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PRINCIPAL INVESTIGATOR: Alvin V. Terry

CONTRACTING ORGANIZATION: Georgia Health Sciences University
AUGUSTA , GA 30912

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14. ABSTRACT The overall goal of this project is to determine the underlying mechanisms for the neurological symptoms associated with Gulf War Illness. The central hypothesis is that subthreshold exposures to organophosphates-OPs (defined as exposures not associated with acute signs of toxicity) may have adversely affected axonal transport and/or myelin integrity in affected individuals. We are studying two OPs, a representative insecticide that was used in the first gulf war, chlorpyrifos (CPF), and a representative, nerve agent, diisopropylfluorophosphate (DFP) in rats. The first two years of this proposal have been primarily dedicated to Specific Aim #1: which has been designed to evaluate OP effects on axonal transport in the living rat brain using manganese-enhanced magnetic resonance imaging (MEMRI) of the optic nerve axonal projections from the retina to the superior colliculus. The following procedures have been conducted to date (N=6): 1) baseline MRI scans; 2) daily injections of vehicle or chlorpyrifos (3.0-18.0 mg/kg) x 14 days; 2) a second MRI scan on the day following the last drug injection; 3) a third scan after a 4 week (OP-free) washout period. For each animal, a separate 6 hour and 24 hour scan was performed after Mn2+ eye injection. For this work a manuscript is currently under review for publication. In this work we also evaluated the effects of an acute (single) exposure to CPF. We have also completed the MR scanning portion for the DFP study and a preliminary analysis indicates similar results (persistent impairments of axonal transport). The experiments for specific aim #2 (devoted to the evaluation of OP-related effects on myelin with diffusion tensor imaging-(DTI) and histology are currently underway.					
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Progress Report W81XWH-12-1-0536 (Year 2)

INTRODUCTION

The overall goal of this project is to determine the underlying mechanisms for the neurological symptoms (particularly cognitive deficits) that have been associated with Gulf War Illness. The central hypothesis we are testing is that subthreshold exposures to organophosphates (defined as exposures not associated with acute signs of toxicity) from insecticides or nerve agents may have adversely affected axonal transport and/or myelin integrity in affected individuals. We are studying two OPs, a representative insecticide that was used in the first gulf war, chlorpyrifos (CPF), and a representative, nerve agent, diisopropylfluorophosphate (DFP) in rats. The first two years of this proposal have been primarily dedicated to Specific Aim #1: which has been designed to evaluate OP effects on axonal transport in the living rat brain using manganese-enhanced magnetic resonance imaging (MEMRI) of the optic nerve axonal projections from the retina to the superior colliculus.

BODY

Specific Aim 1

The experiments for specific Aim 1 are nearing completion (chlorpyrifos and DFP exposure-MEMRI studies). To date we have accomplished the following:

Chlorpyrifos

1. Baseline MRI scans (after Mg^{2+} eye injection) were performed on each test subject.
2. Subjects (N=6) have received daily subcutaneous injections of chlorpyrifos or vehicle (at the following doses) x 14 days:
 - Vehicle
 - CPF 3.0 mg/kg
 - CPF 10.0 mg/kg
 - CPF 18.0 mg/kg
3. A second MRI scan was performed on the day following the last drug injection (after another Mg^{2+} eye injection) in each animal.
4. A third scan was performed after a 4 week (OP-free) washout period (after the third and final Mg^{2+} eye injection) in each animal.
5. For each animal a separate 6 hour and 24 hour scan was performed after Mg^{2+} eye injection.
6. We have completed all of the MEMRI Analyses and have submitted a manuscript for publication (PDF of the submitted paper is included in the appendix).
7. Due to some logistical problems and scanning errors (motion artifacts) which necessitated the elimination of several animals we were not able to include the CPF 10.0 mg/kg cohort in the submitted manuscript. We did, however, add an additional set of animals to evaluate the effects of an acute (single) exposure to CPF (18.0 mg/kg N=6) as well a positive control cohort (N=3, intravitreal injection of colchicine, please see the submitted manuscript in the appendix).

DFP

1. Baseline MRI scans (after Mg²⁺ eye injection) were performed on each test subject.
2. Subjects (N=6) have received daily subcutaneous injections of DFP or vehicle (at the following doses) x 14 days:
 - Vehicle
 - DFP 0.125 mg/kg
 - DFP 0.250 mg/kg
 - DFP 0.500 mg/kg
3. A second MRI scan was performed on the day following the last drug injection (after another Mg²⁺ eye injection) in each animal.
4. A third scan was performed after a 4 week (OP-free) washout period (after the third and final Mg²⁺ eye injection) in each animal.
5. For each animal a separate 6 hour and 24 hour scan was performed after Mg²⁺ eye injection.
6. All of the MR scans have been completed and we are in the process of analyzing these data. In Fig 1 (see Supporting Data section below) we have presented a preliminary figure from the DFP study where appears that our hypothesis is correct (that OP exposure leads to persistent impairments in axonal transport). For the data included in this figure, we have analyzed and compared the data for the middle dose of DFP (0.5 mg/kg versus vehicle N=3 thus far).

Specific Aim 2

The experiments for specific aim #2 are underway (DTI and histology experiments for analyzing the effects of repeated OP exposure on myelin). The conditions for DTI have been optimized and animals are currently in the process of OP dosing. We have already run several cohorts of animals for the myelin histology (Black Gold staining and additional myelin-related methods).

Changes/Problems

As indicated in the last progress report that due to the throughput limitations of the Core Imaging Facility for Small Animals (CIFSA), we are only able to scan a maximum of 4 animals (once) per day (each scan takes from 1-1.5 hours). A few animals have died under anesthesia, which required a full repeat of the dosing and imaging procedures. The limitation of the number of animals imaged each day requires staggering of the cohorts and very tight logistics. We have also encountered some other problems (e.g., motion artifacts) that have required the replacements of animals or the inability to provide a full cohort for the data analysis (e.g., CPF 10.0 mg/kg). While we are a bit behind the schedule originally proposed, we factored in six months at the end of the 3 year project for data analysis, paper submissions, etc. While it is now clear that the imaging portion of this project will take the entire 3 years of the project, we are able to overlap the imaging and histology portions of the study, since the histology experiments are not limited by the capacity of the Core Imaging Facility for Small Animals (CIFSA).

KEY RESEARCH ACCOMPLISHMENTS:

- The experiments for specific Aim 1 are nearing completion. The chlorpyrifos MEMRI experiments are complete (manuscript submitted-please see appendix) and the MR scanning for the DFP MEMRI studies are finished. We are currently in the process of analyzing the data.

- The data collected to date with both chlorpyrifos and diisopropylfluorophosphate support our hypothesis that repeated exposures to OPs leads to persistent deficits in axonal transport.
- The experiments for specific aim #2 are underway (DTI and histology experiments for analyzing the effects of repeated OP exposure on myelin).

REPORTABLE OUTCOMES:

- Publications
 - One Publication is currently under review entitled: (see appendix 1)
- Invited presentations
 - Organophosphate Exposure and Cognitive Deficits: Elucidating the Mechanisms and Identifying Therapeutic Targets”, Presented at the 2014 Southeastern Neuroscience Conference, Augusta Marriott at the Convention Center, Augusta GA, April 19, 2014.
 - “Organophosphates and Cognitive Deficits: Elucidating the Mechanisms and Identifying Therapeutic Targets. Presented at the CounterACT Center of Excellence, University of California, Davis, CA, September 15, 2014.
 - Organophosphates and Cognitive Deficits: Elucidating the Mechanisms and Identifying Therapeutic Targets. Presented to the Department of Pharmacology, Physiology & Neuroscience. University of South Carolina School of Medicine, Columbia, S.C., October 6, 2014.
- Two posters will be presented at the annual Society for Neuroscience Meeting in November and Washington DC.

CONCLUSION

We now have clear evidence with the insecticide OP, chlorpyrifos to support our hypothesis that repeated OP exposures leads to persistent impairments in axonal transport in the brain of living animals. The data from the DFP study analyzed to date also support this hypothesis.

We anticipate that there will be additional adjustments to our experimental protocols in order to finish all of the proposed experiments by the end of the 3 year project. We now expect that the imaging portion of this work will take all 3 years of the project, but with proper management and overlap of the histology experiments we should be able to complete all experiments by the end of the grant period.

