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Development of status epilepticus, sustained calcium elevations and neuronal injury in a rat survival model of lethal paraoxon intoxication

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Abstract

Paraoxon (POX) is an active metabolite of organophosphate (OP) pesticide parathion that has been weaponized and used against civilian populations. Exposure to POX produces high mortality. OP poisoning is often associated with chronic neurological disorders. In this study, we optimize a rat survival model of lethal POX exposures in order to mimic both acute and long-term effects of POX intoxication. Male Sprague-Dawley rats injected with POX (4 mg/kg, ice-cold PBS, s.c.) produced a rapid cholinergic crisis that evolved into status epilepticus (SE) and death within 6-8 min. The EEG profile for POX induced SE was characterized and showed clinical and electrographic seizures with 7-10 Hz spike activity. Treatment of 100% lethal POX intoxication with an optimized three drug regimen (atropine, 2 mg/kg, i.p., 2-PAM, 25 mg/kg, i.m. and diazepam, 5 mg/kg, i.p.) promptly stopped SE and reduced acute mortality to 12% and chronic mortality to 18%. This model is ideally suited to test effective countermeasures against lethal POX exposure. Animals that survived the POX SE manifested prolonged elevations in hippocampal $[Ca^{2+}]_i$ (Ca^{2+} plateau) and significant multifocal neuronal injury. POX SE induced Ca^{2+} plateau had its origin in Ca^{2+} release from intracellular Ca^{2+} stores since inhibition of ryanodine/ IP_3 receptor lowered elevated Ca^{2+} levels post SE. POX SE induced neuronal injury and alterations in Ca^{2+} dynamics may underlie some of the long term morbidity associated with OP toxicity.

Keywords: paraoxon, status epilepticus, survival model, Ca^{2+} dynamics, Sprague-Dawley rats

Introduction

Paraoxon (POX) is an active metabolite of organophosphate (OP) insecticide parathion that has been weaponized and has caused human casualties (Federation of American Scientists, 1998; Gould and Folb, 2003; Croddy *et al.*, 2004; Moorcraft and McLaughlin, 2008). POX is a potent acetylcholinesterase (AChE) inhibitor that prevents the breakdown and causes rapid buildup of the neurotransmitter acetylcholine at the synapse, resulting in a “cholinergic crisis” associated with numerous physiological symptoms, including excessive salivation, respiratory depression, bradycardia and prolonged seizures (status epilepticus, SE) (Bajgar, 2004; Hoffmann and Papendorf, 2006). These symptoms of POX exposure, if left untreated can result in the rapid demise of the OP exposed individuals (McDonough *et al.*, 1995). Survivors of SE and OP compounds poisoning suffer from significant morbidities including spontaneous recurrent seizures, deficits in cognition and depression (Steenland *et al.*, 1994; Garcia *et al.*, 2003; Ruckart *et al.*, 2004; Johnson *et al.*, 2009; de Araujo Furtado *et al.*, 2010; de Araujo Furtado *et al.*, 2012; Rauh *et al.*, 2012; Terry *et al.*, 2012). It is important to develop animal survival models for lethal exposures to evaluate treatments to reduce acute mortality and prevent chronic morbidity after exposure to potent OP agent.

There are studies reporting models of acute POX toxicity and survival shortly after exposure (Petrikovics *et al.*, 2004; Albuquerque *et al.*, 2006; Todorovic *et al.*, 2012); however, to our knowledge there are no studies that evaluate long-term survival after lethal POX induced SE. The goal of this study is to further develop a reliable rat survival model for lethal POX exposure SE that will replicate both the acute and long-term effects of this agent. Such a model could be used screen medical countermeasures to improve survival and also help in studying molecular mechanisms underlying the development of long-term morbidity following OP exposures. Here

we utilized POX to mimic lethal OP poisoning and have followed surviving animals for up to one year after POX intoxication. The mortality, behavioral manifestations and EEG profile for acute POX exposure are characterized in this study as evaluated for other nerve agent and pesticide exposures (McDonough and Shih, 1993; Shih *et al.*, 2007; Deshpande *et al.*, 2010). Our results show that POX exposure produced a reliable response mimicking all the signs and symptoms of OP intoxication. Development of this model resulted in high survival rates following lethal POX exposures as a result of an improved regimen of treatment with atropine, 2-PAM and diazepam. This model could provide a reproducible method to mimic the human survival of POX toxicity.

It has been established that one of the long-term molecular changes that occurs following the survival of SE from either exposure to the OP DFP (Deshpande *et al.*, 2010) or chemoconvulsant pilocarpine (Raza *et al.*, 2004) is the development of sustained elevations in $[Ca^{2+}]_i$ termed the “ Ca^{2+} plateau”. These alterations in Ca^{2+} dynamics along with the associated neuronal injury are thought to underlie the chronic effects of SE on producing acquired epilepsy and behavioral and cognitive impairment (Rice *et al.*, 1998; Raza *et al.*, 2004; Deshpande *et al.*, 2008b). This study will evaluate the effects of POX induced SE on the development of the Ca^{2+} plateau and neuronal damage to determine if the POX SE model produces similar effects on these processes as seen with pilocarpine and DFP SE. We will also investigate the contribution of ryanodine receptor (RyR) or inositol trisphosphate receptor (IP_3R)-dependent regulation of $[Ca^{2+}]_i$ towards the maintenance of the Ca^{2+} plateau utilizing select inhibitors that have been shown to lower elevated Ca^{2+} levels following SE (Nagarkatti *et al.*, 2008; Nagarkatti *et al.*, 2010). Being able to reverse the Ca^{2+} plateau after survival of POX SE with the Ca^{2+} induced Ca^{2+} release (CICR) inhibitors could provide new targets for the

development of agents to reverse the Ca^{2+} plateau and offer a unique insight into the pathophysiology of POX toxicity.

Materials and methods

Animals

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing ~250-300 g and 10-weeks age were used in this study. Animals were housed two per cage at 20-22°C with a 12 Light: 12 Dark hour cycle (lights on 0600-01800 h) and free access to food and water.

Chemicals

All the chemicals were obtained from Sigma Aldrich Company (St. Louis, MO) unless otherwise noted. POX was prepared by dissolving in ice-cold phosphate buffered saline (PBS) just before the experiment and injected subcutaneously (s.c.) and was kept on ice until the time of injections. Time on ice (between diluting in saline to injections) was never more than five minutes. Atropine sulfate and pralidoxime chloride (2-PAM) were dissolved in saline (0.9% NaCl) and sterile filtered. Diazepam injection-USP was obtained from VCU health system pharmacy. All the drugs were prepared fresh on the day of experiment. Dantrolene was dissolved in DMSO. It was applied to the neurons via bath perfusion. Response to dantrolene was recorded for up to 10-mins following the bath perfusion. It took approximately 1 min for the complete exchange of bath solution. Levetiracetam tablets (Lupin; 250 mg) were obtained by VCU division of animal resources from local pharmacy. The water soluble tablets were crushed and

dissolved in saline and sterile filtered to remove the non-soluble binding agents and pharmaceutical excipients before i.p. injection. Carisbamate (RWJ 333369) was a gift from Johnson & Johnson, Pharmaceutical Research & Development L.L.C. (Titusville, NJ, USA), and was suspended in 0.2% methylcellulose for i.p. administration.

