CHLORPYRIFOS EXPOSURE RESULTS IN ALTERED MITOCHONDRIAL DYNAMICS, DEFICITS IN AXONAL TRANSPORT AND IMPAIRMENTS OF SUSTAINED ATTENTION

by

Mary-Louise Risher

(Under the Direction of Alvin V. Terry Jr.)

ABSTRACT

The risk of exposure to significant levels of organophosphate (OP)-based chemicals such as chlorpyrifos (CPF) is substantial for humans worldwide given their widespread use as pesticides in household, agricultural, and industrial environments. The acute toxicity of OPs (including CPF) to target and non-target organisms has been extensively studied and is believed to result from irreversible acetylcholinesterase inhibition and subsequent overstimulation of cholinergic neurons. However, the consequences of repeated exposures to levels of OPs that produce no overt signs of acute toxicity (i.e., subacute levels) are poorly understood. There is substantial epidemiologic evidence that this type of exposure results in prolonged deficits in attention and other domains of cognition long after cholinesterase activity has normalized. However, few prospective studies have addressed this subject and moreover, potential neuronal mechanisms for the reported cognitive deficits have not

been adequately investigated. Therefore, the objectives of this thesis project were to 1) investigate (prospectively) the effects of repeated sub-acute exposures to CPF on cognition (particularly attention) in an animal model and 2) investigate potential neuronal mechanisms for prolonged OP-related effects on cognition *in vitro*. To address the first objective, adult rats were trained to stably perform a task of sustained attention, the five choice serial reaction time task, then treated with vehicle or CPF 18.0 mg/kg for 14 consecutive days or every other day for 30 days. Behavioral testing was performed daily during the CPF-exposure period and throughout a 30 day washout to assess recovery. CPF-treated animals exhibited protracted impairments of sustained attention and an increase in impulsive behaviors without signs of impaired motivation or overt toxicity. For the second objective, rat primary cortical neurons were used to evaluate mitochondrial axonal transport and morphology after 1-24 hours of exposure to various concentrations of CPF and CPFoxon (active metabolite). Imaging studies revealed a concentration-dependent increase in mitochondrial length, a decrease in mitochondrial number, and impaired axonal transport of mitochondria at CPF concentrations that did not inhibit acetylcholinesterase. Given the importance of mitochondrial dynamics and axonal transport to neuronal function, these *in vitro* results provide potential mechanisms for CPF-related deficits in cognitive function observed in vivo.

INDEX WORDS: Chlorpyrifos; Pesticides; Cognition; 5-Choice Serial Reaction Time Task, Attention; Mitochondria; Axonal Transport; Mitochondrial Dynamics

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DEDICATION

To the people in my life (past and present) who believed in me when I could not.

To my husband, the calming water of my life.

And to Dr B.

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CHAPTER 1

INTRODUCTION

Organophosphates (OP) are among the most commonly used pesticides worldwide. They are used not just for agricultural purposes but also for the control of vector borne diseases, pest removal from buildings, on lawns and direct application to stagnant water (Aspelin, 1994). Compounds in this group, such as CPF, irreversibly inhibit cholinesterase activity (the enzyme responsible for halting cholinergic signaling) by phosphorylating the serine residue in the active site, resulting in an accumulation of acetylcholine in the synaptic cleft. This increased interaction between acetylcholine and its receptors on both the pre and post-synaptic terminals results in the over-stimulation of the cholinergic system (Pope et al., 2005;Rohlman et al., 2007). Unfortunately these effects are non-selective for insects, and as a result acute exposure can lead to a number of toxicological symptoms in including seizures, hypersecretory activity, and even death in humans and other non-target organisms (Pope, 1999). In light of the growing evidence that young animals exposed to CPF express developmental and neurobehavioral deficits, the EPA has restricted its use, banning CPF application to households, school playgrounds and other specified areas (US EPA, 2000). However despite a reduction in CPF use in the public domain, its use in agriculture (on grain crops, vegetables and fruits) has dramatically increased amounting to approximately 1.2 billion pounds of pesticide per year, of which 10 million pounds is CPF (EPA, 2009).

OP use in agriculture has greatly improved farming productivity but has made

compounds like CPF virtually ubiquitous in the environment (Weiss, 1997). Therefore it is not surprising that 73% of fresh fruits and vegetables, 61% of processed foods and 66% of drinking water within the U.S. contain residual, yet detectable levels of OPs (USDA, 2005;USDA, 2005) and that 96% of individuals in the US have residual levels of CPF metabolites in their urine (Barr et al., 2005). Recent studies have shown significant levels of CPF within households and farm worker families in many agricultural communities within the US despite the ban on household use (Bradman et al., 2007). Exposure is primarily due to transfer from farming equipment, clothing and air born particles despite U.S. EPA regulations and manufacturer recommendations (McCauley et al., 2003;Bradman et al., 2007). In addition, in many countries there is a lack of regulation, enforcement and/or compliance (Wesseling et al., 1997). Clear evidence of this is in a recent report by Farahat and colleagues (2010) in which Egyptian agricultural workers employ little if any caution when handling and applying CPF. This behavior resulted in up to 90% plasma AChE inhibition without any evidence of acute systemic toxicity. This was surprising since a single dose resulting in such high inhibition would normally result in overt signs of toxicity and hospitalization. Cognitive data are yet to be published, but this report does suggest that there are physiological adaptations that occur as a consequence of repeated exposures. Compounding the problem, especially in third world countries, is the high rate of illiteracy and the limited availability of medical care (Kesavachandran et al., 2009). The end result is high level exposure that would clearly exceed US EPA standards (Dyer et al., 2001;Farahat et al., 2010).

Levels of CPF that are considered safe by the EPA are primarily based on acute studies and/or neurodevelopmental studies and they do not adequately consider the effects of long-term sub-acute exposure (Pope, 1999). In addition, there is very little published data on the effects of repeated sub-acute exposures in human populations. This is because the more subtle symptoms (e.g. flu-like symptoms and headache), can be mistaken for general ill health and they can often go unrecognized and therefore untreated (Weiss, 1997). The work conducted in this thesis project is significant because it addresses potential toxicological effects associated with repeated sub-acute OP exposures, a major concern for high-risk populations such as agricultural workers (who frequently work with large quantities of pesticides).

Literature Review

CPF - Neurobehavioral Effects: Reports have shown that acute exposure to OPs can result in chronic neuropsychiatric symptoms including deficits in short-term memory, learning, attention, information processing and depression (De Silva et al., 2006) (Tan et al., 2009). Similar deficits have been observed after acute and/or repeated exposure to OPs through the use of various tasks including: digit span, continuous performance task, finger tapping, match to sample and reversal learning (Kamel et al., 2003;Rohlman et al., 2007;Steenland et al., 1994) Some of these neurological symptoms can linger for months to years after exposure despite recovery of the AChE activity, suggesting that there are additional mechanisms of action involved.

One of the major drawbacks of human case studies is that they are generally retrospective with a focus on high-level exposure that would result in overt systemic

effects and probable hospitalization. Even in some of the more recent reports (in agricultural workers) that assess chronic sub-acute exposure to CPF, cognitive data are lacking (Dyer et al., 2001;Farahat et al., 2010). Since using a human cohort is not ethically feasible (given the nature of this toxin), prospective data in humans with an emphasis on sub-acute CPF exposure is rare if not absent from the literature. Animal studies are an ideal way to address this problem allowing investigators to assess the effects of sub-acute, long-term and short-term OP exposure in a well defined environment. There are a number of OP-related studies that have been published using rodent models (see Morris, 1984;Sanchez-Santed et al., 2004;Morris, 1984;Moser et al., 2005). Despite the important results of the aforementioned studies, there are few studies that assess the effects of sub-acute exposure, and even fewer that assess the persistence of such OP-related symptoms (Ehrich and Jortner, 2001). Terry and collaborators have previously addressed some of these issues by reporting that rats exposed to repeated sub-acute levels of CPF exhibited impaired performance in the Morris Water Maze (spatial learning) and prepulse inhibition (pre-attentive processing) tasks, suggesting that acute signs of neurotoxicity are not necessarily an antecedent of cognitive impairment (Terry, Jr. et al., 2003;Terry, Jr. et al., 2007).

The 5-choice serial reaction time task (5C-SRTT) is a rodent analog of the human continuous performance task (CPT) that can be used to measure sustained attention, processing speed, impulsive- and compulsive-like behaviors see (Robbins, 2002). These cognitive measures are important since they influence working memory and decision making (i.e., executive function) in daily life, factors that strongly influence our ability to make appropriate and correct responses to a given stimulus (Smulders et

al., 2004). Previous studies have shown that acute exposure to CPF can result in deficits in the CPT, however, the effects of sub-acute exposures to CPF are unknown (Dassanayake et al., 2007). Therefore the 5C-SRTT is an ideal task to gain a prospective insight into the immediate and protracted effects of repeated sub-acute exposure to CPF in a rodent model.

CPF - Novel Mechanisms of Action: As mentioned previously, human exposure to OPs can result in cognitive symptoms that can persist even after cholinesterase levels have returned to normal, suggesting that the deleterious effects of OPs are not necessarily due to the effects on cholinesterase alone.

Several papers published in the 1980-1990's indicated that axonal transport might be impaired as a result of exposure to various toxins including OPs (Reichert and Abou-Donia, 1980;Moretto et al., 1987). In particular, Abou-Donia and collaborators reported an accumulation of tubulovesicular profiles within axons prior to axonal degeneration following OP exposure, consistent with membrane traffic stagnation (Chretien et al., 1981;Souyri et al., 1981; Abou-Donia and Lapadula, 1990). However, only recently has there been any direct evidence relating sub-acute exposures to OPs with deficits in axonal transport. Using the rodent paradigm mentioned previously (Terry, Jr. et al., 2003), it was determined that axonal transport of vesicles in the sciatic nerves (*ex vivo*) was significantly reduced (≥20%) after a 14 day exposure to 18mg/kg CPF which, persisted throughout a 14 day washout period (Terry, Jr. et al., 2003). Later, time course studies showed that a significant reduction in axonal transport could occur within 10 hours of a single 18mg/kg s.c. CPF exposure (Terry, Jr. et al., 2003). These data combined with other studies showing that CPF can inhibit microtubule

formation, covalently modify tubulin, and disrupt kinesin-driven movement, support the argument that axonal transport may be compromised by OPs (Howard et al., 2005;Grigoryan et al., 2008;Prendergast et al., 2007;Gearhart et al., 2007). Such disruptions can reduce the transportation of essential cargo (such as mitochondria) to and from the synaptic terminals, ultimately compromising synaptic maintenance. Moreover, a reciprocal (mutually dependent) relationship appears to exist between mitochondrial function and axonal transport. Specifically, axonal transport requires intact motor proteins moving along cytoskeletal networks (i.e., ATP-dependent processes) that depend on proper mitochondrial placement. Without the appropriate placement of mitochondria, ATP availability and buffering of intracellular Ca^{2+} is compromised resulting in the impairment of a variety of neuronal process (including axonal transport). Such impairments can result in catastrophic effects on neuronal function (Chang and Reynolds, 2006).

Given the recent availability of novel fluorescent markers for identifying and analyzing mitochondria in neuronal culture and the importance of cortical neurons in attention and other domains of cognition, we chose to employ cultured primary cortical neurons as an ideal model system for studying CPF effects on axonal transport and mitochondrial dynamics.

Current Therapeutic Strategies: Other than antidotes for acute toxicity such as atropine and pralidoxime, which target the traditional (cholinesterase based) mechanism of action of OPs, there are no known (effective) treatments for the protracted adverse effects of chronic exposure to OPs. Given that sustained cognitive deficits resulting from chronic exposure to OPs do not appear to be dependent on AChE inhibition, it is

not surprising that the antidotes described above are not effective for this indication. It is anticipated that the results of the experiments described in this thesis may help us to identify new therapeutic targets and design new treatment strategies to alleviate the symptoms of chronic exposure.

Dissertation Objectives

Based on the information presented above we tested two hypotheses. **The first hypothesis** was that repeated, sub-threshold exposures to CPF results in impairment of sustained attention in a rat model that persists throughout a drug-free washout period. **The second hypothesis** was that exposure to CPF disrupts axonal transport, leading to mitochondrial dysfunction and misplacement. The **rationale** for the proposed research was that human populations, particularly pesticide applicators and individuals within farming communities, are exposed to OPs frequently, potentially resulting in long-term cognitive deficits and other neurological sequelae. Elucidating the neuronal mechanisms responsible for these OP-related symptoms is crucial for the development of novel therapeutic strategies.

To test these hypotheses, the following specific aims were addressed:

Specific Aim 1: Determine the consequences of repeated sub-threshold exposures to CPF on sustained attention;

Specific Aim 2. Determine the consequences of CPF exposure on mitochondrial function and axonal transport.

CHAPTER 2

REPEATED EXPOSURES TO LOW-LEVEL CHLOPRYRIFOS RESULTS IN IMPAIRMENTS IN SUSTAINED ATTENTION AND INCREASED IMPULSIVITY IN RATS

M.L. Middlemore-Risher, J.J. Buccafusco, A.V. Terry, Jr. 2010. Neurotoxicology and Teratology. 32(4):415-24
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Abstract

Organophosphates such as chlorpyrifos (CPF) are among the most commonly used pesticides in the world. Therefore, it is not surprising that measurable levels of organophosphates (including CPF) are found in over 50% of fresh fruits, vegetables and grains that we consume and that approximately 80% of adults in the US have detectable levels of CPF metabolites in their urine. It is well known that acute exposure to organophosphates can cause cognitive deficits; however, the effects of daily or intermittent contact with low levels of organophosphates (often reflective of environmental exposures) are not well understood. The objective of this study was to determine if repeated low-level exposures to CPF impaired performance of the 5-Choice Serial Reaction Time Task (5C-SRTT), an animal model of sustained attention. Adult rats were trained to stably perform the 5C-SRTT, then treated with vehicle or CPF 18.0 mg/kg daily for 14 consecutive days or every other day for 30 days. Behavioral testing occurred daily during the CPF-exposure period and throughout a 30 day washout period to assess recovery. All CPF-treated animals exhibited deficits in percent correct, an increase in omissions and premature responses without signs impaired motivation or overt toxicity. Deficits in 5-CSRTT accuracy were apparent well into the 30 day washout period despite significant recovery of cholinesterase activity. These results indicate that repeated exposures to relatively low levels of chlorpyrifos lead to protracted impairments of sustained attention and an increase in impulsive behaviors in rats.

Introduction

Organophosphates are used as pesticides not only in the agricultural industry but also in households, public buildings, on lawns, and by direct application to stagnant water (Aspelin et al., 1994). Uses in the agricultural industry include pest control on grain crops, vegetables, and fruits culminating in approximately 1.2 billion pounds of pesticides per year, 10 million pounds of which is chlorpyrifos (CPF) (Ye et al., 2009;Punzi et al., 2005;EPA, 2009). In 2006 the USDA reported that approximately 73% of fresh fruits and vegetables, 61% of processed foods and 66% of drinking water in the US contained detectable levels of organophosphates (USDA, 2005). Not surprisingly, Barr *et al.,* (2005) reported that 96% of individuals in the US have measurable levels of CPF metabolites in their urine. The FDA has instituted restrictions on the use of many organophosphates in an attempt to prevent exposure to levels that may be detrimental to human health. However these restrictions do not adequately consider the effects of long-term low-level exposure (Pope et al., 1999;EPA, 2009). Moreover, the availability of human data is limited due to the fact that subtle symptoms of low-level organophosphate exposure can go unrecognized and therefore untreated.

Organophosphates such as CPF are known to irreversibly inhibit cholinesterase (ChE) activity by phosphorylating the serine residue within the active site of ChE thus inhibiting the degradation and inactivation of acetylcholine. The resulting accumulation of acetylcholine in the synaptic cleft increases the interaction between acetylcholine and its receptors on both the pre- and post-synaptic terminals, resulting in over-stimulation of the cholinergic system (Pope et al., 2005;Rohlman et al., 2007). Unfortunately these effects are non-selective for insects, and as a result acute exposure to

organophosphates can lead to a number of toxicological symptoms including seizures, hypersecretory activity, and even death in humans and other non-target organisms (Pope et al., 1999). Cognitive symptoms of organophosphate toxicity can include deficits in short-term memory, learning, attention, information processing, reaction time and depression (De Silva et al., 2006). Using a variety of tasks including digit span, the continuous performance task (CPT), finger tapping, match to sample, reversal learning, etc., deficits in processing speed, attention, learning and memory have been reported in humans after acute and/or repeated exposure to organophosphates and other pesticides (Kamel et al., 2003;Rohlman et al., 2007;Steenland et al., 1994;Dassanayake et al., 2007). These symptoms can linger for months to years after exposure despite the recovery of cholinesterase activity.

It should be noted, however, that the majority of the published human literature related to organophosphate exposure and cognition describe the consequences of relatively high-level (acute) exposures that also result in overt symptoms of cholinergic toxicity. Relatively little attention has been given to the subject of chronic, “low-level” exposures to organophosphates that are not associated with acute cholinergic symptoms (Ray et al., 2001). Furthermore, most of the data in humans were obtained via retrospective analyses and/or individual case studies. Hence, the objective of this study was to prospectively evaluate the persistent behavioral effects (i.e., during and after an extended drug-free washout) of repeated, intermittent, and subthreshold exposures to CPF (i.e., studies that for ethical reasons can only be conducted in animal models). This dosing approach was used to model the types of exposure that may be experienced by agricultural and industrial workers, as well as pest control specialists.

We have operationally defined “subthreshold exposures” as doses that do not produce overt signs of cholinergic toxicity, e.g., fasciculations, seizures, diarrhea, excessive urination, salivation, etc. (see reviews Rusyniak et al., 2004;Sungurtekin et al., 2006). The behavioral analyses specifically focused on the effects of CPF on sustained attention using the 5 choice serial reaction time task (5C-SRTT), a rodent analog of the CPT in humans (Robbins et al., 2002).

Materials and Methods

Compound Formulation and Administration.

The organophosphate compound chlorpyrifos (CPF) was used in this study. CPF was obtained from ChemService Inc. (Cat# PS-674, West Chester, PA, USA) and was dissolved in 3% DMSO and 97% peanut oil. CPF and/or Vehicle (3% DMSO + 97% peanut oil (v/v)) were administered by subcutaneous (s.c.) injection in a volume of 0.7ml/kg approximately 5 hours after testing in the 5C-SRTT (see below). CPF and vehicle treated animals were given 18mg/kg CPF (s.c.) and vehicle, respectively, every day for 14 days or every other day for 30 days. A second cohort of animals was exposed to 18mg/kg CPF or vehicle for 14 days everyday or 30 days every other day and euthanized 24 hours after the last day of administration for analysis of ChE activity. This dosing procedure was selected based on previous studies by Terry, Jr. *et al.* (2007) and was defined as subthreshold according to the definition provided above. All other chemicals were reagent grade or better and purchased from Fisher Scientific and Sigma Aldrich (see Gearhart *et al*. (2007) for further details).

