HHS Public Access

Author manuscript

Ann N Y Acad Sci. Author manuscript; available in PMC 2017 June 21.

Published in final edited form as:

Ann N Y Acad Sci. 2016 June ; 1374(1): 176–183. doi:10.1111/nyas.13122.

Role of the calcium plateau in neuronal injury and behavioral morbidities following organophosphate intoxication

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Abstract

Organophosphate (OP) chemicals include nerve agents and pesticides, and there is a growing concern of OP-based chemical attacks against civilians. Current antidotes are essential in limiting immediate mortality associated with OP exposure. However, further research is needed to identify molecular mechanisms underlying long-term neurological deficits following survival of OP toxicity in order to develop effective therapeutics. We have developed rat survival models of OP-induced status epilepticus (SE) that mimic chronic mortality and morbidity following OP intoxication. We have observed significant elevations in hippocampal calcium levels after OP SE that persisted for weeks following initial survival. Drugs inhibiting intracellular calcium–induced calcium release, such as dantrolene, levetiracetam, and carisbamate, lowered OP SE–mediated protracted calcium elevations. Given the critical role of calcium signaling in modulating behavior and cell death mechanisms, drugs targeted at preventing the development of the calcium plateau could enhance neuroprotection, help reduce morbidity, and improve outcomes following survival of OP SE.

Keywords

paraoxon; status epilepticus; cell death; calcium; dantrolene; carisbamate

The increasing risk for organophosphate exposure

Organophosphate (OP) chemicals include nerve agents such as sarin and pesticides such as parathion. These compounds are considered extremely lethal. The civilian population has been exposed to nerve agents under acts of war and terrorism. Recent examples include the reported 2015 sarin gas attack in Ghouta, Syria,[1] the Tokyo subway sarin attack by the Aum Shinrikyo cult in 1995,[2] and the 1988 Halabja chemical attack against Kurdish people in Iraq.[3] OP-based pesticides have also been used against civilians during the Rhodesian War,[4]

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Conflicts of interest

The authors report no conflicts of interest.

and Indian children were accidentally exposed following consumption of pesticide-contaminated lunches.[5] In addition, civilians are exposed to OPs intentionally via suicide attempts, occupationally, or due to industrial accidents. In fact, pesticide ingestion is one of the most common methods for committing suicide in developing nations.[6–8] The military population has also been exposed to OP chemicals. Approximately 30% of the Gulf War veterans suffer from a cluster of symptoms commonly known as Gulf War Syndrome. Prolonged exposure to OP-based pesticides or exposure to sarin gas following demolition of chemical weapon stockpiles are among the possible causes of this syndrome.[9–11] The ease of availability of pesticides make them attractive targets to weaponize and cause mass civilian causalities. Thus, there is a growing threat of OP toxicity in the current geopolitical environment. Research in this field has provided therapeutic antidotes that are critical in limiting immediate mortality associated with lethal OP intoxication.[12] However, further research is needed to identify the molecular mechanisms underlying chronic mortality and morbidity in order to develop effective counteract therapeutics following OP exposure.[13]

Organophosphate poisoning: mechanisms, treatments, and challenges

Paraoxon (POX) is an active metabolite of parathion and is used in laboratory research to reliably model OP pesticide toxicity.[14] Similarly, diisopropyl fluorophosphate (DFP) is used in civilian research as a nerve agent surrogate to model sarin exposure owing to the ease of handling associated with DFP.[15–18] POX, DFP, and other OP chemicals are potent inhibitors of the enzyme acetylcholinesterase (AChE).[19] Inhibition of AChE prevents breakdown of the neurotransmitter acetylcholine (ACh), and ACh levels rapidly build up at the synapses. Overstimulation of ACh receptors leads to the classical cholinergic crisis characterized by salivation, lacrimation, urination, and defecation. This is followed by respiratory depression and bradycardia. Nicotinic receptor stimulation causes muscle fasciculation. This is followed by tonic–clonic seizures and status epilepticus (SE), or prolonged seizure activity that continues unabated and can result in death if left untreated.[20] SE activity is thought to involve recruitment of *N*-methyl-D-aspartate (NMDA) receptors following release of the excitatory neurotransmitter glutamate downstream of the ACh overstimulation.[21–24] Current treatment strategies use atropine to control the cholinergic crisis, pralidoxime to reactivate AChE, and a benzodiazepine, such as diazepam or midazolam, to control seizures.[25,26] While the current antidotes are critical in limiting immediate mortality associated with OP exposure, OP SE survivors are vulnerable to delayed mortality in the critical 2-week period after initial survival and the development of chronic neurological morbidities such as recurrent seizures, depression, and cognitive deficits.[14,22,27–35] Thus, it is essential to develop valid animal models that mimic OP mortality and morbidity and to identify molecular mechanisms underlying long-term neurological deficits from survival of OP toxicity in order to develop effective counteract therapeutics.

