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### Authors

Gao, Jie  
Naughton, Sean X  
Wulff, Heike  
[et al.](#)

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## **Title Page**

# **Diisopropylfluorophosphate impairs the transport of membrane bound organelles in rat cortical axons**

Jie Gao, Sean X. Naughton, Heike Wulff, Vikrant Singh, Wayne D. Beck, Jordi Magrane, Bobby Thomas, Navneet Ammal Kaidery, Caterina M. Hernandez, and Alvin V. Terry, Jr.

Department of Pharmacology and Toxicology, Georgia Regents University, Augusta, Georgia, 30912 (JG, SXN, WDB, BT, NK, CMH, AVT)

Department of Pharmacology, University of California Davis, Davis, California 95616 (HW, VS)

Feil Family Brain and Mind Research Institute, Weill Cornell Medicine, New York, New York 10065 (JM)

## **Running Title Page**

**Running Title:** Diisopropylfluorophosphate impairs axonal transport

### **Corresponding Author:**

Alvin V. Terry Jr., Ph.D.  
Department of Pharmacology & Toxicology  
1120 15th Street, CB-3545  
Georgia Regents University  
Augusta, Georgia 30912  
Phone: 706-721-9462  
Fax: 706-721-2347  
E-mail: aterry@gru.edu

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### **Abbreviations:**

AChE, acetylcholinesterase

APP, amyloid precursor protein

ATR, atropine

COL, colchicine

DFP, diisopropylfluorophosphate

DPZ, donepezil

MEC, mecamylamine

MBO, membrane bound organelle

NMR, nuclear magnetic resonance

OP, organophosphate

OPIDN, organophosphate-induced delayed neuropathies

## **Abstract**

The extensive use of organophosphates (OPs) is an ongoing environmental health concern due to multiple reports of OP-related neurological abnormalities. While the mechanism of the acute toxicity of OPs is attributed to inhibition of acetylcholinesterase (AChE), there is growing evidence that this may not account for all of the long term neurotoxic effects of OPs. In previous experiments (using ex vivo and in vitro model systems) we observed that the insecticide OP chlorpyrifos impaired the movements of vesicles and mitochondria in axons. Here, using a time-lapse imaging technique, we evaluated the OP-nerve agent, diisopropylfluorophosphate (DFP) across a wide range of concentrations (subnanomolar to micromolar) for effects on fast axonal transport of membrane bound organelles (MBOs) that contained the amyloid precursor protein (APP) tagged with the fluorescent marker, Dendra2 (APPDendra2). Both 1 and 24 hours of exposure to DFP and a positive control compound, colchicine, resulted in a decrease in the velocity of anterograde and retrograde movements of MBOs and an increase in the number of stationary MBOs. These effects occurred at picomolar (100 pM) to low nanomolar (0.1 nM) concentrations that were not associated with compromised cell viability or evidence of cytoskeletal damage. Moreover, the effects of DFP occurred at concentrations that did not inhibit AChE activity and they were not blocked by cholinergic receptor antagonists. Given the fundamental importance of axonal transport to neuronal function, these observations may explain some of the long term neurological deficits that have been observed in humans who have been exposed to organophosphates.

**Key Words:** Organophosphate, Pesticide, Nerve Agent, amyloid precursor protein, Gulf War Illness, Alzheimer's disease, Parkinson's disease

## **Introduction**

The chemicals known as organophosphates (OPs) are used for a variety of important agricultural, industrial, and domestic purposes worldwide. However, the prevalence of OPs in the environment has become a public health concern given their toxicity and the number of accidental and intentional poisonings by OPs (e.g., from suicide attempts) (Eddleston et al., 2008). Exposure to OP-based nerve agents from rogue governments and terrorist organizations is an additional risk that was exemplified by the Iraqi military attacks on Kurdish civilians in the 1980s (Macilwain, 1993), the Tokyo Sarin attack in 1995 by domestic terrorists (Nagao et al., 1997), and the recent sarin attacks on civilians in Syria (Sellström et al., 2013).

The mechanism of the acute toxicity of OPs is well established and attributed to the irreversible inhibition of acetylcholinesterase (AChE) which leads to elevations of synaptic acetylcholine and a variety of peripheral, autonomic and central nervous system symptoms collectively described as the “cholinergic crisis”, which can be life threatening (Ecobichon, 2001; Pereira et al., 2014)). A variety of long-term neurological consequences of acute poisonings with OPs have also been documented and include electroencephalogram abnormalities, mood disorders (e.g., anxiety and depression), deficits in psychomotor speed and coordination, and a variety of cognitive deficits (Brown and Brix, 1998; Miyaki et al., 2005; Pereira et al., 2014). A number of epidemiologic studies also suggest that exposures to OPs at levels not associated with acute symptoms of toxicity can result in long term neurobehavioral abnormalities especially cognitive abnormalities (e.g., deficits in attention, working memory, executive function, visuospatial ability and visual memory (Pope et al., 2005; Ross et al., 2013)).