Electrode implantation and seizure monitoring

Rats were stereotaxically implanted with three skull surface electrode screws attached to teflon insulated stainless steel MedWire® (Plastics One, Roanoke, VA, USA.) under general anesthesia with isoflurane/O₂ (5% induction; 2.5% maintenance). Electrode screws were positioned through burr holes above the right and left frontal cortices (AP, 3 mm and ML, ±3 mm from bregma); the third surface electrode screw was positioned over the cerebellum to serve as reference and two additional (non-electrode) skull screws were inserted for structural support. The electrode screws were seated to contact, but not penetrate the dura mater. Female amphenol terminal pins connected to the electrode wire were seated into an electrode pedestal (Plastics One, Roanoke, VA) and this assembly was secured to the skull with Cerebond™ adhesive (Plastics One, Roanoke, VA). Rats were allowed 1 week of recovery time before the start of the experiment. Wire leads were securely connected into the threaded electrode pedestal on the rat and then connected to an electrical-swivel commutator (Plastics One, Roanoke, VA) to allow for free movement of the rat while maintaining continuity of EEG signals. EEG signals were amplified using a Grass model 8-10D (Grass Technologies, West Warwick, RI) and digitized using a Powerlab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO). Evaluation of digitally acquired EEG was performed with Labchart (AD Instruments, Colorado Springs, CO) (Raza *et al.*, 2004; Deshpande *et al.*, 2010).

POX induced status epilepticus

One minute following POX injection animals received 2-PAM (50 mg/kg, i.m.) and atropine (2 mg/kg, s.c.). Rats underwent convulsions and SE-like activity within 5-7 minutes following POX, and behavioral observations and Racine scores were made. Onset of SE was determined by the presence of continuous class 4-5 level seizures as assessed using a modified Racine scale (Racine, 1972), which were objectively and independently determined by three trained observers (Raza *et al.*, 2004; Deshpande *et al.*, 2010). Racine scores were averaged for each animal. Following onset of POX SE, animals were injected with diazepam (5 mg/kg, i.p.) and 2-PAM (25 mg/kg, i.m.) at 1, 3 and 5 h to terminate and control seizures. Surviving animals were then injected with saline (3cc/animal, i.p.) and fed lactose milk as part of supportive care and returned to home cage. Rats may lose up to 10-15 g weight due to fluid loss associated with SE. Animal health was reassessed every day for the next three days and wet-chow and saline was administered if weight gain did not occur. By the fourth-day all the SE rats had recovered from POX induced cholinergic crisis and were ambulatory. Surviving rats were housed individually in temperature and light controlled vivarium. All the rats were visually monitored once a week till their use in behavioral experiments. For Ca^{2+} studies, animals were sacrificed at 1 h following SE onset or at 1, 7 or 30 days after SE. Mortality was assessed at 24, 48 and 72-h. For mechanism of Ca^{2+} plateau studies, levetiracetam (50 mg/kg, i.p.) or carisbamate (90 mg/kg, i.p.) was injected at 1, 6 and 18-h following onset of SE.

Isolation of Hippocampal CA1 Neurons and Loading with Fura-2

Acute isolation of CA1 hippocampal neurons was performed by established procedures (Raza *et al.*, 2004; Deshpande *et al.*, 2010). At specified times following onset of POX induced SE, animals were anesthetized with isoflurane and decapitated. Brains were rapidly dissected and

placed in 4°C oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (aCSF) consisting of (in mM): 201.5 sucrose, 10 glucose, 1.25 NaH₂PO₄, 26 NaHCO₃, 3 KCl, 7 MgCl₂, and 0.2 CaCl₂). MK-801 (1 μM) was added to all solutions to increase cell viability and was removed 15 min prior to imaging. Hippocampal slices (450 μm) were cut on a vibrating microtome (Leica Microsystems, Wetzlar, Germany) and then equilibrated for 10 min at 34°C in a piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES)-aCSF solution containing (in mM): 120 NaCl, 25 glucose, 20 PIPES, 5 KCl, 7 MgCl₂, and 0.1 CaCl₂. Slices were then treated with 8 mg/ml protease in PIPES-aCSF for 6 min at 34°C and rinsed. Enzyme treated slices were visualized on a dissecting microscope to excise the CA1 hippocampal layer which was then triturated with a series of Pasteur pipettes of decreasing diameter in cold (4°C) PIPES-aCSF solution containing 1 μM Fura-2 AM (Invitrogen, Carlsbad, CA). The cell suspension was placed in the middle of 2 well glass-bottomed chambers (Nunc, Thermo Scientific). These glass chambers were previously treated overnight with 0.05 mg/ml poly-L-lysine followed by multiple rinses with distilled water and then further treated with Cell-Tak™ (BD-Biosciences, San Jose, CA) biocompatible cellular adhesive (3.5 μg/cm²) for 30-min, rinsed and air-dried. Neuronal suspension placed in the center of adhesive coated dishes when settled firmly adhered to the bottom. This technique simplified further manipulations on the dissociated neurons. Plates were then incubated at 37°C in a 5% CO₂/95% air atmosphere for 45 min. Fura-2 was washed off with PIPES-aCSF and plates were incubated an additional 15 min to allow for complete cleavage of the AM moiety from Fura-2.

Measurement of [Ca²⁺]_i

Fura-2 loaded cells were transferred to a 37°C heated stage (Harvard Apparatus, Hollington, MA) on an Olympus IX-70 inverted microscope coupled to a fluorescence imaging system (Olympus America, Center Valley, PA) and subjected to [Ca²⁺]_i measurements by

procedures well established in our laboratory (Raza *et al.*, 2004; Deshpande *et al.*, 2010). All experiments were performed using a 20X, 0.7 N.A. water immersion objective and images were recorded by an ORCA-ER high-speed digital CCD camera (Hamamatsu Photonics K.K., Japan). Fura-2 was excited with a 75 W xenon arc lamp (Olympus America, Center Valley, PA). Ratio images were acquired by alternating excitation wavelengths (340/380 nm) by using a Lambda 10-2 filter wheel (Sutter Instruments Co., Novato, CA) and a Fura filter cube at 510/540 emission with a dichroic at 400 nm. All image acquisition and processing was controlled by a computer connected to the camera and filter wheel using Metafluor Software ver 7.6 (MDS Analytical Technologies, Downingtown, PA). Image pairs were captured every 5 s and the images at each wavelength were averaged over 10 frames. Background fluorescence was obtained by imaging a field lacking Fura-2.

Hippocampal CA1 neurons were identified based on their distinct morphology. These neurons displayed pyramidal shaped cell body, long axon and dendrites and have been demonstrated to be devoid of immunoreactivity for specific protein markers for interneurons, including parvalbumin, cholecystokinin, vasoactive intestinal peptide, somatostatin, and neuropeptide Y (Raza *et al.*, 2004; Deshpande *et al.*, 2010). The process of enzymatic treatment and mechanical trituration can add minimal stress during acute dissociation of neurons. However, we have shown previously that the neurons isolated using these procedures exhibit electrophysiological properties identical neurons in slices or in cultures (Raza *et al.*, 2007), are viable, and not apoptotic or necrotic (Sun *et al.*, 2008).

Calcium calibration

We performed Ca^{2+} calibration determinations as described previously (Raza *et al.*, 2004; Deshpande *et al.*, 2010) to provide estimates of absolute $[\text{Ca}^{2+}]_i$ concentrations from the 340/380

ratio values. A Ca^{2+} calibration curve was constructed using solutions of calibrated Ca^{2+} buffers ranging from 0 Ca^{2+} (Ca^{2+} free) to 39 μM Ca^{2+} (Invitrogen, Carlsbad, CA). Values from the calibration curve were used to convert fluorescent ratios to $[\text{Ca}^{2+}]_i$. Final $[\text{Ca}^{2+}]_i$ were calculated from the background corrected 340/380 ratios using the equation (Grynkiewicz *et al.*, 1985):

$$[\text{Ca}^{2+}]_i = (K_d \times S_{f2} / S_{b2}) \times (R - R_{\min}) / (R_{\max} - R)$$

where R is the 340/380 ratio at any time; R_{\max} is the maximum measured ratio in saturating Ca^{2+} solution (39 μM free Ca^{2+}); R_{\min} is the minimal measured ratio Ca^{2+} free solution; S_{f2} is the absolute value of the corrected 380-nm signal at R_{\min} ; S_{b2} is the absolute value of the corrected 380-nm signal at R_{\max} ; the K_d value for Fura 2 is 224 nM.

