CA3 region of the hippocampus was two-fold higher than the colocalization at 3 months (GMR = 2.1, 95% CI = 1.3-3.3, $p = 0.003$) while the percent colocalization at 6 months was lower than at 3

months in the hilus (GMR = 0.4, 95% CI = 0.2-0.7, p = 0.003) and thalamus (GMR = 0.6, 95% CI = 0.4-0.9, p = 0.01).

Overlap of neuronal senescence and neurodegeneration in the hippocampus and thalamus of DFP animal at 3 and 6 months post-intoxication

Our earlier studies characterized the spatiotemporal pattern of DFP-induced neurodegeneration in multiple brain regions, which can be observed as early as 4 h post-intoxication (Li et al., 2011) and remain evident till 6 months post-exposure in the brain (Supasai et al., 2020). To confirm these earlier observations in the current studies, we performed FJC staining on brain sections from DFP and VEH animals to visualize degenerating neurons (Schmued et al., 2005) in the hippocampus and thalamus at 3 and 6 months post-intoxication. Consistent with our previous report (Supasai et al., 2020), negligible FJC labeling was observed in brain sections of VEH animals, while in brain sections of DFP animals, FJC-labeled cells were present in the hippocampus and thalamus (**Figure 3-5A**) at both 3 and 6 months post-intoxication.

Quantitative analysis of the number of FJC-positive cells per unit area as a function of time post-exposure confirmed that acute DFP intoxication resulted in persistent neurodegeneration in the hippocampus and thalamus (**Figure 3-5B**). Two-way and mixed-effects ANOVA indicated a significant main effect of exposure with DFP increasing FJC labeling in the hilus of the dentate gyrus (3 months: p = 0.0065; 6 months: p = 0.0006), CA1 (3 months: p = 0.0065; 6 months: p = 0.0478), CA3 (3 months: 0.0187; 6 months: p = 0.0059) and thalamus (3 months: p = 0.0001; 6 months: p = 0.0238).

To determine whether the spatiotemporal profile of neurodegeneration coincided with that of neuronal senescence, brain sections were immunostained for p16 and then labeled with FJC. There was considerable overlap of p16 immunoreactivity with FJC labeling, as shown in the representative photomicrographs of the hilus and CA3 of the hippocampus of DFP animals

(**Figure 3-6A**). Pearson correlational analysis of all animals from the 3-month cohort demonstrated a positive correlation between the presence of FJC-positive cells and p16-immunopositive cells in the hilus (Pearson r = 0.8894, p = 0.0013), CA3 (Pearson r = 0.8599, p = 0.0014), and thalamus (r = 0.9546, p = 0.0002) (**Figure 3-6B**). At 6 months post-exposure (**Figure 3-6C**), a positive correlation was found for the CA3 (r = 0.7323, p = 0.0249) and thalamus (r = 0.9048, p = 0.0008), but not the hilus (p = 0.0734).

Acute DFP intoxication did not induce amyloid-β (Aβ) plaques

Since neuronal senescence has been associated with Alzheimer's disease in preclinical models (Musi et al., 2018) and chronic repeated exposure to OP pesticides that do not cause cholinergic crisis have been linked to increased risk of Alzheimer's disease in human (reviewed in Jokanović, 2018; reviewed in Sánchez-Santed et al., 2016), we measured Aβ immunoreactivity using the 6E10 antibody to determine whether acute DFP intoxication promoted AD-relevant Aβ proteinopathy. These analyses were performed at the late time points (3 and 6 months) and focused on the hippocampal subregions because these brain regions are involved in the pathophysiology of AD and are critical in learning and memory function (Morrone et al., 2020). We did not observe evidence of plaque formation in the hippocampus of DFP animals at either 3 or 6 months post-intoxication (**Figure 3-S1**).

Discussion

There is increasing discussion about the contribution of cellular senescence in the brain to the pathophysiology of brain aging and neurodegenerative diseases (reviewed in Baker & Petersen, 2018; reviewed in Kritsilis et al., 2018; reviewed in Wissler Gerdes et al., 2020). In various animals models of aging or neurodegenerative diseases, the clearance of senescent cells via pharmacological approaches significantly improves the performance of animals in behavioral tests (the novel object recognition, Stone T-maze, and water maze and Y-maze)

which task cognitive functions (Bussian et al., 2018; Ogrodnik et al., 2021; Zhang et al., 2019), providing proof-of-concept evidence that cellular senescence contributes to the functional deficits associated with aging- and disease-related cognitive impairment. Deficits in learning and memory are also a well-characterized delayed neurologic outcome in rats acutely intoxicated with DFP that manifests during the weeks to months following DFP exposure (Brewer et al., 2013; Flannery et al., 2016; Guignet et al., 2020; Rojas et al., 2016). However, cellular senescence has not been investigated in the context of acute OP intoxication.

To test the hypothesis that acute OP intoxication promotes cellular senescence, we quantified the expression of a well-characterized biomarkers of cellular senescence, p16, in the adult rat model of acute DFP intoxication. Our findings support this hypothesis. Specifically, we observed: (1) p16 immunoreactivity colocalized with a neuron-specific marker but not markers for astrocytes, microglia, or endothelial cells. (2) neuronal senescence in the brain of DFP animals is spatially heterogeneous, with significant p16 immunoreactivity detected in the CA3 and hilus of the hippocampus and in the thalamus, but not in the CA1 of the hippocampus, amygdala, or somatosensory and piriform cortices. (3) Unlike other well-documented neuropathologic consequences such as neuroinflammatory response, oxidative stress, and neurodegeneration which are significantly elevated during the first few days to weeks post-exposure (Flannery et al., 2016; Li et al., 2011; Liang et al., 2018; Rojas et al., 2015; Sisó et al., 2017), p16 expression was not observed during the first month of DFP intoxication. Significant p16 expression was evident at 3 months post-DFP and remained at high levels through 6 months post-intoxication.

Various cell types in the brain have been reported to express features of cellular senescence in brain aging and neurodegenerative diseases including neurons (Jurk et al., 2012; Moreno-Blas et al., 2019; Musi et al., 2018; Vazquez-Villaseñor et al., 2020), astrocytes (Bhat et al., 2012; Chinta et al., 2018; Vazquez-Villaseñor et al., 2020), microglia (Bussian et al., 2018), and oligodendrocyte progenitor cells (Zhang et al., 2019). Our observation of delayed neuronal

senescence in the brain of the DFP rat, adds to the nascent body of literature identifying senescent phenotypes in postmitotic cells, which calls into question the widely held view that cellular senescence is a property of proliferating cells (reviewed in Gorgoulis et al., 2019; reviewed in von Zglinicki et al., 2020).

It was first shown by Jurk et al. that DNA damage leads to a senescence-like state in mature postmitotic neurons *in vivo* via a p21-dependent mechanism (Jurk et al., 2012). Reports of altered expression of cellular senescence-related cell cycle markers in postmitotic neurons challenge the current dogma that neurons exit from the cell cycle once they are terminally differentiated (Barrio-Alonso et al., 2020; Copani et al., 2001; Moreno-Blas et al., 2019; Wani et al., 2017). It is worth noting that aberrant expression of cell cycle proteins in postmitotic cells oftentimes results in abnormal cell cycle reentry and subsequent cell death (reviewed in Xia et al., 2019). For example, differentiated PC12 cells exposed to the OP pesticide dichlorvos for 12 h exhibited increased expression of p53, cyclin-D1 and pRb, but decreased expression of p21(Wani et al., 2017). Further cell cycle analysis via flow cytometry confirmed that dichlorvos shifted the distribution of cells among the different phases of the cell cycle, with fewer in cells in the G0/G1 phase and more in the S and G2/M phases compared to controls. Dichlorvos-activated cell cycle machinery coincided with increased expression of pro-apoptotic proteins Bax and cytochrome c and decreased expression of anti-apoptotic protein Bcl-2, suggesting that dichlorvos caused neuronal cell damage potentially via triggering re-entry of differentiated PC12 cells into the cell cycle, which led to apoptotic cell death.

p16 is a cyclin-dependent kinase inhibitor that prevent cell cycle progression (Sherr & Roberts, 1999). Thus, it is possible that in the DFP rat, increased expression of p16 might be potentially beneficial initially in allowing stressed neurons to enter a senescent state to avoid cell cycle re-entry and the subsequent apoptosis. However, accumulation of senescent neurons in the brain may eventually results in neural network dysregulation and chronic inflammation. In support of this possibility, senescent neurons have been shown to adopt the senescence-

associated secretory phenotype (SASP) and become pro-inflammatory (reviewed in Chinta et al., 2015; Jurk et al., 2012; reviewed in Sah et al., 2021). While we did not measure the SASP in this study, we propose the testable hypothesis that senescent neurons in the DFP brain contribute to persistent DFP-induced neuroinflammation (Supasai et al., 2020) by secreting SASP factors, such as pro-inflammatory cytokines and exosomes, to induce senescence in neighboring neurons in a paracrine fashion as previously demonstrated in other models (Nelson et al., 2012).

In this study, we observed a positive correlation between senescent and degenerating neurons in the hippocampus and thalamus, as indicated by p16 immunoreactivity and FJC labeling. This raises questions as to whether cellular senescence drives neurodegeneration or, conversely, whether neurodegeneration promotes neuronal senescence. However, our observation that not all brain regions previously reported to exhibit significant FJC labeling at 3 and 6 months post-DFP intoxication (Supasai et al., 2020) exhibited p16 immunoreactivity suggests that neuronal senescence and neurodegeneration are likely independent but parallel processes that occur months after acute DFP intoxication.

A key question raised by our study concerns the mechanism(s) by which acute DFP intoxication causes delayed neuronal senescence. Previous studies have demonstrated that intoxication with a single seizurogenic dose of DFP triggers a robust neuroinflammatory response (Flannery et al., 2016; Guignet et al., 2020; Liu et al., 2012; Rojas et al., 2015; Sisó et al., 2017), and upregulates multiple biomarkers of oxidative stress (Guignet et al., 2020; Liang et al., 2018; Putra et al., 2020a; Putra et al., 2020b; Zaja-Milatovic et al., 2009) within hours that persist for weeks post-exposure. Cellular senescence can be induced by neuroinflammation and oxidative stress (reviewed in Baker & Petersen, 2018; reviewed in Gorgoulis et al., 2019; reviewed in Martínez-Zamudio et al., 2017; reviewed in Walton & Andersen, 2019), suggesting potential mechanisms underlying neuronal senescence in the brain of DFP rats. Further studies will be required to assess causal relationships between neuroinflammation, oxidative stress, and

neuronal senescence following acute OP intoxication and assess their contributions to adverse neurological outcomes.