Rat survival models of OP SE

Many OP studies in the literature have focused on the effects of low-dose, chronic OP exposure or the effects of OPs following *in utero* exposure.[36–39] There are also studies reporting models of acute parathion[40,41] and POX exposures.[42–44] However, these models did not focus on evaluating long-term survival after lethal POX SE exposures. Development

of OP SE models is also complicated by their variable pharmacokinetic and pharmacodynamics response, such as the challenges associated with parathion kinetics and differential metabolism.[45–47] We wanted to further develop a reliable rat survival model for lethal OP exposure with SE that would replicate both the acute mortality and chronic morbidity associated with these agents. Such animal models could be very useful for studying the molecular mechanisms of OP toxicities and for screening medical countermeasures to improve survival following OP exposures.

To this end, we have developed two SE survival models of OP toxicity using lethal doses of POX [14] and DFP.[16] The behavioral manifestations and electroencephalography (EEG) profiles for these OP SE models mimicked the signs and symptoms of acute OP intoxication. In this model, rats were exposed to a lethal dose (approximately twice the LD_{50}) of an OP chemical (POX or DFP) and were treated with U.S. Food and Drug Administration–approved drugs to limit immediate mortality.[26] Here, we will discuss the POX model of OP SE. One week before the SE experiments, rats were stereotaxically implanted with skull surface electrodes to record EEG. Briefly, 1 min after POX injection (2 mg/kg subcutaneous (SC)) animals received human-dose equivalents of 2-PAM (25 mg/kg intramuscular (IM) and atropine (0.5 mg/kg IM). Within 5–7 min following POX administration, rats displayed overt cholinergic symptoms and rapidly developed convulsions and SE-like activity. Onset of SE was determined by the presence of continuous class 4–5 level seizures using a modified Racine scale.[48] One hour following onset of POX SE, animals were injected with midazolam (2 mg/kg IM) to terminate seizures. Surviving animals were then injected with saline (3cc/animal intraperitoneal (IP)), fed lactose milk as supportive care, and returned to their home cages. Surviving rats were housed individually in temperature- and light-controlled vivaria. All the rats were visually monitored once a week until their use in Ca^{2+} imaging or behavioral experiments. Chronic mortality (72-h and beyond) in these models of severe OP intoxication was 18–20%.[14,16] These POX and DFP SE survival models manifested the same degree of delayed mortality and morbidity (see below) observed in the human OP-exposure condition.[49–53]

Development of Ca^{2+} plateau following survival from OP SE

One of the important long-term molecular changes that occurs following the survival of SE induced by OPs or chemoconvulsants like pilocarpine is the development of sustained elevations in neuronal calcium levels ($[Ca^{2+}]_i$) known as the "Ca^{2+} plateau."[14,16,54–59] We have developed methodologies to acutely isolate hippocampal CA1 region neurons from brain slices using enzymatic and mechanical trituration. Neurons obtained using these methods show minimal signs of necrosis, exhibit normal electrophysiological membrane properties, and allow us to study Ca^{2+} dynamics in the absence of confounding factors such as glial response. Estimation of neuronal Ca^{2+} levels have revealed the development of a Ca^{2+} plateau wherein hippocampal neurons exhibit significantly elevated Ca^{2+} levels for weeks after the termination of POX SE[14] (Fig. 1A). We have previously shown that, while the induction of Ca^{2+} plateau was dependent on the NMDA receptor during SE,[1654] the maintenance of the Ca^{2+} plateau for several weeks post-SE was independent of NMDA receptor activation and was mediated by persistent Ca^{2+} release from the endoplasmic reticulum through the mechanisms of Ca^{2+}-induced Ca^{2+} release (CICR).[56,57] Indeed,

pretreatment with the NMDA antagonist MK-801 prevented the OP SE–induced elevations in hippocampal Ca^{2+} levels. However, application of MK-801 was not effective in lowering the elevated Ca^{2+} in hippocampal neurons isolated from rats 1 h after SE[16] (Fig. 1B). On the other hand, treatments with dantrolene, levetiracetam, or carisbamate, inhibitors of the CICR mechanisms, were able to lower the elevated Ca^{2+} levels and abolish the Ca^{2+} plateau after SE[14,57,60] (Fig. 1C). It is important to note that, while the NMDA receptor–mediated indiscriminant Ca^{2+} influx turns off after SE is terminated, a sustained Ca^{2+} release from the endoplasmic reticulum continues, owing to a long-lasting activation of molecular components involved in the CICR mechanisms. This is an important aspect of the long-lasting activation of the CICR system. Since Ca^{2+} ions act as major second messengers in multiple signaling cascades, the OP SE–induced prolonged elevations in $[Ca^{2+}]_i$ can trigger neurodegenerative pathways and mediate pathological synaptic plasticity. These alterations in Ca^{2+} dynamics following OP toxicity could therefore underlie the associated neuronal injury, and together they may be responsible for the chronic neurological morbidities following OP SE survival[14] (Fig. 4).