Fluoro-Jade staining

Animals were sacrificed 48-h following POX exposure and received all the antidotes at the appropriate times as outlined in POX SE section above. Briefly, deep anesthesia was induced in rats with ketamine/xylazine (75mg/kg/7.5mg/kg i.p.) mixture. Anesthetized animals were flushed transcardially with saline and perfused with 4% paraformaldehyde in a 100 mM sodium phosphate buffer (pH 7.4). Fixed brains were removed and post-fixed in 4% paraformaldehyde/phosphate buffer overnight, cryoprotected in 30% sucrose/phosphate buffer (pH 7.4) (48 h), flash frozen in isopentane and stored at -80°C until used for sectioning. Coronal sections (40 μm) were cut on a cryostat (Leica Microsystems, Wetzlar, Germany) and stored at -20°C in a cryoprotectant solution of 0.1 M phosphate buffer containing 30% sucrose, 30% ethylene glycol and 1% polyvinyl-pyrrolidone (PVP-40). Sections were washed three times in a 0.1 % triton in PBS and then float-mounted onto microscope slides (Trubond 380; Tru Scientific LLC, Bellingham, WA) and allowed to dry at room temperature overnight prior to staining.

Slides were incubated in a solution of 1% NaOH in 80% ethanol for 5 minutes followed by hydration in a 70% ethanol and then ddH₂O for 2 minutes each. Slides were then incubated in a 0.06% KMnO₄ solution for 10 min followed by washing in ddH₂O for 2 min. Slides were then stained in a 0.0004% fluoro-Jade C solution in 0.1% acetic acid for 20 min. Stained slides underwent three washes in ddH₂O for 2 min each and then dried in a desiccant chamber at 55°C for 30 min. Stained slides were then cleared with xylene for 5 min and cover slipped with DPX mounting agent (Sigma Chem. Co., St. Louis, MO). Stained sections were evaluated with a fluorescent IX70 inverted microscope with a 20X (UAp0 340, 0.7 n.a., water) objective (Olympus America, Center Valley, PA) and excitation/emission filters for visualization of FITC. Greyscale digital images (1324x1024, 16-bit, 1X1 binning) of Fluoro-Jade C staining for select brain regions including dentate gyrus hilus, were acquired with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan).

Data analysis

In dose-response experiments, the response to POX is expressed as the percentage of animals exhibiting SE or mortality. LD₅₀ values representing the estimated doses causing SE or mortality in 50% of animals with their 95% confidence limits (95% CL) were determined by log-probit analysis using the Litchfield and Wilcoxon method.

For comparison of [Ca²⁺]_i between sham and POX-treated animals, Student's *t*-test or One Way Analysis of Variance (ANOVA) was applied when appropriate to compare [Ca²⁺]_i. The Tukey test was used for all post-hoc comparisons. For comparing the distributions of [Ca²⁺]_i levels a Chi-square test was used. Statistical tests were run and graphs generated with SigmaPlot 12.5 (SPSS Inc, Chicago, IL). *p*<0.05 was considered significant.

We used 5-9 animals for each experimental condition or time point studied. Dissociating the hippocampal slices routinely yielded 20-25 healthy, phase-bright neurons that were used for the recordings. The means of each group of neurons from each animal were used to evaluate results and conduct statistical analysis. Viable neurons included in the study had a smooth surface and a pyramidal-like morphology with processes. Non-viable neurons were swollen or circular and the surface was uneven and irregular. Data from each animal were pooled together in respective groups and ultimately represented as total number of cells studied.

Analysis of digital images to count FluoroJade C positive cell staining was carried out with ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, MD) by thresholding for specific stain and obtaining positive cell counts using the particle analysis component (size range in pixel: 25-1000). All parameters for digital acquisition and analysis of staining remained constant throughout. Representative digital images were processed with Adobe Photoshop for figures (Adobe Systems Inc., San Jose, CA).

Results

Effects of POX administration

Paraoxon (POX, 0.1-4 mg/kg, s.c., n= 5-8) was injected and behavioral responses were observed. Rats manifested gross behavioral changes such as increased grooming, sniffing and chewing that were rapidly followed by tremors, wet-dog shakes and Straub tail response. Cholinergic symptoms appeared shortly thereafter which included respiratory distress (croaking, bradypnea), ex-opthalmus, salivation, defecation and urination. Following higher doses of POX, tonic-clonic seizures appeared and rats rapidly went into status epilepticus (SE). Without antidotal treatment of the severe cholinergic crisis, death ensued rapidly in all POX SE animals, demonstrating the lethal effects of this dose of POX exposure. POX induced a dose-dependent

increase in the incidence of SE and mortality. The LD₅₀ for induction of seizure was 0.39 mg/kg (95% CL: 0.32-0.47) and for mortality it was 0.85 mg/kg (95% CL: 0.32-2.19). In addition, the time to onset of POX SE was also dose-dependent. At low dose (0.5 mg/kg) it was 18.7 ± 1.1 min, whereas at high dose (4 mg/kg) it was 3.2 ± 0.2 min. The seizure responses were scored on a five point Racine scale (see methods). At doses 1 mg/kg and above a maximum Racine score of 5 was noted. At low POX doses not associated with mortality, animals spent 40 min in SE in a 1-h observation period. At POX doses associated with mortality, the time of death was also dose-dependent. This data is summarized in Table 1 and 2.

Effects of atropine, 2-PAM and diazepam following acute POX poisoning

The three drug regimen consisting of atropine to treat cholinergic symptoms, 2-PAM as an antidote to AChE inhibition and diazepam for stopping seizures is FDA approved for OP poisoning and being used by military to treat nerve agent exposure (Chemical Hazards Emergency Medical Management, 2013). We investigated the efficacy of these agents in our rat model of lethal POX poisoning. For these experiments we tested the highest dose of POX (4 mg/kg) as this ensured that 100% animals would undergo SE and without intervention resulted in 100% mortality. Such criteria allowed for rigorous testing of the three drug intervention. Mortality was assessed at 1-h, 24-h, 48-h and 72-h following POX injection.

Atropine (2 mg/kg, saline, i.p.) was injected 1-min after POX injection (4 mg/kg, ice-cold PBS, s.c.). All rats (n= 24) went into SE. Mortality was 30% 1-h after SE. At 24-h following SE, mortality was 60% and it was 70% and 90% at 48 and 72-h respectively. Thus, while atropine relieved the cholinergic symptoms and helped in reducing early mortality, the progressive AChE inhibition and SE still led to significant mortality.

Rats (n= 24) were then injected with 2-PAM (25 mg/kg, saline, i.m.) along with atropine (2 mg/kg, i.p.) 1-min after POX injection (4 mg/kg, s.c.). Mortality was almost halved to 15% 1-h after SE when 2-PAM was introduced. At 24-h following SE, mortality was 50% and it was 60% and 80% at 48 and 72-h respectively. Thus, while atropine and 2-PAM administration dramatically lowered the early mortality, the unremitting seizures and SE led to greater mortality in the subsequent days. Racine scores were not affected in the presence of the atropine and 2-PAM either alone or in combination suggesting that these agents did not affect severity of SE.

Rats (n= 24) were administered diazepam (5 mg/kg, i.p.) at 1, 3 and 5-h following POX (4 mg/kg, s.c.) induced SE, in addition to atropine and 2-PAM administration 1-min following POX injection. Mortality rate of 12% at 1-h following POX induced SE was in a similar range as observed above. However, when the SE was terminated with sequential diazepam injections, mortality was dramatically reduced to 8% at 24-h and 17% at 48 and 72-h. These findings suggested that stopping the OP induced SE is critical to reduce the mortality and improve long-term survival. This survival data has been summarized in Table 3. Rats surviving lethal POX exposure at the 72-h time point were returned to the vivarium and housed one per cage. POX treated rats were re-assessed for mortality at 3, 6 and 12-months post rescue from POX SE. Taking into account the normal age-related attritions (<5%) in our rat populations, no further increases in mortality were observed in POX SE survivors. Thus, this lethal POX survival model is ideally suited to prepare animals to study long term effects of POX exposure.