Test Subjects. Male albino Wistar rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN, USA)) approximately 2 months old were housed individually in a temperature controlled room (25^{o}C), maintained on a 12:12h reverse light-dark cycle (lights off at 6am) with free access to water and food during the first week. From week 2 until the end of the study animals were food restricted to approximately 85% of their age-dependent, free-feeding weights based upon Harlan Laboratories growth rate curves. All testing began 2 hours after the initiation of the dark cycle with a minimum of 30 minutes habituation to the light environment prior to testing. All procedures employed during this study were reviewed and approved by the Medical College of Georgia Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain and discomfort in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Behavioral Experiments. All behavioral experiments were conducted in rooms equipped with white noise generators (San Diego Instruments, San Diego, CA) set to provide a constant background level of 70 dB, and ambient lighting of approximately 25-30 Lux (lumen/m^2). Animals in the behavioral cohort were transferred (in their home cages) to the behavioral testing rooms each morning approximately 30 min before the beginning of experiments.

Test Apparatus. Training and testing was conducted using six ventilated, sound attenuated operant chambers (Med Associates, St. Albans, VT, USA). Each operant chamber consisted of 9 nose pokes/apertures, 4 of which were closed off with metal inserts, leaving every other nose poke available (2.5cm wide, 4cm deep). The

apertures were arranged on a curved panel 2 cm above the floor and were equipped with a photocell beam to detect nose pokes. There was a lamp (2.8W) on the rear wall of each aperture that could be illuminated randomly and for varying durations. Food pellets were delivered automatically to a magazine on the opposite wall to the nose pokes. A light inside the food magazine was also turned on to indicate that a pellet (45mg chow pellet, BioServ, Frenchtown, NJ, USA) had been dispensed. The food magazine was equidistant from all nose poke apertures. There was a house light that remained on for the entire session unless an error or omission occurred; the light was located towards the roof of the operant chamber above the magazine. The apparatus was controlled using MedPC software (Med Associates, St. Albans, VT, USA).

Behavioral Procedures. Week one consisted of handling animals to reduce stress and anxiety in preparation for training and testing. Week 2 consisted of handling and food restriction. Week 3 consisted of food restriction, habituation to the apparatus and preparation for training. Specifically, on days 1 and 2 of week 3 subjects were placed in the operant chambers for 15 minutes with the house light and magazine light on and 10 pellets in the magazine dispenser. On days 3-5 of week 3 animals performed a non-spatial training program. Briefly, the animals were placed in the 5C-SRTT operant chamber with the house light illuminated. A pellet was released into the food magazine which was simultaneously illuminated for a maximum of 5 seconds or until the animal collected the pellet. After a 10 second interval another pellet was released from the simultaneously illuminated food magazine. This continued until the 15 minute habituation period expired.

Spatial training in the task began on day 1 of week 4. Animals began with the stimulus duration of 10 seconds, each session being 100 trials or 30 minutes in duration. An initial pellet was delivered to the magazine to facilitate the start of each session. One of the 5 nose poke apertures was illuminated randomly for 10 seconds after which the light was extinguished. The animal was then required to respond correctly by nose poking the previously illuminated aperture within 5 seconds of the light being extinguished. A correct response in the previously established time frame (5 seconds) resulted in a pellet being dispensed into the magazine that was simultaneously illuminated for a maximum of 5 seconds or until the animal retrieved the pellet. Collection of this pellet initiated the intertrial interval, a delay of 5 seconds, before the next trial began. An incorrect response, premature response, or failure to respond (omission) resulted in a 5 second timeout, marked by the extinction of the house light for 5 seconds and no food reward, after which the animal initiated the next trial by a nose poke into the magazine. Animals were trained 7 days a week until they reached stable performance levels at the 10 second stimulus duration (stable performance criterion defined as 2 consecutive days at >80% accuracy, <20% omissions and completion of all 100 trials). Once this criterion was achieved the animals were moved to the next, more challenging stimulus duration and the same performance criterion was applied before the next progression. The following stimulus durations were employed: 5 → 2.5 →1.25 → 1.0 → 0.8 → 0.6 → 0.5 seconds.

The animals were required to meet criterion for a minimum of 3 days at the 0.5 second stimulus duration prior to being moved to the testing groups. Reaching criterion at the 0.5 second stimulus duration required on average 42±1.36 sessions. Once the

animals met criterion they were randomly divided into 1 of 4 groups; every other day 30 day vehicle, every other day 30 day 18mg/kg CPF, everyday 14 day vehicle or everyday 14 day 18mg/kg CPF. Testing began approximately 15 hours after the first injection of CPF or vehicle.

5C-SRTT Performance Assessment. When administration of vehicle or CPF began, rats were assessed daily for signs of acute toxicity. Signs included muscle fasciculations, ataxia, tremors, increased salivation, lacrimation, diarrhea and rhinorrhea. Testing at the 0.5 second stimulus duration continued 7 days a week throughout the administration of CPF/vehicle and for 30 days after the last injection (30 day, CPF-free washout). On day 31 of washout the animals were tested using a randomized, varying stimulus duration of 0.1, 0.25 and 0.5 seconds to increase demands of the task. The following parameters were measured to assess performance before, during and after exposure to CPF/vehicle:

% correct = [# correct /(# correct + # incorrect)]x100

% omissions = [# omissions/(# trials completed)]x100

% Premature responses (Impulsivity) = [(premature responses/trials initiated)] x 100 (specifically, the % of responses made after the trial began, but before onset of the light stimulus, i.e., during the 5 second intertrial interval).

% Perseverative responses (Compulsivity) = [(perseverative responses/correct responses)] x 100 (specifically, the % of nose pokes made after the correct response has been made but before collecting the reward). Responding can occur in the aperture where the responding has just earned a food reward or at another location.

Trials completed = (# correct + # incorrect + # omissions)

Latency to correct = time elapsed from the onset of the light stimulus to making the correct nose poke response.

Latency to incorrect = time elapsed from the onset of the light stimulus to making the incorrect nose poke response.

Latency to reward = time elapsed from making a correct nose poke response to retrieving the food reward from the magazine.

Measurement of Cholinesterase (ChE) Activity. On day 31 of washout (behavioral cohort) or 24 hours after the final CPF/vehicle administration (cholinesterase cohort) the animals were anesthetized with isofluorane. The blood was then drawn directly from the left ventricle, collected in EDTA 4ml Vacutainers (BD, Franklin Lakes, NJ, USA) and centrifuged for 15 min in a clinical centrifuge. The plasma was then removed and quick frozen with liquid nitrogen and stored at -70^{o}C. After the blood draw the animals were quickly decapitated, brains were removed within 3 minutes and quick frozen in liquid nitrogen for storage at -70^{o}C until use.

Preparation of brain homogenates for the ChE activity assay was described in detail in Gearhart *et al.* (2007). Briefly, brains were thawed and the basal forebrain, hippocampus, cortex (excluding prefrontal cortex), prefrontal cortex and striatum were dissected and homogenized in a modified RIPA buffer. Samples were sonicated, filtered and stored at -70^{o}C until needed. ChE activity was assessed using the Ellman method with modifications (Terry et al., 2007). Briefly, tissue homogenate or plasma was added to 96 well plates with 300uL of reaction mixture (4.8uM acetylthiocholine iodide and 321uM dithiobisnitrobenzoate in 0.1 M Na_2HPO_4 buffer, pH 8.0; sigma) and shaken for 30 seconds on a Jitterbug, Boekel Scientific (Feasterville, PA, USA). Plates

were then placed in Biotek uQuant plate reader (Philadelphia, PA, USA) and read at 412nm every 2 minutes for 16 minutes. The rate of ChE activity was then calculated using the formula rate (moles/liter per min) = (Δ absorbance/min)/($1.36X10^4$) (Ellman et al., 1961).

Statistics. Statistical comparisons of treatment groups were conducted using a two way repeated measures ANOVA followed by post hoc analysis using the Student-Newman-Keuls multiple comparison method (SigmaStat 2.03, SPSS Inc., Chicago, IL, USA). Statistical significance was assessed using an alpha level of 0.05. Data are shown as Mean ± SEM.

Results

5C-SRTT - 14 Day Everyday Exposure: CPF vs. VEH

There was a significant decrease in accuracy during exposure to CPF when compared to vehicle matched controls (Fig 2.1) and this deficit persisted well into the 30 day washout period, main effect for treatment ($F_{(1,22)}$= 16.12, p<0.001), session effect ($F_{(43,946)}$= 4.01 p<0.001), treatment x session interaction ($F_{(43,946)}$= 3.57, p<0.001). Post hoc analysis indicated that CPF treated rats were impaired relative to control rats on all sessions from session 3 until session 33 of the study (see the asterisks in Fig 2.1). Further analysis revealed that CPF exposure resulted in a significant increase in % omissions during the exposure period and that this effect persisted throughout the 30 day washout period, main effect for treatment ($F_{(1,22)}$= 18.23, p<0.001). The averaged data for the baseline, drug exposure period, and during the washout period are provided in Table 2.1. There were no significant differences in the total number of trials

completed (Table 2.1). The percentage of premature and perseverative responses is shown in Fig 2.2A and 2.2B, respectively. The figure insets illustrate the data averaged across the sessions during the exposure period and washout periods, respectively. Statistical analysis of premature responses revealed the following results: main effect for treatment ($F_{(1,18)}$= 4.1, p=0.05), session effect ($F_{(43,774)}$= 1.98 p<0.001), treatment x session interaction ($F_{(43,774)}$= 1.01 p=0.42),. Post hoc analysis indicated a significantly higher number of premature responses (p<0.05) in the CPF-treated subjects on sessions 6,18,19,20, and 21. There was also a significant CPF-related decrease in the percentage of perseverative responses primarily associated with the drug exposure period, main effect for treatment ($F_{(1,18)}$= 0.14, p=0.71), session effect ($F_{(43,774)}$= 1.54, p=0.03), treatment x session interaction ($F_{(43,774)}$= 2.29, p<0.001). Post hoc analysis indicated a significant CPF-related decrease in perseverative responses on sessions 6,8,9,10,11, and 12 of exposure, an effect that abated during the 30 day washout period (Fig. 2.2B). The mean latencies associated with correct responses, incorrect responses, and reward collection (magazine latency) at baseline (i.e., before drug exposure), during the drug exposure period, and during the washout period are provided in Table 2.1. There were statistically significant CPF-related increases in the latency to correct and incorrect responses, main effect of treatment ($F_{(1,22)}$= 7.78, p=0.01 and incorrect ($F_{(1,22)}$= 5.58, p<0.03), respectively. Post hoc analyses indicated that CPF was associated with an increased latency to correct response during the exposure period and that this effect persisted during the washout period, while the latency to incorrect response was increased during the exposure period only.

Varying Stimulus Duration (14 day everyday exposure): Day 31 of washout

The effects of a pseudorandom presentation of three different stimulus durations during session 31 of washout from the every day drug exposure regimen are shown in Fig. 2.3 and Table 2.2. For the percent correct assessment statistical analysis revealed the following results, main effect of treatment ($F_{(1,14)}$= 1.32, p=0.27), stimulus duration ($F_{(2,28)}$= 32.17, p<0.001), treatment by stimulus duration interaction ($F_{(2,28)}$= 0.10, p=0.90). Post hoc analysis indicated (as expected) that accuracy in all subjects was increased with an increase in stimulus duration. While visual inspection of Fig. 2.3 indicates that accuracy was slightly lower in the subjects previously exposed to CPF at each stimulus duration, the differences were not statistically significant (p>0.05). There were also non-significant trends (p<0.1) toward increased omissions (Table 2.2) in CPF treated subjects at each of the three stimulus durations (0.1, 0.25, 0.5 seconds). There were no significant differences in any of the latency measures (Table 2.2) or measures of impulsive or compulsive behaviors (Fig. 2.3B, 2.3C).

30 Day Every Other Day Exposure: CPF vs. VEH

As in the case of the daily CPF exposure method, subjects exposed to CPF every other day also demonstrated a significant decrease in accuracy (during the drug exposure period, see Fig. 2.4) and this deficit persisted well into the 30 day washout period, main effect for treatment ($F_{(1,22)}$= 13.47, p<0.001), session effect ($F_{(59,1298)}$= 4.69, p<0.001), treatment x session interaction ($F_{(59,1298)}$= 4.17, p<0.001). Post hoc analysis indicated that CPF treated rats were significantly (p<0.05) impaired relative to control rats during session 7, all sessions from 11 through 42, as well as during sessions 44, 47, and 57

(see the asterisks in Fig 2.4). CPF exposure (every other day) also resulted in a significant increase in % omissions during the exposure period and this effect persisted throughout the 30 day washout period, main effect for treatment ($F_{(1,21)}$= 18.26, p<0.001). The averaged data for the baseline, drug exposure period, and during the washout period are provided in Table 2.1. There were no significant differences in the total number of trials completed (Table 2.1). The percentage of premature and perseverative responses is shown in Fig 2.5A and 2.5B, respectively. The figure insets illustrate the data averaged across the sessions during the exposure period and washout periods, respectively. Statistical analysis of premature responses revealed the following results: main effect for treatment ($F_{(1,22)}$= 13.64, p=0.001), session effect ($F_{(59,1298)}$= 1.10, p=0.28), treatment x session interaction $F_{(59,1298)}$= 1.60, p=0.003. Post hoc analysis indicated a significantly higher number of premature responses ($p<0.05$) in the CPF-treated subjects on nearly all the sessions from session 21 until the end of the study. There were also a significant CPF-related alterations in the percentage of perseverative responses ($F_{(1,21)}$= 0.22, p=0.65), session effect ($F_{(59,1239)}$= 1.22 p=0.13), treatment x session interaction $F_{(59,1239)}$= 4.76, p<0.001. Post hoc analysis indicated a significant biphasic effect of CPF exposure (i.e., perseverative responses were significantly or nearly significantly lower in CPF-treated subjects during sessions 6, 10, 11, 12) during the exposure period, but actually increased relative to vehicle controls during the washout period (i.e., during multiple sessions). The mean latencies associated with correct responses, incorrect responses, and reward collection at baseline, during the drug exposure period and during the washout period are provided in Table 2.1. There were statistically significant CPF-related increases in the latency to

correct and incorrect responses. In the case of the latency to correct responses the main effect of treatment was not significant, ($F_{(1,21)}$= 2.30, p=0.14), however, the treatment x exposure period interaction was highly significant, ($F_{(2,42)}$= 14.85, p<0.001). There was a significant main effect of treatment on incorrect response latencies ($F_{(1,21)}$= 7.64, p=0.01). Post hoc analyses indicated that CPF was associated with an increased latency to correct and incorrect responses during the exposure period, but this effect abated to control levels during the washout period.

Varying Stimulus Duration (30 day every other day exposure): Day 31 of washout

The effects of a pseudorandom presentation of three different stimulus durations during session 31 of washout from the every other day drug exposure regimen are shown in Fig 2.6 and Table 2.2. For the percent correct assessment statistical analysis revealed the following results, main effect of treatment ($F_{(1,14)}$= 0.64, p=0.44), stimulus duration ($F_{(2,28)}$= 43.2, p<0.001), treatment by stimulus duration interaction ($F_{(2,28)}$= 3.85, p=0.03). Post hoc analysis indicated (again, as expected) that accuracy in all subjects was increased with an increase in stimulus duration. However, accuracy in subjects previously exposed to CPF was lower (compared to vehicle controls) at the 0.5 sec stimulus duration. Interestingly, there was a significant CPF-related reduction in the mean latency to collect the reward associated with the 0.25 second stimulus duration (p<0.05). There were no other statistically significant differences in the latencies, omissions or in the measures of impulsive or compulsive behaviors (see Fig. 2.6B, 2.6C, Table 2.2).

ChE Activity

ChE activity was assessed in plasma and selected brain regions in the cholinesterase cohort (i.e., in rats not behaviorally tested) 24 hours after the 14 day everyday CPF-exposure period or after the 30 day every other day exposure period. The behavioral cohort (i.e., animals evaluated in the 5C-SRTT) was used to assess ChE activity at the end of the washout period. The prefrontal cortex, cortex (i.e., the remaining cortical areas), hippocampus, striatum, and basal forebrain were selected for analysis due to their function in the basal forebrain cholinergic system (and the well documented roles of this system in attention and general cognition) and/or their specific roles in performance of the 5C-SRTT (see Harati et al. (2008) for review). Immediately after exposure to CPF, plasma ChE activity was significantly reduced ($F_{(5,60)}$= 1.748, p<0.05) to 21% and 31% of control for everyday and every other day exposed animals respectively (see Fig. 2.7 and 2.8). However, after the 31 day washout period the plasma ChE activity levels recovered to 80% and 89% of control for their respective groups resulting in no significant difference in activity after the 30 day washout period when compared to vehicle matched controls (p=0.782, p=0.62 respectively). ChE activity in brain homogenates immediately after exposure to CPF was significantly decreased in all brain regions when compared to vehicle matched control groups. Specifically, ChE activity in animals exposed to CPF for 14 days (every day) was decreased to 46.6%, 59.4%, 63.3% 55.9%, and 23.5% of control for; cortex, prefrontal cortex, hippocampus, striatum, and basal forebrain, respectively, ($F_{(5,60)}$= 1.748, p<0.05) (Fig. 2.7). Similar results were found in the animals exposed to CPF for 30 days every other day, with ChE activities of 54.5%, 44.6%, 50.9 48.7%, and 25.9% of control for the same respective brain regions ($F_{(5,60)}$= 3.572, p<0.05) (Fig. 2.8). Incorporation of the 31

day washout period resulted in near full recovery of ChE activity in four of the six brain regions analyzed. The striatum and basal forebrain continued to have a small, but significant reduction in ChE activity when compared to control animals after the 31 day washout period for both every day ($F_{(5,60)}$= 3.572, p<0.05) and every other day ($F_{(5,60)}$= 0.750, p<0.05) treatment groups.

Discussion

The results of this study utilizing the 5C-SRTT indicate that repeated exposures to a subthreshold dose of CPF in rats results in protracted impairments in sustained attention and a decrease of inhibitory control (i.e., elevated impulsivity). These observations were evident in animals administered CPF on a daily basis for 14 days or every other day for 30 days so that the cumulative number of exposures was similar. Deficits in sustained attention were indicated by a decrease in accuracy (% correct) and a modest increase in the number of omissions; and increases in impulsivity were suggested by the elevated number of premature responses. It is also interesting to note that the percentage of perseverative responses (surprisingly) was diminished in CPF-treated subjects during the drug exposure period in both the daily exposed and every other day exposed subjects. This effect combined with the increase in response latencies (i.e., for correct and incorrect nose poke selections) observed in both CPF treatment groups might be suggestive of a general motor slowing or performance deficit. However, the effect on perseverative responding abated in the every-day exposed subjects during the washout period, and the effect was actually reversed during washout in the subjects previously treated every other day with CPF. Specifically, in

these subjects, there was a clear increase in perseveratve responses during the washout period (i.e., possibly indicative of an increase in compulsive behaviors).