While AChE inhibition is clearly an important mechanism of the toxicity of OPs, it may not account for all of the long term-neurological alterations associated with these chemicals.

Deleterious effects of OPs that may be additive (or unrelated) to AChE inhibition include oxidative stress, impairments of mitochondrial function, neuroinflammation, and altered neurotrophin responses, etc. (Soltaninejad and Abdollahi, 2009; Banks and Lein, 2012; Terry, 2012). For several years our laboratory has been investigating the possibility that OPs impair axonal transport, a potentially significant issue given the fundamental importance of axonal transport to neuronal maintenance and function. The original impetus for this work was a report by (Reichert and Abou-Donia, 1980) that relatively high doses of certain OPs (phenylphosphonothioate esters and tri-o-cresyl phosphate) known to be associated with OP-induced delayed neuropathies (OPIDN) impaired fast anterograde axonal transport in a rat optic nerve preparation. 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 chlorpyrifos (CPF), an OP not associated with OPIDN except at doses well above the LD<sub>50</sub> (Richardson, 1995). Importantly, the doses used in our CPF study were below the threshold for acute toxicity and, further, the deficits in axonal transport were detected for up to 14 days after the last CPF injection indicating that the impairments were persistent (Terry et al., 2003; Terry et al., 2007).

In a series of subsequent experiments using time-lapse imaging techniques, we also observed impairments in the movement of mitochondria in axons in primary neuronal culture (Middlemore-Risher et al., 2011) associated with both CPF and its metabolite CPF-oxon (CPO). The changes occurred at concentrations of CPF and CPO that did not inhibit AChE activity, they were not blocked by cholinergic receptor antagonists, and they did not appear to be associated with direct (OP-related) effects on mitochondrial viability or function (i.e., mitochondrial membrane potential or ATP production). Most recently, we observed (using a magnetic

resonance imaging technique) that repeated exposures to doses of chlorpyrifos that were below the threshold for acute toxicity led to prolonged impairments of axonal transport in the brains of living rodents (Hernandez et al., 2015).

The purpose of the experiments described here was to evaluate an OP of a different structural class, diisopropylfluorophosphate (DFP) for effects on axonal transport using a new *in vitro* model system (see Discussion for further details). DFP is an alkyl phosphorofluoridate originally synthesized by the German chemist Gerhard Schrader and later evaluated as a potential chemical warfare agent by the Germans, British, and Americans (Pope et al., 2005). It possesses a great deal of structural homology with other highly toxic nerve agents such as sarin and soman, but is less potent (Hobbiger, 1972) and dangerous for laboratory personnel. As a positive control for axonal transport deficits, the tropolone alkaloid colchicine was also evaluated in these studies.

## **Material and Methods**

### ***Drugs***

Atropine (ATR), colchicine (COL), mecamlamine (MEC), deuterium oxide (D<sub>2</sub>O), and diisopropylfluorophosphate (DFP) were obtained from Sigma-Aldrich (St. Louis, MO, USA), stored as recommended by the source vendor and stock solutions were prepared in deionized water. COL and DFP were prepared to use at the following final concentrations (in nM): 0.01, 0.1, 1.0, 10.0, 100.0, 1000.0, and 10000.0). ATR and MEC were prepared to use at 50.0 and 10.0 μM, respectively. All drug stock solutions were prepared at 100-fold higher concentrations in deionized water (≤ 5 (v/v) %, pH 7.0) within 15 minutes of the start of each 1 or 24 hour exposure periods.

### ***Embryonic cortical cultures***

The cerebral cortices from E17-18 Sprague–Dawley rat embryos were extracted and cultured as described previously (Gao et al., 2014) under aseptic conditions. Timed pregnant rats were purchased from Harlan Sprague-Dawley, Inc. Indianapolis, IN and housed and maintained on a 12-hour light/dark cycle in a temperature-controlled room (25°C) with free access to food and water for at least 3 days prior to initiating cultures. All procedures used during this study were reviewed and approved by the Georgia Regents University Institutional Animal Care and Use Committee and are consistent with the Association for Assessment and Accreditation of Laboratory Animal guidelines. Appropriate measures were taken to minimize pain or discomfort in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals et al., 2011). Briefly, cortices were extracted and trypsinized (Trypsin-EDTA #25200, Life Technologies, Carlsbad, CA), then dissociated cellular material was seeded at a density of  $5 \times 10^5$  cell/mL media onto poly-L-lysine (Sigma-Aldrich, St. Louis, MO) coated glass coverslips (25 mm) and 96-well plate (MTT cell proliferation assays). For AChE activity assays, cells were seeded at a density  $1.1 \times 10^6$  cell/mL media on 10 cm petri dishes. Cultures were maintained at 37°C in a 5.0% CO<sub>2</sub> humidified atmosphere in Neurobasal™ (#21103) supplemented with B27 (2.0 (v/v) %; #17504), Glutamax™ (0.5M, #35050) and Penicillin-Streptomycin (100 U/mL; #15140-122). All culture media materials were purchased from Life Technologies (Grand Island, NY, USA).

