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Short title: Calcium dysregulation following organophosphate toxicity

Role of the Calcium Plateau in the 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 outcome following survival of OP SE.

Introduction

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 sub-way 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] and the accidental poisoning in Indian children following consumption of pesticide contaminated lunches [5]. In addition, civilians are exposed to OP's intentionally by suicide attempts or occupationally or due to industrial accidents. In fact, pesticide ingestion is one of the most common method for committing suicide in developing nations[6, 7 8]. The military population has also been exposed to OP chemicals. Approximately 30% of returning soldiers from the Persian Gulf War 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 amongst the possible causes thought to be responsible for this syndrome[9-11]. The ease of availability of pesticides make them attractive target to be weaponized 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 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 fluorophosphates (DFP) is used in civilian research as a nerve agent surrogate to model sarin exposure given 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 rapidly builds up ACh level 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 results in the 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 post 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 literature have focused on effects of low-dose, chronic OP exposure or effects of OP's 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 to study molecular mechanisms of OP toxicities and screen 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 EEG profile 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 2x LD_{50}) of OP chemical (POX or DFP) and were treated with FDA approved drugs to limit immediate mortality[26]. Here we will discuss the POX model of OP-SE. One week prior to SE experiments, rats were stereotaxically implanted with skull surface electrodes to record EEG. Briefly, one minute following POX injection (2 mg/kg, *s.c.*) animals received human-dose equivalents of 2-PAM (25 mg/kg, *i.m.*) and atropine (0.5 mg/kg, *i.m.*). Within 5-7 minutes 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, *i.m.*) to terminate

seizures. Surviving animals were then injected with saline (3cc/animal, *i.p.*) and fed lactose milk as part of supportive care and returned to their home cages. Surviving rats were housed individually in temperature and light controlled vivarium. All the rats were visually monitored once a week till 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 allows 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 NMDA receptor dependent during SE [16, 54], 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 [56, 57]. Indeed, pre-treatment 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 following SE [16] (Fig. 1B). On the other hand, treatments with dantrolene or levetiracetam or carisbamate, inhibitors of the Ca^{2+} induced Ca^{2+} release mechanisms were able to lower the elevated Ca^{2+} levels and abolish the Ca^{2+} plateau post SE [14, 57,60] (Fig. 1C). It is important to note that while NMDA-R mediated indiscriminant Ca^{2+} influx turns off after SE is terminated, there remains a sustained Ca^{2+} release from ER continues due 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 be responsible for the chronic neurological morbidities following OP SE[14] survival (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 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-positive staining neurons were observed within the hippocampus, parietal cortex, and in both 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]. Together, despair, anhedonia, and anxiety constituted 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 the hippocampus[14, 16]. These models provide a reproducible method of mimicking the 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 Persian Gulf War veterans suspected of chronic OP exposure exhibit chronic neurological morbidities such as depression and cognitive impairments[33, 66, 67].

Conclusion

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 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 by brain injury [14, 16, 55, 56]. Given the role of Ca^{2+} induced Ca^{2+} release mechanisms in the maintenance of Ca^{2+} plateau, drugs targeting the molecular components of this signaling mechanisms 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 counteract treatments that prevent or reduce the high mortality and the 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 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 Ca^{2+} induced Ca^{2+} release 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.

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Figure Legends

Figure 1. Development of Ca^{2+} plateau and it's mechanism following OP induced SE

A. Hippocampal CA1 $[Ca^{2+}]_i$ from age-matched control (white bar) and POX 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 base-line even at 30-days post SE (Ca^{2+} plateau). **B.** Hippocampal CA1 $[Ca^{2+}]_i$ from control rats (white bar), DFP rats (black bar), and DFP + MK-801 (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 rats (black bars), and POX + drugs (grey bars) were isolated 48-h after SE. $[Ca^{2+}]_i$ in neurons isolated from POX-SE rats treated with either dantrolene (DANT) or 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 Deshpande, L.S., D.S. Carter, K.F. Phillips, et al. 2014. Development of status epilepticus, sustained calcium elevations and neuronal injury in a rat survival model of lethal paraoxon intoxication. Neurotoxicology.* ***44C****: 17-26).*