REFERENCES

None

APPENDICES

Appendix 1 (Submitted Publication)

SUPPORTING DATA

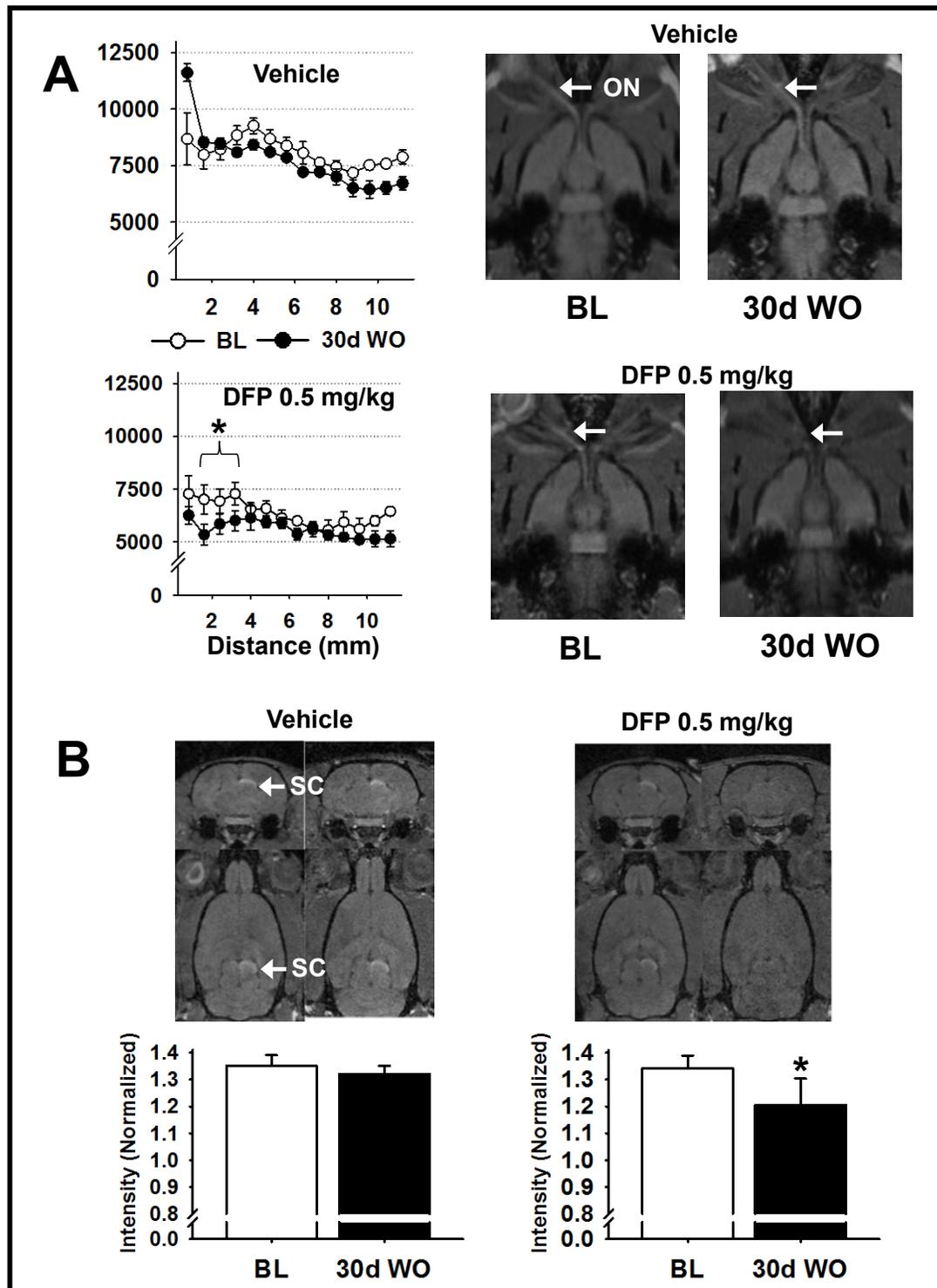


Fig 1. Diisopropylfluorophosphate (DFP) Repeated Exposure Study. $MnCl_2$ was administered by intravitreal injection and MRI scans were collected 6 and 24 hours later to assess Mn^{2+} transport in the optic nerve (ON) and to the superior colliculus (SC), respectively. For each test subject, MRI scans were obtained prior to drug treatment (baseline, BL), and following a 30-day drug-free washout period (30-day washout, 30d WO) that started after 14-

days of exposure to DFP (0.5 mg/kg) or vehicle (both administered s.c. once daily). **(A) Mn²⁺ transport in optic nerve 6 hrs after intravitreal injection.** (Left)-line graphs illustrating normalized intensity values in 0.8 mm bins along the optic nerves from the eyeball to the optic chiasm and (Right) representative horizontal (axial) images illustrating the Mn²⁺ contrast in the optic nerves (indicated by white arrows). Both treatment groups demonstrated progressive decreases in signal intensity the further away from the eyeball, main effect of distance ($F_{13,210}=8.17$, $p<0.001$) 6 hours after intravitreal Mn²⁺ injection. There was also a significant group x distance x time interaction, ($F_{13,210} = 2.16$, $p<0.05$). Within the initial 4.8 mm of the eyeball DFP-treated rats exhibited significantly diminished Mn²⁺ enhancement along the optic nerve at the 30-day drug-free washout period, compared to their baseline values (post hoc $p<0.05$). **(B) Mn²⁺ transport in superior colliculus 24 hrs after intravitreal injection.** Representative rat brain coronal (Top, approximately interaural 2.70 mm, bregma 6.30 mm) and horizontal (Bottom, approximately interaural 6.14 mm, bregma -3.86 mm) MR images at the level of the superior colliculus at (left) baseline (BL) and (right) after a 30 day drug-free washout period. Note the clear Mn²⁺ enhancement in the superior colliculus (indicated by white arrows) of both groups at baseline and reduced enhancement associated with DFP (0.5 mg/kg) 30 days after a drug-free washout period. A histogram illustrating the normalized intensity values (mean \pm s.e.m.) in the superior colliculus are provided below the MR. Statistical results were as follows, group x time effect: $F_{1,16} = 8.42$, $p<0.05$ (post hoc $p<0.05$ in the DFP group after 30 days of washout compared to the respective baseline values). N=3

APPENDIX 1

Submitted Manuscript:

Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain

Ms. No.: NIMG-14-2521

Title: Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain Corresponding Author: Dr. Alvin V. Terry

Authors: Wayne D Beck; Sean X. X Naughton; Indrani Poddar; Bao-Ling Adam; Nathan Yanasak; Chris Middleton

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Your submission, referenced above, has been assigned the following manuscript number: NIMG-14-2521

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Manuscript Number:

Title: Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain

Article Type: Regular Article

Section/Category: Anatomy and Physiology

Corresponding Author: Dr. Alvin V. Terry, Ph.D.

Corresponding Author's Institution: Georgia Regents University

First Author: Alvin V. Terry, Ph.D.

Order of Authors: Alvin V. Terry, Ph.D.; Wayne D Beck; Sean X. X Naughton; Indrani Poddar; Bao-Ling Adam; Nathan Yanasak; Chris Middleton

Abstract: The toxicity of the class of chemicals known as the organophosphates (OP) is most commonly attributed to the inhibition of cholinesterase enzymes. However, there is significant evidence that this mechanism may not account for all of the deleterious neurologic and neurobehavioral symptoms of OP exposure, especially those associated with levels that produce no overt signs of acute toxicity (subthreshold doses). In the study described here we evaluated the effects of the commonly used OP-pesticide, chlorpyrifos (CPF) on axonal transport in the brains of living rats using manganese (Mn^{2+}) -enhanced magnetic resonance imaging (MEMRI) of the optic nerve (ON) projections from the retina to the superior colliculus (SC). T1-weighted MEMRI scans were evaluated at 6 and 24 hours after intravitreal injection of Mn^{2+} . As a positive control for axonal transport deficits, initial studies were conducted with the tropolone alkaloid colchicine administered by intravitreal injection. In subsequent studies both acute (single) and repeated exposures to CPF were evaluated for effects on axonal transport using MEMRI. As expected, intravitreal injection of colchicine (2.5 μ g) produced a robust decrease in transport of Mn^{2+} along the optic nerve (ON) and to the superior colliculus (SC) (as indicated by the reduced MEMRI contrast). An acute subcutaneous (s.c.) injection of CPF (18.0 mg/kg) was not associated with significant alterations in the transport of Mn^{2+} . Conversely, 14-days of repeated s.c. exposure to CPF was associated with decreased in transport of Mn^{2+} along the ONs and to the SC, an effect that was also present after a 30-day (CPF-free) washout period. These results indicate that subthreshold exposures to a commonly used pesticide, CPF can result in persistent alterations in axonal transport in the living mammalian brain. Given the fundamental importance of axonal transport to neuronal function, these observations may (at least in part) explain some of the long term neurological deficits that have been observed in humans who have been exposed to subthreshold doses of OPs.

Suggested Reviewers: Pamela J Lein Ph.D.

Professor, Department of Molecular Biosciences, University of California-Davis
pjlein@ucdavis.edu

Well recognized expert in organophosphate toxicity and has imaging experience

Carey N Pope Ph.D.

Professor, Department of Physiological Sciences, Oklahoma State University

carey.pope@okstate.edu
Well recognized organophosphate expert

Joseph J Gallagher Ph.D.
Research Scientist, Biological Imaging Center, California Institute of Technology
jgg@caltech.edu
Expertise with neuroimaging and MEMRI in particular

Opposed Reviewers:



Medical College
of Georgia

Department of Pharmacology and Toxicology

October 28, 2014

Editor
Neuroimage

Dear Editor,

Please consider our manuscript entitled: "Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain" for publication in Neuroimage.

Thank you.

A handwritten signature in blue ink that reads "Alvin V. Terry Jr." with a stylized flourish at the end.

Alvin V. Terry Jr., Ph.D.
Regents Professor and Chair
Department of Pharmacology and Toxicology

Research Highlights

Acute exposure to cochicine impairs axonal transport in the brain of rats.

Acute exposure to chlorpyrifos does not affect axonal transport in the brain of rats.

Repeated exposure to chlorpyrifos impairs axonal transport in the brain of rats.

**Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in
the living rodent brain**

Caterina M. Hernandez¹, Wayne D. Beck¹, Sean X. Naughton¹, Indrani Poddar¹, Bao-Ling Adam¹, Nathan Yanasak², Chris Middleton², and Alvin V. Terry, Jr.¹

1. Department of Pharmacology and Toxicology, Georgia Regents University, Augusta, Georgia, 30912
2. Core Imaging Facility for Small Animals (CIFSA), Georgia Regents University, Augusta, Georgia, 30912

Corresponding Author:

Alvin V. Terry Jr., Ph.D.
Department of Pharmacology and Toxicology
CB-3545, Georgia Regents University
1120 Fifteenth Street
Augusta, Georgia 30912-2450
Phone 706-721-9462
Fax 706-721-2347
e-mail: aterry@gru.edu

Abstract

The toxicity of the class of chemicals known as the organophosphates (OP) is most commonly attributed to the inhibition of cholinesterase enzymes. However, there is significant evidence that this mechanism may not account for all of the deleterious neurologic and neurobehavioral symptoms of OP exposure, especially those associated with levels that produce no overt signs of acute toxicity (subthreshold doses). In the study described here we evaluated the effects of the commonly used OP-pesticide, chlorpyrifos (CPF) on axonal transport in the brains of living rats using manganese (Mn^{2+})-enhanced magnetic resonance imaging (MEMRI) of the optic nerve (ON) projections from the retina to the superior colliculus (SC). T1-weighted MEMRI scans were evaluated at 6 and 24 hours after intravitreal injection of Mn^{2+} . As a positive control for axonal transport deficits, initial studies were conducted with the tropolone alkaloid colchicine administered by intravitreal injection. In subsequent studies both acute (single) and repeated exposures to CPF were evaluated for effects on axonal transport using MEMRI. As expected, intravitreal injection of colchicine (2.5 μ g) produced a robust decrease in transport of Mn^{2+} along the optic nerve (ON) and to the superior colliculus (SC) (as indicated by the reduced MEMRI contrast). An acute subcutaneous (s.c.) injection of CPF (18.0 mg/kg) was not associated with significant alterations in the transport of Mn^{2+} . Conversely, 14-days of repeated s.c. exposure to CPF was associated with decreased transport of Mn^{2+} along the ONs and to the SC, an effect that was also present after a 30-day (CPF-free) washout period. These results indicate that subthreshold exposures to a commonly used pesticide, CPF can result in persistent alterations in axonal transport in the living mammalian brain. Given the fundamental importance of axonal transport to neuronal function, these observations may (at least in part)

explain some of the long term neurological deficits that have been observed in humans who have been exposed to subthreshold doses of OPs.

Key Words: Pesticide, Organophosphate, Manganese Enhanced Magnetic Resonance Imaging, Gulf War Illness, Alzheimer's disease, Parkinson's disease,

1. Introduction

The chemicals known as the organophosphates (OPs) are used for a wide variety of important applications and they are especially prevalent in the agricultural setting where they have been applied as pesticides for decades. Unfortunately, OPs are highly toxic to humans as well as target organisms and continuing reports of accidental and intentional poisonings (i.e., from suicide attempts) by OPs is an ongoing environmental health concern worldwide (reviewed, Eddleston 2008). The risk of exposure to OP-based nerve agents from rogue governments and terrorist organizations is an additional threat that was recently exemplified by the sarin attacks on civilians in Syria (United Nations Security Council Report, 2013).

The toxic “cholinergic crisis” associated with acute poisoning with OPs and the associated variety of long term neurologic and neurobehavioral consequences have been studied extensively and are primarily attributed to the inhibition of cholinesterase enzymes, especially acetylcholinesterase (AChE) (Ecobichon, 2001, for review see also Pereira et al., 2014), However, there is also significant evidence in the human epidemiological literature (e.g., Ross et al., 2013) that OP exposures not associated with acute toxicity (subthreshold exposures) may also result in prolonged neurological and neurobehavioral deficits including impairments of cognition. Moreover, as an etiological mechanism, AChE inhibition may not account all of the symptoms associated with acutely toxic or subthreshold OP exposures as suggested by the following lines of evidence (reviewed in Banks and Lein, 2012): 1) different OPs can have different toxicological profiles despite having similar effects on AChE activity (Bushnell and Moser, 2006; Jett and Lein, 2006; Pope et al., 2005; Pope, 1999), 2) the OP nerve agent, VX induced neurotoxic effects in AChE knockout mice, in fact the knockout mice were more sensitive to the toxicity than their wild type controls (Duysen et al., 2001); 3) reports in both the

human and animal literature indicate that OP toxicity (especially associated with chronic exposure) can occur in the absence of AChE inhibition (Abou-Donia, 2003; Costa, 2006; Kamel and Hoppin, 2004); 4) human studies of occupational exposures to OPs often fail to find a significant correlation between blood cholinesterase activity and neurobehavioral deficits (Rohlman et al., 2011). Finally, one additional argument against the premise that AChE inhibition (alone) could explain both the acute toxicity and the long-term neurobehavioral deficits (particularly cognitive impairments) associated with OPs lies in the fact that reversible AChEs used to treat Alzheimer's disease (e.g., donepezil) have pro-cognitive effects in humans and animals (e.g., Winblad et al., 2009; Callahan et al., 2013). Moreover, even one OP-based AChE inhibitor (metrifonate) has been documented to have favorable effects on the cognitive symptoms in Alzheimer disease (Becker et al., 1998).

Prospective efforts to further elucidate the long term consequences of OP exposures as well as the mechanisms of the deleterious neurological effects require the use of animals and other model systems. Interestingly, more than 30 years ago experiments in animals indicated that axonal transport is negatively affected by OPs, a potentially notable finding given the fundamental importance of axonal transport to neuronal health and brain function. In these early experiments, OPs known to produce delayed neurotoxicity at relatively high doses (e.g., phenylphosphonothioate esters and tri-o-cresyl phosphate) inhibited fast anterograde axonal transport in an ex vivo rat optic nerve preparation (Reichart and Abou-Donia, 1980). Later studies in our laboratories indicated that both anterograde and retrograde transport of vesicles in the sciatic nerves (ex vivo) was impaired in rats repeatedly exposed to subthreshold doses of the OP insecticide chlorpyrifos (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl] phosphorothionate) (CPF), and, further, these deficits persisted after a 14-day washout period (Terry et al., 2003).

Using the same experimental approach, later time course studies indicated that a significant reduction in axonal transport occurred within 10 hours of a single CPF exposure (18.0 mg/kg s.c.) (Terry et al., 2007).

The purpose of the study described here was evaluate the effects of CPF on axonal transport in the brains of living rats using manganese (Mn^{2+})-enhanced magnetic resonance imaging (MEMRI), a non-invasive imaging method that has gained popularity in the last few years. Thus far, the technique has been successfully used to detect impairments of axonal transport in the brains of aged rats (Cross et al., 2008), mouse models of Alzheimer's disease (Kim et al., 2011; Smith et al., 2007; Smith et al., 2011), frontotemporal dementia (Majid et al., 2014), and mice homozygous for a deletion in the amyloid precursor protein gene (Gallagher et al., 2012). MEMRI exploits both the paramagnetic properties of Mn^{2+} and its ability to serve as a calcium analog in neurons. Due to its paramagnetic properties, Mn^{2+} produces a hyper-intense signal that can be detected in T1-weighted MR images (Lin and Koretsky, 1997) and tracked dynamically over time (Pautler et al., 1998; Pautler and Koretsky, 2002). As a calcium analog, Mn^{2+} enters neurons via voltage-gated calcium channels (Drapeau and Nachshen, 1984, Narita et al., 1990; Sloot and Gramsbergen, 1994; Lu et al., 2007), where it travels within vesicles along microtubules by fast axonal transport (Merritt et al., 1989; Takeda et al., 1998; Silva et al., 2004; Smith et al., 2007; Zhang et al., 2010) in a process that is at least partially dependent on the motor protein kinesin (Bearer et al., 2007; Bearer et al., 2009). Here we utilized MRI to visualize Mn^{2+} enhancement of the optic nerve projections from the retina to the superior colliculus. From intravitreal injection, Mn^{2+} has been shown to enter retinal ganglion cells and to travel within their axons in the anterograde direction along the optic nerve to the superior colliculus and lateral geniculate nucleus (Bearer et al., 2007a; Watanabe et al., 2001). As a

positive control for axonal transport deficits, initial studies were conducted with colchicine administered by intravitreal injection. In subsequent studies, both acute exposure and repeated exposures to CPF administered by subcutaneous injection were evaluated. After the repeated exposure experiments, an extended OP-free washout period was also assessed.

2. Material and Methods

A diagram illustrating the intravitreal injection method and the transport of Mn^{2+} within the axons of retinal ganglion cells in the anterograde direction along the optic nerve to the contralateral superior colliculus and lateral geniculate nucleus is presented in Fig 1 as well as an overview of the three MEMRI studies described in this report (additional details are provided below).

2.1 Animals

Male albino Wistar rats (Harlan, Indianapolis, IN, USA) 2–3 months old were housed in pairs in a temperature controlled room (25 °C), maintained on a standard 12-h light/dark cycle with free access to food (Teklad Global Rodent Diet 2918, Harlan, Madison, WI, USA). All procedures employed during this study were reviewed and approved by the Georgia Regents Sciences University 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. Significant efforts were also made to minimize the total number of animals used while maintaining statistically valid group numbers.