EEG characterization of POX induced SE

POX induced seizures were also confirmed with EEG recordings. Baseline EEG was recorded for 30-min before POX injection (4 mg/kg, s.c.). A representative recording is shown in Fig. 1. POX induced seizure onset was very rapid and the tonic-clonic seizure activity occurred

in parallel with high rhythmic epileptiform EEG activity as shown in Fig 1a (expanded portion). Within 7-8 minutes, animals progressed into electrographic SE. The SE was intense, did not wax or wane and did not lose its intensity throughout the one-hour of seizure activity displaying 7-10 Hz spike activity (Fig. 1a). Animals were injected with diazepam (5 mg/kg) plus 2-PAM (25 mg/kg) at 1, 3 and 5-h following onset of POX SE. This dose of diazepam was effective in completely terminating both behavioral and electrographic SE (Fig. 1, DZP). The EEG pattern for SE termination for up to 24-h following diazepam is shown in Fig. 1. It demonstrates the effectiveness of optimized 3-dose regimen in that seizures remained suppressed upon drug intervention and did not reoccur in days following SE termination.

The electrophysiological progression of POX induced SE is depicted in Fig. 2. EEG alterations following POX administration were rapid and correlated with behavioral seizure manifestations. Fig. 2A shows a baseline EEG recording before POX injection. Low-voltage fast activity started to appear within 2-3 min of POX administration (Fig. 2B). High-voltage slow activity (Fig. 2C) appeared shortly thereafter and rapidly progressed into high-voltage spiking in correlating with behavioral seizure activity by approximately 5-6 minutes (Fig. 2E). By this time animals were in electrographic SE with fully developed seizures. SE continued unabated for one hour, after which time they were terminated using sequential diazepam injections.

POX induced SE caused elevations in hippocampal neuronal $[Ca^{2+}]_i$

To investigate if POX exposure caused long lasting changes in hippocampal neuronal $[Ca^{2+}]_i$, similar to other OPs and chemoconvulsants (Raza *et al.*, 2004; Deshpande *et al.*, 2010), we measured neuronal $[Ca^{2+}]_i$ after 1-h of SE using the high affinity, ratiometric Ca^{2+} indicator Fura-2 in acutely isolated hippocampal neurons from control and POX exposed SE animals. Representative pseudocolor images of neurons from control and POX-SE rats are shown in Fig.

3A. Acutely isolated CA1 hippocampal neurons harvested from animals that underwent 1-h POX induced SE manifested mean $[Ca^{2+}]_i$ of 675 ± 48 nM (n= 6 animals), were significantly higher than $[Ca^{2+}]_i$ in neurons harvested from age-matched controls (163 ± 16 nM, n= 5 animals) as shown in Fig. 3B ($p < 0.001$, *t*-test).

Analysis of the population distributions of $[Ca^{2+}]_i$ revealed only 5% of control neurons exhibited $[Ca^{2+}]_i$ greater than 500 nM, with the majority of neurons falling into the lower Ca^{2+} concentration range. In contrast, approximately 60% neurons isolated from POX SE rats exhibited $[Ca^{2+}]_i$ greater than 500 nM indicating a population shift towards higher Ca^{2+} concentration range. This rightward shift in distribution of $[Ca^{2+}]_i$ levels in POX SE neurons was significantly different from control neurons (Fig. 4, $p < 0.001$, Chi-square test, n= 136 neurons).

Development of calcium plateau following POX induced SE

To investigate the duration of elevated hippocampal $[Ca^{2+}]_i$ following the termination of POX induced SE, we measured $[Ca^{2+}]_i$ at various time points for up to one month. As shown in Fig. 5, at 1, 7 and 30-days following SE termination, $[Ca^{2+}]_i$ (in nM) were 514 ± 14 , 483 ± 32 and 406 ± 28 respectively. These values were significantly higher than $[Ca^{2+}]_i$ of neurons harvested from age-matched control rats ($p < 0.001$, one-way ANOVA, n= 6 animals). These results demonstrate that POX SE in this model produced sustained elevations in intracellular Ca^{2+} -concentrations (Ca^{2+} plateau).

Mechanism for calcium plateau following POX induced SE

We have demonstrated that SE leads to development of Ca^{2+} plateau (Raza *et al.*, 2004; Deshpande *et al.*, 2010) that has its origins in Ca^{2+} release from intracellular stores (Nagarkatti *et al.*, 2010). To investigate contribution of intracellular CICR receptors in the development of

POX Ca^{2+} plateau, $[\text{Ca}^{2+}]_i$ were measured in neurons obtained from POX SE rats in the presence of RyR **blocker** dantrolene (Nagarkatti *et al.*, 2010). As shown in Fig. 6, application of dantrolene (50 μM) to hippocampal neurons isolated from rats 24-h following POX SE resulted in a significant drop in $[\text{Ca}^{2+}]_i$ from 514 ± 14 nM (no drug) to 324 ± 24 nM ($p < 0.01$, $n = 5$ rats). This represent almost 60% reduction in $[\text{Ca}^{2+}]_i$ following dantrolene application suggesting that RyRs are significantly contributing to maintaining the POX SE induced Ca^{2+} elevations.

Following the efficacy of dantrolene in lowering POX SE induced Ca^{2+} elevations in vitro, we next investigated the effects of in vivo administration of levetiracetam and carisbamate. We have shown these drugs to target RyR/ IP_3 receptors and also produce an antiepileptogenic response following SE-like injury (Deshpande *et al.*, 2008a; Nagarkatti *et al.*, 2008). As shown in Fig. 6, $[\text{Ca}^{2+}]_i$ in hippocampal neurons isolated from rats treated with three doses of levetiracetam (50 mg/kg, i.p., 1, 6 and 18-h post POX SE onset) were 290 ± 10 nM that were significantly lower than $[\text{Ca}^{2+}]_i$ in neurons isolated from untreated POX SE rats ($p < 0.001$, $n = 5$ rats). Similarly, $[\text{Ca}^{2+}]_i$ in hippocampal neurons isolated from rats treated with three doses of carisbamate (90 mg/kg, i.p., 1, 6 and 18-h post SE onset) were 348 ± 26 nM that were significantly lower than $[\text{Ca}^{2+}]_i$ in neurons isolated from untreated POX SE rats ($p < 0.001$, $n = 5$ rats) as shown in Fig. 6.

Neuronal injury associated with acute POX intoxication

To assess neuronal injury in animals surviving exposure, brain sections from animals injected with POX or vehicle were labeled with Fluoro-Jade C (FJC) (Li *et al.*, 2011). Across all brain regions examined, there was negligible FJC labeling in brain sections obtained from vehicle controls. In contrast, after POX treatment, FJC-positive cells were observed in select regions throughout the forebrain. Within the hippocampus, FJC-positive staining was observed

in the polymorphic layer and along the hilus/granule cell border of the dentate gyrus.

Additionally, FJC-positive stained neurons were observed throughout layers II and III of the parietal cortex (Fig. 7A). The quantification of the neuronal injury expressed as FJC positive cells is shown in Fig. 7B.