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 previously published in : Deshpande, L.S., D.S. Carter, K.F. Phillips, et al. 2014. Development of status epilepticus, sustained calcium elevations and neuronal injury in a rat survival model of lethal paraoxon intoxication. Neurotoxicology.* ***44C****: 17-26).*

Figure 3. Chronic behavioral morbidities following POX induced SE

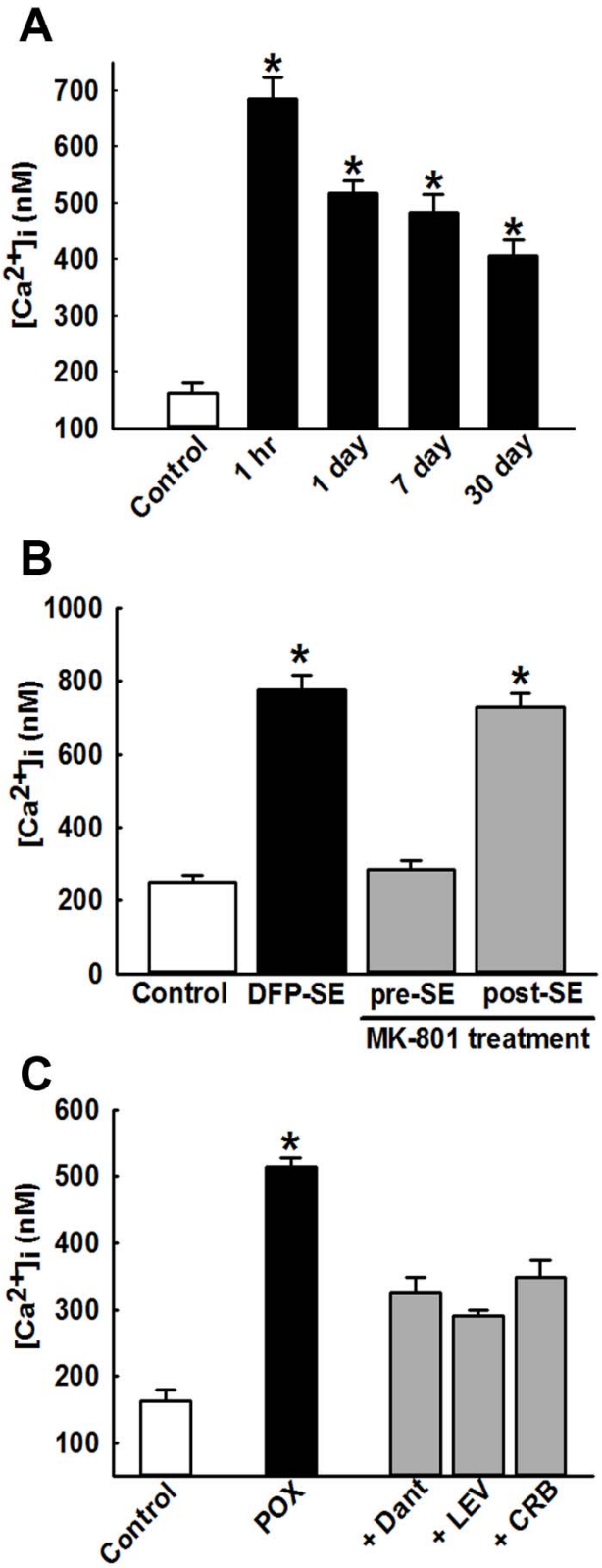
Approximately 3-months following POX SE, surviving rats were tested on various behavioral assays for assessing symptoms of depression and memory impairments. **A.** Increased immobility time in POX SE rats during the Forced Swim Test indicative of behavioral despair. **B.** Decreased sucrose consumption in POX SE rats on the Sucrose Preference Test indicative of anhedonia (lack of feeling 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 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: Deshpande, L.S., K. Phillips, B. Huang, et al. 2014. Chronic behavioral and cognitive deficits in a rat survival model of paraoxon toxicity. Neurotoxicology.* ***44****: 352-357).*