2.2 Drug administration

2.2.1 Colchicine Validation Study

In order to evaluate our ability to detect axonal transport deficits using MEMRI and our specific experimental conditions, a validation (positive control) study was conducted utilizing colchicine, an agent well-documented to reduce axonal transport in several model systems (see Discussion below). Colchicine (Sigma-Aldrich, St. Louis, MO USA) 2.5 μg and MnCl_2 200 μM were co-administered by intravitreal injection in a total volume of 4 μL and MRI scans were collected 6 and 24 hours later (for further details, see sections entitled Intravitreal Manganese Administration and Magnetic resonance imaging, below, respectively).

2.2.2 Chlorpyrifos

Chlorpyrifos (CPF) (ChemService, West Chester, PA, USA) was prepared fresh daily in vehicle, a mixture of 3% DMSO (Sigma-Aldrich, St. Louis, MO, USA) and 97% peanut oil (Kroger® Pure Peanut Oil, obtained locally, Augusta, GA, USA) and administered subcutaneously (s.c.) 0.7 ml/kg body weight. The doses evaluated in this study were identified in previous studies (Terry et al, 2003: 2007: 2011, Middlemore-Risher 2010) and operationally defined as doses not associated with acute signs of cholinergic toxicity (e.g., fasciculations, seizures, diarrhea, excessive urination, salivation, etc., see reviews, Rusyniak and Nanagas, 2004; Sungurtekin et al., 2006).

2.2.2.1 Chlorpyrifos Acute Study

To evaluate the effects of a single subthreshold dose of CPF on axonal transport, subjects were administered CPF (18.0 mg/kg) or vehicle by subcutaneous (s.c.) injection followed immediately with an intravitreal Mn^{2+} (200 μM $\text{MnCl}_2/4$ μL) injection. MRI scans were collected 6 and 24 hours later.

2.2.2.2 Chlorpyrifos Repeated Exposure Study

To evaluate the effects of repeated exposures to CPF, MEMRI baselines scans were first collected in all test subjects (see below) followed by CPF 3.0 mg/kg, CPF 18.0 mg/kg or vehicle (s.c.) injections once daily for 14 days. MEMRI was subsequently conducted on the day after the last CPF injection (24 hours later) then again after a 30-day CPF-free washout period.

In both the acute and repeated CPF-exposure experiments described above, individual rats were weighed and monitored (in their home cages for a period of approximately 5 min each day) for visible cholinergic symptoms (diarrhea, excessive salivation or lacrimation, respiratory difficulties, muscle fasciculations) or other signs of distress throughout the study.

2.3. Intravitreal Manganese Administration

Rats were anesthetized with a mixture of ketamine, 100 mg/kg i.p. and xylazine, 10 mg/kg i.p. prior to each intravitreal injection. MnCl₂ (200 μM/4 μL) was injected with a 30G1½ needle (Beckon-Dickinson Hypodermic #306106) behind the lens to access the vitreous humor of the left eye using care to avoid reflux after removal. Animals were returned to their home cage to be monitored for signs of distress and fully recover prior to each MRI session.

2.4 Magnetic resonance imaging (MRI)

MRI scans were collected 6 and 24 hours following each intravitreal Mn²⁺ injection (see Figure 1A and B). In the CPF repeated exposure study, MRI scans were collected in three separate sessions at the following time points (see Figure 1B): 1) a pre-treatment baseline, 2) at the end of 14 days of CPF exposure, and 3) following a 30-day drug-free washout period. Prior to and throughout the MRI imaging session, rats were anesthetized with a mixture of medical air, oxygen (1:1), and 2.5% isoflurane. Once anesthetized, the head was secured (using medical tape) to a thermo-controlled (37.8°C) cradle while the remainder of the body was unrestrained to promote unrestricted respiration.

Images were acquired on a 7.0T 20cm bore BioSpec MRI spectrometer (Bruker Instruments, Billerica, MA, USA). A standard transmit/receive volume coil (35 mm i.d.) was used for imaging. ECG and respiratory signals were monitored by a physiological monitoring system (SA Instruments, INC., Stony Brook, NY). No gating was necessary considering the head restraint procedures. Initial imaging using a three-plane, 2D T1W FLASH sequence (Fast Low Angle Shot: TE/TR=3.6/145msec; Matrix=128x128; FOV=3.84x3.84cm²; flip angle=30°; 5 3.5-mm thick slices per acquisition plane) was performed to prescribe a subsequent high-resolution T1-weighted image as well as to confirm the successful injection of Mn²⁺ into the eyeball. Visualization of the optic nerve enhancement was realized using a 3D FLASH sequence (TE/TR=3.7/12. msec; # of averages=10; Matrix=192x192x192; field of view = 3.84 x 3.84 x 3.84 cm; flip angle=30°). Total scan time was approximately 60 minutes with total time in the scanner from start to finish being approximately 70 minutes.

2.3.1 MRI Data Analysis

2.3.1.1. *Mn²⁺ Enhancement as a Function of Distance along the Optic Nerve.* Data analysis software was created in-house using MATLAB (The Mathworks Inc., Natwick, MA, USA). During analysis, the following data were used as input for the software: 1) MRI image data, consisting of 192 x 192 x 192 16-bit raw data files; 2) optic nerve localization masks, constructed as 8-bit binary image files (derived from the MRI image data) pinpointing and isolating the optic nerve from the left eye to the optic chiasm; and 3) cerebellum voxel intensity normalization values, calculated using a modification of a method reported by Minoshima et al 1993. The normalization factors were calculated as follows. First, the cerebellum was outlined to measure the mean, minimum and maximum voxel intensities. Second, the maximum voxel intensity values were sorted to isolate the middle two-thirds for which the mean was calculated.

Third, the mean was multiplied by 65% to generate a ‘cerebellum voxel normalization value’ for each MRI scan in order to minimize voxel intensity differences across scans obtained at different time points within and between animals.

For each animal at each time point, the analysis software used input data listed above to generate a matching voxel enhancement value at various locations along the optic nerve. First, the optic nerve mask is used to extract optic nerve voxels from the MRI image. Next, a distance algorithm determines the distance between a voxel and the beginning of the optic nerve. Given the distance along the optic nerve for each voxel, voxel intensities are averaged within 0.8 mm bins along the length of the optic nerve. Finally, intensities for all animals were normalized using the ratio of cerebellar intensity to a value of 8000, an estimated mean cerebellum intensity value.

Considering that the optic nerve has a non-zero width, the distance between a voxel and the eyeball was operationally defined as the length along the optic nerve parallel to a curve running through the center of the optic nerve. To begin, the distance algorithm groups pixel locations within a certain range along the head-to-tail axis in the optic nerve, and calculates a local centroid with coordinates in all three dimensions. A linear interpolation of all local centroids yields a set of locations that serve as the curve along the center of the optic nerve, divided into one thousand sections (i.e. less than the width of a voxel). Given any voxel in the optic nerve map, the closest point along this curve to the voxel is considered to give the distance of the voxel along the optic nerve, where the distance is measured from a coordinate at the beginning of the curve (i.e., the terminus at the eye). A plot of mean voxel intensity as a function of distance along the nerve (originating at the eye) was used to localize the region within which peak enhancement was encountered 6 hours after intravitreal Mn^{2+} injection.

2.3.1.2. *Mn²⁺ Enhancement in the superior colliculus.* Each MRI image set was imported as a raw data file (16-bit, 192 x 192 x 192 pixels) and analyzed using a semi-automated method with ImageJ software (Abramoff et al., 2004). Mn²⁺-enhancement in the superior colliculus (SC) was localized and identified with reference to the Rat Brain Atlas (Paxinos and Watson, 4th Edition) to establish neuroanatomical boundaries. Both Mn²⁺ enhanced and contralateral (non-enhanced) SC were manually outlined and analyzed to obtain a mean intensity value, defined as the sum of each voxel's raw intensity value contained within an outline divided by the total number of voxels. For each section, the enhanced SC intensity value was divided by the contralateral non-enhanced SC intensity value to obtain a normalized SC intensity value. In turn, the mean normalized SC intensity is obtained from all sections (n = 10 -11 sections) within each time point for each subject. Data collected from an MRI image set was excluded if one of three events were present, 1) no visible Mn²⁺ enhancement in eyeball or optic nerve in comparison to contralateral side, 2) distorted boundaries of SC or optic nerve due to movement (i.e. motion artifact), and 3) no Mn²⁺ enhancement in SC during baseline scan (repeated exposure CPF group only).