Neuronal injury following OP SE

Neuronal loss in several brain regions has been observed following SE,[54,55] OP SE,[14,15] and exposure to other chemical threat agents.[61] We have observed widespread neuronal loss induced by POX SE, as assessed using the Fluoro Jade (FJC) labeling technique.[14,62] FJC^+ neurons were observed within the hippocampus, parietal cortex, and in both the amygdala and thalamic nuclear regions of POX SE rats (Fig. 2). Damages to these critical brain areas have been implicated in memory impairment, depression, anxiety, epilepsy, and other neurological morbidities.[27,63,64]

Chronic morbidity following survival from OP SE

We have also analyzed OP SE survivors in these animal models for the development of neurological morbidities (Fig. 3). We have observed symptoms of chronic depression and memory impairments in these OP-exposed rats.[62,65] OP SE survivors displayed increased immobility in the forced swim test, indicative of despair; reduced sucrose consumption in the sucrose preference test, indicative of anhedonia; and spend less time in the open arm of elevated plus maze, indicative of high anxiety.[62,65] Despair, anhedonia, and anxiety are symptoms of depression. In addition, these rats performed poorly in the novel object recognition task, indicative of memory impairment.[62,65] Survival from OP SE was also associated with significant neuronal damage throughout the limbic system, particularly in the hippocampus.[14,16] These models provide a reproducible method of mimicking human survival of OP toxicity. In addition to lethal OP intoxication, chronic low-dose OP exposures have also been implicated in long-term neurological morbidities. For example, agricultural pesticide applicators and Gulf War veterans suspected of chronic OP exposure exhibit chronic neurological morbidities, such as depression and cognitive impairments.[33,66,67]

Conclusions

Ca^{2+} ions are second messenger molecules in various signaling cascades that modulate behavior, memory, and cell death.[55,56,68–72] The development of THE Ca^{2+} plateau is therefore a critical substrate for inducing neuronal damage and triggering many of the long-term plasticity changes following OP SE–induced brain injury.[14,16,55,56] Given the role of CICR mechanisms in the maintenance of the Ca^{2+} plateau, drugs targeting the molecular components of this signaling mechanism could prove to be effective agents in extending neuroprotection following survival from OP SE. We have demonstrated neuroprotective and antiepileptogenic effects of dantrolene[57] and carisbamate[73] in an *in vitro* model of SE-induced acquired epilepsy. The ability of dantrolene, levetiracetam, and carisbamate to reduce or abolish the Ca^{2+} plateau could make them attractive neuroprotective adjuvant treatments following OP SE. These agents could also prove beneficial in reducing the chronic neurological morbidities observed in OP SE survivors. We are actively exploring these possibilities in our laboratories (Fig. 4).

Despite advances in developing more effective agents for controlling the cholinergic crisis associated with OP SE, there is a pressing need to develop treatments that prevent or reduce the high mortality and chronic morbidity associated with OP SE. This is an important area of research that has direct translational implications for clinical treatment.[74,75] Development of animal models of OP SE are critical to identifying the molecular mechanisms underlying symptoms of OP toxicity. This knowledge can provide molecular targets that can be used to develop effective therapies for the treatment of OP SE (Fig. 4). This research indicated that agents that inhibit CICR and can reduce or prevent the Ca^{2+} plateau may be an innovative area for development of medical countermeasures that can lower mortality and morbidity following SE and OP SE.

Acknowledgments

This work was supported by the CounterACT Program, National Institutes of Health Office of the Director (NIH OD), and the National Institute of Neurological Disorders and Stroke Grant No. U01NS058213-10 to R.J.D. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. This work was also supported by the Office of the Assistant Secretary of Defense for Health Affairs through the Gulf War Illness Research Program under Award No. (W81XWH-14-1-0478) to L.S.D. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense. Portions of this work were presented at the 9th Annual NIH Countermeasures against Chemical Threats (CounterACT) Network Research Symposium.