There are several additional observations that lead us to conclude that the protracted effects of CPF on performance of the 5C-SRTT are related to deficits of information processing and/or attention as opposed to general deficits in motivation, locomotor activity, malaise etc. For example, we did not observe any alterations in the magazine latency (i.e., the latency to collect food rewards) which has been described as an index of motivation in the 5C-SRTT (see review, Robbins, 2002). In addition, we did not observe any significant alterations in the total number of trials completed. In the 5C-SRTT, subjects must nose poke the food magazine after an incorrect response (i.e., after an incorrect nose poke into one of the stimulus apertures) or after an omission (i.e., when 5 seconds expired after the stimulus presentation without a nose poke) in order to initiate the next trial. Therefore, significant delays in initiation would be exemplified by a decrease in the total number of trials completed (which, as noted above, we did not observe). As reviewed by Robbins (2002) changes in the response latencies in the absence of changes in latencies to collect earned food reinforcers (as observed in the present study) likely reflect alterations of information processing and/or decisional processes. In addition, increases in omissions in the absence of changes in magazine latencies (which we also observed) are most likely due to gross impairments in attention. Finally, it is also important to note that on day 31 of washout, when a CPF - related sensitivity to the presentation of the variable stimulus durations was observed (i.e., accuracy was decreased), there was a clear trend toward decreases in response

latencies in CPF treated animals (statistically significant for the magazine latency associated with the 0.25 sec stimulus duration).

The data described in this report complement previous studies in our laboratory (Terry et al., 2007) where a series of behavioral tests were conducted during a washout period after CPF was injected (dose range, 2.5-18.0 mg/kg) every other day over the course of 30 days. In that study, dose-dependent decrements in a water maze hidden platform task and a prepulse inhibition (PPI) procedure were observed, without significant effects on open field activity, rotorod performance, or grip strength. Those results further support the argument presented here that repeated exposure to 18.0 mg/kg of CPF is not associated with deleterious motivational or locomotor effects that might confound the interpretation of (cognition-related) behavioral results. The study also suggested that repeated exposures to CPF results in prolonged impairments of other domains of cognition. The deficits in PPI observed in that study may be particularly relevant to the 5C-SRTT results presented here given that PPI has been widely used as a neurophysiological measure of the early pre-attentive stages of information processing (Braff et al., 1990). Further, PPI has been shown in animals to be sensitive to cholinergic manipulations (Hohnadel et al., 2007). Our observations of prolonged elevations in impulsivity in animals previously exposed to CPF (in the current study) would appear to agree with the previous report of Cardona *et al.* (2006) who demonstrated that one acute dose of CPF is associated with increases in impulsivity in rats 6 months after exposure.

The biochemical basis for the effects on 5C-SRTT performance noted here may be multifactorial. As observed in Figures 1 and 4, there was a relatively rapid decrease

in accuracy of the task associated with both CPF-exposure methods, an effect that appears to correlate with previous publications of CPF-related time courses of ChE inhibition. For example, we (Terry et al., 2007) previously observed that the 18mg/kg CPF every other exposure method was associated with maximal plasma ChE inhibition beginning around day 7 of dosing. This level of ChE inhibition correlates reasonably well with the performance deficits in the 5C-SRTT in the current study that begin around day 10 of dosing (see Fig 4). In the every day CPF exposure approach where the cumulative dose is higher at an earlier time point, the decrease in 5C-SRTT accuracy occurred earlier (see Fig 1). This behavioral effect (i.e., performance deficits beginning around day 4 of dosing) would appear to correlate with Bushnell et al., (1993) who reported that maximal inhibition of whole blood cholinesterase activity occurred four days after a single subcutaneous injection of CPF (albeit at considerably higher doses than used in the present study). It should be noted here that the CPF-related effects in the 5C-SRTT described above were evident when cholinesterase inhibition was maximal as well as when it had significantly recovered toward baseline levels of activity in both plasma and brain (i.e., during the CPF-free washout period). Conversely, in the Bushnell (1993) study cite above, functional deficits in working memory and motor function in rats administered single (relatively high) doses of CPF were apparent within two days of administration, but recovered within three weeks, long before ChE activity had returned to normal. Collectively, the results of that study and ours suggest that the behavioral deficits associated with CPF may not be fully explained by ChE inhibition alone.

A variety of studies employing cholinergic agonists and antagonists as well as cholinergic lesions (i.e., of the nucleus basalis magnocellularis, NBM) have clearly documented an important role of the cholinergic system in performance in the 5C-SRTT (Harati et al., 2008;Robbins, 2002). We (Terry et al., 2007) have previously observed protracted decreases in several cholinergic proteins in multiple brain regions after chronic subthreshold exposure to CPF (i.e., effects that may contribute to the deficits in 5C-SRTT in the present study). Our previous observations of reduced expression of the α_7 nicotinic acetylcholine receptor (nAChR) in multiple brain regions including the basal forebrain and prefrontal cortex may be of particular importance in light of findings in other laboratories. For example, Hoyle *et al.* (2006) previously found that α_7 nAChR knockout mice exhibited decreases in accuracy in the 5C-SRTT and an increase in impulsivity. Similar findings (i.e., performance deficits and elevations in impulsivity) have been reported in α_7 nAChR mutant mice in a signaled nose poke task designed to measure appetitive learning and behavioral impulsivity (Keller et al., 2005). Collectively, such studies support a role of α_7 nAChRs in sustained attention and behavioral disinhibition. Thus, it is interesting to speculate that the mechanism for the CPF-related attentional deficits and elevations in impulsivity (observed in our study) are related to α_7 nAChR deficits. Interestingly, Smulders *et al.* (2004) have also made the argument (as we have, see above) that persistent neurobehavioral effects of organophosphates (including CPF) cannot be explained by acetylcholinesterase inhibition alone, and, that such effects may involve direct actions at nAChRs.

From a translational perspective there are several points that should be considered. Substantial evidence suggests that a variety of OPs (including CPF) can

persist in the environment for extended periods after use and as a result they represent a threat to numerous people, especially from a low-level chronic or intermittent exposure standpoint. Many insecticides were (by design) manufactured to persist in soil to eradicate pests such as termites. Thus, (as noted in the Introduction) the short term (daily) and intermittent (every other day) dosing regimens used in this study provided a model for the types of exposures that might be experienced by agricultural and industrial workers, as well as pest control specialists. Our dosing approach in rats was subthreshold for acute signs of toxicity; however, it resulted in plasma and brain inhibition of cholinesterase ranging between 40-80%. This level of inhibition is likely to be significantly higher than that associated with most environmental exposures in the general population or that encountered by most agricultural workers. However, Dyer et al. (2001) previously reported that termiticide applicators in Australia chronically exposed to organophosphates had serum cholinesterase inhibition levels of approximately 50% with no signs of acute toxicity, suggesting that (like in our animals studies) physiological adaptations may take place over time such that acute symptoms are not manifested. Further, in a study of termiticide applicators in North Carolina, levels of the CPF metabolite, TCP (3,5,6-trichloro-pyridinol) were found to exceed that of the general population by an average factor of 70 (Hines et al., 2001), suggestive of relatively high levels of CPF exposure. It is interesting to note that termiticide applicators (i.e., specifically exposed to chlorpyrifos) more commonly complain of attention and memory-related difficulties compared to non-exposed controls (Steenland et al., 1994). It should be noted, however, that (with a few exceptions) such deficits

have not be objectively confirmed when termiticide applicators have been clinically evaluated using quantitative tasks designed to evaluate specific domains of cognition.

Conclusion

In conclusion, the results of this rodent study support the premise that repeated, low-level exposures to commercial organophosphate pesticides like CPF may lead to protracted deficits in sustained attention and to increases in impulsivity in the absence of acute (cholinergic) side effects or motivational deficits. The observations are significant given the widespread use of OP insecticides in household, agricultural, and commercial environments worldwide and the importance of attention and impulse control to human cognition and general function in society. The results also emphasize the critical need for additional (prospective) investigations to determine more definitively how specific OPs at low-levels (over time) affect specific domains of cognition (as well as the neurobiological substrates of the cognitive effects, i.e., experiments that can only be conducted in animal models).

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Table 2.1. Effects of Chlorpyrifos on Latencies, Omissions, and Trials Completed in the Five Choice Serial Reaction Time Task. Data are presented as the mean ± SEM. Baseline = before the drug exposure period; exposure = during the drug exposure period; washout = during the 30 day drug-free washout period * = statistically significant difference ($p<0.05$) between vehicle and chlorpyrifos-treated group during the same treatment period. (N=8-10).

Group/Dosing Frequency	Treatment Period	Latency Correct (s)	Latency Incorrect (s)	Magazine Latency (s)	% Omissions	Trials Completed
Vehicle (Every Day)	Baseline	0.70±0.03	1.73±0.13	1.53±0.12	4.06±0.47	100.00±0.00
	Exposure	0.70±0.02	1.72±0.07	1.44±0.13	2.94±0.37	99.83±0.17
	Washout	0.69±0.03	1.72±0.07	1.55±0.10	2.85±0.0.62	100.00±0.00
Chlorpyrifos (Every Day)	Baseline	0.64±0.02	1.37±0.10	1.66±0.14	5.23±1.03	100.00±0.00
	Exposure	0.90±0.03*	2.24±0.07*	1.53±0.07	22.99±0.3.14*	88.73±2.58
	Washout	0.89±0.12*	1.87±0.05	1.53±0.10	15.24±3.60*	97.28±0.66
Vehicle (Every Other Day)	Baseline	0.69±0.03	1.72±0.16	1.62±0.07	3.59±1.37	100.00±0.00
	Exposure	0.70±0.04	1.72±0.08	1.70±0.07	3.28±0.59	99.99±0.01
	Washout	0.66±0.05	1.73±0.09	1.58±0.08	2.00±0.44	100.00±0.00
Chlorpyrifos (Every Other Day)	Baseline	0.67±0.02	2.13±0.08	1.39±0.11	3.62±0.69	100.00±0.00
	Exposure	0.83±0.03*	2.01±0.05*	1.65±0.12	12.01±1.51*	96.45±1.40
	Washout	0.73±0.03	1.75±0.07	1.54±0.11	5.18±0.67*	98.69±0.07

Table 2.2. Effects of Chlorpyrifos on Latencies, Omissions, and Trials Completed in the Variable Stimulus Duration Version of the Five Choice Serial Reaction Time Task. Data shown here were collected on day 31 of the drug free washout period. All data are presented as the mean ± SEM. * = statistically significant difference ($p<0.05$) between vehicle and chlorpyrifos-treated group at the same stimulus duration. (N=6-8).

Group/Dosing Frequency	Stimulus Duration (s)	Latency Correct (s)	Latency Incorrect (s)	Magazine Latency (s)	% Omissions	Trials Completed
Vehicle (Every Day)	0.10	0.70±0.05	1.40±0.13	1.40±0.09	6.57±2.29	
	0.25	0.74±0.12	1.72±0.20	1.51±0.18	4.70±1.72	(total)
	0.5	0.75±0.06	1.62±0.29	1.37±0.11	6.79±2.39	98.75±1.25
Chlorpyrifos (Every Day)	0.10	0.77±0.12	1.51±0.18	1.42±0.13	11.57±5.33	
	0.25	0.71±0.06	1.46±0.19	1.35±0.09	8.34±5.42	(total)
	0.5	0.68±0.07	1.65±0.25	1.32±0.05	10.67±4.89	100.00±0.00
Vehicle (Every Other Day)	0.10	0.70±0.08	1.13±0.18	1.76±0.36	3.70±1.21	
	0.25	0.73±0.08	1.31±0.22	2.13±0.34	1.34±0.53	(total)
	0.5	0.69±0.06	1.29±0.25	1.62±0.30	4.10±1.17	100.00±0.00
Chlorpyrifos (Every Other Day)	0.10	0.46±0.04	0.96±0.20	1.33±0.13	2.60±1.35	
	0.25	0.52±0.07	1.19±0.38	1.06±0.09*	2.85±0.95	(total)
	0.5	0.52±0.06	1.29±0.35	1.23±0.17	5.73±4.03	96.63±3.38

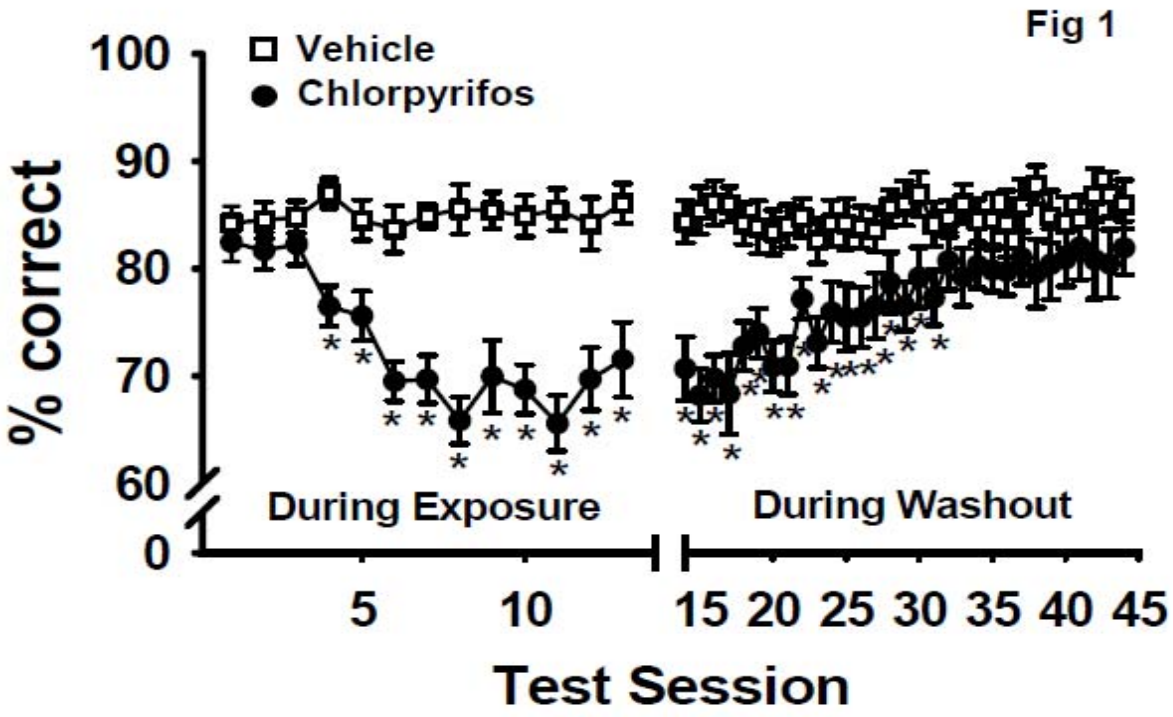

Figure 2.1. The effects of CPF (18.0 mg/kg) exposure on accuracy (% correct) in the 5C-SRTT during the 14 day every day exposure period and during the 30 day (drug-free) washout period. All data are presented as mean ± SEM. * = Statistically significant difference ($p<0.05$) between CPF-treated and vehicle-treated controls. (N=8-10).

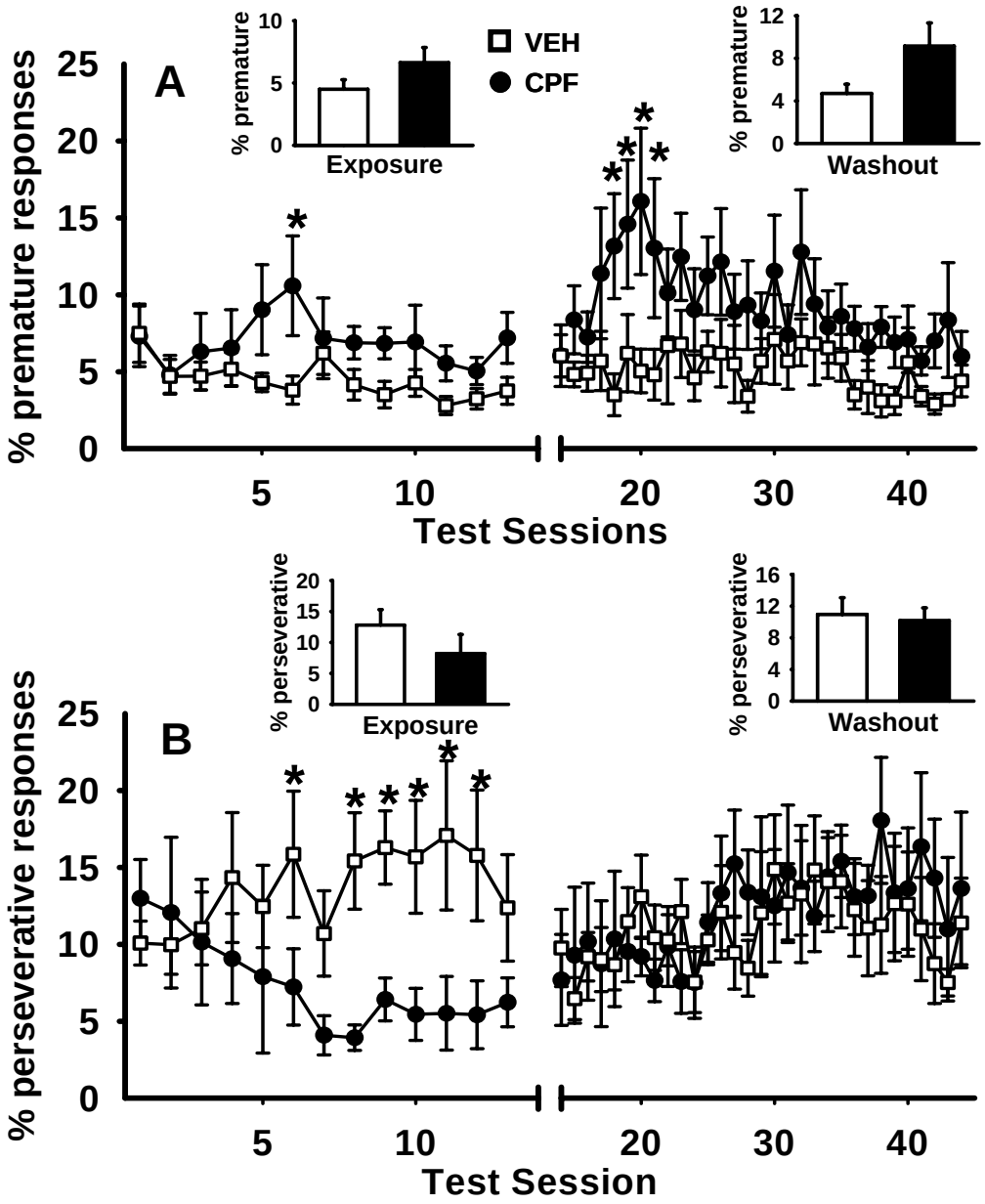

Figure 2.2. A. The effects of CPF (18.0 mg/kg) exposure on the percentage of premature responses (A) and perseverative responses (B) in the 5C-SRTT during the 14 day every day exposure period and during the 30 day (drug-free) washout period. The histogram insets illustrate the overall effect (i.e., responses averaged across the sessions) during the drug exposure and washout periods. * = Statistically significant difference ($p<0.05$) between CPF-treated and vehicle-treated controls. (N=8-10).