Discussion

The ready availability of pesticides and other toxic agricultural and industrial chemicals make these agents attractive target for terrorists to weaponize them and cause civilian casualties (Federation of American Scientists, 1998; Gould and Folb, 2003; Central Intelligence Agency, 2004; Croddy *et al.*, 2004; Moorcraft and McLaughlin, 2008). It is therefore important to study mechanisms for reducing mortality and morbidity upon exposure to OP agents. Compounds like DFP and POX mimic the actions of sarin, soman and VX, and are therefore utilized in a controlled experimental setting to model the effects of nerve agent exposure in civilian laboratories (Kadriu *et al.*, 2009; Wright *et al.*, 2009; Zaja-Milatovic *et al.*, 2009; Deshpande *et al.*, 2010; Li *et al.*, 2011; Todorovic *et al.*, 2012). The POX lethal exposure and survival model optimized in this study provides an ideal animal model to develop medical counter measures to POX toxicity in a reliable and cost effective manner. Our survival model after lethal POX exposure offers a valuable contribution to the field by providing a reproducible system to study the chronic effects of POX toxicity and the development of OP exposure co-morbidities. In addition, this study demonstrates comparable findings to other OP agents such as DFP (Kadriu *et al.*, 2009; Wright *et al.*, 2009; Zaja-Milatovic *et al.*, 2009; Deshpande *et al.*, 2010; Li *et al.*, 2011), the chemoconvulsant pilocarpine (Raza *et al.*, 2004), and chemical threat agents such as Tetramethylenedisulfotetramine (TETS) (Cao *et al.*, 2012), that POX exposure produces elevated neuronal $[Ca^{2+}]_i$ and injury. These changes in intracellular Ca^{2+} concentrations

persisted for over a month following the termination of POX SE, and in combination with the observed with the multifocal neuronal injury, may underlie some of the long-term effects of OP toxicity on neuronal function and behavioral impairments (DeLorenzo *et al.*, 2005; Filbert *et al.*, 2005; de Araujo Furtado *et al.*, 2012). Furthermore, identification of mechanisms responsible for injury-induced Ca^{2+} plateau may elucidate novel targets and candidate drugs for the potential treatment of long-term morbidity associated with POX and other OP exposures.

Following high dosages of POX, the enzyme AChE is rapidly inhibited, resulting in a massive buildup of acetylcholine at the synapses. A cholinergic crisis ensues that result in the progression of bradypnea, bradycardia and convulsions, followed by rapid death (Bajgar, 2004; Hoffmann and Papendorf, 2006). The three drug regimen presented in this study consisting of atropine, 2-PAM and diazepam is FDA approved and used by US military (Mark I kit), and is the mainstay treatment for OP poisoning/ nerve agent toxicity (Chemical Hazards Emergency Medical Management, 2013). A higher survival rate was obtained following lethal POX exposures by optimizing the standard three drug regimens used for treating OP poisoning. The mortality following lethal POX intoxication was most effectively reduced by atropine, in agreement with previous nerve agent studies (Shih *et al.*, 2007). A lower dose of atropine (0.1-0.2 mg/kg), similar to auto injector doses used in human pre-exposure situations for OP intoxication, was effective in reducing mortality by 20% in our animal model (data not shown). However, since we wanted to develop an animal model of lethal OP exposure survival in order to study long-term morbidities, we increased atropine concentration to 2 mg/kg. This higher dose of atropine, along with 2-PAM and diazepam intervention, resulted in a significantly higher survival rate of 85%. This is in line with findings from our previous report following lethal DFP exposure (Deshpande *et al.*, 2010). Our results show that while each drug by itself is critical to

limit acute mortality, stopping the SE with diazepam is by far the most important intervention to significantly reduce long-term mortality. This is in agreement with the clinical literature that reports a high mortality in patients undergoing SE (DeLorenzo *et al.*, 2009).

POX induced SE produced prolonged elevations in neuronal Ca^{2+} concentrations similar to those seen with DFP and pilocarpine (Raza *et al.*, 2004; Deshpande *et al.*, 2010). These elevations were sustained and lasted for up to one-month following the termination of SE. This Ca^{2+} plateau had its origin in Ca^{2+} release from intracellular stores, since intervention with CICR receptor inhibitors dantrolene (Nagarkatti *et al.*, 2010) or levetiracetam (Nagarkatti *et al.*, 2008) abolished the Ca^{2+} plateau when administered in vitro or in vivo. The Ca^{2+} plateau following SE is thought to trigger plasticity changes during the period of epileptogenesis that ultimately results in the development of acquired epilepsy (DeLorenzo *et al.*, 2005). Indeed, treatment with the purported antiepileptogenic agent carisbamate prevented the development of spontaneous epileptiform discharges in hippocampal neurons following SE (Deshpande *et al.*, 2008a). These epileptiform discharges are a result of altered Ca^{2+} dynamics following SE-like injury (DeLorenzo *et al.*, 2005). In line with these studies, treatment with carisbamate lowered POX SE mediated elevations in intracellular Ca^{2+} levels. It will be interesting to evaluate if Ca^{2+} levels revert back to higher concentrations following removal of drugs. This may necessitate multiple drug injections to maintain lower Ca^{2+} levels post SE in order to prevent development of Ca^{2+} mediated altered synaptic plasticity.

It has been demonstrated that SE is associated with significant cell death in various brain regions (DeLorenzo *et al.*, 2005). This neuronal death is thought to be Ca^{2+} dependent since manipulations that lower neuronal Ca^{2+} entry before or during SE are effective in producing significant neuroprotection. Thus, NMDA receptor antagonist MK-801 given before SE prevents

the protracted elevations in $[Ca^{2+}]_i$ inhibits epileptogenesis and other SE related morbidities (Rice *et al.*, 1998; Raza *et al.*, 2004; DeLorenzo *et al.*, 2005; Deshpande *et al.*, 2010). We have also demonstrated that while NMDA receptor mediated Ca^{2+} entry is essential for generation of the Ca^{2+} plateau; it does not play a role in its maintenance (Raza *et al.*, 2004; Deshpande *et al.*, 2010). Thus, MK-801 administered 1-h after SE cannot prevent the development of Ca^{2+} plateau and does not afford neuroprotection (Raza *et al.*, 2004; DeLorenzo *et al.*, 2005). However, CICR from intracellular stores contributes to the maintenance of the Ca^{2+} plateau such that inhibition of RyRs not only abolishes Ca^{2+} plateau, but it also prevents the development of SE-like injury induced spontaneous epileptiform discharges in hippocampal neurons in vitro (Nagarkatti *et al.*, 2010). We hypothesize that it is this Ca^{2+} entry occurring during SE and not the Ca^{2+} plateau generated after one-hour of SE that contributes to neuronal death. It will be interesting to investigate if the other brain regions associated with neuronal death also manifest similar Ca^{2+} elevations. The CICR agents used in this study are administered one-hour after SE at which point they may not be able to prevent the on-going neuronal death processes. However, by inhibiting the sustained Ca^{2+} elevations post SE we predict that they may be able lower the morbidities associated with SE. This possibility is currently being evaluated by our laboratory.

We have observed POX intoxicated rats for up to one-year following their rescue with the three-drug regimen optimized in this study. After taking into consideration the normal age-related attrition in the population of rats housed in our vivarium, we did not observe increases in mortality percentage in POX SE rescued rats. To our knowledge this is the first study that has followed the mortality in rats surviving lethal POX exposure for an extended period of time out to one year. An SE survival model of POX intoxication should also exhibit long-term consequences of OP exposure indicative of cognitive deficits and co-morbidities (Steenland *et*

al., 1994; Brown and Brix, 1998; Ivens *et al.*, 1998; Wesseling *et al.*, 2002; Ruckart *et al.*, 2004; Edwards and Tchounwou, 2005; de Araujo Furtado *et al.*, 2012; Terry *et al.*, 2012). In our preliminary results we have observed the development of chronic seizures, depressive symptoms and memory impairments in POX SE survivor rats (data not shown). The complete assessment of chronic neurological disorders in POX survivors is currently underway in our laboratory. However, these preliminary observations further validate our proposed model.