2.4 *Cholinesterase activity*

Cholinesterase activity was assessed in brain using the method of Ellman (Ellman 1961) with modifications to accommodate a 96-well microplate format at 25°C (Terry et al., 2007). Brains were collected in separate sets of animals in parallel with the animals in the MEMRI studies at four time points, 1) 6 and 2) 24 hours following a single CPF (18.0 mg/kg, s.c.) injection, 3) 24 hours after completing 14 days of repeated CPF exposure (3.0 or 18.0 mg/kg, s.c.), and 4) 30 days after completing 14 days of repeated CPF exposure (3.0 or 18.0 mg/kg, s.c.). Subjects were anesthetized with isoflurane and transcardially perfused with ice-cold phosphate-

buffered saline (PBS) to thoroughly clear the brain of blood, particularly peripheral borne butyrylcholinesterases. Brains were extracted, rinsed with PBS and stored at -80°C until use. Brain tissue was homogenized in ice-cold PBS (wt/vol: 1 g wet brain tissue/4 mL PBS) using a motor driven glass-teflon tissue grinder and total protein concentration was measured using a Micro BCA Protein Assay Kit (ThermoFisher Scientific Inc., Rockford, IL, USA) according to manufacturer's instructions. Brain homogenates (20–50 µg protein/µl) were assayed in triplicate for AChE using 0.48 mM acetylthiocholine (substrate) and 0.52 mM dithiobisnitrobenzoic acid diluted in 1.0 mM sodium phosphate buffer (see Terry et al., 2007). The formation of reaction product (yellow color) was monitored by measuring absorbance values (in optical density) at 412 nm every 2 min for 16 min (µQuant™ Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, VT, USA). The cholinesterase-mediated reaction rate (moles/L per min) was calculated by dividing the change in absorbance per min by 13,600 (Ellman 1961).

2.5 Statistical Analyses

All statistical analyses were performed using NCSS 2001 (NCSS, Kayesville, UT, USA). Mn²⁺-enhancement, body weights and cholinesterase activity were compared by Student's t-tests or analysis of variance with repeated measures when indicated to compare treatment (vehicle vs. colchicine or chlorpyrifos), time (baseline, washout 1 or washout 2) and/or distance along optic nerve (in 0.8 mm bins). Student Newman-Keuls multiple comparison procedures were used to examine post hoc differences when indicated. Statistical significance was assessed using an alpha level of 0.05.

3. Results

3.1 Body Weights

Test subjects were weighed upon arrival arrival, then again on the day of dosing in the colchicine and acute CPF study. The weights of the animals in these studies ranged from approximately 325 to 370 grams at the time of dosing and were not significantly different. In the repeated exposure study, the subjects were weighed at different time points (i.e., at baseline, the end of CPF dosing, and at the end of the 30-day CPF-free washout period, see Table 1A). Both vehicle and CPF-treated subjects gained weight as expected over the course of the repeated exposure study (effect of weighing day, $F_{13,295} = 92.83$, $p < 0.001$). CPF-treated subjects weighed slightly less than vehicle after 14 days treatment (treatment x day effect, $F_{26,295} = 10.44$, $p < 0.001$), but were similar after the 30-day CPF-free washout period.

3.2 Signs of distress or cholinergic toxicity

As noted in the Methods, in both the acute and repeated CPF-exposure experiments, individual test subjects were weighed and monitored daily for visible cholinergic symptoms and/or other signs of distress throughout the study. There were no cases where such signs were detected in any portion of the study.

3.3 Acetylcholinesterase (AChE) Activity

The effects of acute and repeated exposure to CPF on AChE activity are provided in Table 1B. Acute exposure to CPF was not associated with significant inhibition of AChE activity at 6 or 24 hours post injection. In the CPF (14-day) repeated exposure study, AChE activity was reduced (24 hours after the last injection) in CPF-treated rats ($F_{2,12} = 159.30$, $p < 0.001$) to approximately 60% and 20% of control in the 3.0 and 18.0 mg/kg group, respectively (post hoc $p < 0.05$ for both doses). After the 30-day CPF-free washout period, AChE activity had fully returned to control levels in the lower dose group, whereas it was still reduced

to approximately 74% of control in the higher dose group ($F_{2,12} = 43.46$, $p < 0.001$, post hoc $p < 0.05$).

3.4 Colchicine Study

The effects of colchicine administered by intravitreal injection on axonal transport are illustrated in Fig 2. Both treatment groups (Fig 2A) demonstrated progressive decreases in signal intensity in the optic nerves the further away from the eyeball, main effect of distance, ($F_{13,76}=8.25$, $p < 0.001$) 6 hours after intravitreal Mn^{2+} injection. However, colchicine-treated rats exhibited significantly diminished Mn^{2+} enhancement along the optic nerve from the eyeball to the optic chiasm compared to vehicle-treated rats, main effect of group, ($F_{1,76} = 9.00$, $p < 0.05$). Post hoc analysis indicated that enhancement was significantly lower ($p < 0.05$) at all points past the initial 0.8 mm segment in the colchicine-treated subjects compared to vehicle-treated controls. This effect on the Mn^{2+} transport is also clearly apparent 24 hours later in the superior colliculus (Fig 2B), where enhancement is visibly reduced in colchicine-treated rats compared to vehicle-treated controls ($p < 0.001$).

3.5 Chlorpyrifos Acute Study

The effects of an acute (subcutaneous) injection of CPF (18.0 mg/kg) on axonal transport are illustrated in Fig 3. Six hours after intravitreal injection of Mn^{2+} both treatment groups demonstrated progressive decreases in signal intensity in the optic nerves the further away from the eyeball, main effect of distance ($F_{13,167}=3.48$, $p < 0.001$), particularly the latter 5.6 mm most proximate to the optic chiasm (Fig 3A). However, there were no statistically significant effects of CPF on the transport of Mn^{2+} (main effect of group $p > 0.05$). In addition, there were also no differences in signal intensity observed in the superior colliculus 24 hours after intravitreal injection of Mn^{2+} (Fig 3B).

3.6 Chlorpyrifos Repeated Exposure study

The effects of repeated exposure to CPF (3.0 mg/kg, 18.0 mg/kg, or vehicle administered by subcutaneous injection once daily for 14 days) on axonal transport are illustrated in Figs 4-5. In each of the figures the following conditions are illustrated: baseline (BL)-before CPF or vehicle exposure, washout 1 (WO1)-one day after the last drug injection; washout 2 (WO2)-30 days after the last drug injection. For each condition, Mn^{2+} contrast (six hours after intravitreal injection) along the optic nerve (Fig 4) was greatest within the initial 4.0 mm from the eyeball and then significantly decreased closer to the optic chiasm, main effect of distance, ($F_{13,949}=57.07$, $p<0.001$). The following main effects were also observed for the optic nerve comparisons, group x time x distance, $F_{52,949} = 1.91$, $p<0.001$. Post hoc analyses indicated that in the subjects treated with CPF 3.0 mg/kg, enhancement was significantly decreased (compared to their respective baseline values, $p<0.05$) at washout 1 in the initial four, 0.8 mm bins from the eyeball (i.e., at 0.8, 1.6, 2.4, and 3.2 mm). At washout 2 significant decreases were observed in the CPF 3.0 mg/kg treated subjects at the 1.6 and 3.2 mm distances from the eyeball. While a trend toward reduced signal intensity can be observed in the subjects treated with CPF 18.0 mg/kg between the 2 and 2 mm distances from the eyeball at washout 2, the differences did not meet the required level of significance (all p values were >0.05). In the superior colliculus (24 hours after intravitreal Mn^{2+} injection, Fig 5), the following main effects were observed, group effect, $F_{2,60} = 4.012$, $p<0.05$. Post hoc analysis indicated that signal intensity was lower ($p<0.05$) at WO1 for both the 3.0 and 18.0 mg/kg CPF groups compared to vehicle-treated controls. In addition, within group comparisons indicated that CPF 18.0 mg/kg, but not 3.0 mg/kg was associated with reduced Mn^{2+} enhancement ($p<0.05$) in the superior colliculus at both WO1 and WO2 compared to the respective baseline values.

4. Discussion

As noted in the Introduction, the purpose of this study was to determine if subthreshold exposures to a commonly used OP (CPF) were associated with impairments in axonal transport in the brain of living rats as we have previously observed in peripheral nerve preparations in the rat (ex vivo). The initial experiments with colchicine were conducted in order to confirm our ability to use MEMRI as a suitable method for detecting axonal transport deficits in the brains of rats. Colchicine is a tropolone alkaloid that binds tightly to tubulin thus impairing tubulin polymerization and the assembly of microtubules. The consequent disruption of microtubule dynamics impairs the ability of motor proteins to transport cargo in axons (Hastie, 1991; Uppuluri et al., 1993, Han et al., 1998). The intravitreal administration of colchicine at a dose of 2.5 μg was chosen for use in our experiments based on the work of Karlsson et al., 1971, as it was the lowest dose associated with impaired axonal transport in the retinal ganglion cells of rabbits. In each of the 3 studies described in this report, MRI scans were collected 6 and 24 hours following each intravitreal Mn^{2+} injection so that quantitative comparisons of transport could be made in the optic nerve and superior colliculus. This was based on previous studies where, using intravitreal Mn^{2+} injections and MEMRI of the rat visual pathway, the rate of Mn^{2+} transport within retinal ganglion cell axons was estimated at approximately 1 mm/hr and the entire visual projection from the retina to the superior colliculus was enhanced within 24 hours (Thuen et al., 2005; Thuen et al., 2008).

The next series of experiments were conducted to determine if a single (acute) exposure to a subthreshold dose of CPF affected axonal transport of Mn^{2+} in the visual pathway. This was based on a previous series of experiments where we observed a significant reduction in anterograde and retrograde axonal transport of vesicles in the sciatic nerves (ex vivo) of rats

within 10 hours of a single (s.c.) injection of CPF 18.0 mg/kg (Terry et al., 2007). Surprisingly, we did not observe any statistically significant alterations of Mn^{2+} transport in the optic nerve at 6 or 24 hours after intravitreal injection of Mn^{2+} nor did we observe significant differences in Mn^{2+} enhancement in the superior colliculus at the 24 hour time point. The basis for this unexpected observation is unclear, but could be related to some anatomical differences between retinal ganglion cells and neurons in the sciatic nerve or the easier (or more rapid) access to neurons by CPF in the periphery versus the CNS.