### ***Cell transfection and treatments***

All culture transfections were conducted at 37°C after 5-7 days in vitro (DIV) with APPDendra2-cDNA (Magrane et al., 2012) and Lipofectamine® 2000 (Life Technologies). All

time-lapse imaging studies (see below) were conducted 24-36 hours post-transfection. Doses of COL or DFP employed for 1 hour pre-exposures were (in nM): 0.001, 0.01, 0.1, 1.0, 10.0, 100.0, 1000.0 or 10,000.0 and for 24 hours pre-exposures were (in nM): 0.01, 0.1, 1.0, 10.0 or 100.0. Cultures were treated along the same timeline with deionized water 5.0 (v/v) % as a control and are indicated as vehicle (VEH). Prior to all live imaging experiments, culture media was exchanged with phenol-free Neurobasal™ (#12348-017, Life Technologies)

### ***Live imaging and Measurements of Axonal Transport***

Transfected cells were located in primary cortical cultures using an inverted epifluorescent microscope (Deltavision, Deconvolution Olympus IX71; Olympus, Bothell, WA) connected to a CCD camera (Photometrics Cool SNAP HQ<sup>2</sup>, Roper Scientific, Tucson, AZ, US). Axons successfully transfected with APPDendra2 were identified by their fluorescence and morphological features (i.e., long neurites, constant thin diameter, no branching, perpendicular emergence from the cell body). Throughout the duration of the imaging session, cultures were maintained at 37°C under 5% CO<sub>2</sub> conditions within an environmental (i.e., heat and mixed gas controller) chamber. Once the APPDendra2 transfected neurons were localized (under 60X magnification, 1.42 numerical aperture), axons were video recorded and frames captured every 5 seconds for 5 minutes (SoftWoRx; Applied Precision, Issaquah, WA) to track the movements of dynamic particles and identify stationary particles. Using NIH ImageJ (<http://rsb.info.nih.gov/ij/>) with input/output and kymograph plug-ins, images were compressed into audio video interleaved (AVI) animation files. Briefly, kymographs (a graphic representation of a sample's position vs time) were generated to analyze the nature of APPDendra2 particle transport and directionality (i.e., anterograde, retrograde or stationary). Particle transport in the anterograde direction was identified by its movement away from the cell body and transport in the retrograde direction was

identified by its movement towards the cell body. Stationary particles were identified by the absence of all movement. The distance traveled by each particle was measured in micrometers ( $\mu\text{m}$ ). Individual particle velocities were measured in  $\mu\text{m}$  per second during periods of sustained dynamic movement (i.e.  $\geq 5$  consecutive frames). Data were plotted as the ratio of the number of particles (retrograde, anterograde or stationary) to the total number of analyzed particles. Fig 1 provides an example of an APPDendra2 transfected neuron and a representative kymograph.

### ***Mecamylamine and atropine co-incubation experiments***

The method described above for assessing axonal transport was also used to determine the effects of co-incubation of DFP with the muscarinic antagonist atropine or the nicotinic antagonist mecamylamine. Specifically, cortical neurons were co-incubated (for 24 hours) with either atropine (50  $\mu\text{M}$ ) or mecamylamine (10  $\mu\text{M}$ ) and a representative concentration of DFP shown to impair axonal transport (10 nM) in the first set of experiments (see results below). The representative concentrations of atropine or mecamylamine were based on previous in vitro studies (Middlemore-Risher et al., 2011).

### ***Evaluation of cultured cell viability and toxicity***

The concentration and time-dependent effects of COL or DFP on cell viability (total number of viable cells) were assessed using a 96-well plate format Vybrant®MTT Cell Proliferation Assay kit (Life Technologies) according to the manufacturer's instructions. Cell viability measurements are reported as the percentage of viable cells in comparison to the vehicle-treated cultures (i.e. control). In order to measure cellular toxicity associated with COL or DFP, a Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich® St. Louis, MO, USA. Lot# B9B270726V) was also used according to the manufacturer's instructions. Data for both cell viability and toxicity assays are expressed as percentage of output value (i.e. absorbance

values) for COL or DFP-exposed compared to the output value of vehicle- or untreated cell cultures (i.e. control).