We have demonstrated that CNS injuries such as SE, stroke or traumatic brain injury result in neuronal injury and protracted elevations in hippocampal $[Ca^{2+}]_i$ (Rice *et al.*, 1998; Raza *et al.*, 2004; Deshpande *et al.*, 2008b; Deshpande *et al.*, 2010). Since Ca^{2+} is a major second messenger molecule, such sustained increases in its levels activate multiple signaling cascades, affect protein transcription and alter gene expression of proteins involved in controlling membrane excitability and synaptic plasticity (DeLorenzo *et al.*, 2005). These Ca^{2+} mediated alterations are thought to underlie the long-term morbidity following CNS insults (DeLorenzo *et al.*, 2005; Deshpande *et al.*, 2008b). OP exposure has been reported to be associated with neurological problems such as seizures, cognitive deficits and other behavioral disorders (de Araujo Furtado *et al.*, 2010; de Araujo Furtado *et al.*, 2012). Inhibition of the POX SE induced Ca^{2+} plateau with a regimen of FDA approved drugs could prove to be novel countermeasure agents to potentially prevent the morbidity associated with OP poisoning.

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Figure Legends

Figure 1. A representative continuous 24-h EEG recording from a rat before, during and after POX induced SE. Baseline EEG activity is noted before POX exposure. This leads to intense SE minutes after POX administration (4 mg/kg, s.c.). (a) Expanded Depicts an expanded EEG segment of SE characterized by high frequency spiking. SE is robust, intense and doesn't wax and wane for the entire 1-h. SE is stopped at 1-h following administration of diazepam (DZP). (b) Expanded EEG segment after 1st DZP (5 mg/kg, i.p.) depicting rapid termination of SE. Two additional DZP and 2-PAM injections are administered at 3 and 5-h after SE onset. SE remained suppressed and seizures did not reoccur for the 24-h period following POX toxicity symptoms. Traces are representative of n= 6 animals.

Figure 2. EEG progression of POX induced SE. (A) Baseline activity prior to POX injection. (B) Low-voltage fast activity appears within 2-3 min of POX administration. (C) High-voltage slow activity appears within the next minute that progresses to (D) high frequency and high-voltage spiking and (E and F) sustained continuous seizure activity. EEG activity at 15-min following POX administration (F) demonstrates electrographic SE with fully developed continuous seizures. Spike frequency during SE was sustained at greater than 7- 10 Hz. SE was induced by POX within 5-7 min following injections. EEG activity correlated with behavioral observations.

Figure 3. Effect of POX induced SE on $[Ca^{2+}]_i$. (A) Pseudocolor ratiometric images of representative control and POX treated isolated neurons. Control neurons had bluish color that corresponds to lower Fura-2 ratio while POX-SE neurons had orange-red color that corresponds to higher Fura-2 ratio. (B) Elevated $[Ca^{2+}]_i$ in CA1 hippocampal neurons acutely isolated from

animals that had experienced 1-h of POX induced SE compared to neurons from control animals. (* $p < 0.001$, t -test, $n = 6$ and 5 animals respectively). Data are represented as mean \pm SEM.

Figure 4. Distribution of $[Ca^{2+}]_i$ for control and POX-SE hippocampal neurons. Control neurons demonstrated a normal distribution for $[Ca^{2+}]_i$ with approximately 95% of neurons exhibiting $[Ca^{2+}]_i$ less than 500 nM and only 5% neurons exhibiting very high $[Ca^{2+}]_i$. In contrast, POX-SE neurons demonstrated a rightward shift towards higher $[Ca^{2+}]_i$ with approximately 60% neurons exhibiting $[Ca^{2+}]_i$ greater than 500 nM ($n = 136$ neurons).

Figure 5. Development of Ca^{2+} plateau following POX induced SE. CA1 hippocampal $[Ca^{2+}]_i$ from control (white bar) and POX rats were isolated immediately after (1-h) and 1, 7 and 30 days after SE (black bars). $[Ca^{2+}]_i$ in control animals at each time point were not significantly different from the control values shown and thus were omitted from the graph for clarity. $[Ca^{2+}]_i$ in neurons isolated from POX-SE rats was significantly higher than control values (* $p < 0.05$, one-way ANOVA, post hoc Tukey test, $n = 6$ rats at each time). Data represented as mean \pm SEM.

Figure 6. Mechanism of Ca^{2+} plateau following POX SE. CA1 hippocampal $[Ca^{2+}]_i$ from control (white bar) and POX rats were isolated 24-h after SE (black bars). Dantrolene (Dant, 50 μ M, bath application) caused a significant decrease in elevated post SE Ca^{2+} levels. In separate experiments, rats were injected with either levetiracetam (LEV, 50 mg/kg, i.p.) or carisbamate (CRB, 90 mg/kg, i.p.) at 1, 6 and 18-h post SE onset. CA1 hippocampal neurons were isolated 30-mins post the last drug injection. $[Ca^{2+}]_i$ in neurons isolated from POX-SE rats treated with either LEV or CRB were significantly lower than comparable POX SE rats (no drugs) values at the respective time point. (* $p < 0.05$, compared to POX, one-way ANOVA, post-hoc Tukey test, $n = 5$ animals for each treatment). Data represented as mean \pm SEM.

Figure 7. POX SE induced neuronal injury. **A.** Representative photomicrographs of Fluoro-Jade C (FJC) staining in the (a) dentate gyrus-hilus region, (b) thalamus, (c) parietal cortex and (d) temporal/ peri-rhinal cortex of a control rat (left panel, c) and a POX rat 2 days after POX SE (4 mg/kg, s.c.) Scale bars, 200 μ m. **B.** Quantitative analyses of FJC labeling. FJC positive cells indicative of neuronal injury were observed in hilus, thalamus and cortex of POX rats 48-h after SE termination. Control rats did not exhibit any FJC labeling. (* $p < 0.05$ compared to control, t-test, $n = 4-6$ rats).

Table 1: Dose dependent effects of POX on SE and mortality

Dose of POX (mg/kg, s.c., n= 5-8)	% SE	% Mortality
0.1	7 ± 2	0
0.5	42 ± 7	26 ± 5
0.8	71 ± 6	50 ± 6
1	81 ± 4	88 ± 4
2	90 ± 2	100
4	100	100
8	100	100

Table 2: Characteristics of POX induced SE

POX dose (mg/kg, s.c., n=8)	SE onset time (min.)	Racine Score (scale: 0-5)	Time of death (min.)
0.1	37 ± 3	2	-
0.5	19 ± 1	2	32 ± 3
0.8	11 ± 1	4	26 ± 4
1	7 ± 0.5	5	12 ± 1
2	5 ± 0.5	5	7 ± 0.5
4	3 ± 0.2	5	5 ± 0.2
8	2 ± 0.4	5	3 ± 0.2

Table 3: Role of three-drug regimen in promoting survival following lethal POX intoxication

Time post SE	Percent survival following POX SE in the presence of			
	No drug	Atropine	Atropine + 2-PAM	Atropine + 2-PAM + diazepam
1-h	0	71 ± 4	83 ± 6	88 ± 4
24-h	-	41 ± 4	50 ± 6	83 ± 6
48-h	-	33 ± 6	41 ± 4	79 ± 8
72-h	-	12 ± 4	21 ± 4	79 ± 8