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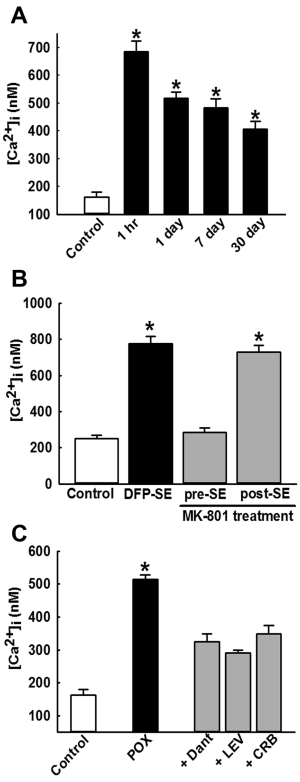

Figure 1.

Development of Ca^{2+} plateau and its mechanism following OP-induced SE. (A) Hippocampal CA1 $[Ca^{2+}]_i$ from age-matched control (white bar) and POX-exposed rats were isolated 1 h and 1, 7, and 30 days after SE (black bars). $[Ca^{2+}]_i$ in POX-SE rats was significantly higher than control values at all the time points and did not return to baseline, even at 30 days post-SE (Ca^{2+} plateau). (B) Hippocampal CA1 $[Ca^{2+}]_i$ from control rats (white bar), DFP-treated rats (black bar), and DFP + MK-801–treated rats (grey bars) were isolated 1 -h after SE. MK-801 pretreatment prevented the elevations in $[Ca^{2+}]_i$ that occur following DFP-induced SE. However, MK-801 treatment 1 h after DFP-induced SE did not significantly affect DFP-SE induced $[Ca^{2+}]_i$ elevations. (C)Hippocampal CA1 $[Ca^{2+}]_i$ from control rats (white bar), POX-treated rats (black bars), and POX + drugs rats (grey bars) were isolated 48 h after SE. $[Ca^{2+}]_i$ in neurons isolated from POX-SE rats treated with either dantrolene (DANT), levetiracetam (LEV), or carisbamate (CRB) were significantly lower than POX SE rats (no drugs) values at the respective time point. All data represented as mean ± SEM. $*P < 0.05$ Data in 1A and 1C reproduced from Ref. 14, with permission.

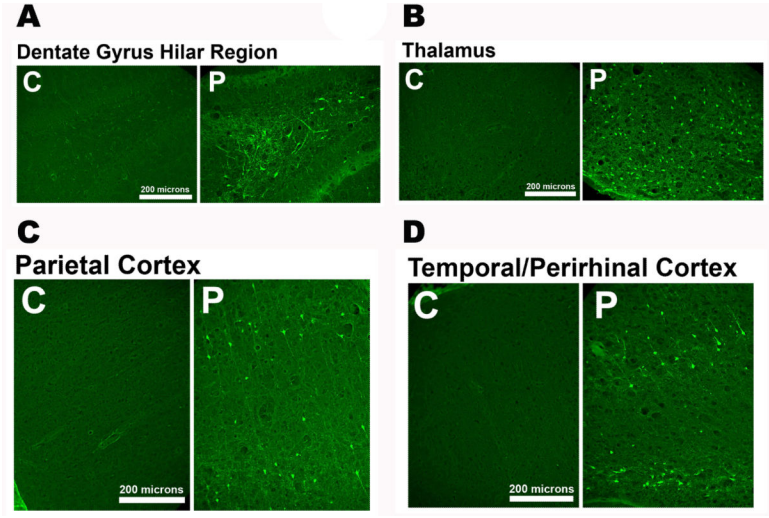

Figure 2.

Neuronal injury following POX-induced SE. Representative photomicrographs of Fluoro-Jade C (FJC) staining in the dentate gyrus–hilus region, parietal cortex, amygdala, and thalamus 2 days after POX SE. Scale bars, 200 μm. Data adapted from Ref. 14.