While we observed delayed but persistent neuronal senescence in brain regions that are critically involved in cognitive behavior after acute DFP intoxication, there are several limitations of this study that need to be addressed to strength this interpretation. First, we need to corroborate these findings based on analysis of only one biomarker of cellular senescence by characterizing expression of additional biomarkers such as p21, lamin B1, and SASP. Second, in order to develop testable hypotheses regarding the functional significance of neuronal senescence to the chronic neurotoxic effects of acute OP intoxication, it would be of value to determine which neuronal cell type(s) are expressing p16 and whether treatments targeting cellular senescence mitigates not only neuronal senescence but also cognitive impairment. Third, further studies are warranted to determine whether cellular senescence is a phenotype generalized across OPs.

In summary, we present novel data demonstrating that acute DFP intoxication caused delayed cellular senescence, specifically in neurons. Spatiotemporal characterization of neuronal p16 expression indicated that senescence developed between 1 and 3 months post-DFP exposure and was limited to the hippocampus and thalamus. In addition, we confirmed that acute DFP intoxication induces persistent neurodegeneration as previously reported (Supasai et al., 2020). While there was a positive correlation between neurodegeneration and neuronal senescence in the hippocampus and thalamus, it is not clear whether there is a causal relationship between these two pathogenic outcomes because neurodegeneration was observed in brain regions that exhibited negligible p16 immunoreactivity up to 6 months post-exposure. These findings implicate cellular senescence as a potential pathogenic mechanism linking acute OP intoxication to chronic neurotoxic outcomes and as a potential therapeutic target for protecting brain functions in individuals acutely intoxicated with OPs.

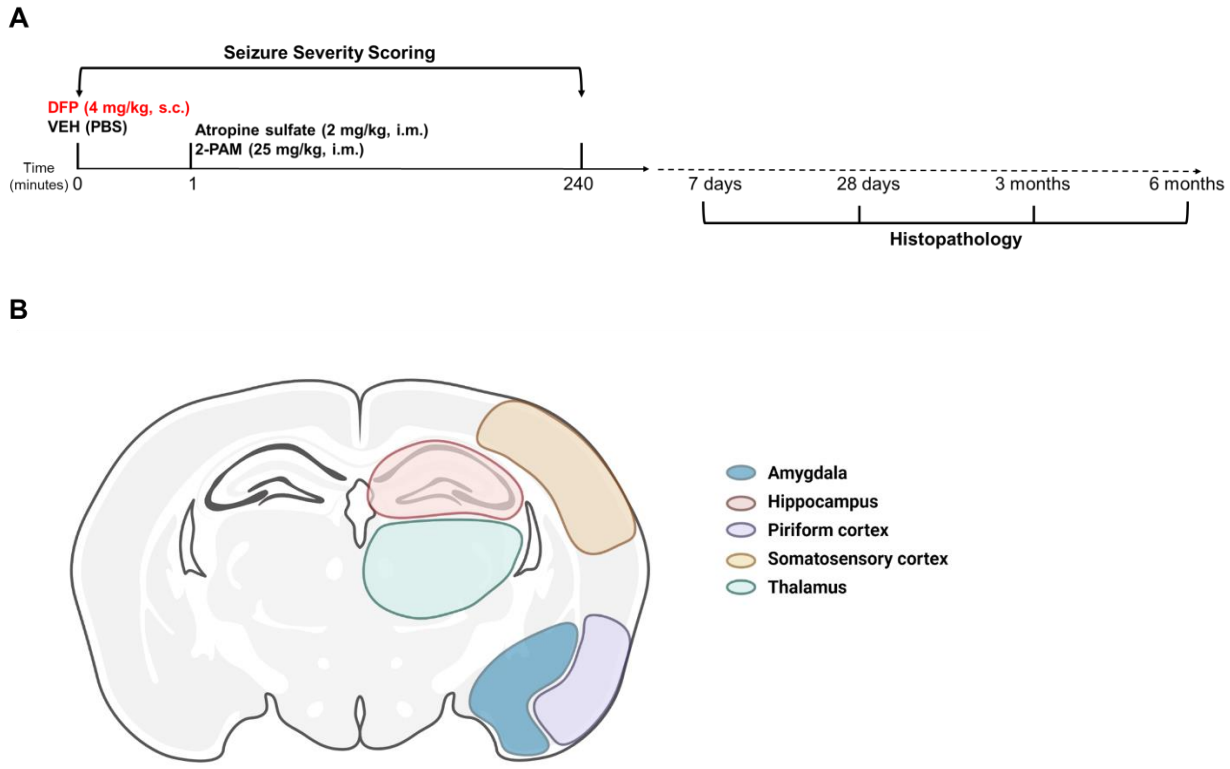


Figure 3-1. Schematic illustrating the experimental design for DFP exposure. ***(A)*** Adult male Sprague-Dawley rats were treated with DFP (4 mg/kg, s.c.) or equivalent volume of vehicle (300 µl of PBS) at time 0. Both groups were administered atropine sulfate (2 mg/kg, i. m.) and 2-PAM (25 mg/kg, i. m.) 1 min later. Animals were euthanized at varying time points (7 and 28 days, and 3 and 6 months post-DFP intoxication) to collect tissues for immunohistochemistry. ***(B)*** Illustration of the brain regions of interest examined for neuropathology following acute DFP intoxication. Created with BioRender.com.

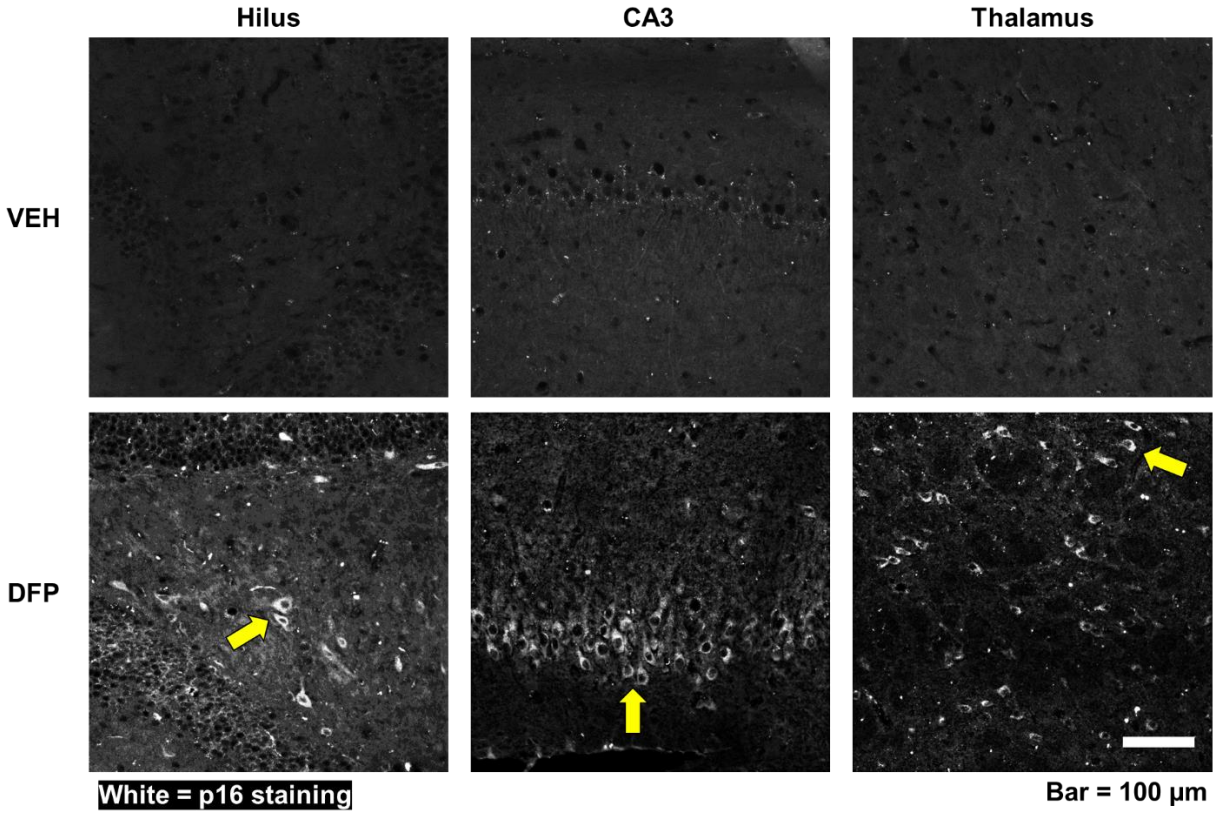


Figure 3-2. p16 immunoreactivity is detected in the rat brain at 3 months post-DFP intoxication. Representative photomicrographs showing p16-immunopositive cells (illustrated by the yellow arrows) in the hilus of the dentate gyrus, CA3 of the hippocampus, and thalamus at 3 months post-intoxication. Scale bar = 100 µm.