Immunocytochemistry - Cultures (on 25 mm coverslips) were also processed to evaluate gross changes in cellular morphology and structure following both 1 (10.0  $\mu$ M) or 24 hours (100.0 nM) exposure to COL or DFP compared to vehicle-treated controls. Immediately following COL or DFP treatment, coverslips were washed 3 times in phosphate-buffered saline (PBS) sequentially decreasing in temperature from 37°C to 4°C, gradually fixed for 30 minutes in formalin (Thermo Fisher Scientific, Waltham, MA) (from 2.5 (v/v)% to 5 (v/v)% then a final 10 (v/v)% and thoroughly washed with PBS. To assess whole cell and axon morphology, antibodies targeting microtubule associated protein (MAP2A/2B, #MAB364, Millipore, Temecula, CA) and doublecortin (DCX; #4604, Cell Signaling Technology, Danvers, MA) were utilized as somato-dendritic and axonal markers, respectively. Briefly, coverslips were blocked in goat (MAP2A/2B) or donkey (DCX) serum-based blocking buffer, in % (v/v): 10 serum, 1.0 bovine serum albumin, 0.1 Triton X-100, 0.1 cold fish gelatin and 0.05 Tween-20 for 30 minutes at 25°C, then incubated in anti-MAP2A/2B (1:250) or DCX (1:400) overnight at 4°C. Following PBS washes, the ABC method was employed to amplify and detect the primary antibody signal, followed by streptavidin-conjugated AlexaFluor594 (Life Technologies/Molecular Probes, Eugene, OR). Coverslips were incubated for 2 hours at 25°C in Acti-stain™ 555 (Cytoskeleton, Denver, CO), a fluorophore-conjugated phalloidin, to assess cytoskeletal structure. All coverslips were mounted in DAPI-mounting media (Vectashield, Vector Laboratories, Burlingame, CA) to counterstain nuclei. Using confocal microscopy, all fluorescent staining was localized and identified under low magnification ( $\leq$  40X) followed by image acquisition at 63X magnification (Zeiss 780 upright, Zeiss, Thornwood, NY). Image processing (i.e. conversion between formats,

background noise reduction, pseudo-coloring, etc.) was completed using ZEN (Zeiss) and GIMP (<http://gimp.org>) 2.8 and Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA) softwares.

### ***Preparation of cell lysates for AChE activity assays***

Following transfections and/or drug exposures, cultures were washed with PBS, transferred to a microtube after scraping from coverslips into PBS (pH 8.0) containing Triton X-100 (final concentration = 1.0 (v/v) %) (PBS-TX100) and stored at -80°C. Samples were solubilized in PBS-TX100 at 4°C for 2 hours with constant agitation. To remove cellular debris, samples were centrifuged at 13,400  $\times$  g for 1 minute at 4°C to clarify samples and collect the supernatant. Total protein for each supernatant collected was measured using detergent-compatible protein assay (Pierce Micro BCA™ Protein Assay Kit, ThermoFisher Scientific Inc. Rockford, IL) according to manufacturer's instructions.

### ***Measurement of Acetylcholinesterase activity***

Cell lysates (yielding a minimum of 8.0 ug/uL total protein) were assayed in duplicate to measure acetylcholinesterase (AChE) activity in samples after exposure to DFP. AChE activity was assessed using the Ellman method with modifications to accommodate a 96-well microplate format at 25°C (Prendergast et al., 2007; Middlemore-Risher et al., 2011). The specific concentrations utilized for each chemical (Sigma-Aldrich, St. Louis, MO) in the reaction mixture (prepared in 1.0 mM sodium phosphate buffer (pH 7.0  $\pm$  0.05) were as follows (in mM): 0.48 acetylthiocholine, 7000 tetraisopropyl pyrophosphoramidate (iso-OMPA, a butyrylcholinesterase inhibitor) and 0.52 mM 5,5'-dithiobis(2-nitrobenzoic acid). The formation of reaction product was monitored by measuring absorbance values at 412 nm every 2 minutes for 16 minutes (Mx synergy Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, VT, USA). The rate

of AChE activity was then calculated for each time point of measurement using the formula (change in absorbance/min)/ (1.36 x 10<sup>4</sup>), then normalized to the intra-experiment vehicle-treated control by exposure time (i.e., 1 or 24 hours).

### ***DFP stability studies***

Stock solutions of DFP were prepared by dissolving 20  $\mu$ L DFP in 600  $\mu$ L of deuterium oxide (D<sub>2</sub>O) (Cambridge Isotope Laboratories, Andover, MAUSA) or PBS (pH=7.4) Diisopropyl phosphoric acid (DIPA), which arises after hydrolytic P-F bond cleavage, was obtained by the hydrolysis of DFP. NMR studies were performed at University of California Davis Nuclear Magnetic Resonance (NMR) Facility using an 800 MHz Avance III (for <sup>1</sup>H and <sup>13</sup>C) and 500 MHz Avance DRX (for <sup>31</sup>P and <sup>19</sup>F) spectrophotometers (Bruker, Billerica, MA USA). All NMR were recorded at different time points (see Fig 4). The extent of DFP hydrolysis was assessed from the area of the DFP and DIPA proton signals. All parameters (shim, gain and number of scans etc.) were kept constant during data acquisition for any particular matrix and are as follows,: **DFP** <sup>1</sup>H-NMR (800 MHz, CDCl<sub>3</sub>):  $\delta$  = 4.77 (octet, 2 H,  $J$  = 6.40 Hz), 1.38 (d, 12 H,  $J$  = 6.40 Hz); <sup>13</sup>C-NMR (200 MHz, CDCl<sub>3</sub>):  $\delta$  = 75.1 (d, CH  $J$  = 5.9 Hz), 23.5 (m, CH<sub>3</sub>); <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>):  $\delta$  = -10.78 (dt,  $J_{P-F}$  = 974.8 Hz,  $J_{P-H}$  = 6.4 Hz);. <sup>19</sup>F-NMR (470 MHz, CDCl<sub>3</sub>):  $\delta$  = -76.87 (d,  $J_{F-P}$  = 974.8 Hz), and **DIPA** <sup>1</sup>H-NMR (800 MHz, D<sub>2</sub>O):  $\delta$  = 4.51 (octet, 2 H,  $J$  = 6.40 Hz), 1.28 (d, 12 H,  $J$  = 6.40 Hz); <sup>13</sup>C-NMR (200 MHz, D<sub>2</sub>O):  $\delta$  = 72.3 (m, CH), 22.8 (m, CH<sub>3</sub>); <sup>31</sup>P-NMR (202 MHz, D<sub>2</sub>O):  $\delta$  = -1.64 (t,  $J_{P-H}$  = 6.9 Hz).