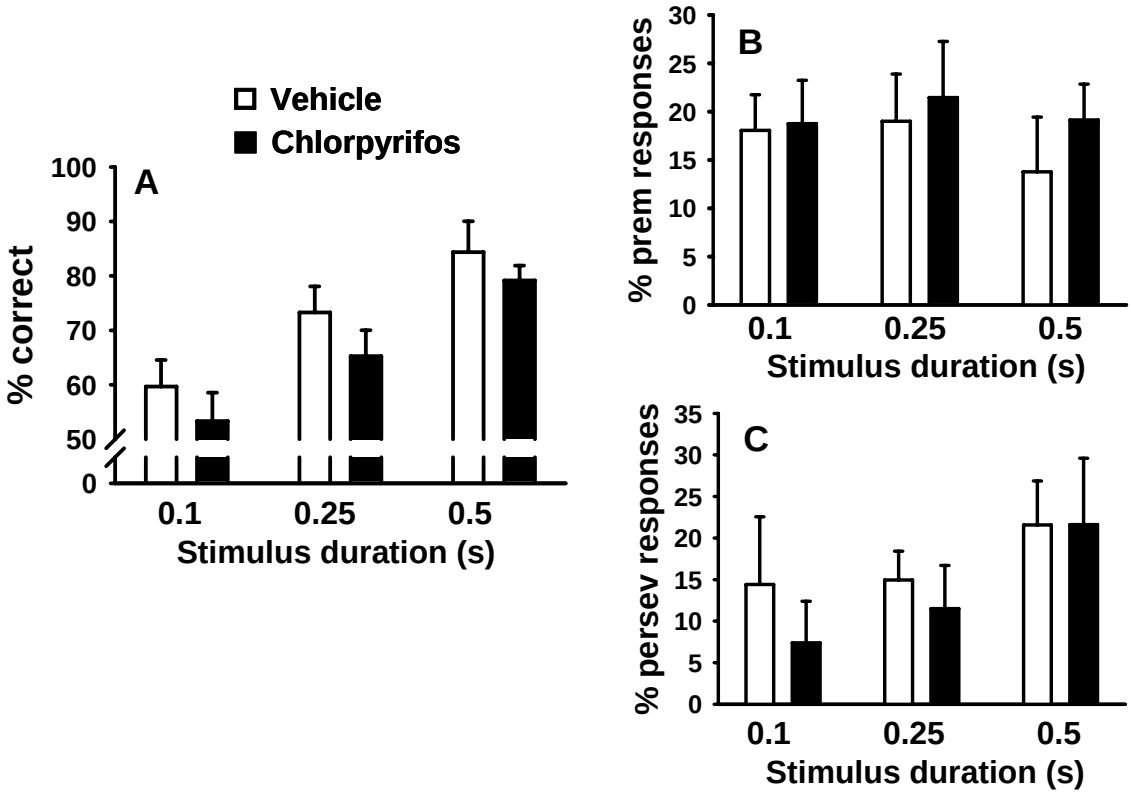

Figure 2.3. The residual effects of CPF (18.0 mg/kg) exposure (14 day every day exposure method) in a variable stimulus duration version of the 5C-SRTT on day 31 of a drug-free washout period. Dependent measures include: % correct (A), percentage of premature responses (B) and the percentage of perseverative responses (C). Data are presented as mean ± SEM for each stimulus duration. (N=6-8).

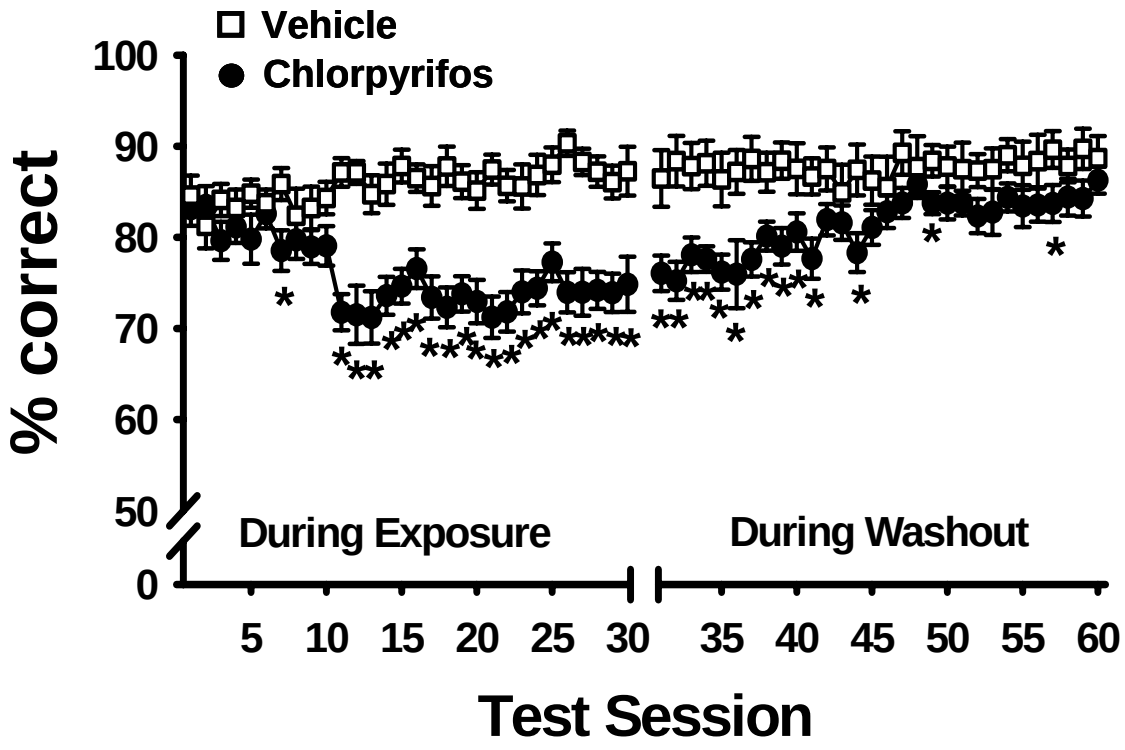

Figure 2.4. The effects of CPF (18.0 mg/kg) exposure on accuracy (% correct) in the 5C-SRTT during the 30 day every other day exposure period and during the 30 day (drug-free) washout period. All data are presented as mean ± SEM. * = Statistically significant difference ($p<0.05$) between CPF-treated and vehicle-treated controls. (N=8-10).

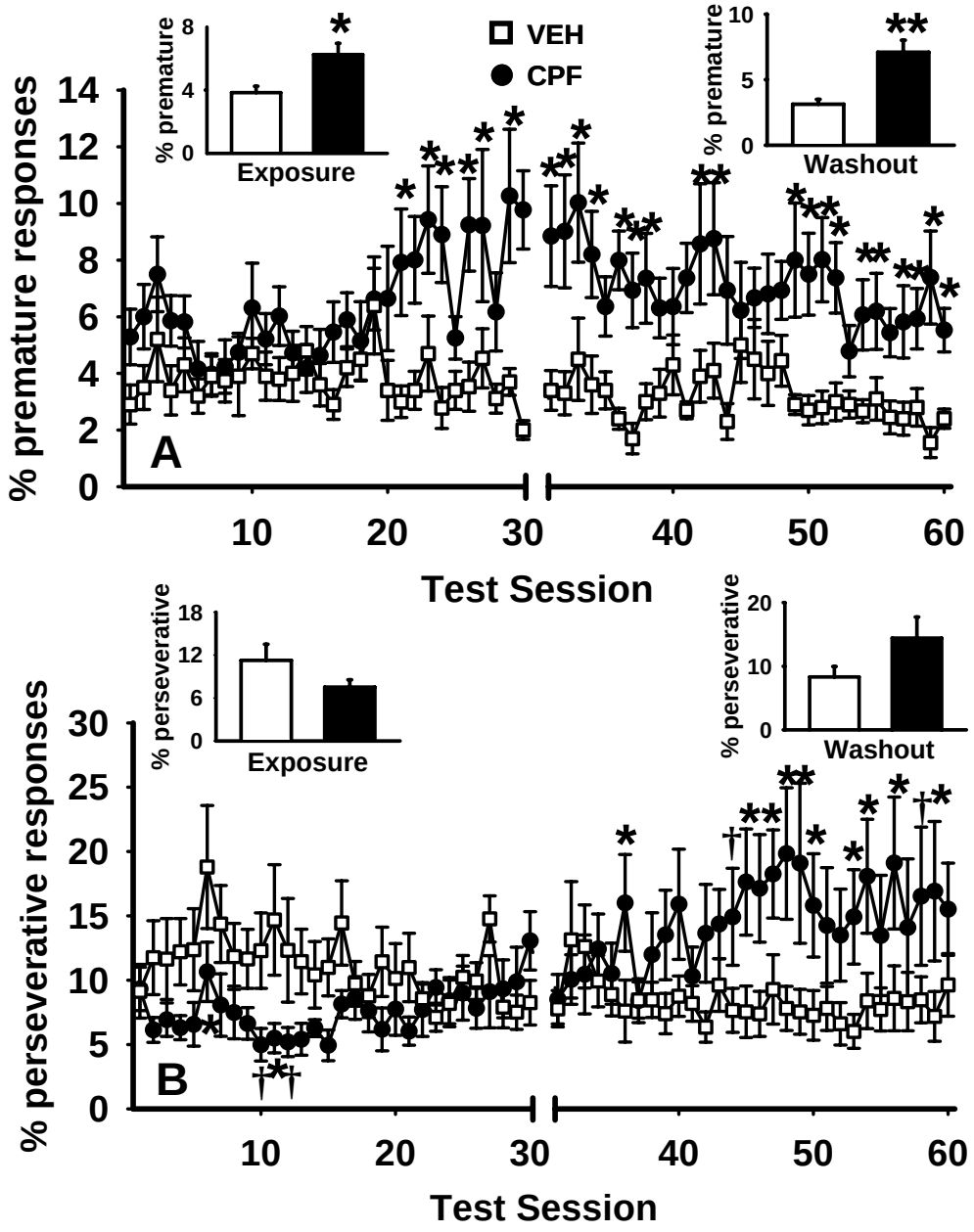

Figure 2.5. The effects of CPF (18.0 mg/kg) exposure on the percentage of premature responses (A) and perseverative responses (B) in the 5C-SRTT during the 30 day every other day exposure period and during the 30 day (drug-free) washout period. The histogram insets illustrate the overall effect (i.e., responses averaged across the sessions) during the drug exposure and washout periods. * = Statistically significant difference ($p<0.05$) between CPF-treated and vehicle-treated controls. ** = $p<0.01$; † = $p<0.06$. (N=8-10).

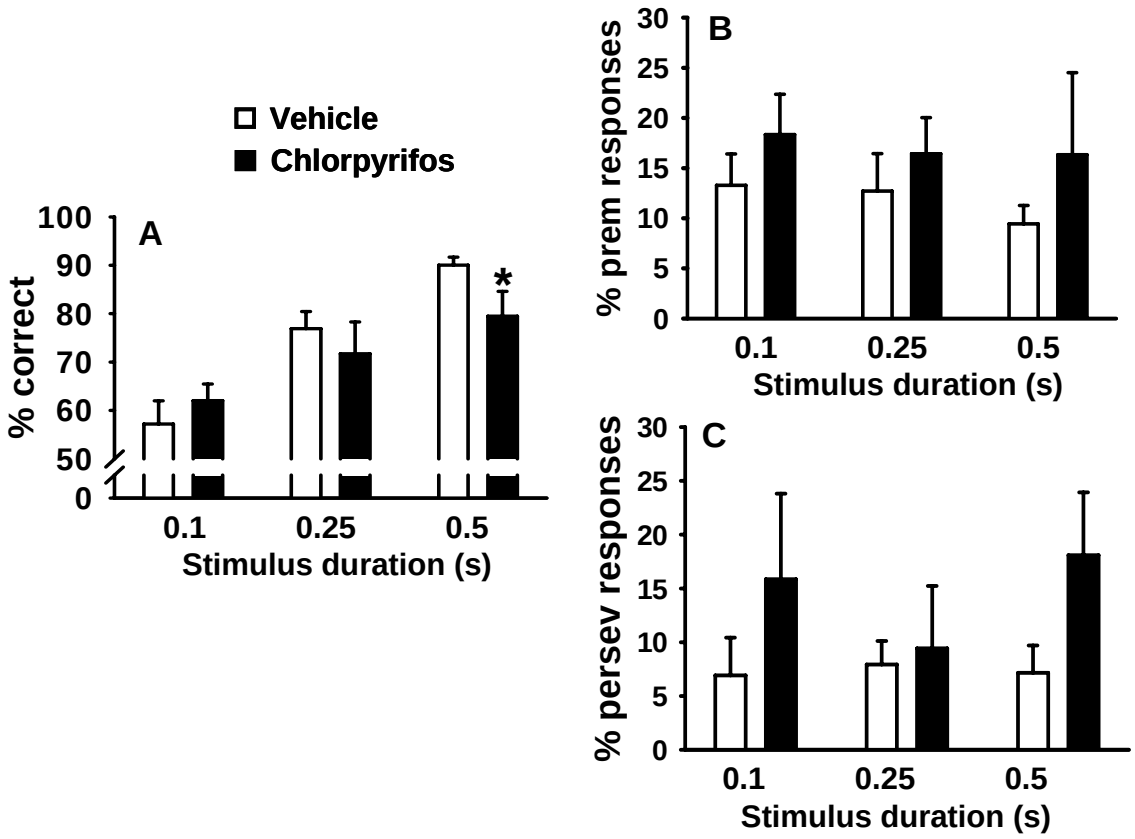

Figure 2.6. The residual effects of CPF (18.0 mg/kg) exposure (30 day every other day exposure method) in a variable stimulus duration version of the 5C-SRTT on day 31 of a drug-free washout period. Dependent measures include: % correct (A), percentage of premature responses (B) and the percentage of perseverative responses (C). Data are presented as mean ± SEM for each stimulus duration. * = Statistically significant difference ($p<0.05$) between CPF-treated and vehicle-treated controls. (N=6-8).

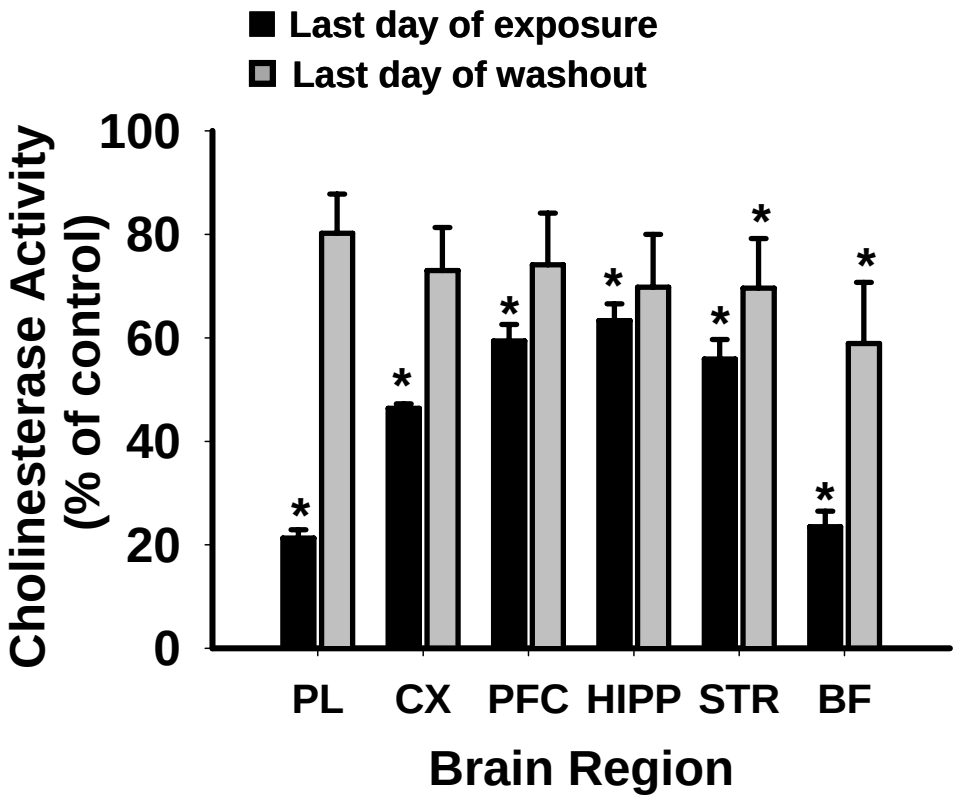

Figure 2.7. The effects of CPF (18.0 mg/kg) exposure (14 day every day exposure method) on cholinesterase activity. Data (mean ± SEM) are presented as % of vehicle matched control levels immediately following 14 consecutive days of exposure to CPF (black bars) and 31 days after the last exposure to CPF (gray bars) Cholinesterase activity was obtained from: plasma (PL); CX (cortex); PFC (prefrontal cortex); HIPP (hippocampus); STR (striatum); BF (basal forebrain). *Statistically significant at $p<0.05$. (N=6).