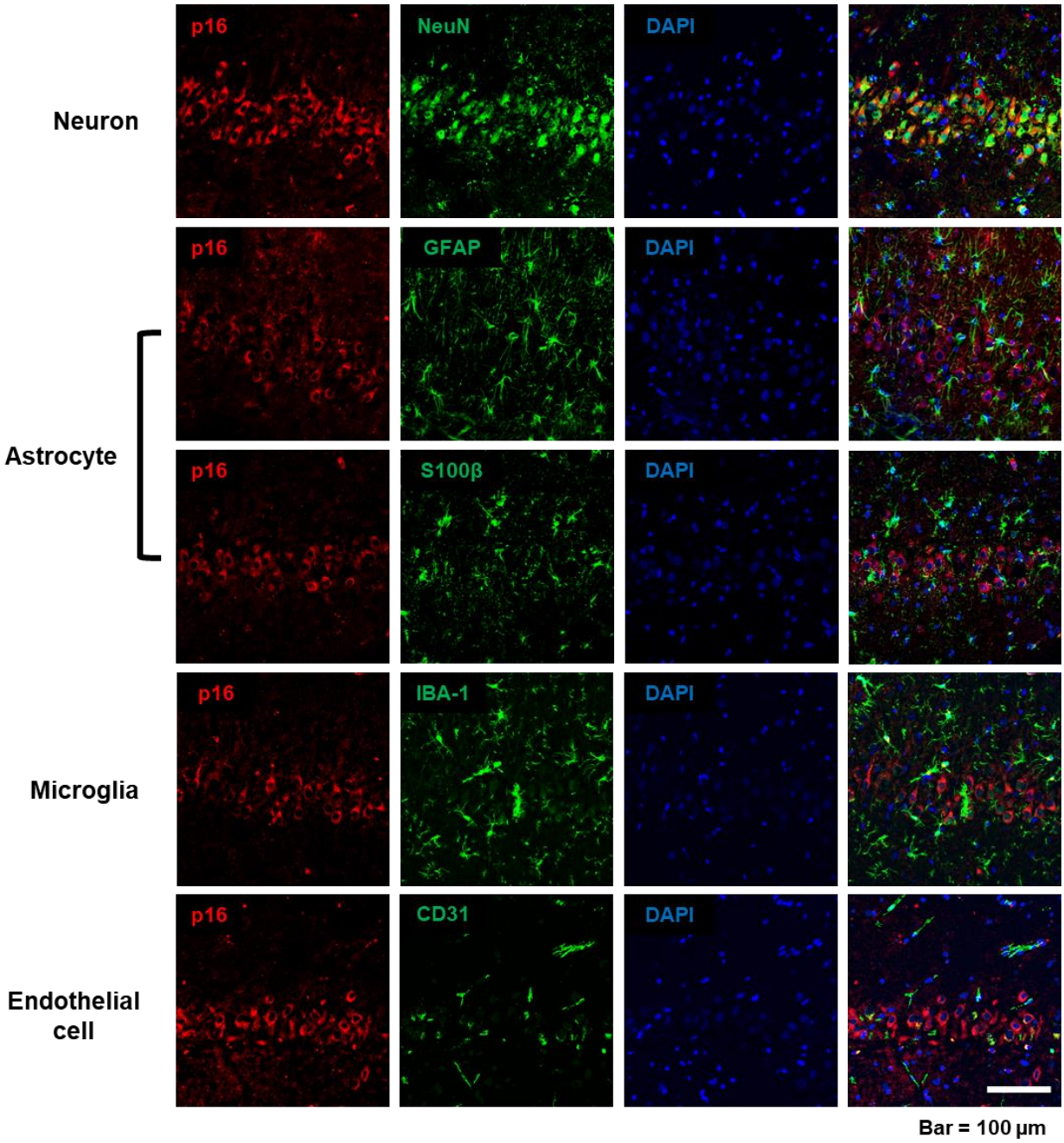
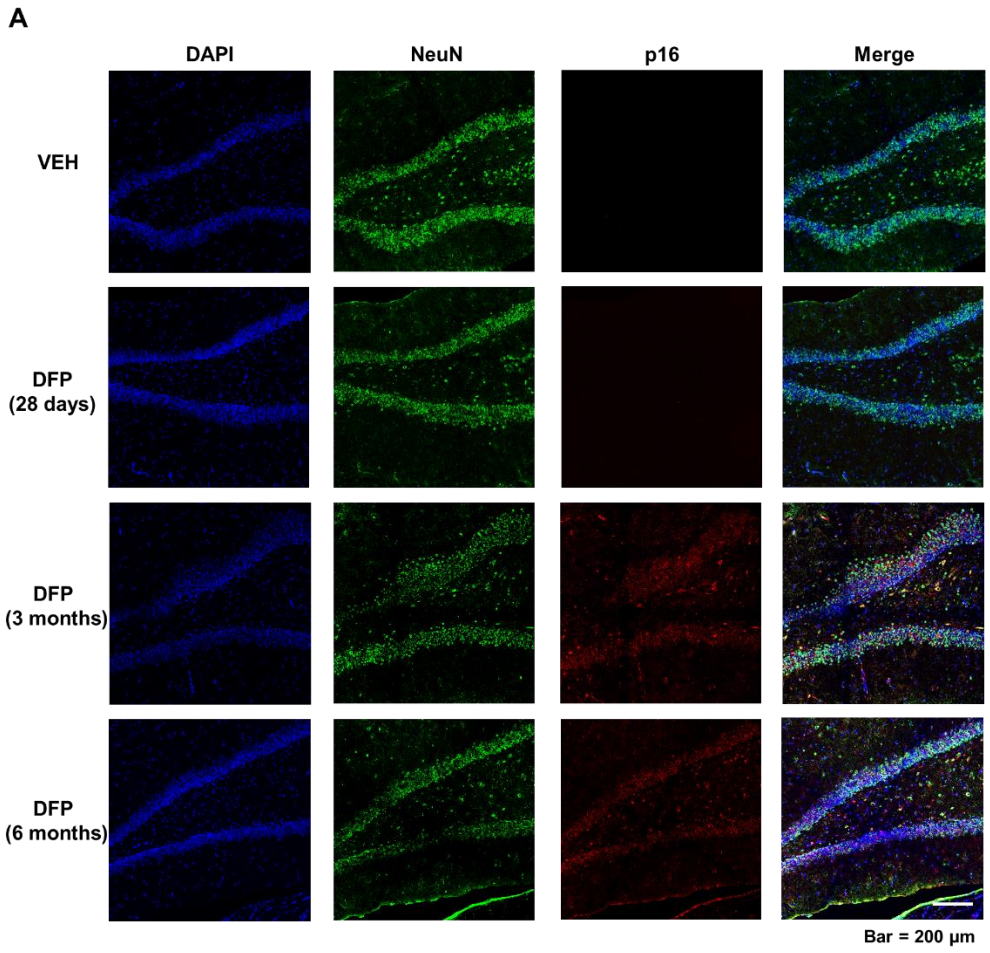
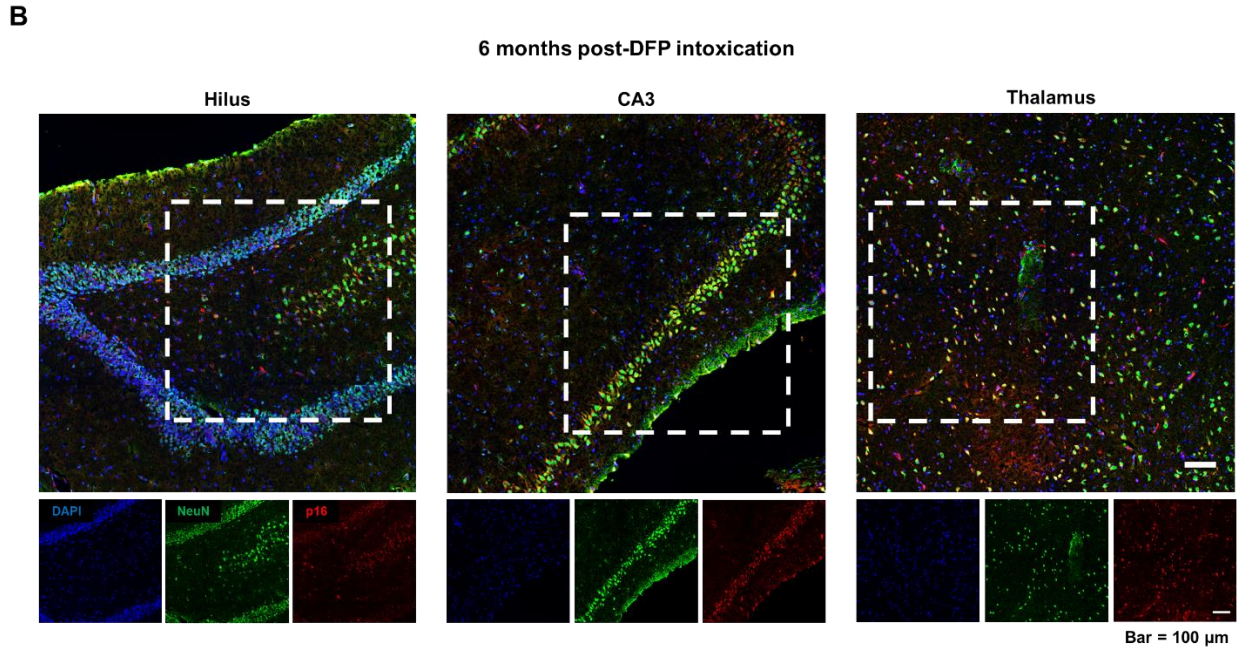


Figure 3-3. Cell specificity of p16 immunoreactivity in the acute DFP rat model.

Representative photomicrographs of the CA3 of the hippocampus at 3 months post-DFP intoxication immunostained for biomarkers of senescence, p16 (red); neurons NeuN (green); astrocytes GFAP or S100β (green); microglia IBA-1 (green); or endothelium CD31 (green). Sections were counterstained with DAPI to identify cell nuclei. Scale bar = 100µm.