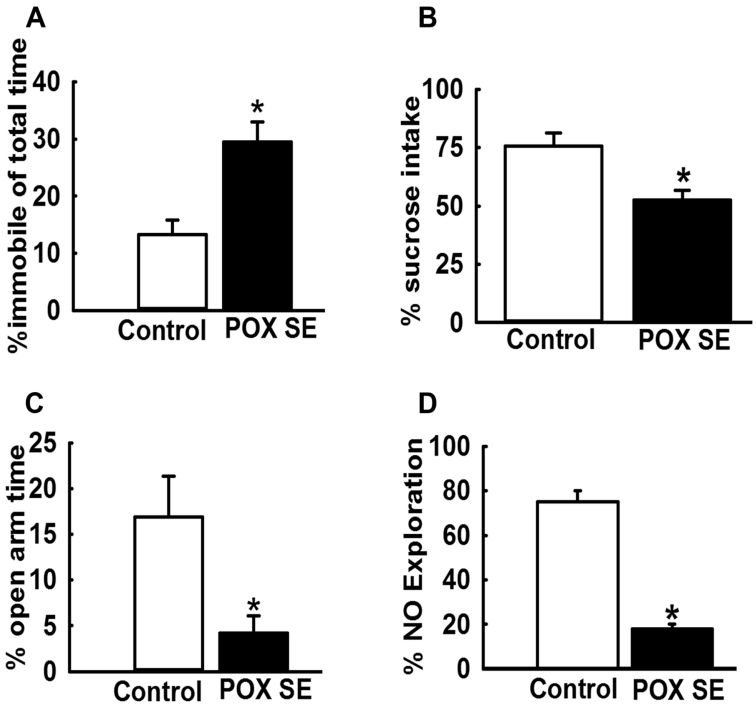

Figure 3.

Chronic behavioral morbidities following POX-induced SE. Approximately 3 months following POX SE, surviving rats were tested on various behavioral assays to assess symptoms of depression and memory impairments. (A) Increased immobility time in POX SE rats during the forced swim test, indicative of behavioral despair. (B) Decreased sucrose consumption in POX SE rats on the sucrose preference test, indicative of anhedonia (inability to feel pleasure). (C) Enhanced anxiety in POX SE rats as characterized by significantly less time spent in the open arm of the elevated plus maze. (D) Impaired recognition memory in POX SE rats on the novel object recognition test, as rays displayed significantly less time spent exploring the novel object. All data expressed as mean ± SEM, $*P < 0.05$, *t*-test, $n = 8$ rats. Data adapted from Ref. 65.

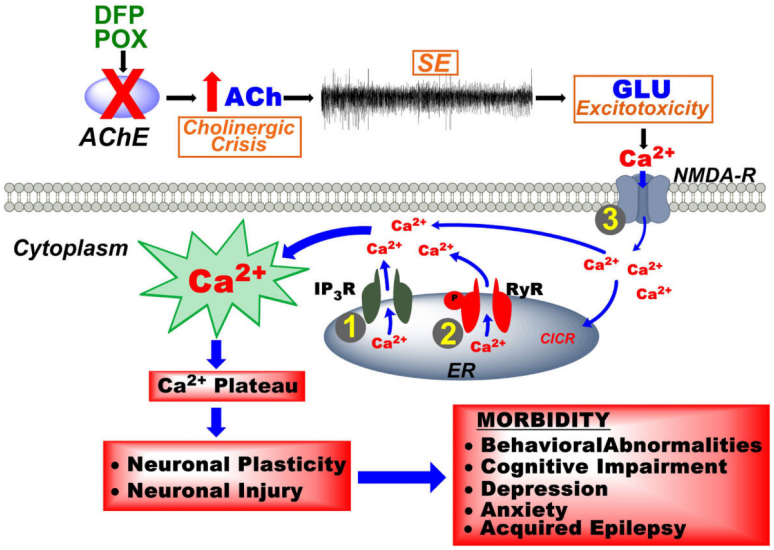

Figure 4.

Development of the calcium plateau following OP-induced SE and possible targets for countermeasures therapy. OP chemicals such as DFP or POX inhibit the enzyme acetylcholinesterase (AChE), initially producing a cholinergic crisis that propagates into self-sustaining SE and ultimately leads to glutamate excitotoxicity. Downstream activation of NMDA receptors leads to massive influx of Ca^{2+} ions into the postsynaptic neurons. Activation of Ca^{2+}-induced Ca^{2+}-release (CICR) mechanisms leads to release of Ca^{2+} into the cytoplasm from the endoplasmic reticulum (ER) via the ryanodine receptor (RyR) and the inositol–trisphosphate receptor (IP_3R). While NMDA activation is required for genesis of the Ca^{2+} plateau, the maintenance is dependent on sustained Ca^{2+} release via CICR mechanisms. After SE is terminated, NMDA activation is shut off, but the Ca^{2+} release from ER continues, due to long-lasting activation of the CICR mechanisms. The Ca^{2+} plateau triggers neurodegenerative pathways leading to neuronal injury and activates nuclear signaling that can lead to the neuronal plasticity that underlies chronic morbidities characterized by the development of acquired epilepsy, memory deficits, and psychiatric impairments. Inhibiting the critical targets (1, 2, or 3) in the Ca^{2+} plateau cascade with pharmacological agents (dantrolene, levetiracetam, or ketamine) can exert neuroprotective effects and can decrease or prevent the development of the chronic neurological morbidities associated with OP SE survival.