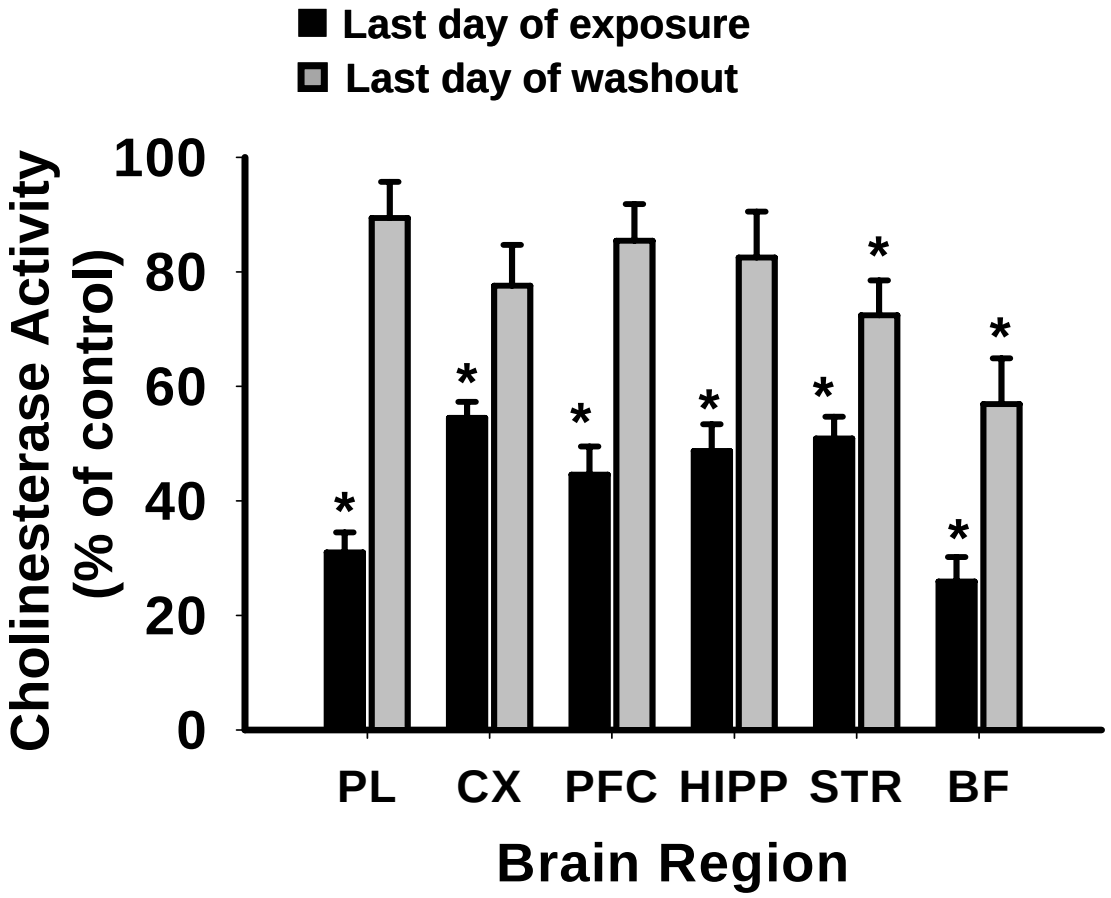

Figure 2.8. The effects of CPF (18.0 mg/kg) exposure (30 day every other day exposure method) on cholinesterase activity. Data (mean ± SEM) are presented as % of vehicle matched control levels immediately following the last exposure to CPF (black bars) and 31 days after the last exposure (gray bars) Cholinesterase activity was obtained from: plasma (PL); CX (cortex); PFC (prefrontal cortex); HIPP (hippocampus); STR (striatum); BF (basal forebrain). *Statistically significant at $p<0.05$. (N=6).

CHAPTER 3

EXPOSURE TO CHLORPYRIFOS AND CHLORPYRIFOS-OXON RESULTS IN ALTERED MITOCHONDRIAL DYNAMICS AND DEFICITS IN AXONAL TRANSPORT IN RAT CORTICAL

M-L. Middlemore-Risher, A.V. Terry Jr. To be submitted to Journal of Neuroscience.

Abstract

The organophosphate chlorpyrifos (CPF) is one of the most commonly used insecticides in the world with multiple pest-control related applications in agriculture and industry. We have previously reported that repeated, sub-acute exposures to CPF (i.e., at doses not associated with signs of acute toxicity) resulted in protracted impairments in the performance of attention and memory-related tasks in rodents as well as deficits in axonal transport ex-vivo (i.e., in the sciatic nerve). Here we investigated the effects of CPF and its active metabolite CPFoxon (CPO) on neuronal function at the mitochondrial level (specifically mitochondrial dynamics and axonal transport) in rat primary cortical neurons using time-lapse confocal techniques. Exposure to CPF (1-20μM) or CPO (5nM-20μM) for 1-24 hours resulted in a decrease in axonal transport accompanied by mitochondrial redistribution (at 20uM CPF/CPO), a concentration-dependent increase in mitochondrial length and decrease in mitochondrial number (indicative of increased fusion versus fission events). Further experiments indicated that these changes occurred at concentrations of CPF/CPO that did not inhibit acetylcholinesterase activity (known to be the primary mechanism of action). Additionally, the changes were not affected by cholinergic receptor ligands (atropine or epibatidine) and did not appear to be associated with direct effects on mitochondrial function (i.e., diminished ATP production, mitochondrial membrane potential or superoxide production). Given the importance of mitochondrial dynamics and axonal transport to neuronal function, these in vitro results provide potential mechanisms for the CPF-related deficits in cognitive function previously observed in vivo.

Introduction

Chlorpyrifos (CPF) belongs to a group of broad spectrum organophosphorous (OP) insecticides that are used worldwide for agriculture, control of vector borne diseases and pest removal. The primary mechanism of action for CPF as an insecticide is irreversible inhibition of cholinesterases by the active metabolite chlorpyrifos-oxon (CPO). The resulting elevation of acetylcholine leads to over-stimulation of cholinergic synapses (Pope et al., 2005;Rohlman et al., 2007). Unfortunately these effects are non-selective for insects, and as a result acute exposure to CPF can lead to a number of toxicological symptoms including seizures, hypersecretory activity, and even death in humans and other non-target organisms (Pope, 1999). Human case reports and rodent studies have documented sustained cognitive deficits after OP exposure even when signs of acute toxicity were absent (De Silva et al., 2006;Terry, Jr. et al., 2003;Terry, Jr. et al., 2007;Middlemore-Risher et al., 2010). Symptoms of such exposures can include impaired attention and decreased reaction time that can persist for years after an event, however, the specific mechanism of this phenomenon remains unknown (De Silva et al., 2006).

We have previously shown that axonal transport of vesicles is significantly reduced in the sciatic nerve (ex-vivo) within 10 hours of a single 18mg/kg s.c. injection of CPF and that protracted impairments of axonal transport can be observed after repeated CPF exposures (Terry, Jr. et al., 2003;Terry, Jr. et al., 2007). These observations appear to complement previous studies showing that CPF can impair microtubule-associated protein function, covalently modify tubulin and disrupt kinesin-dependent microtubule motility (Howard et al., 2005;Grigoryan et al., 2008;Prendergast

et al., 2007;Gearhart et al., 2007), key components of the axonal transport process . Such disruptions can reduce the transportation of cargo to and from synaptic terminals, weakening synaptic maintenance and plasticity (Chang and Reynolds, 2006). The role of mitochondria in axonal transport is of particular interest since ATP is necessary to drive motor protein dependent transport (including mitochondrial axonal transport). Therefore both the direct effects of CPF on axonal transport as well as indirect effects through the disruption of mitochondrial dynamics, including ATP synthesis could have devastating consequences on neuronal function and cognition (Chang and Reynolds, 2006).

In recent years microscopy and time lapse imaging techniques have greatly improved in conjunction with an increased availability of novel fluorescent markers for identifying and analyzing mitochondria in neuronal culture. Therefore we examined the effects of CPF/CPO exposure on mitochondrial transport and morphology in primary cortical neurons (since cortex is an area important for attention and other domains of cognition), to determine the roles that direct cholinergic receptor interactions and mitochondrial function may play. This work was conducted to elucidate potential novel mechanisms for CPF-related deficits in cognitive function previously observed in vivo (Terry, Jr. et al., 2003;Terry, Jr. et al., 2007;Middlemore-Risher et al., 2010).

Materials and Methods

Cell culture. Instructions for culturing neurons were obtained from Poindron et al., (2005) with modifications. Briefly, the cortex from postnatal day zero Sprague Dawley pups was removed, placed in dissecting media + trypsin (500mL HBSS, 2.5g glucose,

2.4g HEPES, 3.5g sucrose, pH 7.3-7.4. Sterile filtered and stored at 4^{o}C) and incubated at 37^{o}C in a water bath with gentle shaking for 25 minutes. The reaction was halted by adding 5mL of culture medium with serum (500mL RPMI-1640 media**,** 25mL FBS**,** 50mL horse serum (heat inactivated)**,** 1.25mL of 100 U/ml penicillin, and 100 µg/ml streptomycin). The tissue was centrifuged for 5 minutes at 1000xg and the supernatant was removed and replaced with 1mL culture medium with serum. Mechanical dissociation was performed using and the dissociated neurons were filtered (BD Falcon 352340) into a sterile tube and centrifuged for 5 minutes at 1000xg. The neuronal pellet was resuspended in 1mL of serum culture medium and the cells were counted and added to poly-L-lysine coated plates at the desired concentration. After 1 hour incubation at 37^{o}C/5%CO2 the serum media was removed and replaced with serum free media (500mL Neurobasal Media, 2% B27, 300µL of 100 U/ml penicillin, and 100 µg/ml streptomycin, 75µL L-glutamine. Sterile filtered and stored at 4oC). The neurons were used for experiments 7 days later.

Drugs and chemicals. CPF and CPO were obtained from ChemService (PS-674, MET-674B, West Chester, PA, USA). CPF was dissolved in 0.5% DMSO and used immediately. CPO was dissolved in methanol (80mM) and stored at -80oC until needed. The final concentrations of DMSO and methanol that were used in the cell cultures (for vehicle and compound exposures) was 0.01%. Atropine (Invitrogen A0257) and Epibatidine (Invitrogen E1145) were dissolved in water for immediate use.

Measurement of mitochondrial axonal transport and morphology. Neurons were grown on 8 well 1.5 German glass bottom chamberslides (Nalge Nunc 155409). 0, 1, 5, 10, 20µM of CPF or 0, 0.005, 1, 5, 10, 20µM CPO was added to the media for 1 or 24

hours. After the desired exposure period the neurons were fluorescently tagged with (50nM) mitotracker CMXRos (Invitrogen M7512) and placed in phenol red-free neurobasal media. The chamberslides were placed on the confocal microscope (Deltavision, Deconvolution Olympus IX71, Washington, USA) in a Precision Control Weather Station (37oC, 5% CO2) for 5 minutes to equilibrate before imaging. Images of axonal mitochondria were captured every second for 8 seconds or every second for 1 minute using a 60X, 1.42 NA objective (SoftWoRx, Applied Precision, Washington, USA). The images were compressed into AVI animation files using NIH Image J (http://rsb.info.nih.gov/ij/). Mitochondrial length and number were measured within the axon (>100μm from the cell body) from the first still frame and the number of moving mitochondria were counted over 5 sequential frames using LSM Image Browser. The specific criteria for mitochondrial movement was determined based on previously published literature and on our own observations (Ligon and Steward, 2000;Kaasik et al., 2007). The working definition for mitochondrial movement: 1) Mitochondrion must be moving in the same direction for a minimum of 3 out of 5 total frames observed. 2) Mitochondrial leading and trailing edges must be moving in the same direction. The results were expressed as the number of mitochondria/μm, mean mitochondrial length and number of mitochondria moving/μm as a percent of control.

Atropine and epibatidine co-incubation experiments. The method described previously for assessing mitochondrial axonal transport, length and number was used here to determine the effects of co-incubation of CPF or CPO with atropine and/or epibatidine. Specifically, neurons were co-incubated with 3, 30 and 90μM epibatidine (Sigma Aldrich E1145) and/or 1, 10 and 50μM atropine (Sigma Aldrich A0257) with 1

and 20μM CPF or 0.005μM and 20μM CPO for 24 hours. The same criteria (as previously described) was used to obtain images and for analysis.

Mitochondrial distribution experiments. Neurons were grown on 25mm glass coverslips. 0 and 20μM CPF or CPO was added to the media for 24 hours. The neurons were incubated with mitotracker deep red 633 (Invitrogen M22426), fixed in 4^{o}C methanol, permeabilized with 0.2% triton in PBS and blocked with 10% goat serum in PBS. The neurons were then incubated with an antibody against Tau (Sigma Aldrich T8326, 1:2000) for 1 hour followed by incubation with FITC (Jackson Immuno-Research 115-095-003, 1:75) for 1 hour at room temperature. The neurons were rinsed twice with PBS and placed on glass slides (Fisher Scientific 12-550-15) using PVA-DABCO (polyvinyl alcohol, 1,4, diazabicyclo [2,2,2,] octane, Tris-HCl, Glycerol). The slides were stored flat at 4^{o}C until imaging using confocal microscopy. The slides were placed on the stage of a Leica SP2 scanning confocal microscope and imaged using a 63X, 1.4 NA objective. Cells were excited using 488nm (for FITC) or 633nm (for mitotracker) laser lines. Average pixel intensity was determined within the specified region (30μm^2 area, 5μm from the nucleus), normalized with background subtraction using the Zeiss LSM Image Analysis software and expressed as percent of control.

AChE Inhibition Assays. Neurons were grown on clear 96 well plates (Nunc, Thermo Scientific, Rochester, NY) and treated with CPF or CPO (≥1μM and ≥0.005μM respectively) for 24 hours. AChE inhibition was determined based on the Ellman assay (1961) with modifications by Schuh et al., (2002) and Prendergast et al., (2007). Briefly, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide were added to the wells and allowed to equilibrate for 1 minute. Plates were then loaded into the

Beckman Coulter DTX 880 multimodal detector (Fullerton, CA, USA) and absorbance at 412nm was measured every 2 minutes for 16 minutes. The rate of AChE activity was then calculated for each time point of measurement using the formula (Δ absorbance/min)/($1.36x10^4$).

Mitochondrial membrane potential, ATP synthesis, and superoxide production. Neurons were grown on 96 well plates ((Nunc, Thermo Scientific, Rochester, NY) and 50mm glass coverslips (Fisher 22050238) and treated with 0-500μM CPF or CPO for 24 hours. The media/toxin was removed and the neurons were incubated with DePsipher to determine mitochondrial membrane potential ($\Delta\Psi m$) (Trevigen 6300-00K) or MitoSOX to determine changes in superoxide production (Invitrogen M36008). To obtain representative images of DePsipher fluorescence the neurons were imaged using the Leica SP2 scanning confocal microscope and imaged using a 63X, 1.4 NA objective. A 485nm excitation filter and 535nm and 590nm emission filters were used to measure green and red fluorescence respectively. Quantification was performed using the photon-counting multimode plate reader (Mithras LB940; Berthold Technologies GmbH Wildbad, Germany) (for more details see Hollins et al., 2009) and calculated as green fluorescence divided by red fluorescence to determine a ratio and expressed as a percent of control. MitoSox emission was measured using the plate reader mentioned above at 515/590nm a/e and expressed as a percent of control.

To determine changes in ATP production the bioluminescent somatic cell assay kit was used (Invitrogen FLASC). Luminescence quantification was determined using the Microplate Fluorescence Reader (FLx800 and KC Junior software, BioTek Instruments, Inc.) and was expressed a percent of control.

Statistics. Comparisons between treatment groups were made using analysis of variance (ANOVA) followed by the Student-Newman-Keuls method for post hoc analysis (SigmaStat 2.03, SPSS Inc., Chicago, IL, USA). Statistical significance was assessed using an alpha level of 0.05. Data are shown as Mean ± SEM. *p* values reflect differences between treatments unless stated otherwise.

Results

CPF exposure disrupts mitochondrial axonal transport, distribution and morphology

Live cell imaging revealed that after 24 hour exposure to CPF there was a decrease in mitochondrial axonal transport that plateaued at approx. 50% of control ($p<0.05$) (Fig.3.1A). Representative time lapse images of single axons (found in supplemental movies 1 and 2) clearly indicate a reduction in mitochondrial transport after 24hr exposure to 1μM CPF. This was confirmed by a significant redistribution of mitochondria as seen in Figure 3.1B ($p=0.003$). The reduction in transport was accompanied by a dose dependent increase in mitochondrial length (approx. 180% of control) and decrease in mitochondrial number (approx. 60% of control) suggestive of an increase in mitochondrial fusion versus fission events (Fig. 3.1C,D). To determine how rapid the deficits in transport and changes in morphology occurred we looked at the 1 hour time point. Here we observed axonal transport deficits (approx. 35% of control) and more robust changes in mitochondrial dynamics (i.e. increase in mitochondrial length (approx. 250% of control) and decrease in mitochondrial number (approx. 40% of control) suggesting that these changes can occur rapidly and persist for 24 hours (Fig. 3.2A-C).

Representative images of 1 hour and 24 hour exposure to CPF are provided in Fig. 3.3A,B. The images show a significant dose dependent increase in mitochondrial elongation. This is accompanied by a decrease in surrounding mitochondria supporting the previous findings that show redistribution with a reduction in total mitochondria. Interestingly, the changes in mitochondrial length appear to be more robust than the decreases in mitochondrial number (at 1 and 24 hours) suggesting that mitochondrial elongation begins prior to the induction of mitochondrial fusion. Therefore the results show that CPF exposure promotes a dose dependent increase in elongation and fusion as well as deficits in axonal transport as early as 1 hour that can persist for up to 24 hours.

Exposure to the metabolite CPO disrupts mitochondrial axonal transport, distribution and morphology

It has been suggested that the majority of CPF activity (as a cholinesterase inhibitor-based insecticide) is expressed via its conversion to CPO; therefore it was important to determine if the changes in mitochondrial dynamics and transport occurred in the presence of CPO alone. 24 hour exposure to CPO resulted in a similar decrease in mitochondrial axonal transport (approx. 40% of control) and was accompanied by a redistribution of mitochondria to the initial segment ($p<0.001$) (Fig. 3.4A,B). Figure 3.4C,D show an increase in mitochondrial length (approx. 350% of control) however the decrease in mitochondrial number was more subtle with a maximum reduction of 40% ($p=0.058$). Representative time lapse images of single axons showing mitochondrial transport can be found in supplemental movies 3 and 4 clearly indicating a reduction in

mitochondrial transport after exposure to 1.0μM versus control. At the 1 hour time point there were significant deficits in axonal transport (approximately 40% of control, Fig. 3.5A) and an increase in mitochondrial length (225% of control) (Fig. 3.5B). The decrease in mitochondrial number (65% of control) was consistent with 24 hour CPO exposure (60% of control) (Fig. 3.5C). Representative images of 1 hour and 24 hour exposure to CPO are shown in Fig. 3.6A,B revealing an increase in elongated/tubular mitochondria versus the shorter more globular mitochondria present in the control images.

AChE inhibition is not responsible for the deficits in axonal transport and changes in mitochondrial dynamics previously observed

Subsequent experiments were conducted to determine if the effects on mitochondrial axonal transport, length and number were due to the metabolism of CPF to its active metabolite CPO. Since the presence of CPO results in AChE inhibition, determining the level of inhibition following exposure to varying concentrations of CPF/CPO allowed us to indirectly assess relative levels of CPO. Following 24 hour exposure to CPF there was significant dose dependent AChE inhibition at 5, 10, 20μM respectively (Figure 3.7A). However, there was no detectable AChE inhibition at 1μM CPF (p=0.899) despite finding significant deficits in axonal transport and increased mitochondrial fusion (Fig. 3.7A). As expected, 24 hour exposure to CPO resulted in a more pronounced AChE inhibition from 0.01-20μM (Fig. 3.7B). Further results showed that changes in mitochondrial dynamics and deficits in axonal transport persisted even after 24 hour exposure to 0.005μM where no AChE inhibition was observed (Fig. 3.9 & 3.11 A-C).

These results, therefore, suggest that the deficits in axonal transport and changes in mitochondrial dynamics are not dependent on AChE inhibition.

Elevated cholinergic receptor interactions are not necessary to promote the deficits in axonal transport and changes in mitochondrial dynamics previously observed.