A
DAPI
NeuN
p16
Merge
VEH
DFP
(28 days)
DFP
(3 months)
DFP
(6 months)
Bar = 200 µm


B
6 months post-DFP intoxication
Hilus
CA3
Thalamus
DAPI
NeuN
p16
Bar = 100 µm

C

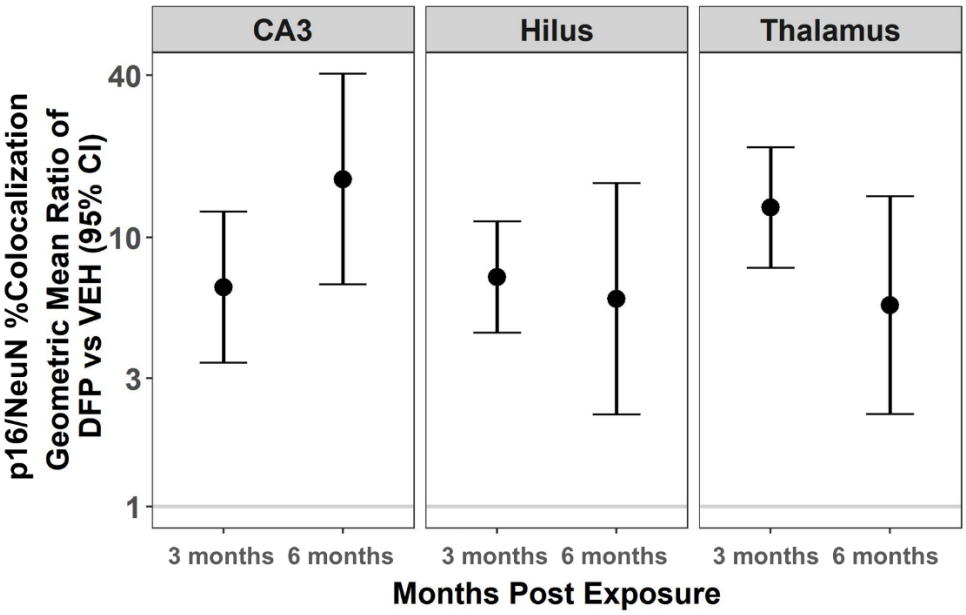


Figure 3-4. Temporal profile of p16 expression following acute DFP intoxication. ***(A)*** Representative photomicrographs of p16 (red) and NeuN (green) dual immunostaining of the dentate gyrus of the hippocampus at 28 days, 3, and 6 months post-DFP intoxication. Scale bar = 200 µm. ***(B)*** Neuronal senescence persists through 6 months post-DFP intoxication in the hippocampus and in the thalamus. Scale bar = 100 µm ***(C)*** Dots represent point estimates of the geometric mean ratios (GMRs) of % NeuN immunopositive cells co-labeled with p16 in DFP vs. VEH; bars represent the 95% confidence intervals. The difference in neuronal p16 expression between DFP and VEH animals varied by brain region and time post-exposure ($p = 0.007$). When the confidence interval includes 1, there is no statistical evidence of a significant difference between the two groups. All significant results remained significant after correction for false discovery (FDR).

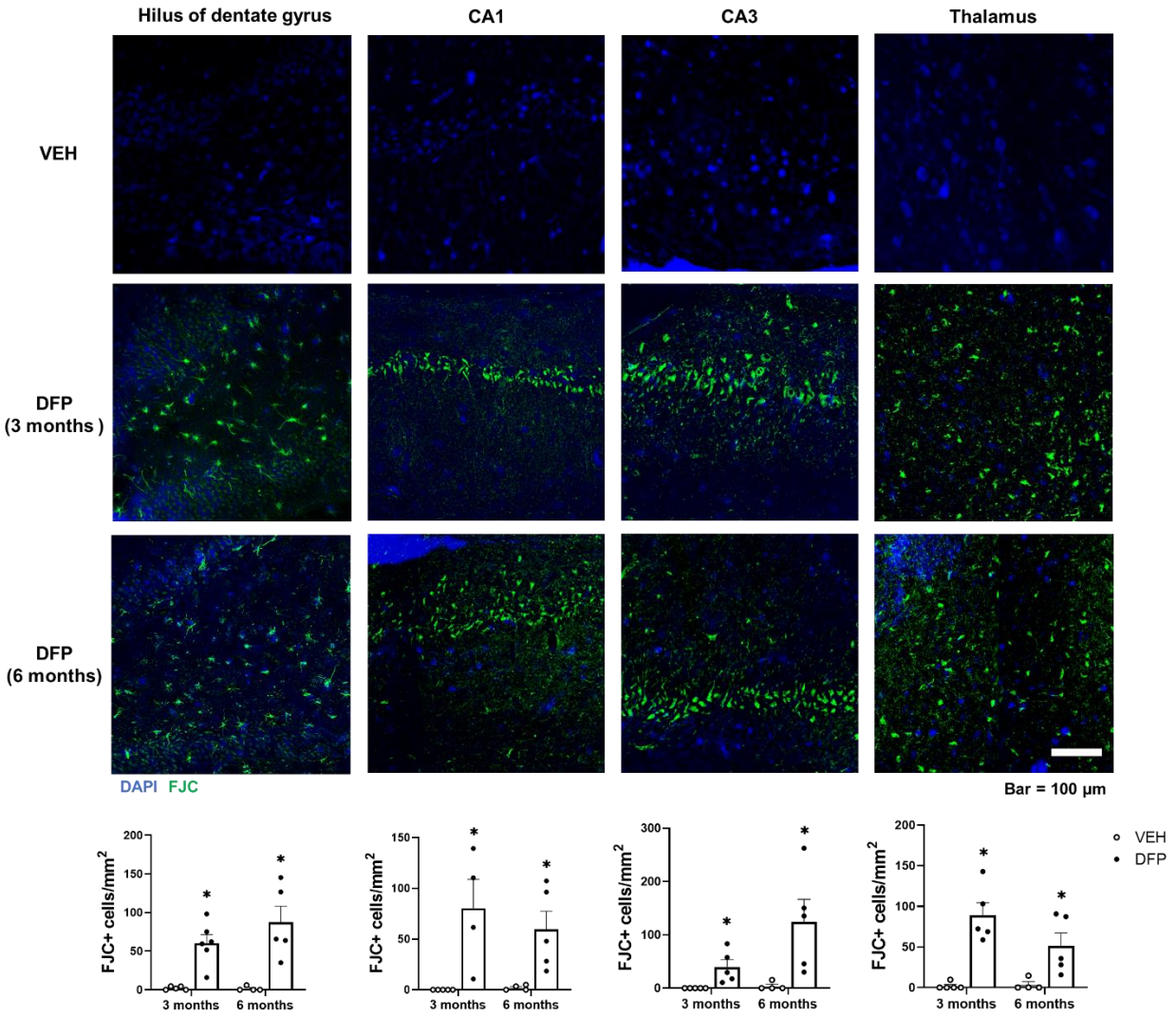
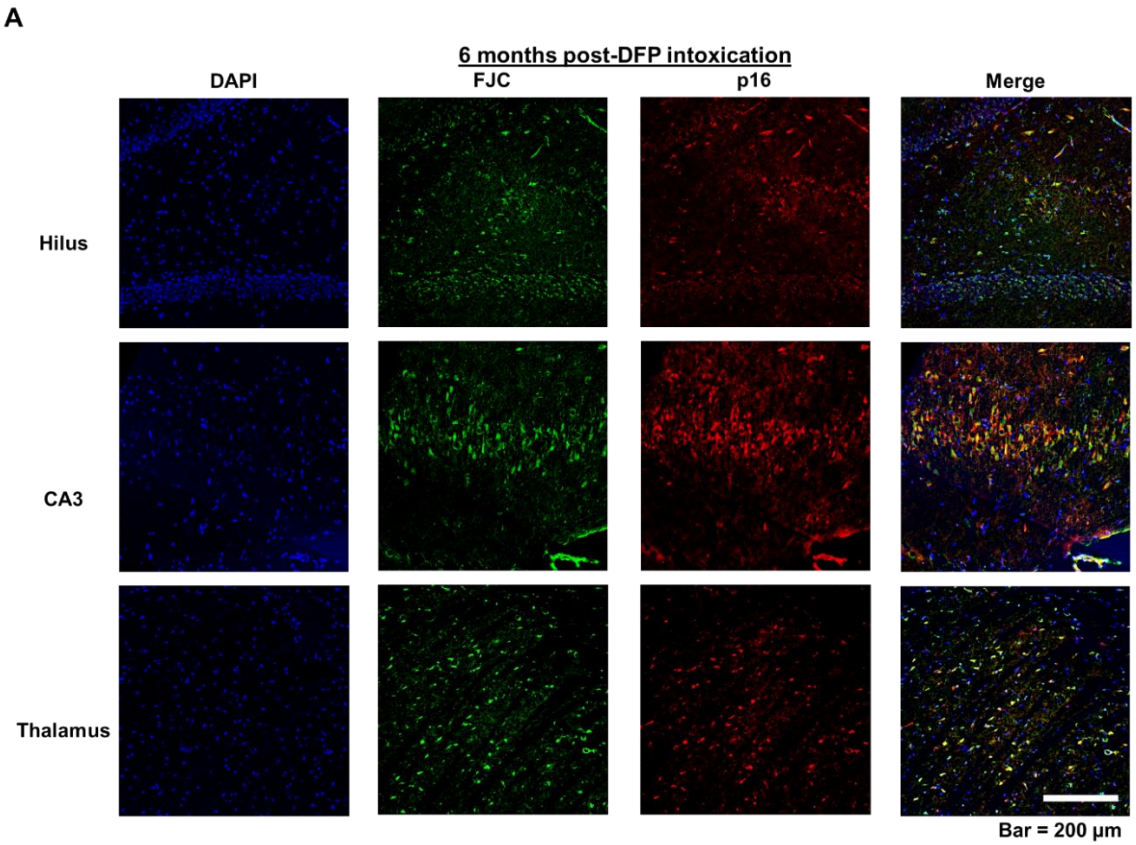


Figure 3-5. DFP caused neurodegeneration that persisted at 3 and 6 months post-exposure. ***(A)*** Representative photomicrographs of FJC labeling (green) in the hilar dentate gyrus, CA1 and CA3 region of the hippocampus, and thalamus of rats at 3 and 6 months following exposure to vehicle (VEH) or DFP. All sections were counterstained with DAPI (blue) to identify cell nuclei. Scale bar = 100 µm. Quantification of FJC-labeled cells per unit area. Data presented as mean + SE (N = 4 - 6 animals / group). * Significantly different from vehicle at $p \leq 0.05$ as determined by two-way or mixed-effects ANOVA with Bonferroni's multiple comparisons test.