Conversely, in the 14-day repeated exposure experiments, the 3.0 mg/kg (but not the 18.0 mg/kg) dose of CPF was associated with significant decreases in transport of Mn^{2+} along the optic nerves (6 hours after Mn^{2+} injection). Transport to the superior colliculus (at the 24 hour time point), was also decreased by CPF (most notably at the 18.0 mg/kg dose), an effect that was also present after a 30-day (CPF-free) washout period. The source of the dose discrepancy in the optic nerves is unclear, although from visual inspection of Fig 4 it does appear that signal intensity is reduced (albeit non-significantly) at all points in washout 2 in the CPF (18.0 mg/kg)-treated subjects. Overall, these would appear to support the aforementioned studies (Terry et al., 2003; Terry et al., 2007) where repeated exposures to subthreshold doses of CPF impaired the transport of vesicles in the sciatic nerves of rats, deficits that persisted throughout a 14 day washout period.

While this study was not designed to investigate potential molecular mechanisms for OP-related alterations in axonal transport, one hypothesis is that OPs might (in some manner) alter the function of motor proteins such as kinesin and dynein and/or components of the neuronal cytoskeleton (e.g., microtubules) that are important for axonal transport (reviewed, Terry 2012). As noted in the Introduction, previous studies have indicated that the transport of Mn^{2+} in

vesicles along microtubules is at least partially dependent on the motor protein kinesin (Sloot and Gramsbergen, 1994; Takeda et al., 1998). The hypothesis that OPs negatively affect kinesin-driven axonal transport is supported by our previous studies as well those of as other laboratories. Specifically, using in vitro microtubule motility assays, we observed an increase in the number of locomoting microtubules that detached from kinesin-coated glass when kinesin was preincubated with the OPs chlorpyrifos, chlorpyrifos-oxon, or diisopropylfluorophosphate (Gearhart et al., 2007). These data suggested that OPs might covalently modify kinesin, thereby weakening the kinesin-microtubule interactions that are necessary for anterograde axonal transport. This hypothesis was supported by another study where (using the biotin-tagged OP agent, FP-biotin) OP binding to tyrosine in the human kinesin 3C motor domain was demonstrated (Grigoryan et al., 2009).

An alternative (or perhaps complementary) hypothesis is that (like colchicine) OPs impair tubulin polymerization leading to the disruption of microtubule assembly which in turn leads to impairments of axonal transport. This hypothesis is supported by our previous collaborative studies (Prendergast et al., 2007) where (utilizing a spectrophotometric method) we demonstrated that chlorpyrifos-oxon inhibited the polymerization of tubulin, and (utilizing organotypic slice cultures of rodent brain and histological methods) caused a marked decrease in the concentration of microtubule associated protein-2. Studies in other laboratories appear to support these findings. For example, utilizing atomic force microscopy, Lockridge and colleagues observed that chlorpyrifos oxon disrupted tubulin polymerization and further (utilizing mass spectrometry), that chlorpyrifos oxon covalently binds to tubulin, an effect that may explain the disruptions in tubulin polymerization (Grigoryan and Lockridge, 2009; Jiang et al., 2010).

While it is difficult to make causal connections between these observations in animals and in vitro models and the wide variety of long-term neurological symptoms that have been associated with OP exposure in humans, OP-related effects on axonal transport may represent one attractive hypothesis. Axonal transport is an essential process in neurons that is responsible for the movement of a variety of important macromolecules (e.g., mitochondria, receptor proteins, growth factors) to and from a neuron's cell body (reviewed, Duncan and Goldstein, 2006) and further, impairments in axonal transport have been implicated in the pathology of a wide variety of neurological illnesses (e.g., amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, Parkinson's disease, Pick's disease, and progressive supranuclear palsy, see Stokin and Goldstein, 2006 for review). It is noteworthy that many of these illnesses are characterized by similar neurobehavioral deficits that have been observed in people who have been exposure to OP-based pesticides. As an example, a recent meta-analysis of 14 studies and data from more than 1600 participants, found an association between subthreshold OP exposures and impairments in attention, working memory, executive function, visuospatial ability, and visual memory (Ross et al., 2013). Here it is also important to note there is a small but growing body of literature to suggest that OP exposure may even represent a potential risk factor for Alzheimer's disease as well as some of the other neurodegenerative disorders mentioned above (Hayden et al., 2010; Hancock et al., 2008, Zaganas et al., 2013).

A similar constellation of chronic neurologic symptoms to that discussed in the preceding paragraph has also been associated with the syndrome now known as Gulf War Illness (GWI, Lange et al., 2001) which has been consistently observed in about 25-30% of Gulf War veterans, or about 175,000 to 250,000 of the 700,000 troops deployed to the war in 1990-91 (RAC 2014). Interestingly, among the potential contributing factors to GWI, exposure to acetylcholinesterase

inhibitors (AChEIs) has been suggested as a plausible explanation for several of the neurological-based symptoms (Golomb et al., 2008). It has been estimated that at least 41,000 United States (US) military personnel were exposed to insecticides that contained either carbamate or OP-based AChEIs (Fricker et al., 2000; US Department of Defense, 2003) and as many as 100,000 may have been exposed to low (i.e., non-acutely toxic) levels of the nerve agents sarin and cyclosarin following the destruction of an Iraqi munitions storage complex at Khamisiyah, Iraq, in March 1991 (Berardocco, 1997).

It is also important to note that several prospective behavioral studies in our laboratories and others have found OP-related deficits in behavioral tasks that map well onto the domains of cognition that have been found to be affected in humans with GWI and/or those who are known to have been previously exposed to subthreshold doses of OPs. These animal tasks include delayed matching (working memory), water maze (spatial learning and memory) novel object recognition (recognition memory), and the performance of the five-choice serial reaction time task (sustained attention) (Bushnell et al., 1991, Terry et al., 2003; Terry et al., 2007; Terry et al., 2011; Middlemore et al., 2010; Yan et al., 2012; Terry et al., 2014).

In conclusion, the results of this study indicate that repeated, subthreshold exposures to a commonly used OP pesticide, CPF can result in persistent alterations in axonal transport in the living mammalian brain. Given the fundamental importance of axonal transport to neuronal function, these observations may (at least in part) explain some of the long term neurological deficits that have been observed in humans who have been exposed to subthreshold doses of OPs. These findings may complement other studies which have identified other deleterious effects of OPs that may be additive (or unrelated) to AChE inhibition and include oxidative

stress, impairments of mitochondrial function, neuroinflammation, altered neurotrophin responses, etc. (reviewed, Soltaninejad and Abdollahi, 2009; Banks and Lein 2012; Terry 2012).

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Figure Legends

Fig 1. Diagram illustrating the intravitreal injection method used in this study and the transport of Mn^{2+} within the axons of retinal ganglion cells (retina) in the anterograde direction (red line) along the optic nerve to the contralateral superior colliculus (SC) and lateral geniculate nucleus (LGN). Below the diagram is the experimental design for each of the three studies described in this report. Abbreviations: chlorpyrifos (CPF), manganese (Mn^{2+}), subcutaneous (s.c.), hours (hrs), magnetic resonance imaging (MRI),

Fig 2. Colchicine Validation Study. Colchicine 2.5 μ g and $MnCl_2$ 200 μ M were co-administered by intravitreal injection and MRI scans were collected 6 and 24 hours later. **A.** Representative horizontal (axial) T1-weighted MR images of rat brain (proximate to interaural line) of the optic nerve emerging from the eyeball (6 hour). Note the clear Mn^{2+} enhancement along the full length of the right optic nerve (indicated by white arrows) to the optic chiasm of the vehicle but not in the colchicine-treated subject. The line graphs to the right illustrate normalized intensity values (see methods) in 0.8 mm bins along the optic nerve from the eye to the optic chiasm. **B.** Representative rat brain coronal (Top, approximately interaural 2.70 mm, bregma 6.30 mm) and horizontal (Bottom, approximately interaural 6.14 mm, bregma -3.86 mm) MR images. Note the clear Mn^{2+} enhancement in the superior colliculus (indicated by white arrows) of vehicle but not in the colchicine-treated subject. A histogram illustrating the normalized intensity values (mean \pm s.e.m.) is provided in the right part of the figure. *, $p < 0.05$, colchicine vs vehicle, ***, $p < 0.001$ initial 0.8 mm vs remaining 10.4 mm (in 0.8 mm bins) of optic nerve. Abbreviations: colchicine (COL), hour (hr), magnetic resonance imaging (MRI), manganese (Mn^{2+}), optic nerve (ON), superior colliculus (SC), vehicle (VEH). N=3

Fig 3. Chlorpyrifos (CPF) Acute Study. Intravitreal Mn^{2+} injections were immediately followed by a single dose of CPF (s.c., 18.0 mg/kg), then MRI scans were collected 6 and 24 hours later.