Epibatidine and atropine (nicotinic and muscarinic-acetylcholine receptor ligands, respectively) were co-incubated with CPF and CPO to determine whether CPF/CPO-induced changes in mitochondrial dynamics are due to the secondary effects of elevated synaptic ACh at its receptors. The concentrations we used were based on previous studies by Egea et al., (2007) and Heppner and Fiekers (1992). Two higher concentrations were also used to ensure that no protection was left undetected.

Epibatidine: Co-incubation of 1μM CPF with 3, 30 and 90μM epibatidine for 24 hours did not reverse (or attenuate) the OP-related axonal transport deficits (Fig. 3.8A). Mitochondrial elongation was also still present and the mitochondrial number remained elevated as was previously observed ($p<0.001$) (Fig. 3.8B,C). Co-incubation of 0.005μM CPO with 3, 30, 90μM epibatidine for 24 hours also failed to reverse axonal transport deficits or changes in mitochondrial morphology (i.e. length and number) (Fig. 3.9A-C). Additional experiments were conducted co-incubating 20μm CPF/CPO with epibatidine, and as with the lower doses of CPF/CPO there was no improvement in transport or recovery of mitochondrial dynamics (data not shown). The data show that co-incubation with epibatidine offers no protection against the effects of low level CPF/CPO.

Atropine: When 1μM CPF was co-incubated with 1, 10, 50μM atropine there was no reversal of the mitochondrial axonal transport deficits. The mitochondrial number remained low and length remained high relative to vehicle controls (Fig. 3.10A-C). Similarly, co-incubation of 0.005μM CPO with 1, 10, 50μM atropine failed to reverse the axonal transport deficits and mitochondrial elongation/fusion (Fig. 3.11A-C). Additional experiments co-incubating 20μm CPF/20μm CPO with atropine failed to improve transport and recovery of mitochondrial dynamics (data not shown).

Our findings indicate that changes in mitochondrial axonal transport and mitochondrial dynamics are not dependent on ACh-receptor interactions.

Changes in axonal transport and mitochondrial dynamics are independent of direct mitochondrial toxicity

Having ruled out cholinergic-receptor effects as a possible mechanism for the deficits in mitochondrial transport and morphology previously observed, we then determined whether these changes were secondary to direct effects of CPF/CPO on mitochondrial function.

Mitochondrial Membrane Potential: $\Delta\Psi m$ was assessed by staining with DePsipher (Trevigen) since the collapse of the electrochemical gradient is a key indicator of cell death. This is a lipophilic cationic dye that exists as a monomer at low concentrations in the neuron (emission peak 527nm; green) however in the presence of mitochondria (with an intact $\Delta\Psi m$) the dye accumulates and aggregates (emission peak 590nm; red) allowing a ratio of green/red fluorescence to be determined. After 24 hour exposure to 1-20μM CPF (concentrations relevant to the deficits in axonal transport and changes in

mitochondria dynamics) there were no significant changes in ΔΨm (Fig. 3.12A,B,D). As expected, significant compromise of ΔΨm was observed at concentrations that are known to be cytotoxic i.e., 500μM CPF (p=0.003, p=0.002 respectively) (Fig. 3.12C,D). There was no significant decrease in ΔΨm after 24 hour exposure to 1-20μM CPO (Fig. 3.13A,B,D). As with the 24 hour exposure to 500μM CPF there was a significant decrease in ΔΨm at 500μM CPO (Fig. 3.13C,D).

ATP synthesis: Given the importance of ATP synthesis for motor protein dependent axonal transport and general neuronal function, ATP production was assessed after 24 hour exposure to 1-20μM CPF and CPO. The results indicate that there is no significant reduction in ATP production after exposure to the parent compound (CPF) or active metabolite (CPO) (p=0.116-0.291, p=0.203 respectively) (Fig. 3.14A,C).

Superoxide Production: Lipid peroxidation has been observed after exposure to high levels of CPF and relies on the presence or lack of clearance of superoxide, so we investigated whether there were elevated levels of mitochondrial superoxide after exposure to 1-20μM CPF and CPO. Results show no significant increase in superoxide production with the parent compound or active metabolite when compared to vehicle controls (p=0.130, p=0.236 respectively) (Fig. 3.14B,D).

We therefore conclude that CPF/CPO exposure results in changes to mitochondrial dynamics and impaired axonal transport that are not consequences of impaired mitochondrial function or increased ROS production.

Discussion

The experiments described in this report indicated that cultured cortical neurons exposed to CPF or CPO results in a concentration-dependent decrease in axonal transport (accompanied by mitochondrial redistribution within the axon initial segment), an increase in mitochondrial length, and a decrease in mitochondrial number (indicative of increased fusion versus fission events). The neuronal changes occurred at concentrations of CPF/CPO that did not inhibit acetylcholinesterase activity, they were not by attenuated or blocked by cholinergic receptor ligands, and they did not appear to be associated with directly toxic effects on mitochondria as would be suggested by diminished ATP production, alterations in mitochondrial membrane potential, or elevations in superoxide production.

The effects of CPF/CPO on axonal transport may be a particularly important finding given the fundamental importance of this process to neuronal function. Interestingly deficits in axonal transport have been implicated in many neurodegenerative diseases (Chang and Reynolds, 2006;Trushina and McMurray, 2007;Lin and Beal, 2006), with symptoms ranging from cognitive decline to peripheral neuropathies (Pigino et al., 2003;Misko et al., 2010;Trushina et al., 2004). The processes by which these deficits occur appear to vary and remain to be elucidated in many of these neurodegenerative diseases. However, there is a consensus that axonal transport impairments result in the depletion of newly synthesized proteins and decreased removal of damaged proteins and organelles from the synaptic terminals. Such transport deficits and a decreased ability of mitochondria to meet the spatial and local transient demands of the cell can compromise neuronal function and promote

programmed cell death (Chen and Chan, 2006;Chang and Reynolds, 2006;Iijima-Ando et al., 2009).

In the current study a decrease in mitochondrial axonal transport and an increase in the number of mitochondria in the initial segment were observed, suggestive of a change in mitochondrial distribution. One explanation for this observation could be that intracellular Ca^{2+} has increased in particular areas, resulting in redistribution. However, work by Saotome and colleagues (2008) have shown that mitochondria undergo enhanced fission when relocated to areas of high Ca^{2+}, whereas exposure to CPF/CPO resulted in enhanced mitochondrial fusion. Another possibility is that redistribution is due to an unequal disruption of anterograde versus retrograde mitochondrial transport**.** Previous studies have shown that bidirectional transport is affected following OP exposure through the direct covalent modification of specific tyrosine residues located near GTP binding sites or within regions of protofilament-protofilament interactions, thus inhibiting microtubule formation (Grigoryan et al., 2008;Prendergast et al., 2007). Additionally, co-incubation of kinesin (anterograde motor protein) with CPF and CPO has been shown to disrupt kinesin-dependent transportation along microtubules, (Gearhart et al., 2007). The effects of OPs on dyneins/dynactin (retrograde motor proteins) have not been investigated to date. It is possible that disruptions in either kinesins or dyneins/dynactin may impair bidirectional transport, however the relative inhibition of anterograde versus retrograde mitochondrial transport by CPF/CPO is unclear at present (Brady et al., 1990;Martin et al., 1999;Deacon et al., 2003).

The dose dependent decrease in mitochondrial number and increase in mitochondrial length after CPF/CPO exposure observed in this study could indicate an

increase in fusion versus fission events (i.e. hyperfusion). When considering the OP-related changes in mitochondrial dynamics and the axonal transport deficits previously discussed, there are three potential scenarios that should be considered: 1. They are independent events; 2. Mitochondrial fusion occurs and the resultant elongation impedes axonal transport; 3. Axonal transport deficits actually promote mitochondrial fusion in some manner. These are challenging questions that remain unanswered in the literature. In contrast with the idea that these events are independent of each other, it is possible that the changes in mitochondrial dynamics are a stress-induced event and/or due to disruption of key fusion and fission proteins such as mfn2/opa1 or drp1 leading to deficits in mitochondrial transport. This idea may hold credence since Russo and colleagues (2009) recently reported that mfn2 interacts with Miro (an essential member of the complex that links mitochondria to kinesin motor proteins) to assist with bidirectional transport. More recently Misko and colleagues (2010) determined that disruption of mfn2 can selectively disrupt mitochondrial transport/distribution and is suggested to be the cause of long peripheral axon degeneration in Charcot Marie Tooth disease (Cartoni and Martinou, 2009). Further studies will be necessary to determine whether CPF/CPO have direct effects on these crucial fusion/fission proteins and whether deficits in axonal transport can promote alterations in mitochondrial dynamics.

Another goal of this study was to determine how CPF/CPO might directly affect mitochondrial viability and ATP production (important for motor protein function, facilitating the transport of cargo to and from the pre-synaptic terminals). Therefore, we addressed whether $\Delta\Psi m$ (an early marker of cell death) or ATP production were compromised after CPF/CPO exposure. Our studies indicate that $\Delta\Psi m$ and ATP

production matched vehicle control levels, suggesting that direct mitochondrial toxicity or impairments in the ability of mitochondria to generate ATP were not responsible for the dose dependent axonal transport deficits observed.

It is important to note that mitochondrial fusion and the formation of mitochondrial networks have been described as a pro-survival mechanism whereby $\Delta\Psi m$ (and therefore ATP production) are maintained under stressful conditions, even when Bax has translocated to the mitochondrion (Lee et al., 2004;Tondera et al., 2009). Furthermore, impaired mitochondrial fusion can result in loss of $\Delta\Psi m$ and a reduction in oxidative phosphorylation (OXPHOS) (Olichon et al., 2003;Chen et al., 2005). Despite the normal levels of ATP reported here, we did not differentiate between OXPHOS and glycolysis. However, based on previous data, ATP production via OXPHOS can result in the production of highly networked mitochondria whereas glycolysis dependent ATP production can produce more spherical mitochondria though this has yet to be established in neuronal cells (Plecita-Hlavata et al., 2008). Therefore we can conclude that either 1) there is no compromise of $\Delta\Psi m$ or OXPHOS-dependent ATP production or 2) mitochondrial fusion is induced in order to maintain $\Delta\Psi m$ and ATP production therefore disguising any underlying dysfunction.

It is accepted that OPs can elicit oxidative stress and DNA damage at high concentrations (Bagchi et al., 1995;Soltaninejad and Abdollahi, 2009). Slotkin and colleagues (2005) have shown that CPF can evoke lipid peroxidation in the developing rat brain at concentrations that only cause mild signs of systemic toxicity. Additionally, PC12 cells (commonly used as a neuronal developmental model) show a concentration dependent increase in ROS formation in response to CPF exposure (Crumpton et al.,

2000). We were unable to detect significant increases in production of superoxide in this study (at the concentrations of CPF and CPO evaluated), but it is still possible that low levels of superoxide (i.e., below our limits of detection) could promote fusion.

There are many other factors that are yet to be elucidated: the long-term implications of CPF/CPO-induced chronic hyperfused mitochondria; whether CPF/CPO exposure influences dendritic function, plasticity and connectivity; whether there is a role for astrocytes in the clearance of CPF/CPO, including buffering the effects of this toxic insult; and whether repeated exposure to these compounds leads to increased vulnerability to stress-induced cell death. It has been suggested that CPF may decrease neuronal resistance to other oxidative stressors, increasing vulnerability to disease states such as ALS, Huntington's, Alzheimers Disease (AD) and Parkinson's Disease (Crumpton et al 2000;Pantelidou et al., 2007;Trushina et al., 2004;Rui et al., 2006;Beal, 2003;Hayden et al., 2010) all of which have been previously linked to impaired axonal transport and altered mitochondrial dynamics (Chevalier-Larsen and Holzbaur, 2006;Chevalier-Larsen and Holzbaur, 2006;Ferreirinha et al., 2004;Gunawardena et al., 2003) though direct evidence is still lacking.

Conclusion

In conclusion, the results of this study indicate that cultured cortical neurons exposed to the commonly used OP insecticide CPF and its oxidative metabolite CPO results in a concentration-dependent decrease in axonal transport of mitochondria, an increase in mitochondrial length and a decrease in mitochondrial number (indicative of increased fusion versus fission events). These neuronal changes occurred at

concentrations of CPF/CPO that did not inhibit AChE activity, they were not attenuated by cholinergic receptor ligands, and they did not appear to be associated with directly toxic effects on mitochondria. Given the importance of mitochondrial dynamics and axonal transport to neuronal function, these in vitro results provide potential mechanisms for the CPF-related deficits in cognitive function previously observed in vivo.

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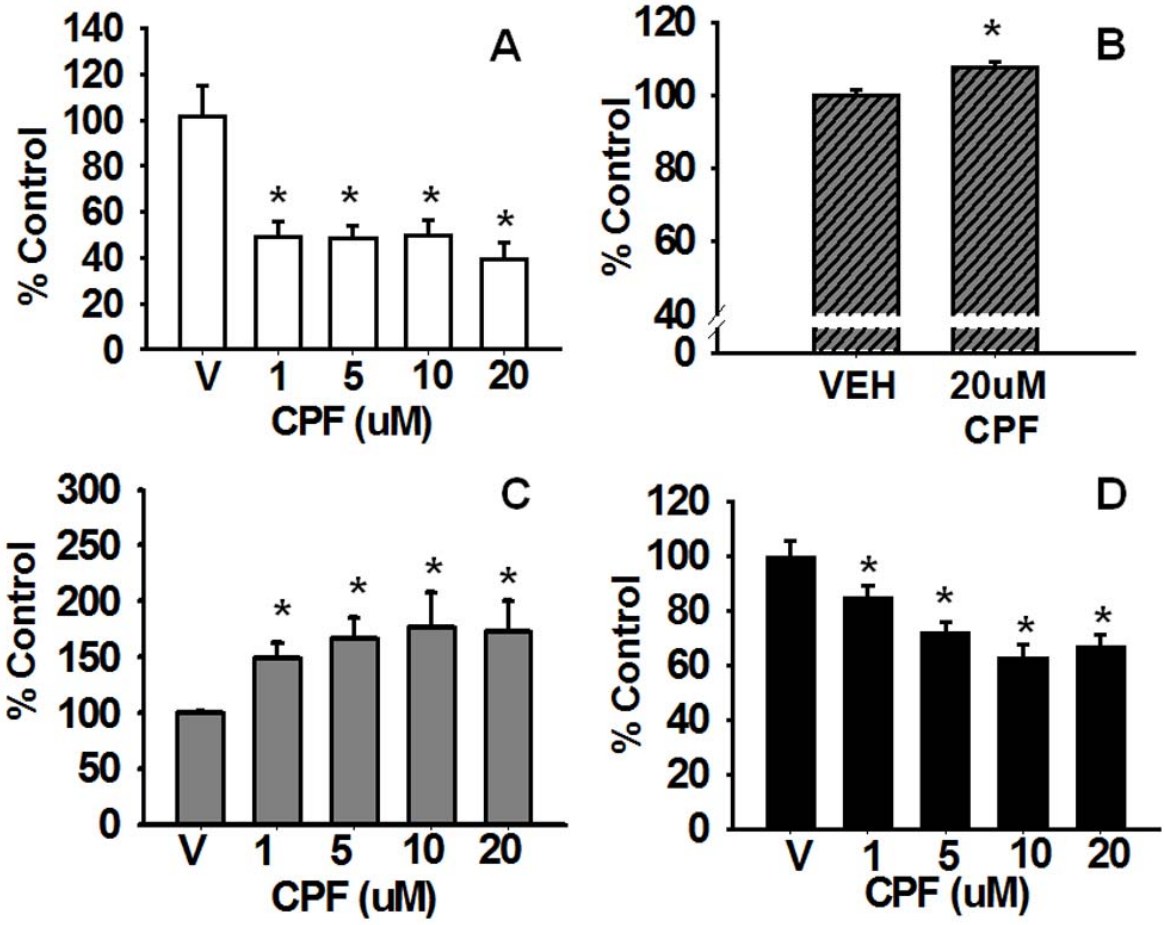

Figure 3.1. Exposure to CPF disrupts mitochondrial transport and alters mitochondrial dynamics. **A, C, D -** 24 hour exposure to CPF was associated with a decrease in axonal transport , a dose dependent increase in mitochondrial length, and decrease in mitochondrial number respectively. **B,** Increased number of mitochondria in the initial segment presents a change in mitochondrial distribution. Data are presented as mean (% control) ± SEM (approx. 25 neurons from 3 experiments). *****Significant difference from vehicle (V) control (p<0.005).

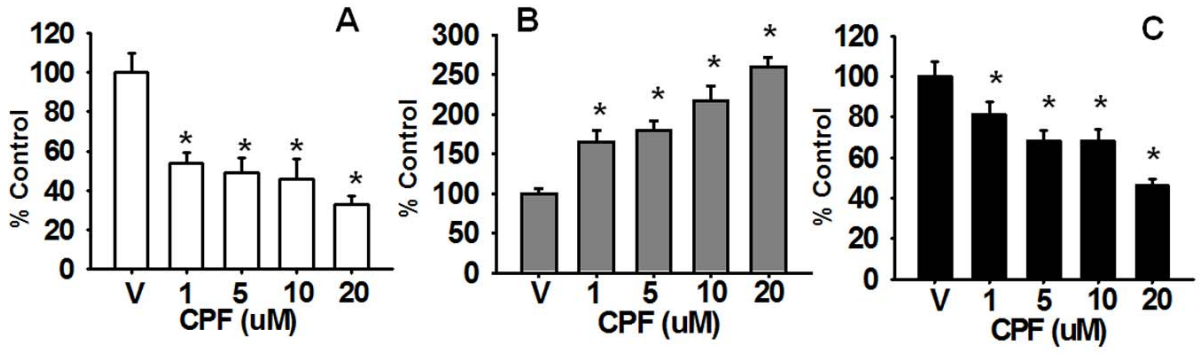

Figure 3.2. Exposure to CPF disrupts mitochondrial transport and alters mitochondrial dynamics. **A, B, C -** 1 hour exposure to CPF was associated with a dose dependent decrease in axonal transport, an increase in mitochondrial length, and a decrease in mitochondrial number respectively. Data are presented as mean (% control) ± SEM (25 neurons from 3 experiments). *****Significant difference from vehicle (V) control (p<0.005).