A
6 months post-DFP intoxication
DAPI
FJC
p16
Merge
Hilus
CA3
Thalamus
Bar = 200 µm

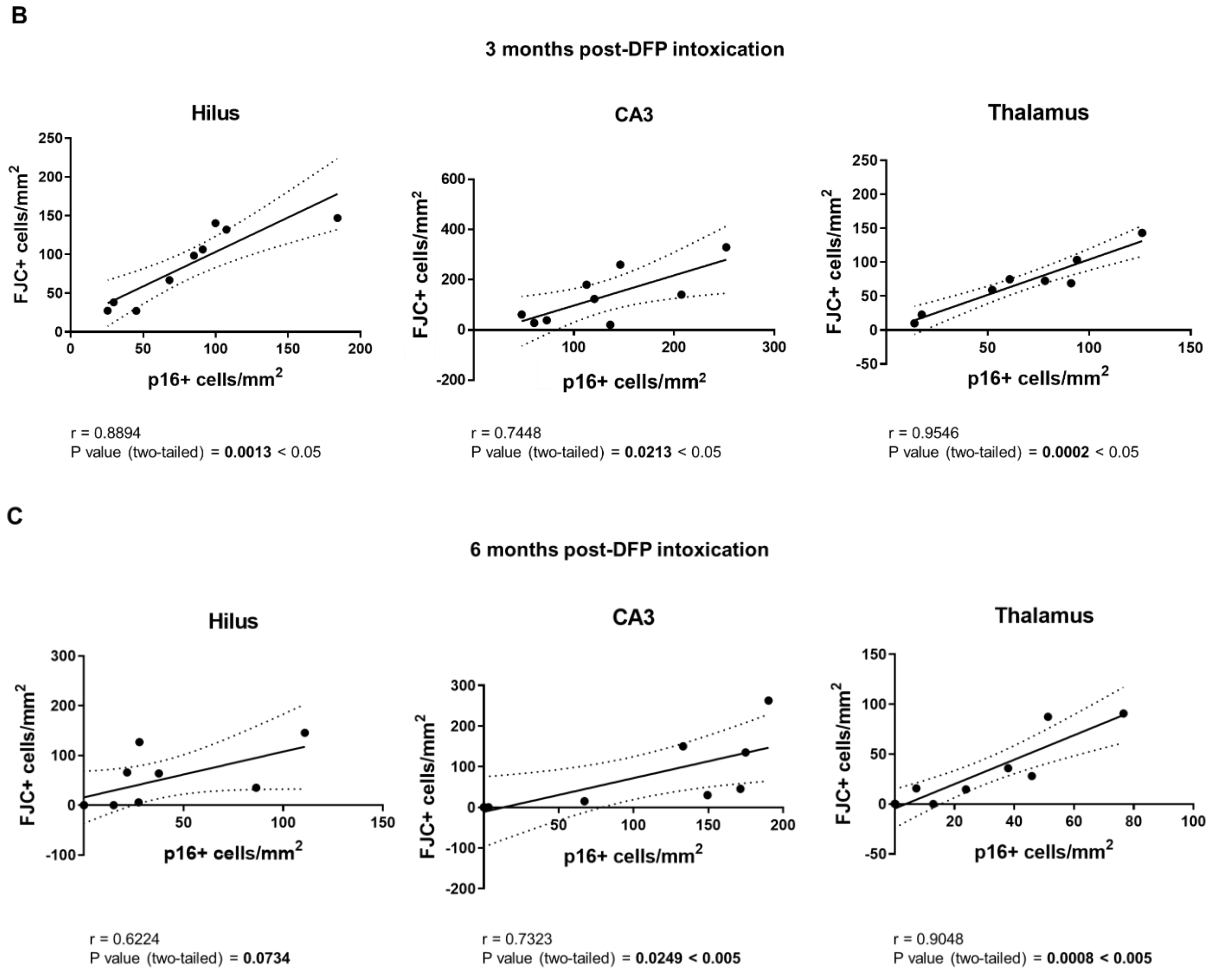


Figure 3-6. Neuronal senescence and neurodegeneration in the hippocampus of DFP-intoxicated animals are positively correlated. ***(A)*** Representative photomicrographs of p16 immunoreactivity (red) and FJC staining (green) in the rat hippocampus and thalamus at 6 months post-DFP intoxication. Sections were counterstained with DAPI (blue) to identify cell nuclei. Scale bar = 200 µm. ***(B)*** Pearson correlation of p16-immunopositive versus FJC-positive cells in the hilus, the CA3 of the hippocampus, and the thalamus at 3 months post-DFP intoxication. ***(C)*** Pearson correlation of p16-immunopositive versus FJC-positive cells in the hilus, the CA3 of the hippocampus, and the thalamus at 6 months post-DFP intoxication.

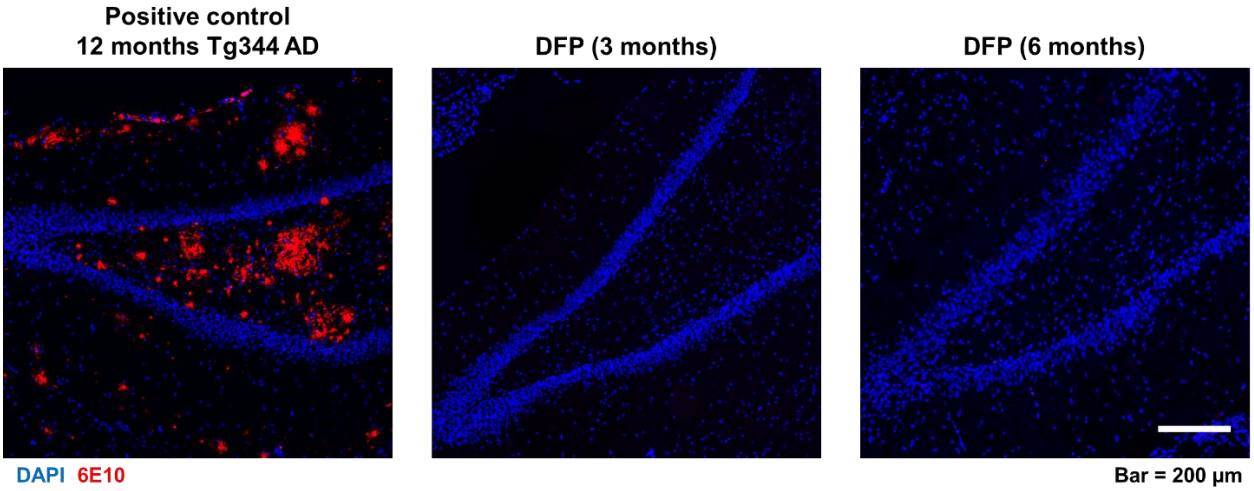
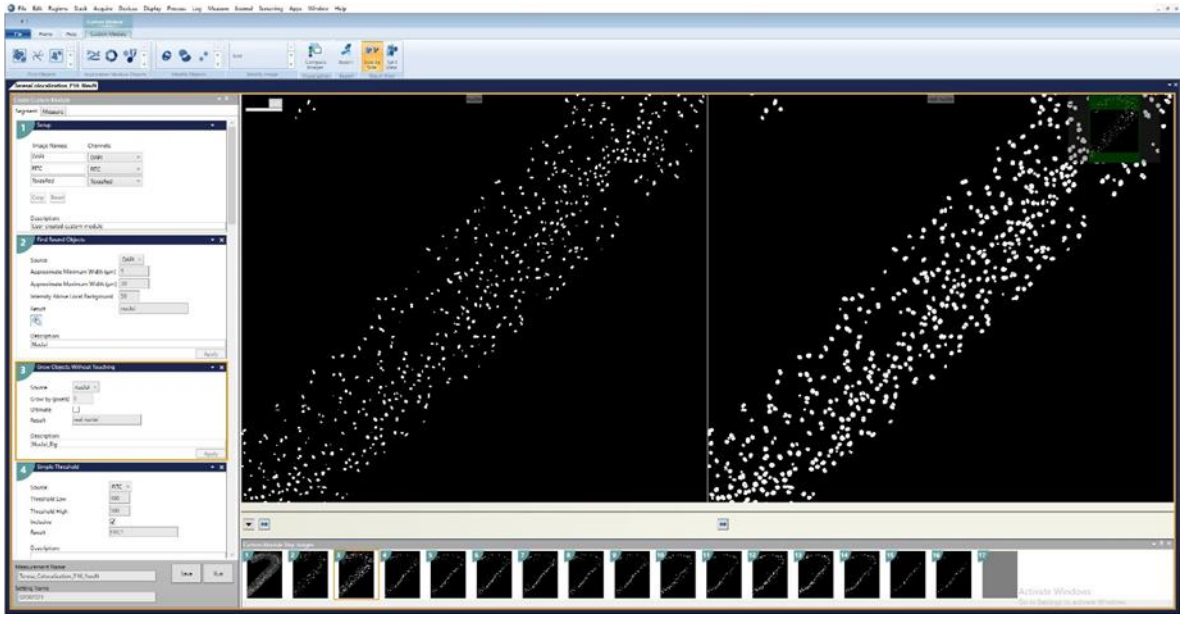
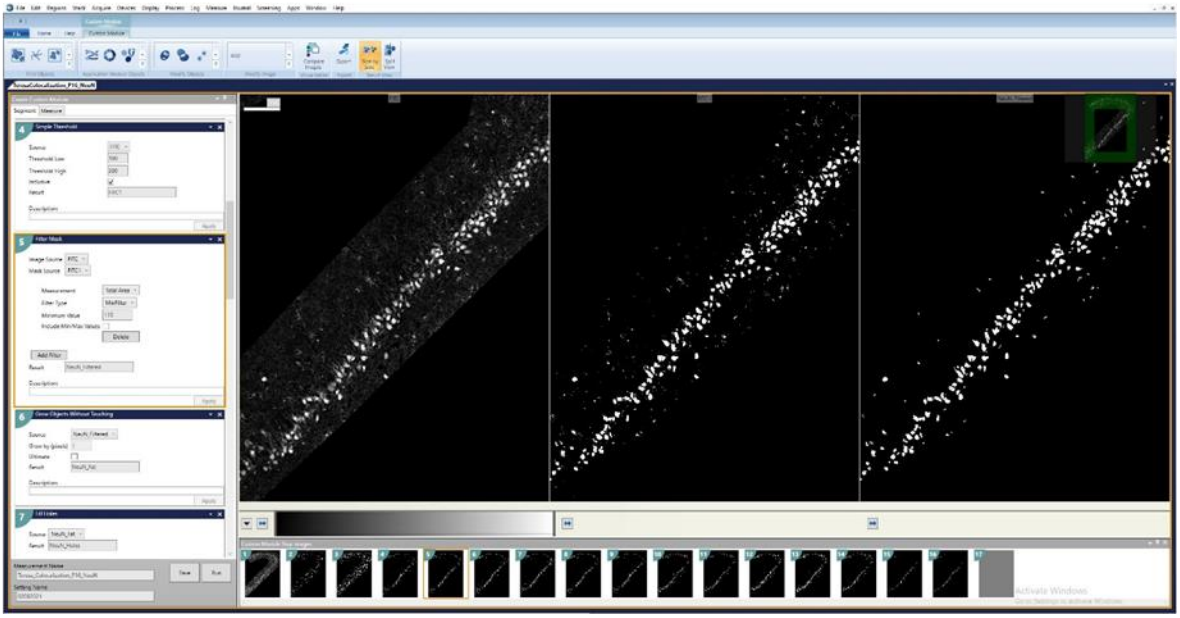


Figure 3-S1. Absence of Alzheimer's disease (AD) phenotype as determined by immunostaining with Ab 6E10 to identify Aβ plaques. Representative photomicrographs of Aβ plaques indicated by 6E10 immunolabeling reveals no evidence of Aβ proteinopathy in the hippocampus of DFP rats. Brain sections from a 12-month-old Tg344 AD were immunostained with 6E10 as a positive control.