n= 8 for no drug condition and n= 24 for each separate drug combination

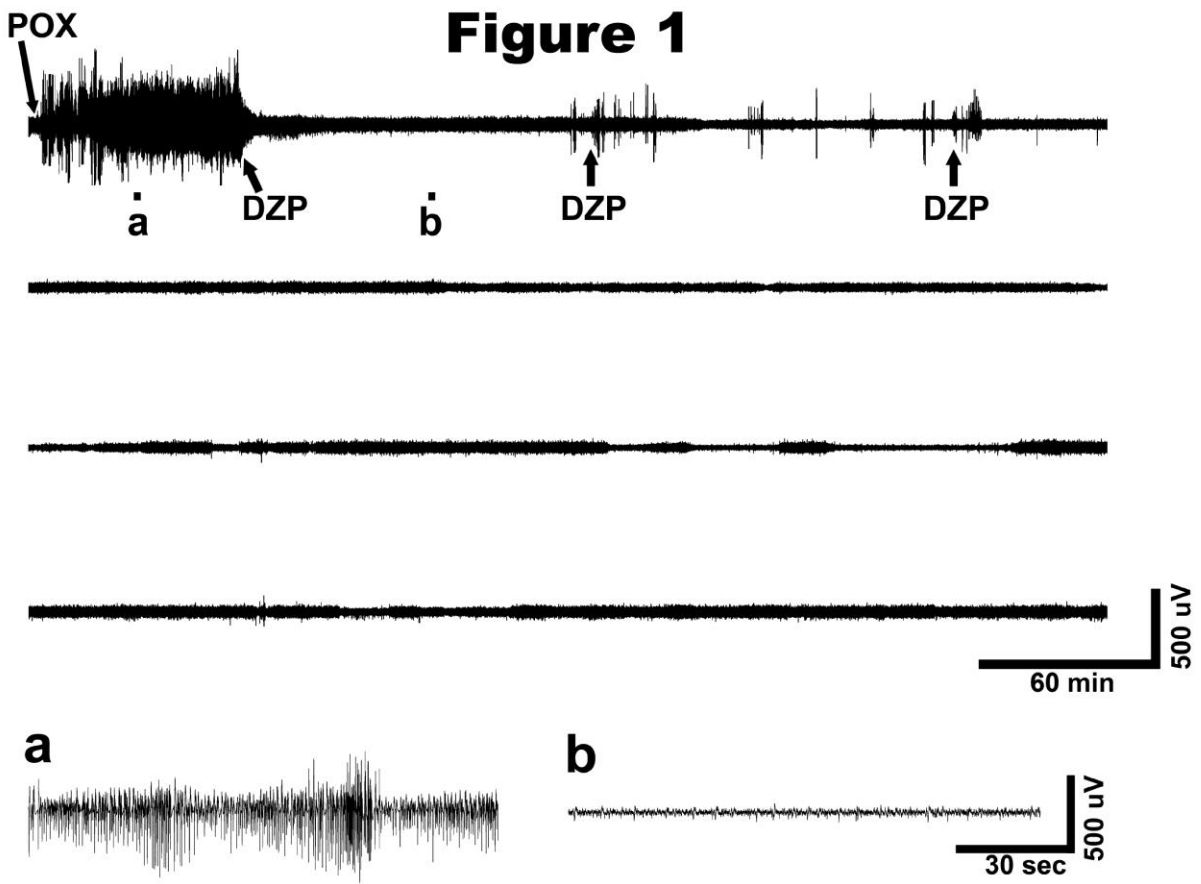
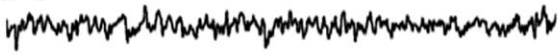
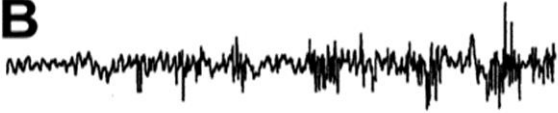