### **Statistical Analyses**

All statistical analyses were performed using SigmaPlot (Systat Software, San Jose, CA) Analysis of variance (ANOVA) was used to compare the concentration-dependent effects of drug treatments to vehicle-treated controls and the method of Holm-Sidak was used to examine

post hoc differences when indicated. Statistical significance was assessed using an alpha level of 0.05. Values depicted in the figures reflect the mean  $\pm$  s.e.m. The number of independent experiments conducted for each drug evaluation and the number of replicates per drug concentration are indicated in the figure legends.

## **Results**

### ***APPDendra2 transfection and live imaging***

In the current study, we adapted and optimized previous methods for transfecting APPDendra into rat primary motor neuron cultures (Magrane et al., 2012) for APPDendra2 to rat primary cortical neuron cultures for live imaging. Twenty-four hours following transfection, cultured neurons exhibited clear expression of APPDendra2 in the soma and axons (both of which were identified morphologically) (Figure 1A). For imaging, individual APPDendra2-labeled MBO's in axons were identified under lower magnification as distinct (green fluorescent) structures with a circular or tubular shaped appearance (see the arrows in Figure 1A). At higher magnification (63X), definitive proximal and distal axonal regions of each axon were identified and corresponding kymographs (see Fig 1B for a sample) demonstrate that many MBOs were highly mobile, moving in both the anterograde and retrograde directions, while others remained stationary.

### ***Effects of colchicine and DFP on cell viability, neuronal morphology and cytoskeletal integrity***

The effects of COL and DFP on cell viability (across the same range of concentrations that were later evaluated in the axonal transport studies; see results below) were initially assessed using an MTT assay. As shown in Fig. 2A, 1 hour incubation with COL significantly decreased cell survival (by about 10%) only at the highest concentration that was evaluated (10uM), while

there were no significant effects of DFP at any of the concentrations that were evaluated (Fig. 2B). As a secondary method for evaluating the effects of COL and DFP on neuronal cell viability, an assay to measure lactate dehydrogenase (LDH) levels in the media was used. Compared to the control group treated with vehicle only, 1 hr incubation with COL did not significantly increase LDH release except at the highest concentration evaluated (10 $\mu$ M) where it increased by about 15% (Fig 2C). Conversely, 1hr exposure to DFP was not associated with significant increases in LDH release at any of the concentrations that were evaluated (Fig 2D). Visual (qualitative) analyses of the immunocytochemistry images (actin, DCX, and MAP, see Fig 2E-G) revealed no overt alterations in cellular morphology or structure after one hour of exposure to 10  $\mu$ M of COL or DFP.

Exposure to concentrations of COL of 0.1 nM and above for 24 hours were associated with modest impairments in cell viability in the MTT assay (e.g., ~30% decrease in viability at 100 nM, see Fig 3A). Likewise, LDH levels were increased at the 10 and 100 nM concentrations of COL (see Fig 3C). In the DFP (24 hr exposure) experiments, the two highest concentrations (10 and 100 nM) were also associated with modest impairments in cell viability (i.e., by approximately 15-20%) as determined in the MTT assay (Fig 3B). Similar to COL, LDH levels were also increased at the 10 and 100 nM concentrations of DFP (see Fig 3D). Visual analyses of the immunocytochemistry images (actin, DCX, and MAP, Fig 3E-G) revealed some alterations in the cellular morphology and structure after 24 hr of exposure to 10  $\mu$ M COL or DFP.

#### *DFP is Stable at pH 7.4 for 24 Hours*

The Merck Index (Stecher, 1968) and several product sheets have indicated that DFP degrades in DMSO and at a pH of 7.5, therefore, we checked both the purity and the stability of

DFP obtained from Sigma-Aldrich (St. Louis, MO USA). A combination of  $^1\text{H}$ -,  $^{13}\text{C}$ ,  $^{19}\text{F}$  and  $^{31}\text{P}$ -NMR was found to be a good way of detecting impurities in commercially available DFP (see Fig 4A for the  $^1\text{H}$ -NMR). While most batches we analyzed were of a reasonable purity, one out of five patches contained significant amounts of an unidentified phosphorous containing impurity (data not shown) and we would therefore advise experimenters using commercially available DFP to check individual batches for purity by NMR before use.