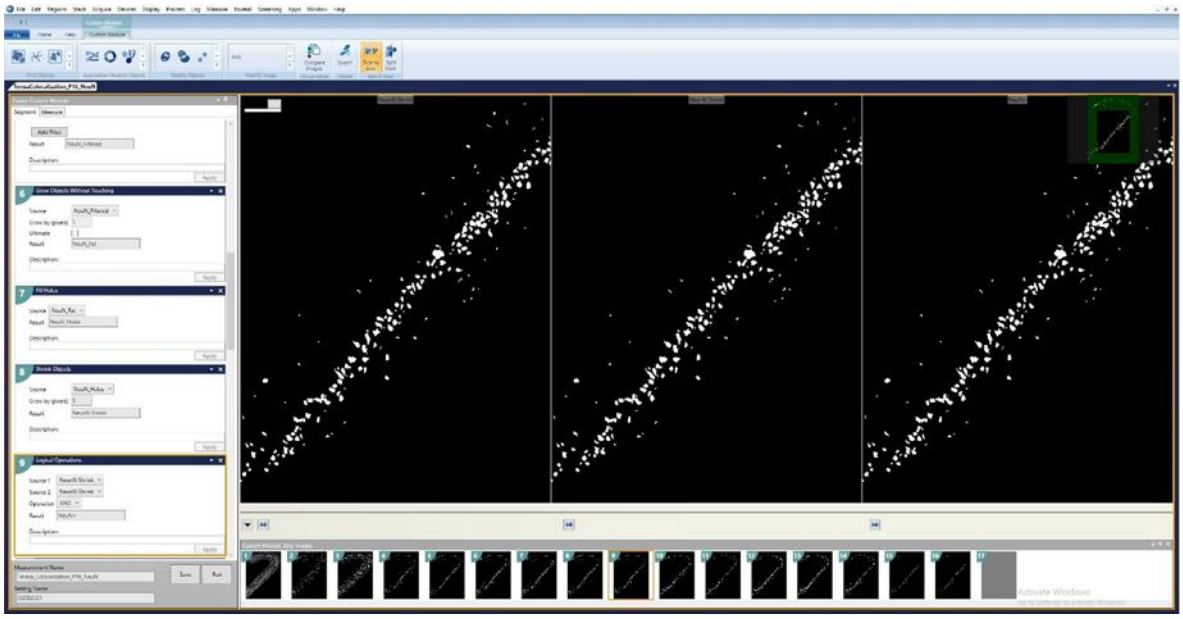
Step 1 - 3



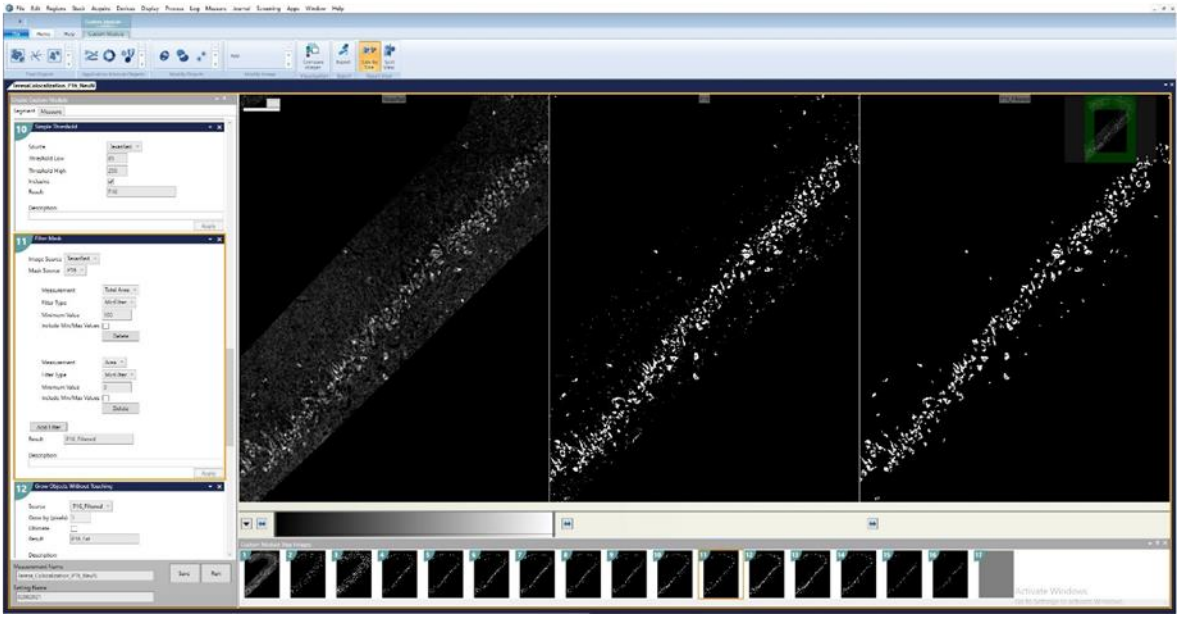
Step 4 - 6



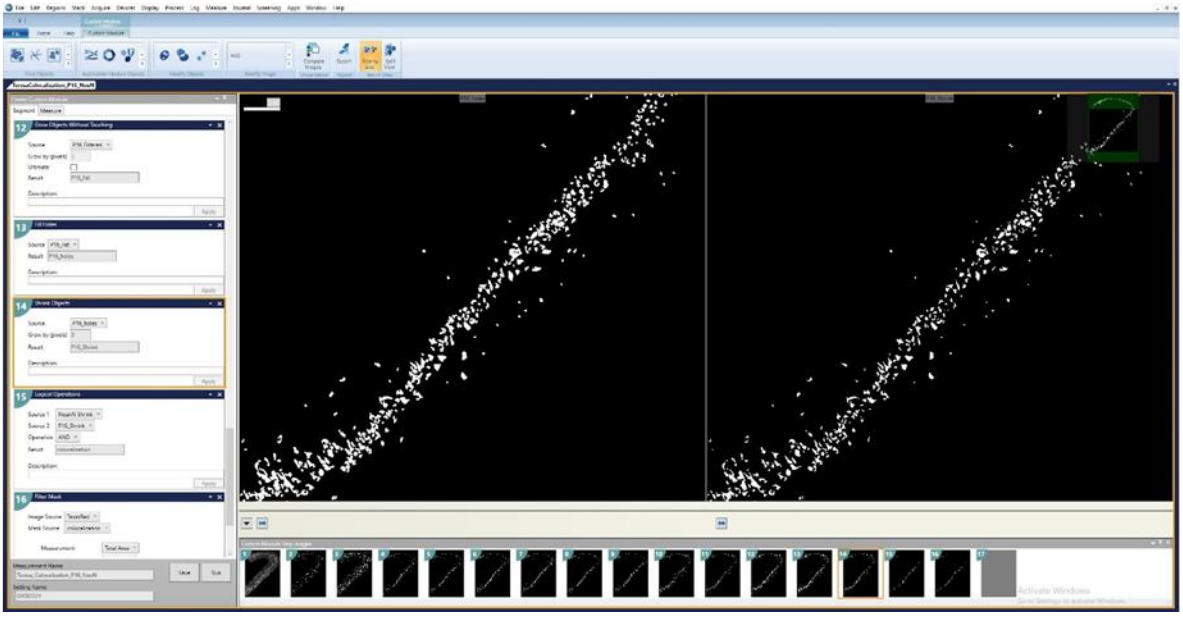
Step 7 - 9



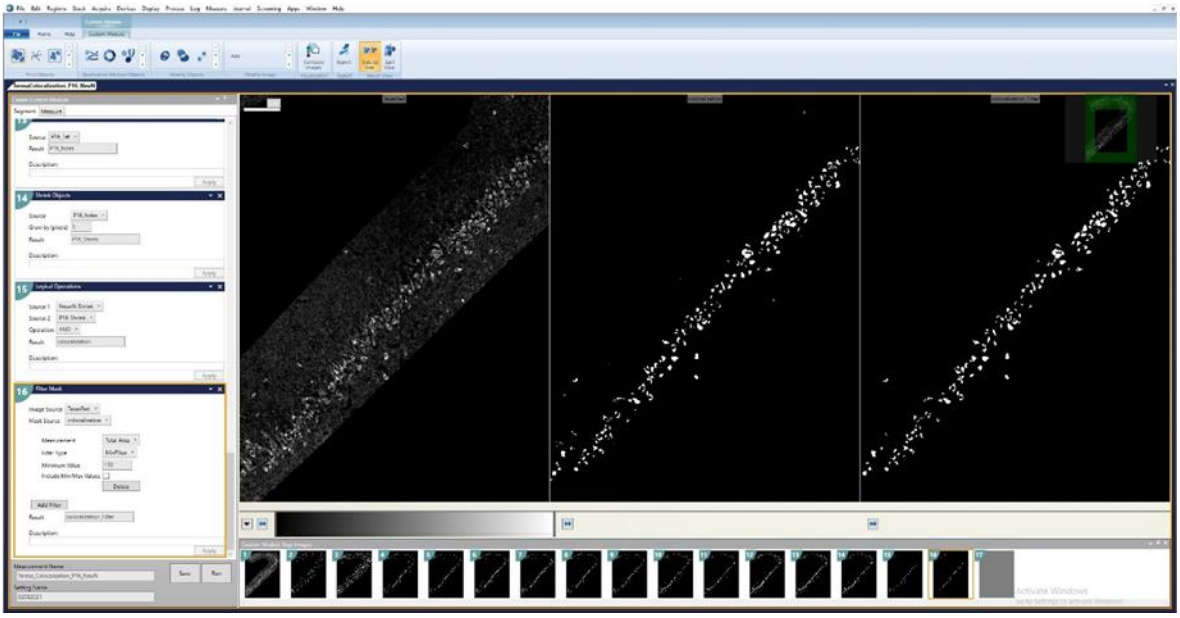
Step 10 - 11



Step 12 - 14



Step 15 - 16



Supplemental method. Steps used to measure p16/NeuN colocalzation using Custom Module Editor from Molecular Devices. Below are the steps included for building the analysis: 1) Setup with DAPI, FITC, and TexasRed wavelengths; 2) Find Round Objects: Identify nuclei on the DAPI image; 3) Grow Objects Without Touching: Enlarge the nuclei by 8 pixels; 4)