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 N-methyl-D-aspartate receptors (NMDA-R) leads to massive influx of Ca^{2+} ions into the post-synaptic 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 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 a long lasting activation of CICR mechanisms. The Ca^{2+} plateau triggers neurodegenerative pathways leading to neuronal

injury and activates nuclear signaling that can lead to 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.

Figure 1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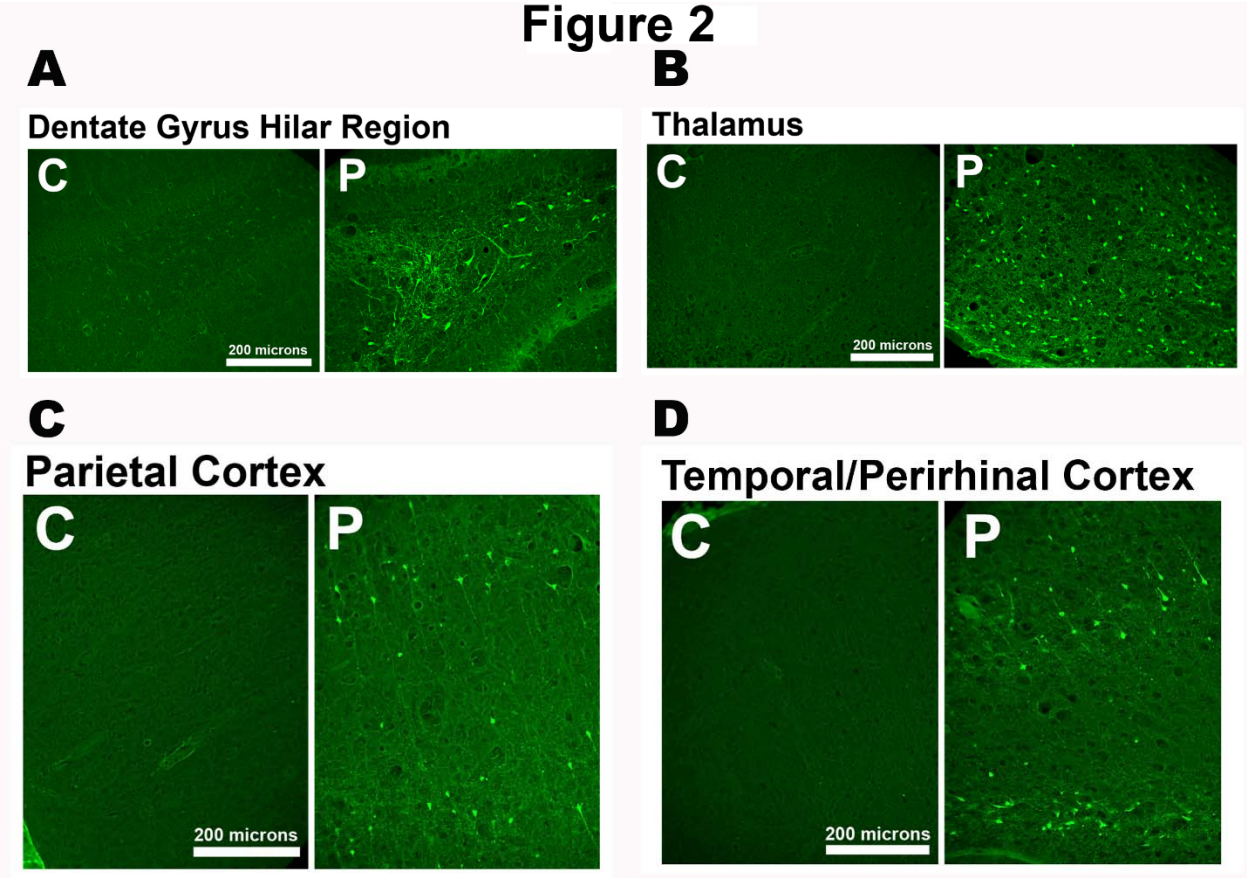
400
300
200
100
Control
1 hr
1 day
7 day
30 day
B
[Ca2+]i (nM)
1000
800
600
400
200
0
Control
DFP-SE
pre-SE
post-SE
MK-801 treatment
C
[Ca2+]i (nM)
600
500
400
300
200
100
Control
POX
+ Dant
+ LEV
+ CRB