We next investigated the stability of DFP in an aqueous-based solution and the speed at which it hydrolyzed into diisopropyl phosphoric acid (DIPA) through nucleophilic substitution of the F by an  $-\text{OH}$  group. This hydrolysis can be traced by the diminishing octet at 4.77 ppm and the appearance of a shifted octet at 4.51 ppm in the 800 Hz  $^1\text{H}$ -NMR spectrum (Fig 4B; Please note that the signals are to the left and right of the  $\text{H}_2\text{O}$  signal). Another indication of the hydrolysis is a change of the  $^{31}\text{P}$  signal from a doublet induced by the coupling to the F in DFP to a singlet in the hydrolysis product that no longer contains F. While DFP was highly stable in DMSO and showed no signs of hydrolysis even after 19 days (data not shown), it hydrolyzed in pure water ( $\text{D}_2\text{O}$  for the NMR experiments) with a half-life of roughly 10 hrs (Fig 4B). In phosphate buffered solutions ( $\text{D}_2\text{O}$  phosphate buffered saline for the NMR experiments), DFP was stable and showed no signs of hydrolysis up to 20 hrs (Fig 4C) after introduction to an aqueous solution, suggesting that it would be sufficiently stable throughout the period of exposure used for our vitro studies. However, based on the observation that DFP hydrolysis was associated with its preparation in unbuffered deionized water, we always freshly prepared aqueous DFP stocks within 15 minutes of diluting into buffered culture media prior to all experiments to avoid any hydrolysis.

### ***Colchicine Impairs APP Axonal Transport***

In order to validate the methods used for measuring fast axonal transport in primary cortical neurons, we evaluated COL, a compound known to impair tubulin polymerization and to impair fast axonal transport in multiple model systems (see further details in the Discussion). Kymograph analysis revealed that, after 1 hr of exposure to COL, there was a significant ( $p \leq 0.05$ ) decrease in the velocity of APP transport in both the anterograde and retrograde direction associated with most of the doses (0.1 nM–10  $\mu$ M) that were evaluated (Fig. 5A). Notably, compared to the vehicle-control group, COL at concentrations as low as 0.1 nM, significantly ( $p < 0.05$ ) decreased MBO velocity (by ~21%) in the anterograde direction. Representative time lapse images of single axons exposed to vehicle or COL 10.0 nM for 1 hr are provided in supplemental movie files 1 and 2, respectively. The reduction in velocity was accompanied by a concentration-dependent decrease in the overall percentage of MBOs moving in the anterograde direction (Fig 5B) and an increase in the percentage of stationary MBOs (Fig 5D). Interestingly, COL did not significantly affect the overall percentage of APP particles moving in retrograde direction (Fig 5C). Subsequent experiments were conducted to determine whether the deficits in transport associated with COL occurred after a longer exposure period (24 hr). The same general trend of effects were observed, i.e., concentration dependent decreases in the velocity of anterograde and retrograde transport of MBOs (Fig 6A), a decrease in the overall percentage of particles moving in the anterograde (but not retrograde) direction (Fig 8B and C respectively), and an increase in the overall percentage of stationary particles (Fig 6D). Here it is important to note that axonal transport measurements were conducted only in neurons that appeared healthy with moving MBOs, an important consideration for the higher concentrations of COL (and DFP, see below) at the 24 hr time point where some evidence of compromised cell viability was detected (as noted above).

### ***DFP Impairs APP Axonal Transport***

Kymograph analysis indicated that 1hr of exposure to DFP (similar to COL) resulted in a concentration-dependent decrease in the velocity of APP transport in both the anterograde and retrograde direction. Concentrations of DFP as low as 0.1nM resulted in ~20% decrease MBO velocity in the anterograde direction and ~30% decrease in velocity in the retrograde direction (Fig. 7A). A representative time lapse image of a single axon exposed to DFP 10.0 nM for 1 hr is provided in supplemental movie file 3. Like COL, DFP exposure resulted in a decrease in the overall percentage of particles moving in the anterograde (but not retrograde) direction (Fig 7B and C respectively), and an increase in the overall percentage of stationary particles (Fig 7D). Subsequent experiments were also conducted to determine whether the deficits in transport associated with DFP at 1 hr of exposure occurred after a longer exposure period (24 hr). Again, the same general trend of effects were observed, i.e., concentration dependent decreases in the velocity of anterograde and retrograde transport of MBOs (Fig 8A), a decrease in the overall percentage of particles moving in the anterograde (but not retrograde) direction (Fig 8B and C respectively), and an increase in the overall percentage of stationary particles (Fig 8D).