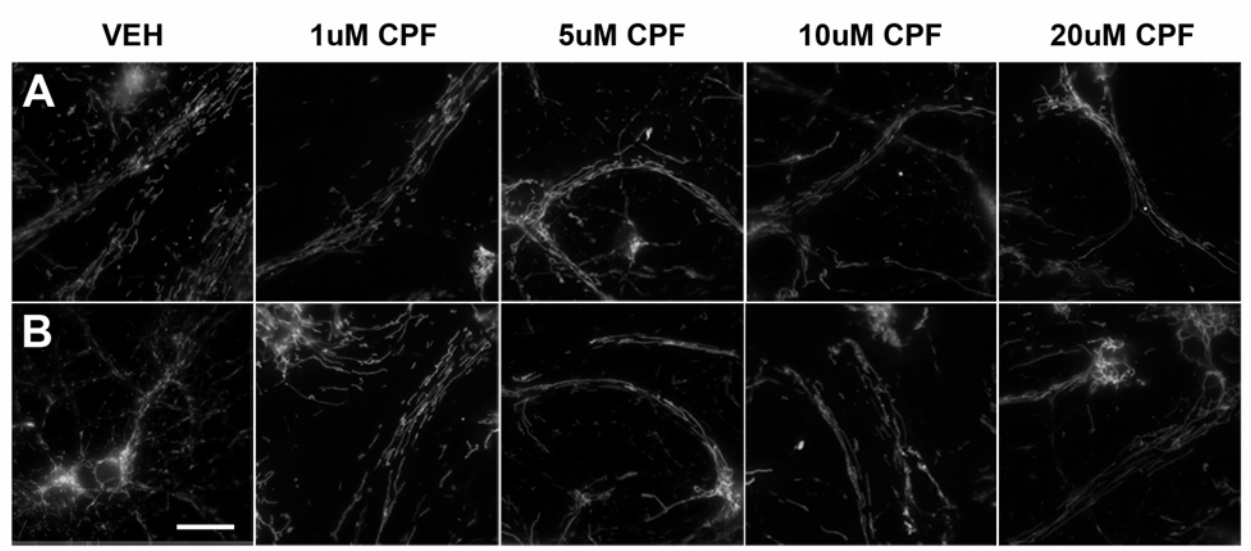

Figure 3.3. Exposure to CPF disrupts mitochondrial morphology and fusion/fission dynamics. Shown here are representative images of neuronal cultures after **A,** 1 hour and **B,** 24 hour exposure to increasing concentrations of CPF. Images reveal an increase in mitochondrial length and mitochondrial redistribution. Scale bar = 100μm.

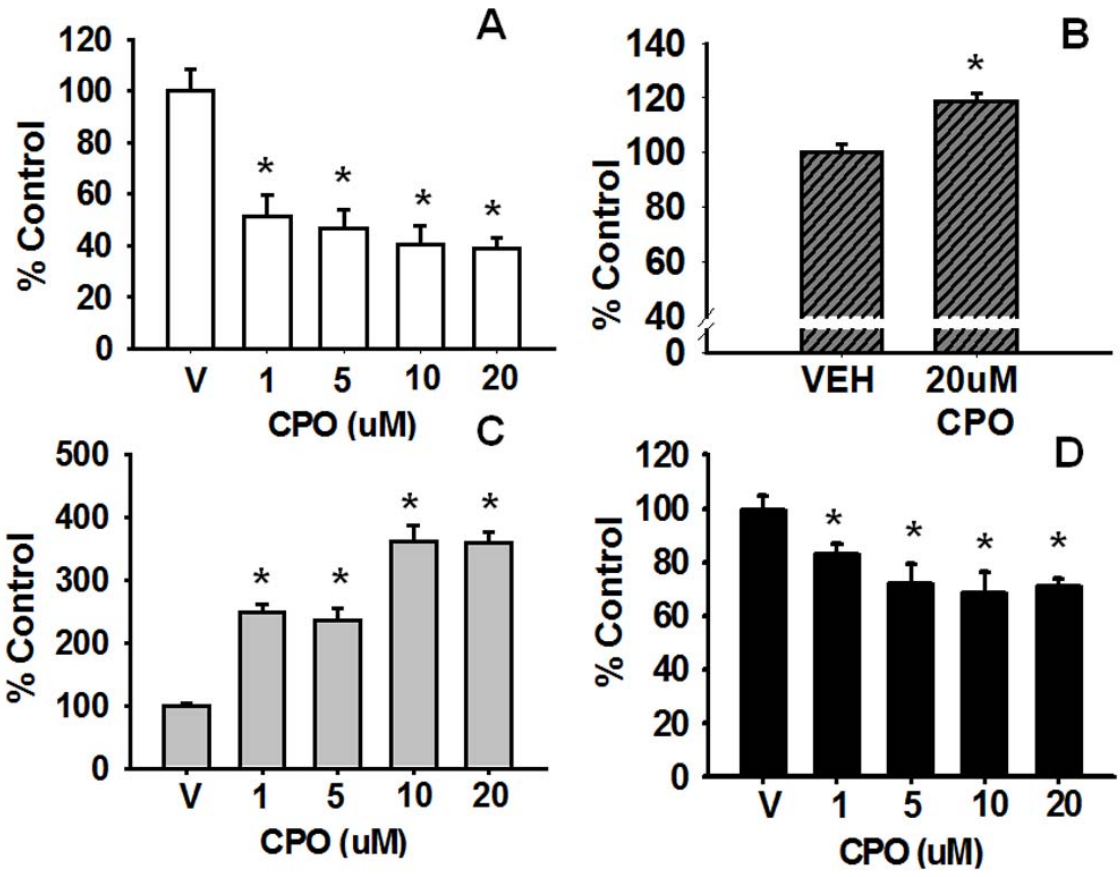

Figure 3.4. Exposure to CPO (active metabolite) disrupts mitochondrial transport and alters mitochondrial dynamics. **A, C, D -** 24 hour exposure to CPO was associated with a decrease in axonal transport, an increase in mitochondrial length, and a non-significant decrease in mitochondrial number (p=0.058) respectively. **B,** Increased number of mitochondria in the initial segment represents a change in mitochondrial distribution. Data are presented as mean (% control) ± SEM (25 neurons from 3 experiments). *****Significant difference from vehicle (V) control (p<0.005).

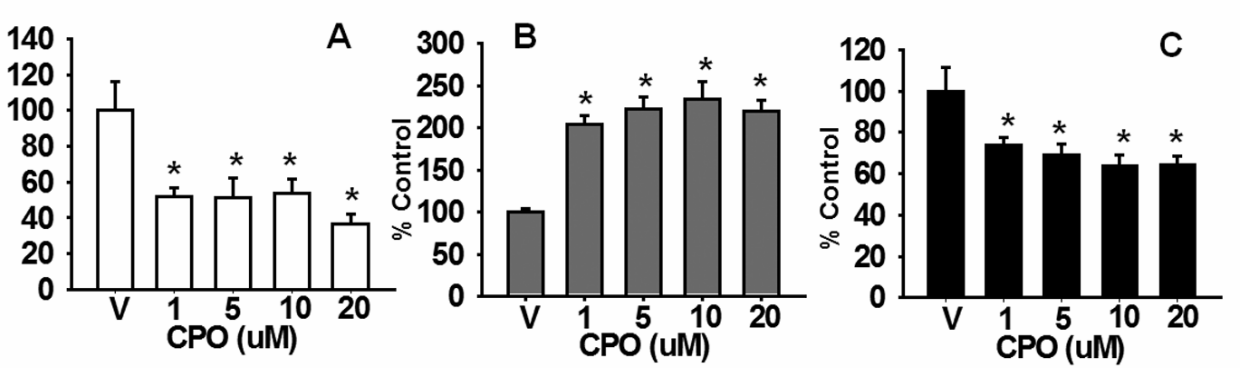

Figure 3.5. Exposure to CPO (active metabolite) disrupts mitochondrial transport and alters mitochondrial dynamics. **A, B, C -** 1 hour exposure to CPF was associated with a decrease in axonal transport, an increase in mitochondrial length, and a decrease in mitochondrial number respectively. Data are presented as mean (% control) ± SEM (25 neurons from 3 experiments). *****Significant difference from vehicle (V) control (p<0.005).

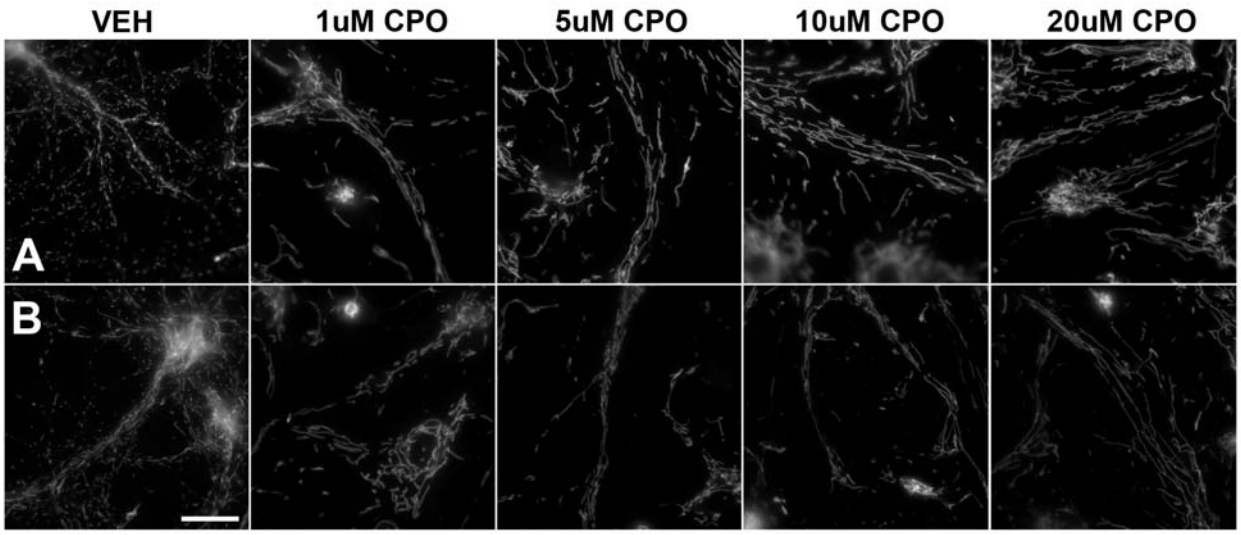

Figure 3.6. Exposure to CPO (active metabolite) disrupts mitochondrial morphology and fusion/fission dynamics. Shown here are representative images of neuronal cultures after **A,** 1 hour and **B,** 24 hour exposure to increasing concentrations of CPO. Images reveal an increase in mitochondrial length and mitochondrial redistribution. Scale bar = 100μm.

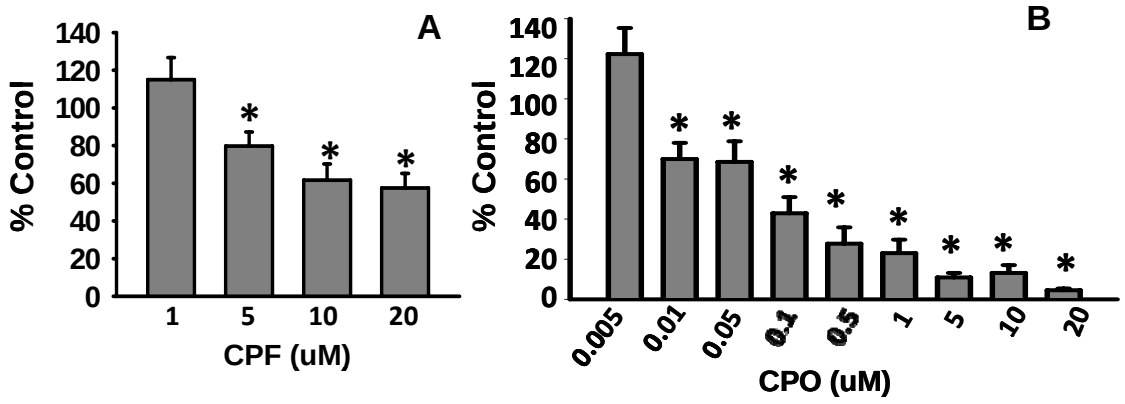

Figure 3.7. Concentration-dependent effects of CPF/CPO on AChE activity. Cortical neurons were exposed to increasing concentrations of CPF and CPO for 24 hours. AChE inhibition was determined using the Ellman assay. **A,** Concentration dependent increase in AChE inhibition from 5-20uM CPF. **B,** Concentration dependent increase in AChE inhibition from 0.01-20uM CPO. At concentrations of 1μM CPF or 0.005μM CPO (shown to cause changes in axonal transport and altered mitochondrial dynamics), no AChE inhibition was seen. Data presented as mean (% control) ± SEM (min. of 4 wells from 3 different experiments. *****Significant difference from vehicle control (p<0.005).

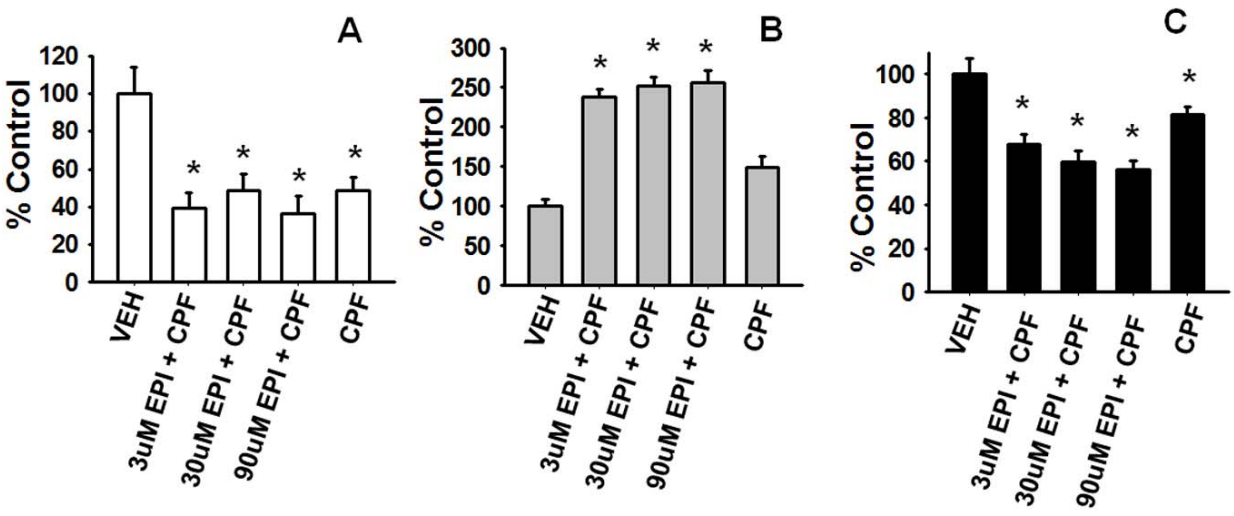

Figure 3.8. Co-incubation with epibatidine does not cause reversal of the CPF effects on mitochondrial transport and dynamics. Increasing concentrations of epibatidine were co-incubated with 1μM CPF for 24 hours. Afterwards there was no reversal of the axonal transport deficits **(A)**, increased mitochondrial length **(B)** , or decreased mitochondrial number **(C)** when compared to 24 hour exposure to 1μM CPF alone. Data are presented as mean (% control) ± SEM (min. 20 neurons from 3 different experiments). *****Significant difference from vehicle (V) control (p<0.005).

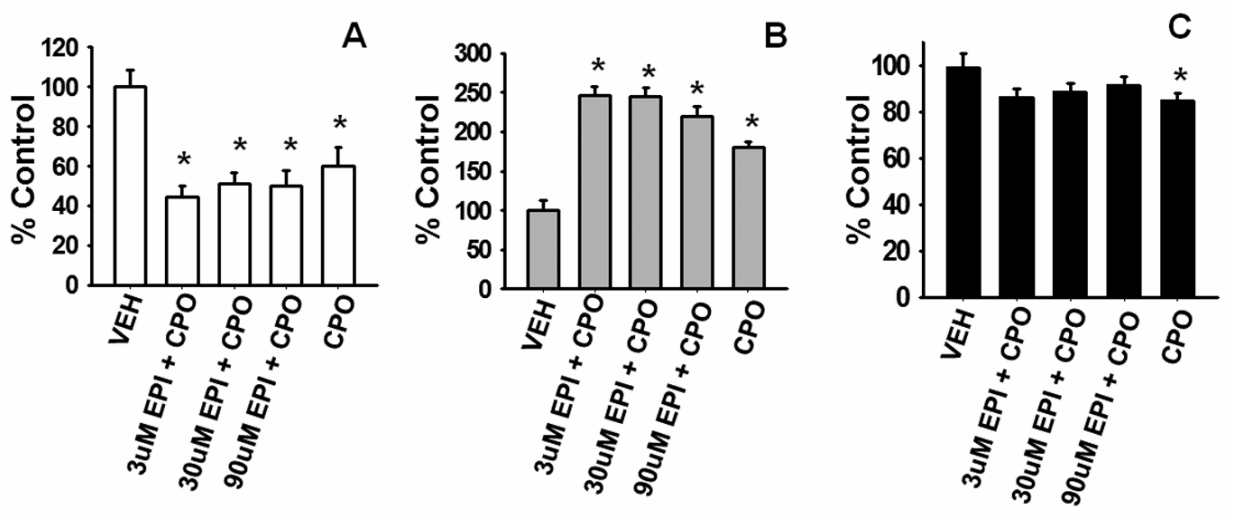

Figure 3.9. Co-incubation with epibatidine does not cause reversal of the CPO effects on mitochondrial transport and dynamics. Increasing concentrations of epibatidine were co-incubated with 0.005μM CPO for 24 hours. After exposure there was no reversal of the axonal transport deficits **(A)**, increased mitochondrial length **(B)**, or decreased mitochondrial number **(C)** when compared to 24 hour exposure to 0.005μM CPO alone. Data are presented as mean (% control) ± SEM (min. 20 neurons from 3 different experiments). *****Significant difference from vehicle (V) control (p<0.005).

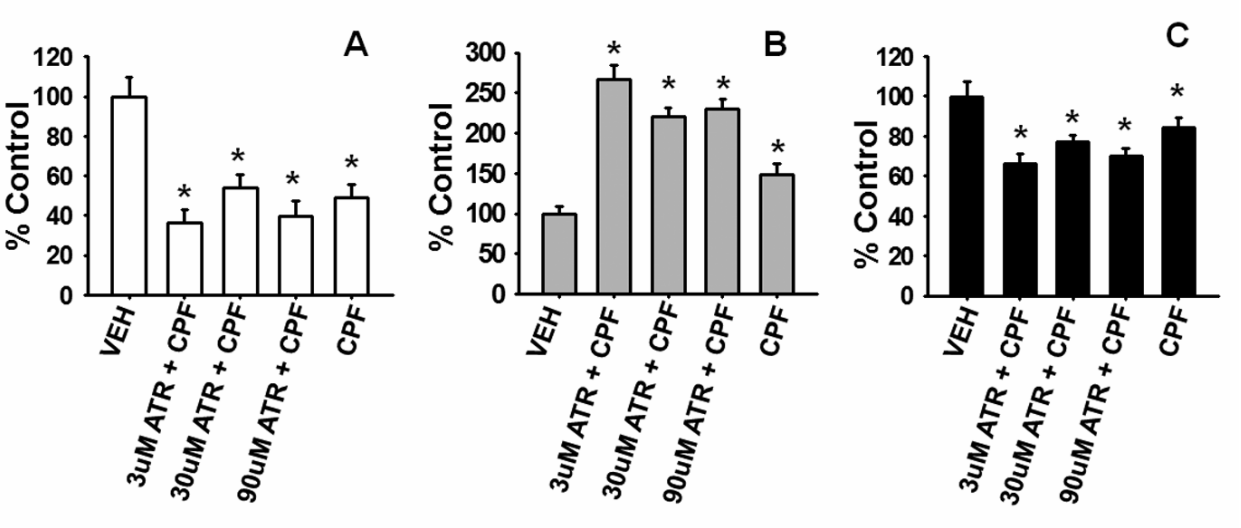

Figure 3.10. Co-incubation with atropine does not cause reversal of the CPF effects on mitochondrial transport and dynamics. Increasing concentrations of epibatidine were co-incubated with 1μM CPF for 24 hours. Afterwards there was no reversal of the axonal transport deficits **(A)**, increased mitochondrial length **(B)**, or decreased mitochondrial number **(C)** when compared to 24 hour exposure to 1μM CPF alone. Data are presented as mean (% control) ± SEM (min. 20 neurons from 3 different experiments). *****Significant difference from vehicle (V) control (p<0.005).