Figure 2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Dentate Gyrus Hilar Region
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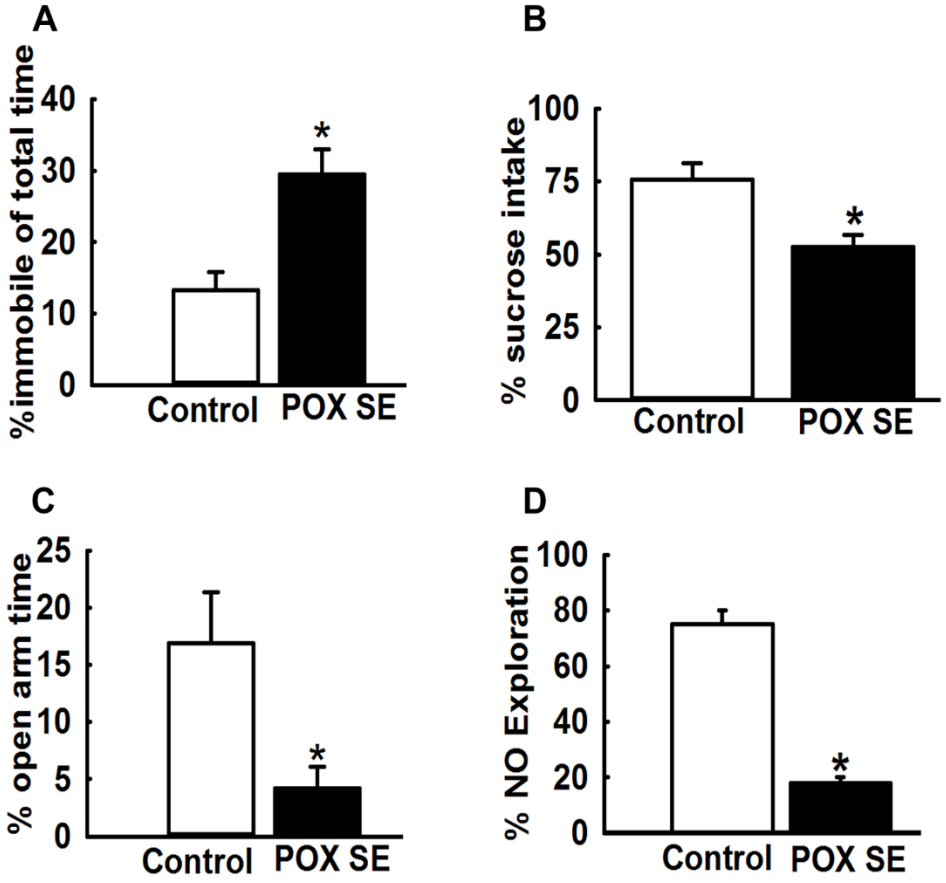
200 microns
B
Thalamus
C
P
200 microns
C
Parietal Cortex
C
P
200 microns
D
Temporal/Perirhinal Cortex
C
P
200 microns