### ***Muscarinic and nicotinic antagonists do not affect DFP-related impairments in axonal transport***

Additional experiments were also conducted (at the 24 hr exposure period) to determine if either the muscarinic acetylcholine receptor antagonist atropine or the nicotinic acetylcholine receptor antagonist mecamylamine would affect the DFP-related impairments of axonal transport. The results of these experiments are illustrated in Fig 9 and they clearly show that the DFP-related deficits in anterograde and retrograde axonal transport persisted in the presence of either atropine or mecamylamine (Fig 9A). Likewise, neither atropine nor mecamylamine

changed the DFP-related effects on the number of moving (Fig 9B and C) or stationary particles (Fig 9D). Moreover, neither atropine nor mecamylamine (when administered alone) affected anterograde or retrograde axonal transport (Fig 9A) or the number of moving or stationary particles (Fig 9B-D).

### ***Effects of DFP on AChE Activity***

The effects of DFP on AChE activity in vitro are shown in Fig 10. In Fig 10A, the effects of one hr of exposure to DFP on neuronal cell lysates across the range of concentrations evaluated in the axonal transport studies indicate that the  $IC_{50}$  was approximately 90 nM and that the threshold for inhibition was likely to be somewhere just below 10 nM. In Fig 10B we evaluated a concentration of DFP that was close to the  $IC_{50}$  (100 nM) across a time course that ranged from 1-24 hrs. While there was some variation in activity, the stability of DFP (as indicated by its ability to decrease AChE activity) was retained for up to 24 hrs. For comparison purposes, we also evaluated a wide range of concentrations of DFP in a well-established assay in our laboratory using purified eel AChE. Here the  $IC_{50}$  for DFP was considerably higher (~ 1.75  $\mu$ M) when compared to neuronal cell lysates. Likewise, the threshold for inhibition was considerably higher (i.e., between 0.1 and 0.3  $\mu$ M). This difference in the effects of DFP in the two assays may be a function the relatively low levels of AChE in embryonic cortex. For example, Thomas 1985 reported that the percentage of AChE-positive neurons in E15 rat cortex (cells cultured for two weeks) was approximately 2%. As noted above, our cultures were prepared from E17-18 rat cortex and (cells cultured for 5-7 days prior to transfection and subsequent OP studies at day 8-9).

## **Discussion**

The major findings of this study can be summarized as follows: 1) Using a novel in vitro model system and time-lapse imaging techniques, we observed that exposure to the nerve agent DFP resulted in a concentration-dependent decrease in the anterograde and retrograde transport of MBOs in cortical axons. 2) The neuronal changes occurred at concentrations of DFP that did not inhibit AChE activity and were below the threshold for neurotoxicity (as would be suggested by compromised cell viability or evidence of cytoskeletal damage), and they were not blocked by cholinergic receptor antagonists. The later observation further supports the argument that the DFP effects on axonal transport are not directly related to AChE inhibition and consequent elevations in synaptic levels of acetylcholine. Moreover, DFP was found to be stable in the buffers used in the culture conditions as indicated in the NMR studies as well as the AChE activity assays where a DFP concentration close to the  $IC_{50}$  retained its inhibitory activity up to 24 hrs.

The new method used in this study to measure axonal transport employs time-lapse microscopy techniques to measure the trafficking of MBOs containing a transfected fluorophore-tagged amyloid precursor protein (APP) cDNA construct (Magrane et al., 2012). This particular cDNA construct, referred to as APPDendra2, is derived from the APP695 isoform which has several desirable properties for in vitro studies such as those described in this report. For example, it is known to be expressed in the human brain, it is preferentially expressed in neurons (as opposed to astrocytes) (Rohan de Silva et al., 1997), and, moreover, it is highly expressed in cholinergic forebrain neurons (Harkany et al., 2002). In addition, APP is well documented to travel in neurons by fast axonal transport (Koo et al., 1990; Sisodia et al., 1993) in a process that is dependent on conventional kinesin (Amaratunga et al., 1993; Ferreira et al., 1993; Buxbaum et al., 1998). To visualize the transport of APP in neuronal axons in culture, we transfected rat

cortical neurons with a plasmid encoding APP695 tagged with Dendra2. Dendra2 provides a unique combination of advantageous properties including a monomeric state suitable for protein labeling and efficient chromophore maturation at 37°C in mammalian cells. These properties make Dendra2 an ideal tool for tracking the movements of proteins labeled with Dendra2 in real time.

The velocity of APP movements in cortical axons observed in this study (under control conditions for the 1 hr drug exposure experiments) was similar to that described by Magrane et al 2012 for motor neurons (0.5-0.7µm/sec in anterograde and 0.4-0.5µm/sec in retrograde direction). In the current study and the previous Magrane study, axonal transport assessments were made 24h after transfection with lipofectamine 2000 (Magrane et al., 2012). Compared to the 24h transfection, however, the APP particle velocity was decreased by about 20% after the 48h transfection (~0.4µm/sec) a time period necessary to accommodate our 24 drug exposure experiments.