A. Representative horizontal (axial) T1-weighted MR images of rat brain (proximate to interaural line) of the optic nerve emerging from the eyeball (6 hr). Note the clear Mn^{2+} enhancement along the full length of the left optic nerve (indicated by white arrows) to the optic chiasm of the vehicle and CPF-treated subjects. The line graphs to the right illustrate normalized intensity values (see methods) in 0.8 mm bins along the optic nerves to the optic chiasm. **B.** Representative rat brain coronal (Top, approximately interaural 2.70 mm, bregma 6.30 mm) and horizontal (Bottom, approximately interaural 6.14 mm, bregma -3.86 mm) MR images. Note the clear Mn^{2+} enhancement in the superior colliculus (indicated by white arrows) 24 hrs following injection of the vehicle and CPF-treated subjects. A histogram illustrating the normalized intensity values (mean \pm s.e.m.) is provided in the right part of the figure. N=6

Fig 4. Chlorpyrifos (CPF) Repeated Exposure Study-Optic Nerve Images. Baseline MRI scans were obtained for each test subjects and then CPF (3.0 and 18.0 mg/kg), or vehicle was administered by subcutaneous injection once daily for 14 days. One day (24 hours) after the last drug injection $MnCl_2$ was administered by intravitreal injection and MRI scans were collected 6 hours later. After a 30-day CPF-free washout period, the MEMRI procedure was repeated. In the figure, representative horizontal (axial) images illustrating the Mn^{2+} contrast in the optic nerves (ON, indicated by white arrows) are provided. From left to right, the following conditions are illustrated: baseline (BL), before CPF or vehicle exposure, washout 1 (WO1)-one day after the last drug injection; washout 2 (WO2)-30 days after the last drug injection and a

corresponding line graph illustrating normalized intensity values (see methods) in 0.8 mm bins along the optic nerves from the eyeball to the optic chiasm for each treatment group: (A) vehicle, (B) CPF 3.0 mg/kg and (C) CPF 18.0 mg/kg. Note the reduced signal intensity within the first 4 mm of the optic nerves in the line graphs in the CPF 3.0 mg/kg-treated subjects at WO1 and WO2 compared to their respective baseline values. N=6

Fig 5. Chlorpyrifos (CPF) Repeated Exposure Study-Brain Images. Baseline MR images were obtained from test subjects and then CPF (3.0 and 18.0 mg/kg), or vehicle was administered by subcutaneous injection once daily for 14 days. One day (24 hours) after the last drug injection and 30-days after a CPF-free washout period, MRI scans were collected 24 hours after intravitreal Mn^{2+} injection.. Representative rat brain coronal (Top, approximately interaural 2.70 mm, bregma 6.30 mm) and horizontal (Bottom, approximately interaural 6.14 mm, bregma -3.86 mm) MR images from each group and time point. From left to right, the following conditions are illustrated: baseline (BL), before CPF or vehicle exposure, washout 1 (WO1)-one day after the last drug injection; washout 2 (WO2)-30 days after the last drug injection. Note the clear Mn^{2+} enhancement in the superior colliculus (indicated by white arrows) of all groups at baseline and reduced enhancement associated with CPF (18.0 mg/kg) at WO1 and WO2. Histograms illustrating the normalized intensity values (mean \pm s.e.m.) are provided to right of each MRI. * $p < 0.05$; ** $p < 0.01$ CPF- WO1 and WO2 versus baseline values, respectively. + $p < 0.05$ versus vehicle control at the same washout period. N=6

Table 1 Body Weights and Acetylcholinesterase Activity**A. Body Weights**

Group	Repeated Exposure Study		
	Baseline	Washout 1	Washout 2
Vehicle	328.89±7.16	392.00±5.74	481.00±9.43
CPF 30. mg/kg	331.89±6.89	382.50±5.95**	479.33±9.82
CPF 18.0 mg/kg	343.89±12.02	357.88±14.44**	462.29±16.52

B. Acetylcholinesterase Activity

Group	Acute Exposure Study			
	6 hr Time Point	% of Control	24 hr Time Point	% of Control
Vehicle	37.59±1.80		33.10±4.88	
CPF 18.0 mg/kg	40.57±1.52	107.93±4.04	33.94±4.06	104.20±8.83

Group	Repeated Exposure Study			
	Washout 1	% of Control	Washout 2	% of Control
Vehicle	37.38±1.27		34.35±0.86	
CPF 3.0 mg/kg	22.56±1.52**	60.36±4.01	34.85±0.83	101.46±2.42
CPF 18.0 mg/kg	7.32±0.58**	19.59±1.56	25.44±0.71**	74.06±2.06

Acetylcholinesterase activity is expressed as nmoles acetylthiocholine hydrolyzed/min/mg protein

Abbreviations = CPF, Chlorpyrifos "***", p<0.001

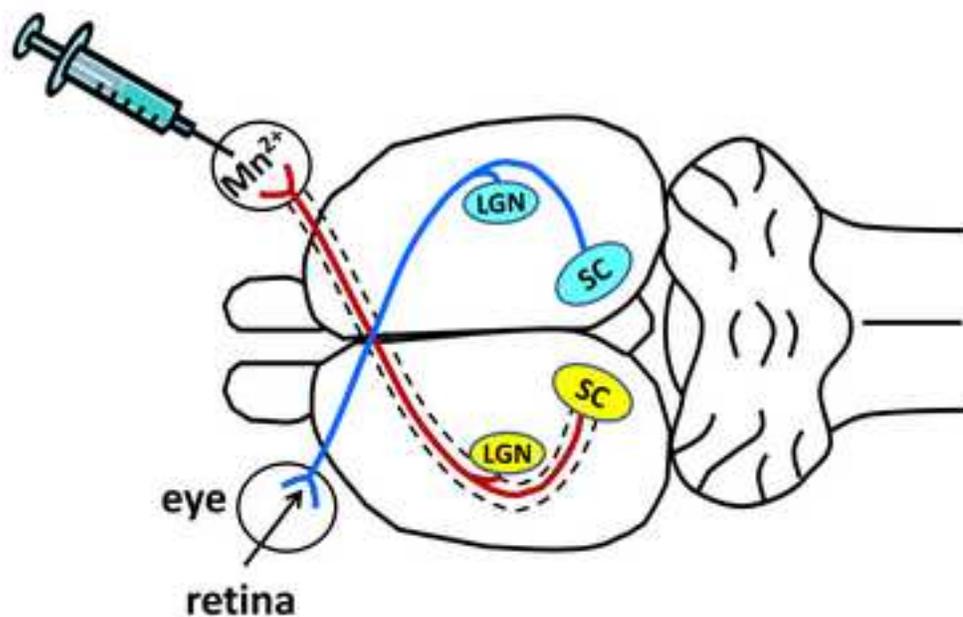


Fig 1

Study	Procedure	Imaging	
		6 hrs	24 hrs
1: Colchicine Validation	Mn ²⁺ + Colchicine Combined Intravitreal Injection	MRI	MRI
2: CPF Acute Exposure	Mn ²⁺ Intravitreal Injection CPF s.c. Injection	MRI	MRI
3: CPF Repeated Exposure	Baseline Evaluation Mn ²⁺ Intravitreal Injection	MRI	MRI
	↓		
	14 days of Daily CPF Treatment		
	↓		
	24 hr CPF-Free Washout Mn ²⁺ Intravitreal Injection	MRI	MRI
	↓		
	30 day CPF-Free Washout Mn ²⁺ Intravitreal Injection	MRI	MRI