Simple Threshold for FITC (set low at 100 and high at 300): Enable and adjust the threshold of the image manually to help segment the features from the background on the FITC image; 5) Filter Mask: Remove objects from the mask based on measurement values of the objects by using Total Area and MinFilter value at 115; 6) Grow Objects Without Touching: Enlarge the identified objects on the FITC image by 3 pixels (“NeuN_Fat”); 7) Fill Holes: Fills holes in objects identified by Step 6, which allows the filled area to be included in measurement data (“NeuN_Holes”); 8) Shrink Objects: Shrink objects from “NeuN_Holes” by 3 pixels (“NeuN_Shrink”); 9) Logical Operations: Have the results for NeuN+ objects; 10) Simple Threshold for TexasRed (set low at 85 and high at 250): Enable and adjust the threshold of the image manually to help segment the features from the background on the TexasRed image (“P16”); 11) Filter Mask: Remove objects from the mask based on measurement values of the objects by using Total Area and MinFilter value at 100; 12) Grow Objects Without Touching: Enlarge the identified objects on the Texas Red image by 3 pixels (“P16_Fat”); 13) Fill Holes: Fills holes in objects identified by Step 12, which allows the filled area to be included in measurement data (“P16_holes”); 14) Shrink Objects: Shrink objects from “P16_holes” by 3 pixels (“P16_Shrink”); 15) Logical Operation: Have the colocalization results for NeuN+ objects with P16 objects; 16) Filter Mask: Remove objects from the mask based on the measurement from Step 15 by using Total Area and MinFilter value at 150 (“colocalization_Filter”)

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Chapter 4

Conclusion

Organophosphates (OPs) were first synthesized for their insecticidal properties, but they were later developed as lethal warfare agents during World War II (Costa, 2018). OP pesticides are readily accessible (Nicolopoulou-Stamati et al., 2016; Trasande, 2017) and OP poisoning as a consequence of accidental or intentional exposures constitutes a serious threat to global public health (Haley, 2018; OPCW, 2017; UN, 2017). Multiple epidemiology studies have reported acute and chronic OP exposures are associated with deficits in neurobehavioral performance (Anger et al., 2020; Farahat et al., 2003; Figueiredo et al., 2018; Loh et al., 2010; Miyaki et al., 2005; Nishiwaki et al., 2001; reviewed in Rohlman et al., 2011; Roldán-Tapia et al., 2005). However, finding a universal antidote that is effective against all OP chemicals has proven difficult due to the diversity of OPs in respect to their physicochemical properties and reactivity, and the varying degrees in the severity of neurotoxic outcomes depending on the type and duration of exposure. Furthermore, a number of neurotoxic mechanisms beyond acetylcholinesterase (AChE) inhibition have been implicated in the context of acute and chronic OP exposures (reviewed in Naughton & Terry, 2018; Tsai & Lein, 2021; reviewed in Voorhees et al., 2017). Therefore, a thorough understanding of the relationship between the neurologic consequences following OP exposures and the underlying neurotoxic mechanisms are necessary in order to better treat or protect affected individuals.

Research has taken advantage of preclinical animal models that recapitulate human responses to OP intoxication to investigate pathogenic mechanisms underlying both the acute and chronic toxicity of OPs. From the findings reported in this dissertation, several overarching conclusions may be drawn: 1) different OPs and exposure paradigms cause different neurotoxic effects; therefore, robust characterization of the mechanism(s) and neurologic outcomes in preclinical animal models of OP intoxication are needed to identify therapeutic targets and strategies; 2) oxidative stress is involved in the cognitive effects caused by repeated exposures to chlorpyrifos (CPF) at doses that do not cause cholinergic crisis, as exemplified by our finding that treatment with the antioxidant Trolox significantly alleviates CPF-induced oxidative stress

and astrogliosis across multiple brain regions coincident with preventing cognitive deficits in CPF-exposed animals; and 3) cellular senescence may be a novel mechanism contributing to the chronic neurological consequences observed following acute diisopropylfluorophosphate (DFP) intoxication. Collectively, the observations from both data chapters extend a growing literature (reviewed in Costa, 2018; reviewed in Naughton & Terry, 2018; Tsai & Lein, 2021) implicating non-cholinergic mechanisms (i.e. oxidative stress and cellular senescence) in mediating the neurotoxic effects of OPs.

Oxidative stress as a molecular mechanism underlying cognitive deficits associated with occupational CPF intoxication

Chapter 2 of this dissertation tested the hypothesis that oxidative stress mediated the cognitive deficits seen in humans repeatedly exposed to OPs in an occupational setting. Repeated exposures to OPs at doses that do not elicit cholinergic crisis have been linked to impaired neurobehavioral performances and neuropsychiatric conditions in humans, and observation that has been corroborated in preclinical animal models (reviewed in Ross et al., 2013; reviewed in Voorhees et al., 2017). However, the paucity of evidence linking behavioral changes to biomarkers of OP exposures, such as OP metabolites or reduced acetylcholinesterase (AChE) levels, and the failure of most human studies to identify a dose-response relationship have caused some to question whether occupational OP exposures cause neurotoxicity (reviewed in Meyer-Baron et al., 2015; reviewed in Rohlman et al., 2011). A dose-related effect of occupational OP exposure on human neurobehavior was recently demonstrated in an assessment of pesticide application teams in Egypt who were primarily exposed to a single OP, CPF (Anger et al., 2020). Importantly, blood cholinesterase activity was not found to link to performance on the Trail Making Test, which assesses cognitive function. This is in accordance with previous epidemiologic studies that found a link between occupational OP exposures and neurotoxic effects, but not between blood cholinesterase

activity and neurobehavioral outcomes (Meyer-Baron et al., 2015; reviewed in Rohlman et al., 2011). These findings suggest that non-cholinergic pathways may play a significant role in the neurotoxicity of repeated lower-level OP exposures, which has been supported by a considerable body of preclinical evidence (reviewed in Silva, 2020; reviewed in Terry, 2012; reviewed in Voorhees et al., 2017). In order to investigate the cause-effect relationships between repeated OP exposures and neurobehavioral outcomes, we took advantage of a recently characterized adult rat model of human occupational exposures to CPF (Lee et al., 2016; Voorhees et al., 2019).

The data presented in chapter 2 is among the first to provide a cause-effect relationship between oxidative stress and cognitive deficits. Chapter 2 adds to the knowledge base of occupational OP exposure by demonstrating that rats exposed to CPF repeatedly for 21 days manifest cognitive impairments (as examined by the context and cue fear conditioning tests), delayed but persistent AChE inhibition, and upregulated expression of biomarkers of oxidative stress and neuroinflammation (varying oxylipins in the plasma and whole brain, hippocampal prostaglandin E_2 (PGE2) levels, neuronal 3-nitrotyrosine (3-NT) expression, and astrogliosis). The activation of oxidative stress pathways has been implicated as a molecular mechanism that contributes to neurotoxicity caused by chronic OP exposures (Fereidouni et al., 2019; Hussein et al., 2018; Ki et al., 2013; Mahmoud et al., 2019), suggesting antioxidants as potential therapeutic candidates for mitigating CPF-induced oxidative stress and its subsequent adverse outcomes on cognitive function. To specifically target the role of oxidative stress following repeated CPF intoxication, daily treatments with a vitamin E-derivative antioxidant Trolox was used to scavenge free radicals and examined for its neuroprotection efficacy against the neurotoxic effects caused by CPF. We found that not only did Trolox treatment reduce indices of oxidative stress, but it also significantly improved cognitive deficits and alleviated astrogliosis. Of note, Trolox treatment had no effect on CPF-induced AChE inhibition, supporting the hypothesis that oxidative stress is mechanistically linked to neurobehavioral deficits caused by occupational

CPF exposures.

In addition to the identification of the causal role of oxidative stress in CPF-induced cognitive deficits following repeated exposures, increased astrogliosis was also found to coincide with elevated oxidative stress and was significantly alleviated by antioxidant Trolox treatment in CPF animals. Therefore, in follow-up research, it would be important to determine the mechanistic role of neuroinflammation, specifically astrogliosis, in the context of occupational CPF exposure. The downstream mechanism(s) that link oxidative stress/astrogliosis to cognitive impairment remain unknown. Interestingly, there is evidence that altered hippocampal synaptic structure/transmission (Speed et al., 2012) could potentially be associated with neurobehavioral deficits following repeated CPF exposures. Given the close relationship between redox balance, astrocytes, and synapses at the functional and structural levels (reviewed in Beckhauser et al., 2016; reviewed in Papouin et al., 2017; reviewed in Santello et al., 2019), it would be informative to further investigate the functional association between oxidative stress and/or astrocyte activation and synaptic connectivity in the animal model of occupational OP exposure.