Figure 3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%immobile of total time
40
30
20
10
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Control
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B
% sucrose intake
100
75
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25
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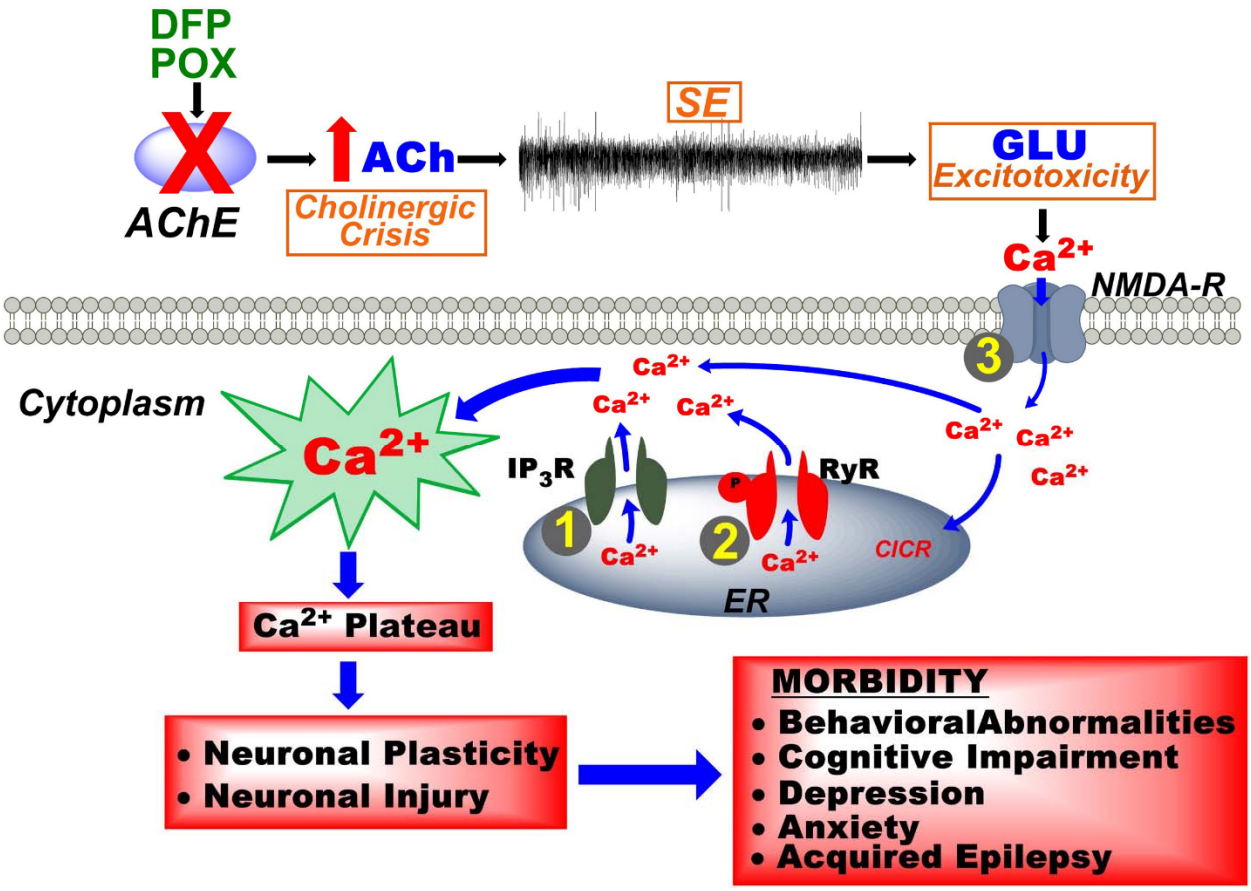
DFP
POX
AChE
ACh
Cholinergic Crisis
SE
GLU
Excitotoxicity
Ca2+
NMDA-R
3
Cytoplasm
Ca2+
IP3R
RyR
1
2
P
CICR
ER
Ca2+ Plateau
• Neuronal Plasticity
• Neuronal Injury
MORBIDITY
• BehavioralAbnormalities
• Cognitive Impairment
• Depression
• Anxiety
• Acquired Epilepsy