Fig 2

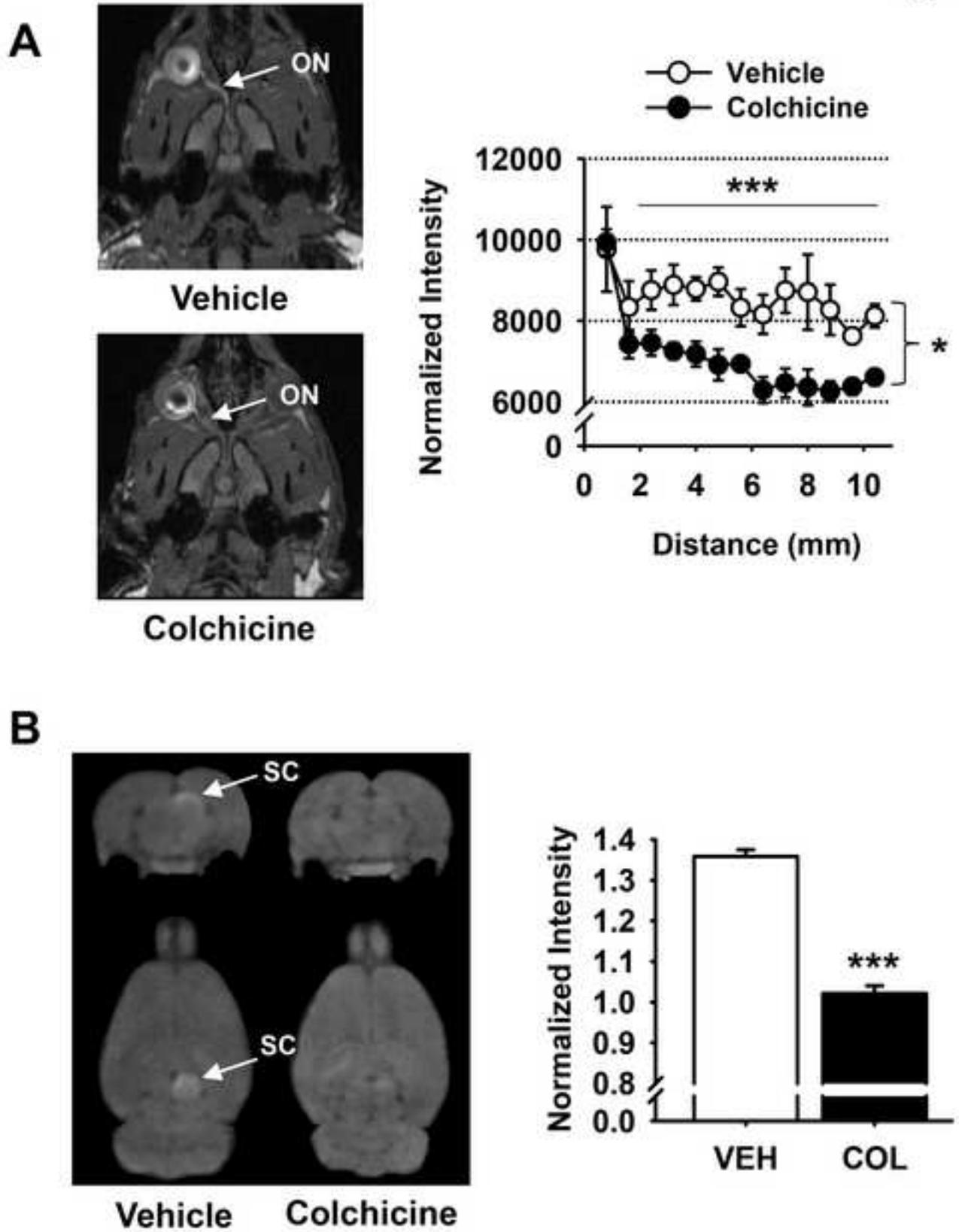


Fig 3

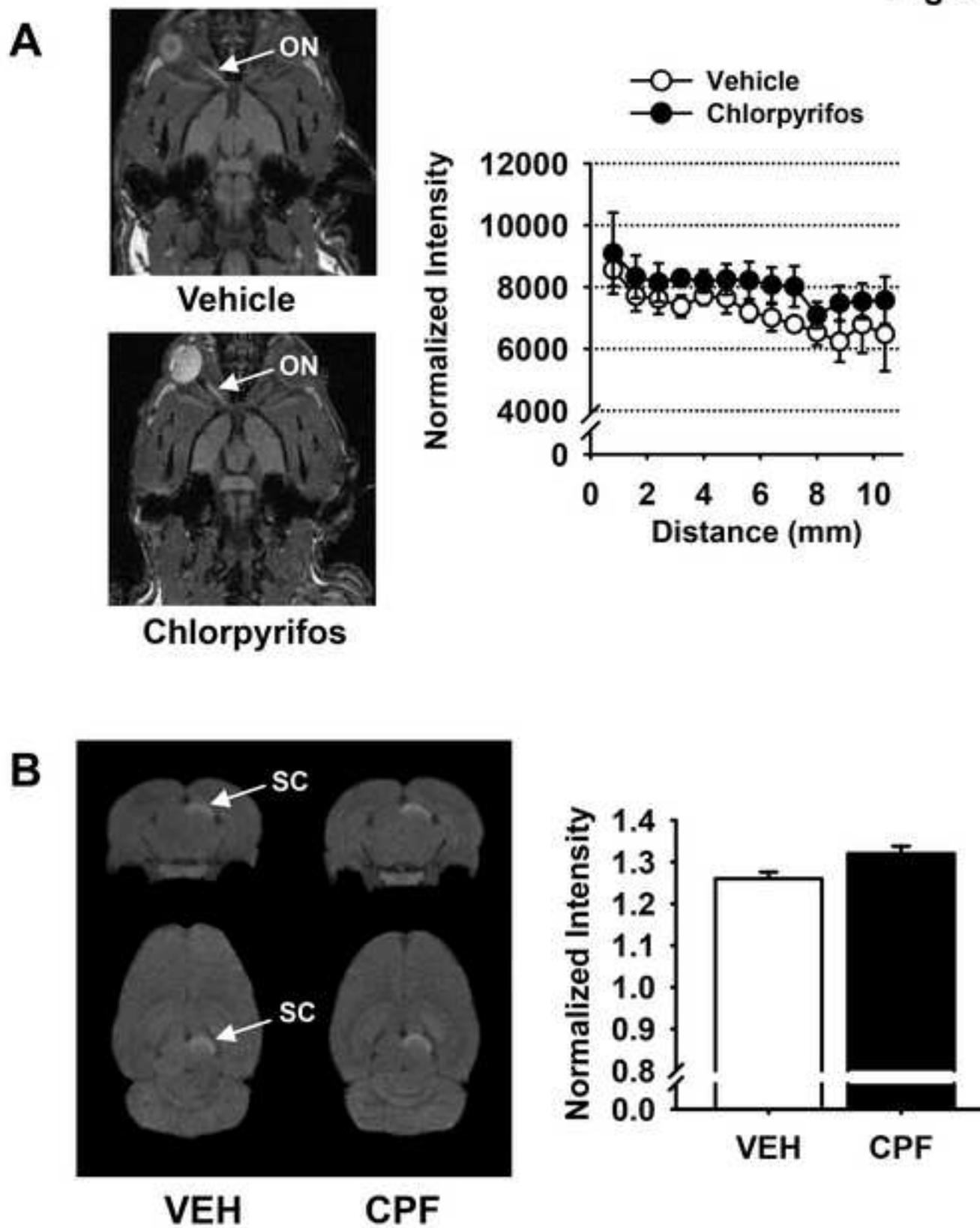


Figure 4
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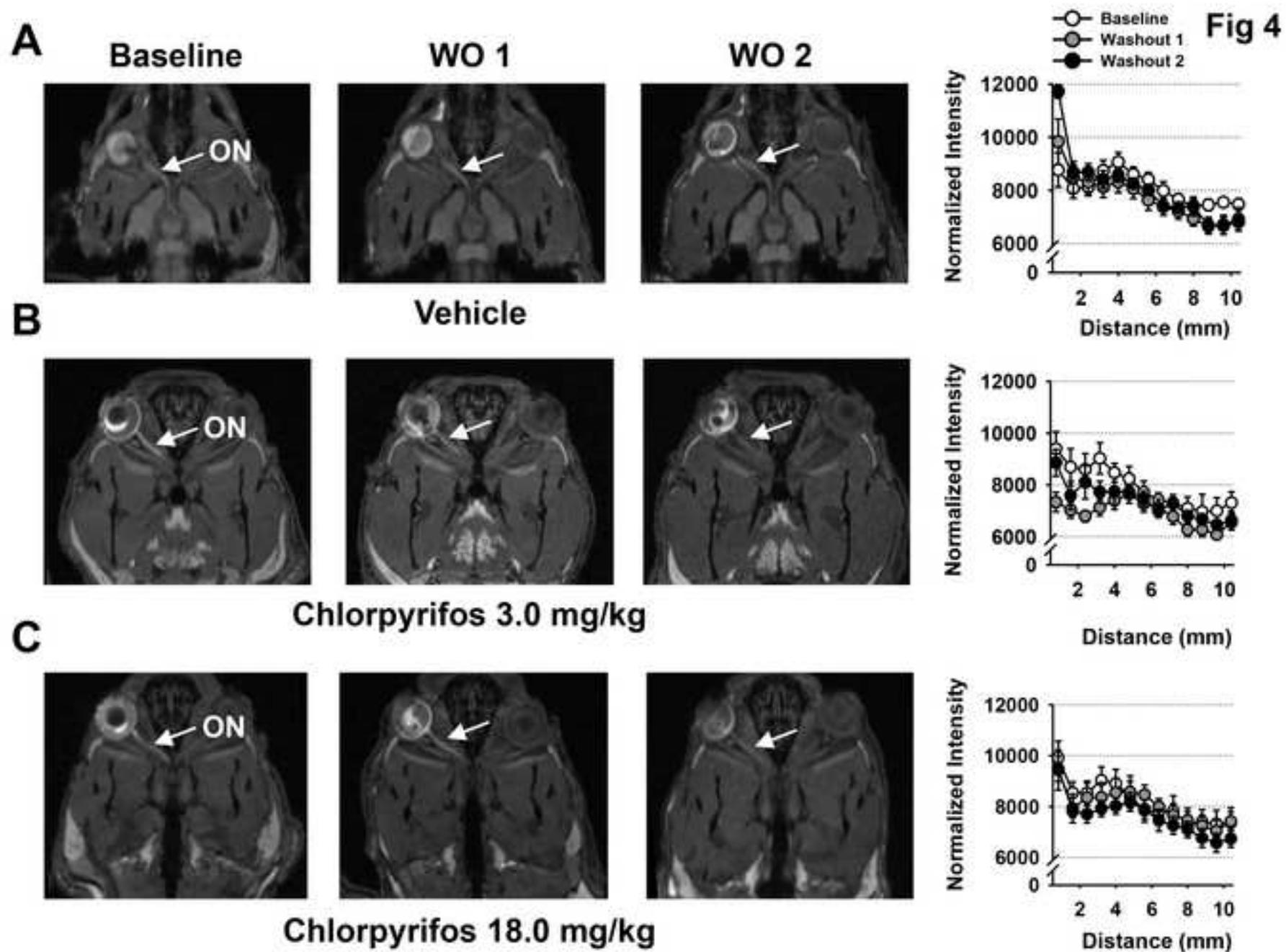


Figure 5
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Fig 5