Figure 2

A



B



C



D



E



F



Figure 3

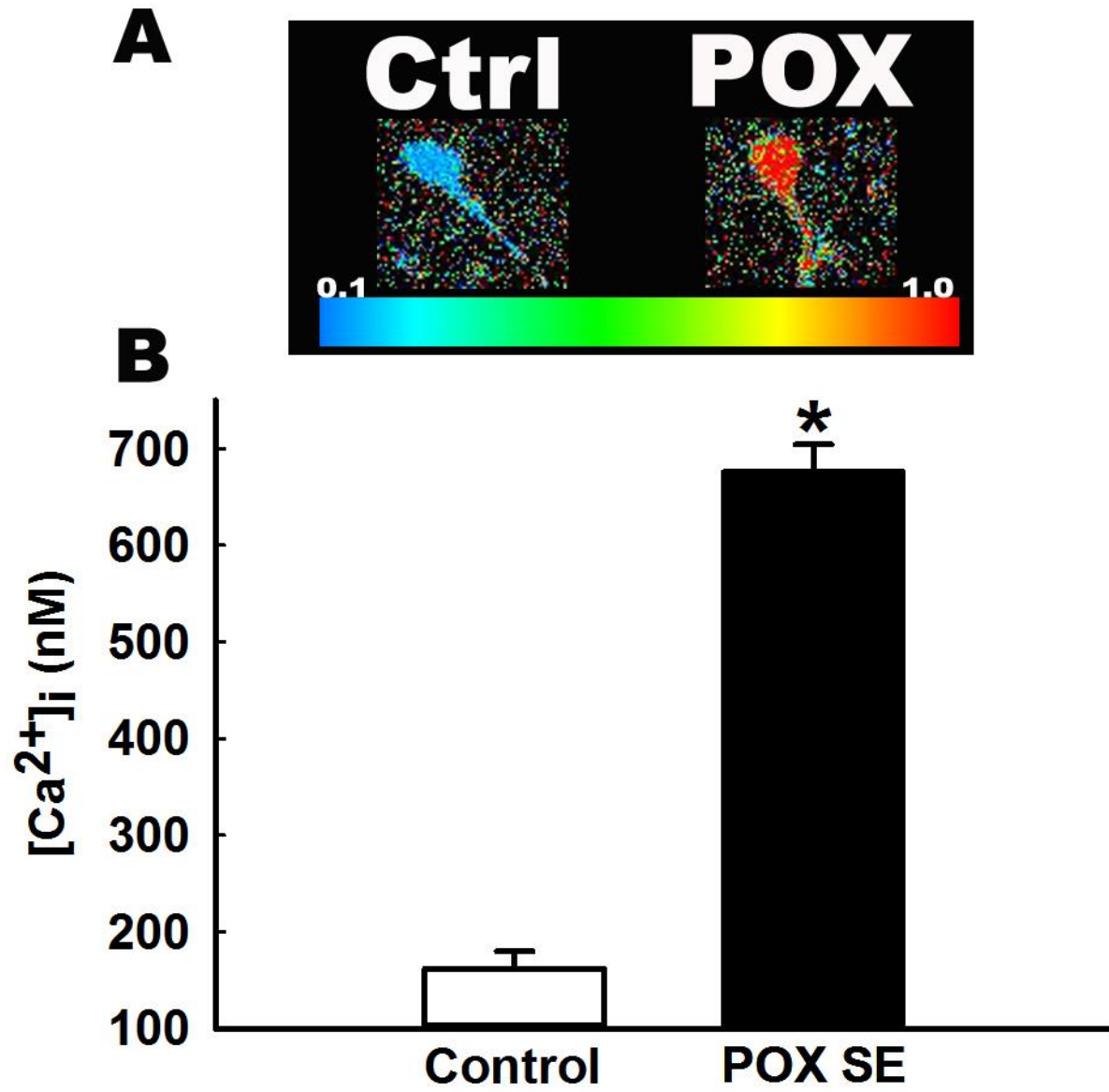


Figure 4

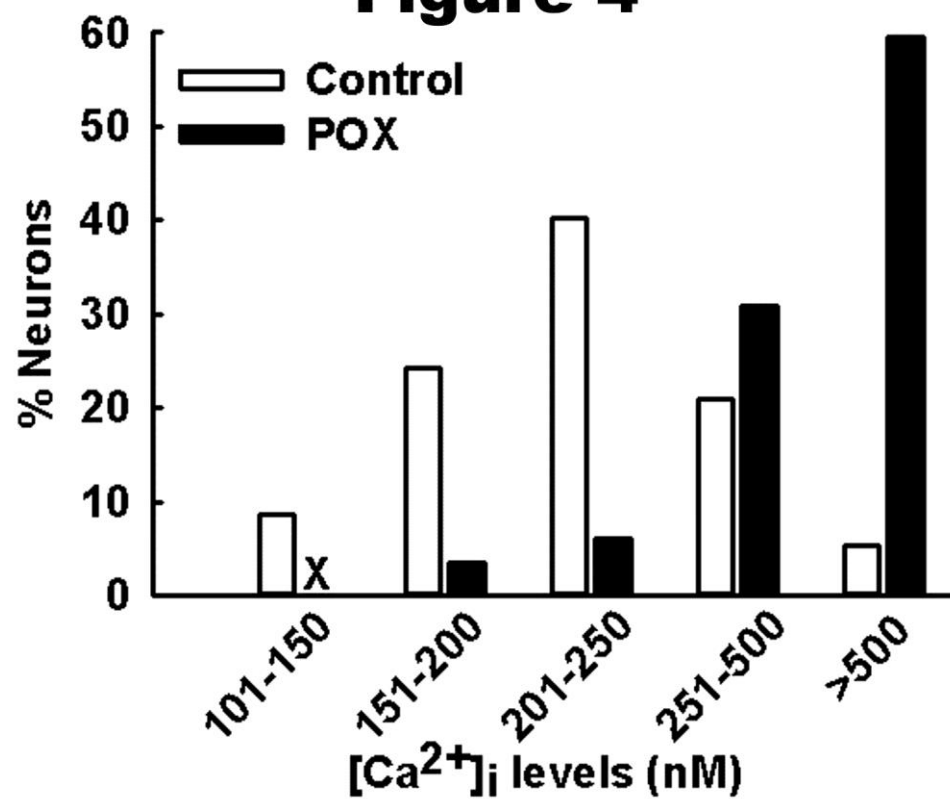


Figure 5

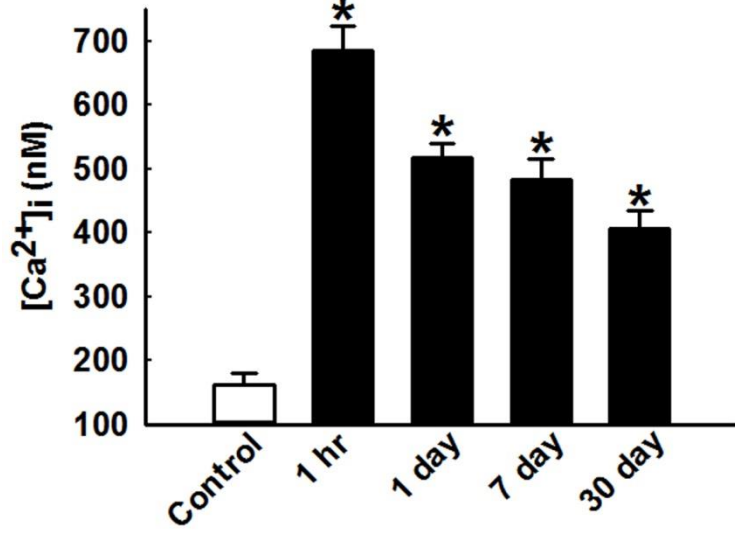


Figure 6

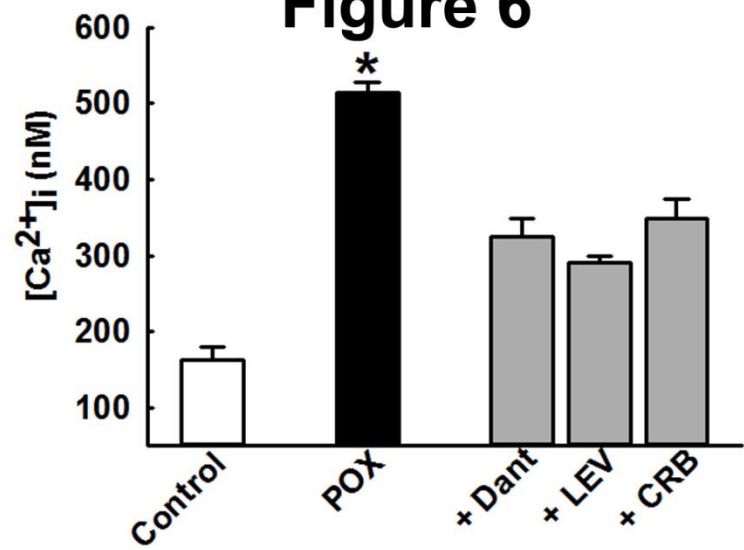
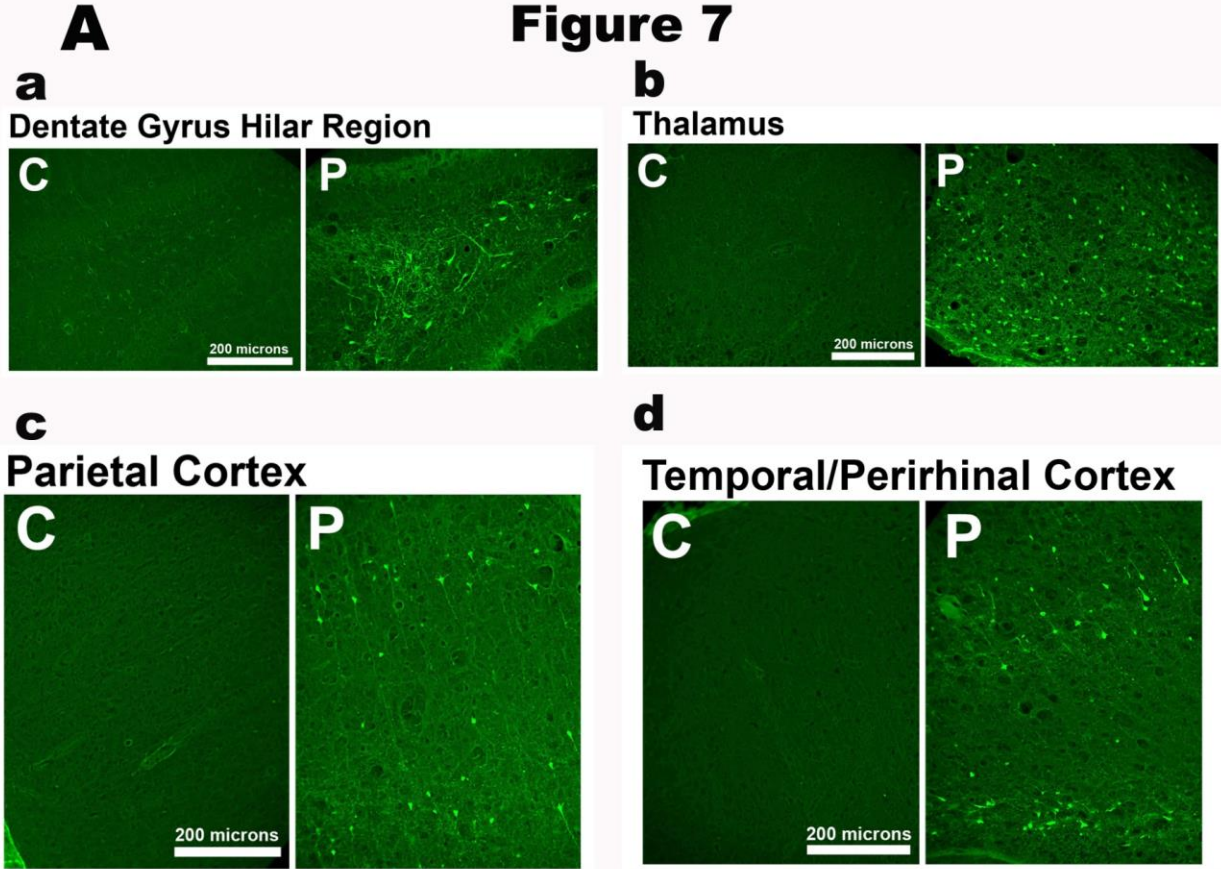


Figure 7



B