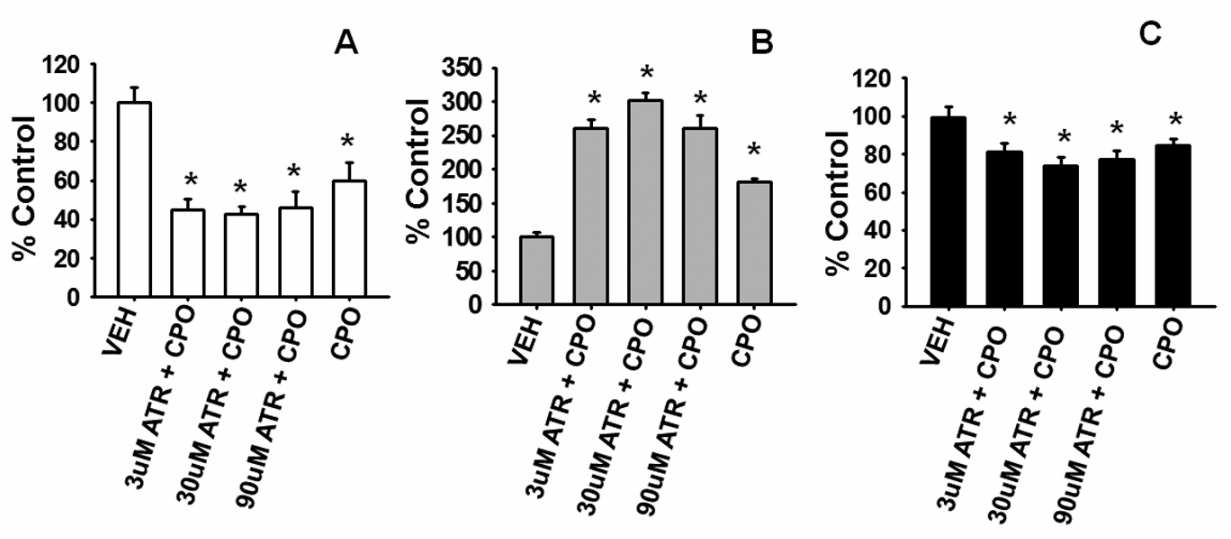

Figure 3.11. Co-incubation with atropine does not cause reversal of the CPO effects on mitochondrial transport and dynamics. Increasing concentrations of epibatidine were co-incubated with 0.005μM CPO for 24 hours. After exposure there was no reversal of the axonal transport deficits **(A)**, increased mitochondrial length **(B)**, or decreased mitochondrial number **(C)** when compared to 24 hour exposure to 0.005μM CPO alone. Data are presented as mean (% control) ± SEM (min. 20 neurons from 3 different experiments). *****Significant difference from vehicle (V) control (p<0.005).

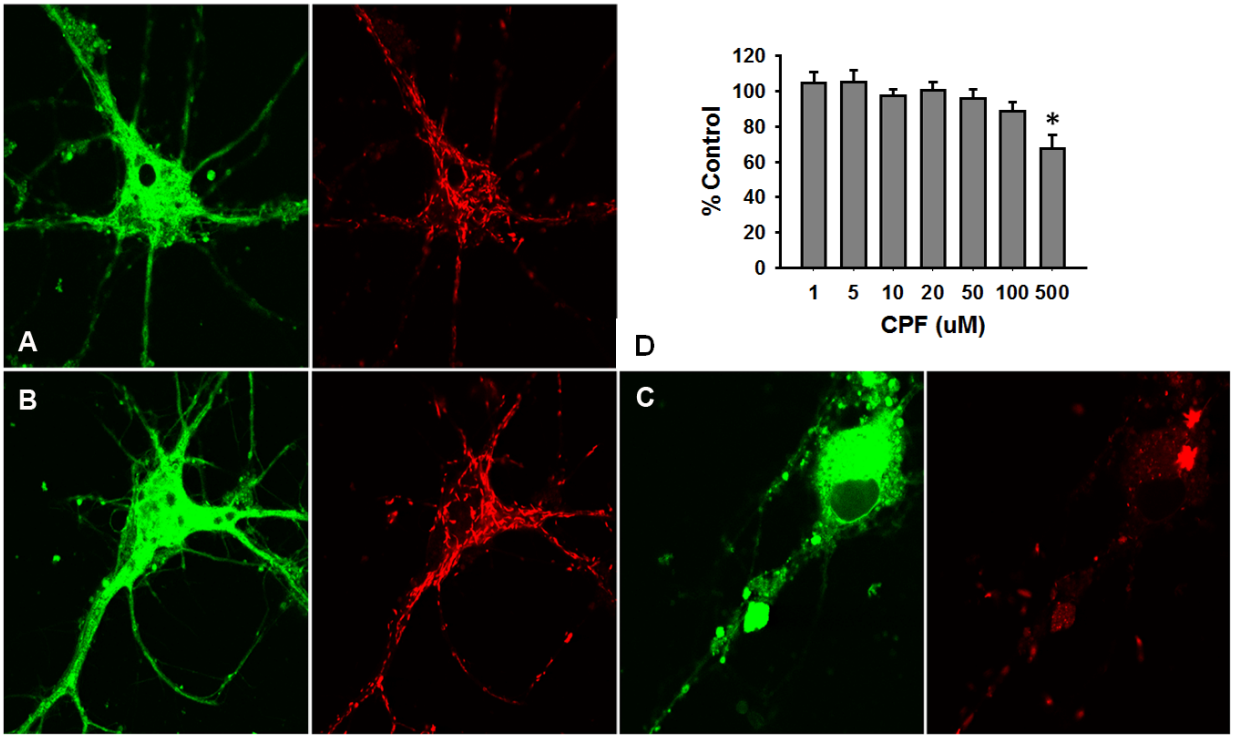

Figure 3.12. Exposure to 1-20μM CPF does not induce mitochondrial membrane potential depolarization. Cortical neurons were exposed to increasing concentrations of CPF for 24 hours. Green represents neuronal staining, red represents mitochondrial staining when the membrane potential is intact. **A.** After 24 hour exposure to vehicle, **B.** After 24 hour exposure to 20μM CPF, **C.** After 24 hour exposure to 500μM CPF showing a significant decrease in ΔΨm. Green (neuronal staining-left). Red (mitochondrial staining when ΔΨm is intact-right). **D.** Shows quantification ΔΨm ratio (green:red) with a significant decrease in ΔΨm at 500μM CPF (a concentration known to cause cell death). Data are presented as mean (% control) ± SEM (min. 6 wells from 3 different experiments). *****Significant difference from vehicle (V) control (p<0.005).

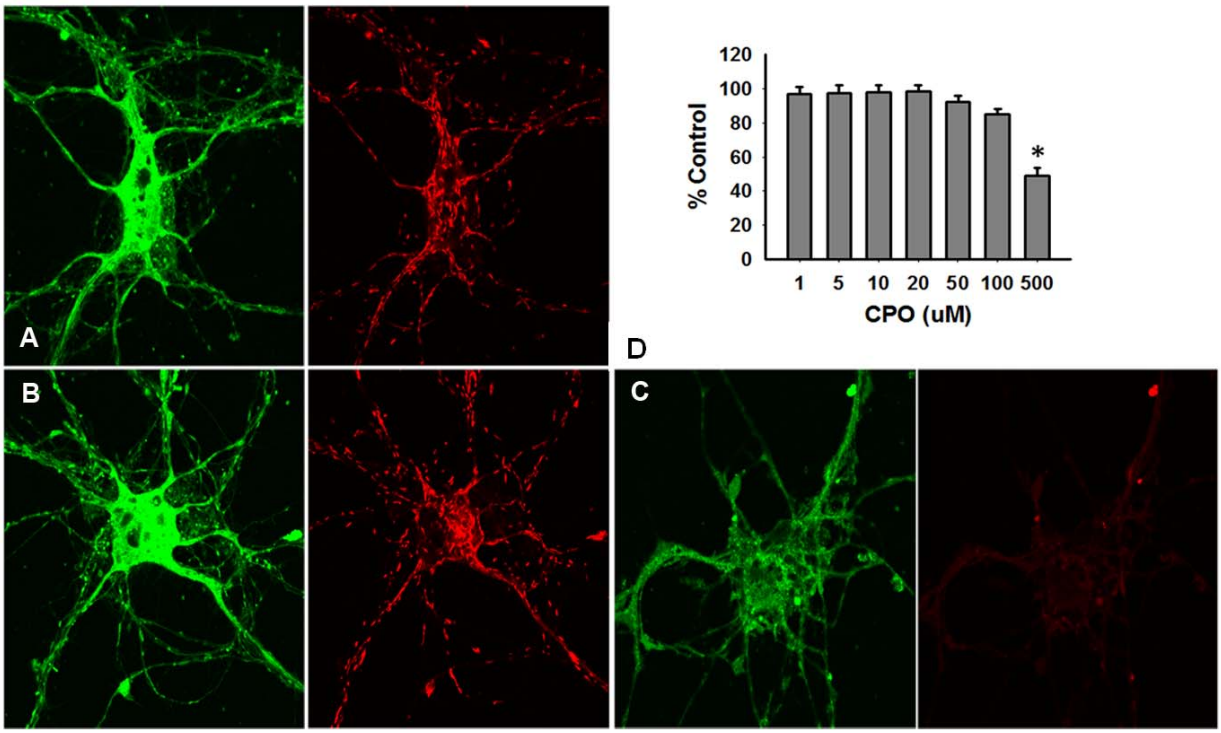

Figure 3.13. Exposure to 1-20μM CPO does not induce mitochondrial membrane potential depolarization. Cortical neurons were exposed to increasing concentrations of CPO for 24 hours. Green represents neuronal staining, red represents mitochondrial staining when the membrane potential is intact. **A.** After 24 hour exposure to vehicle, **B.** After 24 hour exposure to 20μM CPO, **C.** After 24 hour exposure to 500μM CPO showing a significant decrease in ΔΨm. Green (neuronal staining-left). Red (mitochondrial staining when ΔΨm is intact-right). **D.** Shows quantification ΔΨm ratio (green:red) with a significant decrease in ΔΨm at 500μM CPO (a concentration known to cause cell death). Data are presented as mean (% control) ± SEM (min. 6 wells from 3 different experiments). *****Significant difference from vehicle (V) control (p<0.005).

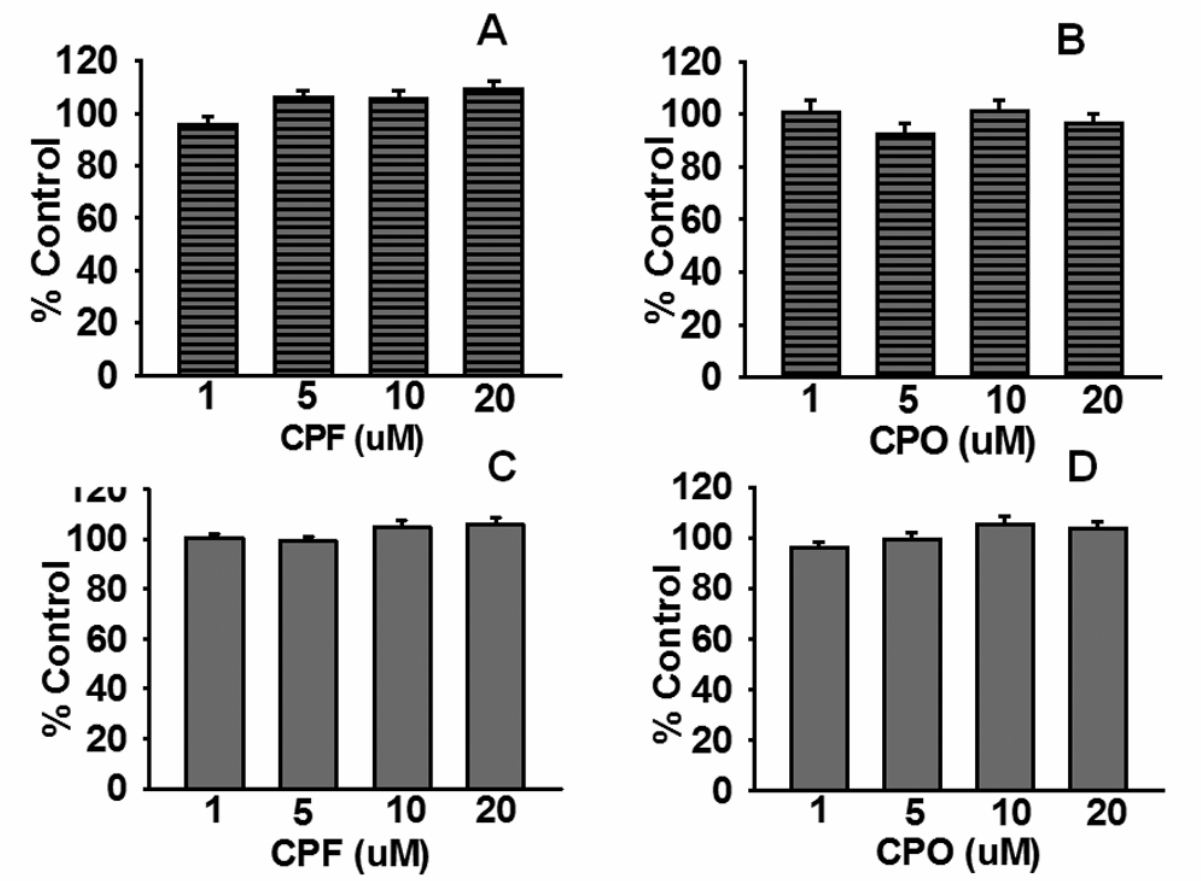

Figure 3.14. Exposure to CPF and CPO do not induce changes in ATP synthesis or superoxide production. Cortical neurons were incubated with 20μM CPF or CPO for 24 hours. **A&B**. Show that exposure to 20μM CPF and 20μm CPO respectively does not impair ATP production. **C&D**. Show that exposure to 20μM CPF and 20μM CPO respectively does not induce elevated superoxide production. Data are presented as mean (% control) ± SEM (min. 6 wells from 3 different experiments). *****Significant difference from vehicle/control (p<0.005).

Movie File Download Instructions

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Supplemental Movie 1. Time-lapse microscopy after 24 hour exposure to vehicle (0.01% DMSO). Images were captured every second for 20 seconds and presented at 3 frames per second. http://www.yourfilelink.com/get.php?fid=564230

Supplemental Movie 2. Time-lapse microscopy after 24 hour exposure to 1μM CPF. Images were captured every second for 20 seconds and presented at 3 frames per second. http://www.yourfilelink.com/get.php?fid=564229

Supplemental Movie 3. Time-lapse microscopy after 24 hour exposure to vehicle (0.01% methanol). Images were captured every second for 20 seconds and presented at 3 frames per second. http://www.yourfilelink.com/get.php?fid=564228

Supplemental Movie 4. Time-lapse microscopy after 24 hour exposure to 1μM CPO. Images were captured every second for 20 seconds and presented at 3 frames per second. http://www.yourfilelink.com/get.php?fid=564227

CHAPTER 4
CONCLUDING REMARKS

This dissertation addressed two aims. **Aim 1** was to determine the consequences of repeated sub-acute exposures to CPF on sustained attention using the 5C-SRTT. The results of this study support the premise that repeated, low-level exposures to commercial OP pesticides like CPF may lead to protracted deficits in sustained attention and an increase in impulsivity in the absence of acute (cholinergic) side effects or motivational deficits. This supports the **first hypothesis** that repeated, sub-threshold exposures to CPF results in impairment of sustained attention in a rat model that persists throughout a drug-free washout period.

Aim 2 was to determine the consequences of CPF exposure on mitochondrial function and axonal transport in primary cortical neurons. We determined that exposure to CPF and CPO resulted in axonal transport deficits that were accompanied by mitochondrial redistribution and changes in mitochondrial dynamics. These changes did not appear to be directly associated with cholinergic (receptor-based) mechanisms, disruption of OXPHOS, mitochondrial membrane depolarization or superoxide production. These data support the **second hypothesis** that exposure to CPF and CPO disrupts mitochondrial axonal transport, leading to mitochondrial dysfunction through misplacement.

This work represents one of only a few studies that have been designed to prospectively examine the effects of repeated, sub-acute exposures to CPF and it is the first to show that such exposures can result in protracted deficits in sustained attention and inhibitory control in an animal model. These results are significant given the

widespread use of OP-based insecticides worldwide and they raise concerns regarding other types of cognitive deficits that may develop in people who are repeatedly exposed to these chemicals. Clearly, additional prospective animal studies are needed to definitively determine the effects of CPF on other domains of cognition, such as working memory and cognitive flexibility, since they are crucial components of executive function in everyday life.

The cell culture experiments conducted in the latter portion of this project are the first to show that mitochondrial dynamics and mitochondrial axonal transport are disrupted after exposure to CPF and CPO. The long-term consequences of such changes are still to be fully elucidated, but chronic disruption of mitochondrial movement and redistribution will inevitably result in compromised neuronal function as has been shown in many neurodegenerative diseases.

The field of mitochondrial dynamics has expanded greatly over the last decade, and the role of fusion/fission processes in the function of mitochondria is becoming clearer. However, how mitochondrial dynamics and axonal transport processes interact and how a delicate balance in these processes is maintained under normal conditions is still not well understood. Until these processes are better elucidated, understanding how OPs affect this delicate balance will not be fully realized. Whether CPF and CPO have direct affects on the important fusion/fission proteins (e.g. abnormal phosphorylation of dynamin related proteins) is yet to be determined. However, as biological techniques improve, we will able to expand on our knowledge of pesticide actions at the molecular level in mammalians (as well as target organisms). It is this understanding that will be fundamental to drug development for combating the

deleterious effects of OPs, for optimizing guidelines for use and safety of OPs in the agricultural industry worldwide, and for designing better pesticides that minimize human risk while optimizing crop sustainability.