Cellular senescence, an uncharacterized phenotype, as a potential mechanism contributing to neurotoxicity of acute DFP intoxication

While a positive correlation between seizure activity and neuropathology has been demonstrated in acute OP models (Hobson et al., 2017; McDonough & Shih, 1997; Rojas et al., 2021), it is important to note that seizure activity is not the sole determinant of the spatiotemporal profile and magnitude of the neuropathologic response to acute OP intoxication as it was observed that antiseizure efficacy did not necessarily correlate with neuroprotection in preclinical animal models of acutely intoxicated with OPs (González et al., 2020; Kuruba et al., 2018; Spampanato et al., 2020; Supasai et al., 2020). For example, studies have shown that delayed treatments with benzodiazepines fail to provide full protection of the brain against long-

term consequences, despite significant reduction of seizure activity (Kuruba et al., 2018; Spampanato et al., 2020; Supasai et al., 2020). Further, a recent study of a subpopulation of adult male Sprague Dawley rats that exhibit minimal to no seizure behavior when exposed to seizurogenic doses of DFP (referred to as “low responders”) were found to have the same extent of cholinesterase inhibition in the brain and comparable levels of neuropathology (neurodegeneration and mineralization) as normal DFP responders (González et al., 2020). These findings indicate the importance of understanding seizure-independent mechanisms of acute OP exposure and not solely focusing on terminating seizure activity when assessing therapeutic candidates.

A variety of mechanisms in addition to cholinergic pathways and seizures have been proposed to contribute to the delayed and persistent neurological damage observed following acute OP intoxication (reviewed in Guignet & Lein, 2019; reviewed in Naughton & Terry, 2018; Tsai & Lein, 2021). The neuropathological consequences of acute OP intoxication such as neuroinflammatory responses, oxidative stress, and neurodegeneration, persist up to 6 months post-exposure in rats (Guignet et al., 2020; Putra et al., 2020a; Supasai et al., 2020) and probably longer in humans. However, there are critical research questions that remain to be answered such as the causal roles of neuroinflammation and oxidative stress to chronic neurological sequelae, and the causal relationships between pathogenic mechanisms (such as neuroinflammation and oxidative stress) and neurodegeneration/neurobehavioral impairments (reviewed in Guignet & Lein, 2019; Tsai & Lein, 2021). The understanding regarding pathogenic mechanisms that link acute OP poisoning to chronic neurotoxic outcomes remains an active area of research so the research community could develop more effective therapeutic strategies for mitigating the adverse neurological sequelae associated with acute OP intoxication.

An adult rat model of acute intoxication with DFP was employed in chapter 3 to investigate whether acute OP intoxication causes cellular senescence in brain regions associated with the functional deficits observed following acute OP intoxication, specifically spontaneous recurrent

seizures and cognitive impairment. Via immunohistochemical analysis of one of the widely acknowledged senescence biomarkers, p16, DFP animals, but not vehicle controls, were found to have significant p16 expression in the hilus of the dentate gyrus and CA3 of the hippocampus, and the thalamus, but not other brain regions. Perhaps more important are the observations that neurons, but not astrocytes, microglia or endothelial cells, expressed p16 and neuronal p16 expression did not occur until 3 months post-DFP intoxication. Furthermore, senescent neurons were also found to be positively correlated with neurodegeneration at these late time points. However, the fact that not all brain regions previously reported to have significant FJC labeling at 3 and 6 months post-DFP intoxication also had p16 immunoreactivity suggests that neuronal senescence and neurodegeneration are likely separate but parallel processes that take place months after acute DFP intoxication.

Historically, a key distinguishing feature of cellular senescence was irreversible cell cycle arrest. Thus, cellular senescence was largely thought to occur predominantly in mitotic cell types. This likely explains why cellular senescence in the brain, especially in the context of postmitotic cells like neurons, has been relatively understudied until recently. It has been a subject of debate whether cellular senescence in postmitotic cells plays a protective or exclusively harmful role (reviewed in Burton & Krizhanovsky, 2014). It could be speculated that it may be advantageous to induce cellular senescence in postmitotic cells as an escape from stressor-induced potential tissue degeneration and to preserve integrity. This view can be attributed to the appreciation that mature neurons have increased restrictions to apoptosis (Annis et al., 2016; reviewed in Hollville et al., 2019), and supported by intriguing findings over the past decades suggesting that progressive loss of neurons might not necessarily be linked to age-related cognitive decline (reviewed in von Bartheld, 2018). However, accumulation and persistence of senescent neurons may paradoxically cause adverse effects and contribute to disease-related pathologies by producing inflammatory mediators known as the senescence-associated secretory phenotype (SASP) (Jurk et al., 2012; Musi et al., 2018; Riessland et al.,

2019). Therefore, understanding the role of cellular senescence in postmitotic cells is an active area of research.

The dual roles of cellular senescence emphasize the need for future studies to unveil the role of senescent neurons in the context of acute OP intoxication. Whether treatments targeting senescence-related pathways and/or elimination of senescent cells directly could offer neuroprotection in DFP-intoxicated animals will provide further insights to the functional role of cellular senescence in the context of acute OP exposures. In addition, it is unknown whether cellular senescence is a phenotype observed across different OPs and exposure paradigms.

Yet another outstanding question regarding cellular senescence in postmitotic cells is whether canonical measures of cellular senescence can be applied to postmitotic cell entities like neurons. Published literature has cautioned against the use of lipofuscin and SA-βal as markers of neuronal senescence since these biomarkers are observed in neurons as a function of age and/or disease independent of other biomarkers of cellular senescence (Piechota et al., 2016; reviewed in Sah et al., 2021; reviewed in Wengerodt et al., 2019). This caution is supported by the early presence of SA-βgal in hippocampal neurons in 3-month-old mice (Piechota et al., 2016) and neurons in the forebrain and cerebellum of 1-month-old mice (Musi et al., 2018). Therefore, this highlights the need for further research to provide clearer roles of senescence-related markers expressed in postmitotic cell types as there might be distinct properties that are different from senescent mitotic cells.

Insights regarding targeting oxidative stress as a therapeutic candidate for neuroprotection in different OP exposure paradigms

It is widely acknowledged that OPs can cause oxidative stress in cells, especially at acute, high doses (reviewed in Farkhondeh et al., 2020; reviewed in Pearson & Patel, 2016; reviewed in Vanova et al., 2018). Recent studies of repeated low-level OP exposure in animal models have also reported upregulated indices of oxidative stress; and shown that targeting oxidative

stress via treatment with antioxidants can significantly decrease biomarkers of oxidative stress and increase levels of enzymatic antioxidants (Fereidouni et al., 2019; Hussein et al., 2018; Mahmoud et al., 2019; Singh et al., 2018). However, very few studies have included an examination of neurobehavioral performances as a functional readout to evaluate whether oxidative stress is mechanistically contributing to behavioral deficits associated with repeated CPF exposures. In chapter 2, antioxidant therapy given as both prophylactic supplement and therapeutic treatments was found to improve cognitive impairment but not the inhibition of AChE activity in rats receiving repeated CPF exposure. This suggests oxidative stress as a mechanism, in addition to or other than AChE inhibition, contributes to neuropathological outcomes in a rat model of occupational CPF exposure. Our findings support and expand on prior literature implicating the involvement of oxidative stress in chronic OP exposures, and suggest that oxidative stress represents a promising therapeutic target for ameliorating neuropathological consequences caused by occupational CPF exposures.

Cellular senescence has been acknowledged as a stress response that can be triggered by varying inducing stressors including oxidative stress (Barascu et al., 2012; Correia-Melo et al., 2016; Hutter et al., 2004; reviewed in Walton & Andersen, 2019; Wiley et al., 2016), in addition to proliferation-related signaling, telomere shortening, and oncogenes (Campisi, 2013). Additionally, it is suggested that neurons expressing features of cellular senescence can adapt pro-oxidant and pro-inflammatory phenotypes and become a source that contributes to oxidative and inflammatory stress (Jurk et al., 2012; Lüth et al., 2000). While recent studies have focused on the application of senolytics to clear senescent cells as a powerful strategy to alleviate pathological phenotypes of aging and neurodegenerative diseases (Bussian et al., 2018; Chinta et al., 2018; Musi et al., 2018), it remains to be investigated whether probing potential inducers of cellular senescence, such as oxidative stress, could also offer neuroprotection against senescence-induced pathologic outcomes. Therefore, encouraged by the neuroprotective effects of immediate treatment with antioxidants following acute OP intoxication (Liang et al.,

2019; Liang et al., 2018; Putra et al., 2020a; Putra et al., 2020b) and based on the observation of elevated neuronal p16 expression in chapter 3, future investigation assessing senescence phenotypes following antioxidant treatment will be informative in assessing whether there is a causal relationship between oxidative stress and cell senescence in the context of acute OP intoxication.

Concluding thoughts

Recent data have suggested multiple mechanisms that contribute to OP neurotoxicity (reviewed in Farkhondeh et al., 2020; reviewed in Guignet & Lein, 2019; Tsai & Lein, 2021). The data presented in this dissertation highlight the critical role of oxidative stress in mediating the neurological outcomes following OP exposures, and suggest that oxidative stress may be a promising therapeutic target. Chapter 2 expands on our understanding of oxidative stress in occupational CPF exposures by providing some of the first evidence of a cause-effect relationship between oxidative stress and cognitive deficits and suggests the neuroprotective effects offered by antioxidant treatment. While the precise mechanism and biological explanation underlying neuronal senescence as well as the induction of neuronal senescence in response to acute OP intoxication remain to be further investigated, chapter 3 revealed a novel phenotype, a significantly increased expression of the senescence marker p16 in neurons, after acute DFP intoxication. The findings from chapter 3 also suggested that follow-up studies further detailing the senescence profile following acute DFP intoxication will likely provide a new therapeutic strategy against the long-term neurotoxic outcomes caused by acute OP exposures. In conclusion, while the complexity of the pathogenic mechanisms involved in OP neurotoxicity may impede the progress of identifying promising therapeutic strategies for OP poisoning, the findings from this dissertation expand the field by providing supporting evidence of the mechanistic role of oxidative stress and presenting the novel finding on cellular senescence.

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