The initial experiments with COL were conducted in order to confirm our ability to use this tracking method for detecting fast axonal transport deficits associated with drug exposure. COL 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). As described in the Results, the use of colchicine as a positive control was validated. Specifically, exposure to COL for 1 or 24 hr was associated with a concentration-dependent impairment in the velocity of APPDendra2-labeled MBOs both in the anterograde and retrograde direction and these effects were accompanied by an increase in the percentage of stationary MBOs. While there was little evidence that COL exposure for 1 hr was associated

with compromises in cell viability, morphology or cytoskeletal integrity, there was some evidence of compromised cell viability after 24 hr. The results of the DFP experiments followed a similar pattern as that observed with COL across all of the experiments, except that DFP was not as toxic to the neurons.

While the molecular mechanisms underlying the COL-related impairments in axonal transport are assumed to involve impairments in tubulin polymerization and consequent microtubule disruption (as described above), the mechanism of the DFP effects are unclear. However (like COL), some OPs have been observed to impair tubulin polymerization in previous studies. For example utilizing a spectrophotometric method, Prendergast et al., 2007 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. Moreover, 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).

An alternative (or perhaps complementary) hypothesis is that OPs like DFP might (in some manner) alter the function of motor proteins such as kinesin and dynein to impair axonal transport (Terry, 2012). 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 DFP (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). Our observation that retrograde (as well as anterograde) axonal transport was impaired by DFP suggested that the retrograde motor protein dynein might also be (in some manner) affected by OPs. To our knowledge no studies have (to date) addressed this possibility.

While it is difficult to make causal connections between these observations using 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. Interestingly, among the potential contributing factors to gulf war illness (GWI), which is characterized by multiple neurological and neurobehavioral symptoms, exposures to OP-based insecticides and nerve agent-OPs (following the destruction of an Iraqi munitions storage complex at Khamisiyah, Iraq, in March 1991) have been implicated (RAC, 2014). 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 (Hancock et al., 2008; Hayden et al., 2010; Zaganas et al., 2013).

Potential effects of OPs on molecules that are directly involved in axonal transport (e.g., kinesin, tubulin) may be added to the growing list of non-cholinesterase targets for OPs which now includes a variety of esterase and non-esterase enzymes, neurotransmitter receptors, and elements of cell signaling pathways including carboxylesterase, acylpeptide hydrolase, adenylyl cyclase, neuropathy target esterase, muscarinic receptors, cannabinoid receptors, albumen, transferrin, and ATP Synthase (Duysen et al., 2001; Casida and Quistad, 2005; LoPachin and DeCaprio, 2005; Terry, 2012). Importantly, it has been suggested that interactions of OPs with such non-cholinesterase targets may contribute to the more delayed and persistent effects observed following chronic exposure to OPs (Lotti and Moretto, 2005; Costa, 2006).

In conclusion, the results of this *in vitro* study suggest that one underlying mechanism of the wide variety OP-based neurological deficits that have been reported might involve alterations in axonal transport. Future studies designed to determine the molecular basis of this effect of OPs may lead to the development of therapeutic strategies for the neurological deficits associated with OP exposure.

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## Figure Legends

**Fig 1. Methods for assessing the effects of diisopropylfluorophosphate (DFP) on axonal transport in vitro.** (A) Representative image demonstrating successful transfection of APPDendra2 in rat primary cortical neurons. Green fluorescent membrane bound organelles (MBOs) in the axon are indicated by the arrows. (B) Kymograph recorded at 5 sec intervals for 5 min generated from APPDendra2-labeled MBOs after treatment with DFP. MBOs are categorized in one of three ways: anterograde (A), retrograde (R) or stationary (S). Velocity information was obtained from the slope of the lines. Scale bar = 20  $\mu\text{m}$ .

**Fig 2. One hour of exposure to colchicine (COL), but not diisopropylfluorophosphate (DFP) is associated with concentration-dependent impairments in cell viability.** Following an acute (1 hour) exposure to COL (A,C) or DFP (B,D), cell viability was measured by both MTT colorimetric and lactate dehydrogenase (LDH) release assays, presented as the percentage of cell survival or release, respectively, compared to vehicle-treated controls. Each bar represents the mean  $\pm$  S.E.M from 2-3 independent experiments. “\*”,  $p < 0.05$  compared to control. Representative immunocytochemistry images (pseudo-colored for contrast and to facilitate identifying stains in the figure and indicated structures) comparing the cellular morphology and structure after acute 10 $\mu\text{M}$  COL or DFP exposure. (E) fluorophore-conjugated actin (Acti-stain 555, red) and nuclei (blue), (F) anti-doublecortin (DCX, axon, blue) and nuclei (yellow), (G) anti-microtubule associated protein 2 (MAP2, somato-dendritic, green) and nuclei (red). Scale Bar = 10  $\mu\text{m}$ .

**Fig 3. Twenty four hours of exposure to colchicine (COL) and diisopropylfluorophosphate (DFP) are associated with concentration-dependent impairments in cell viability.** Following 24 hours of exposure to COL (A,C) or DFP (B,D), cell viability was measured by both MTT colorimetric and LDH release assays, presented as the percentage of cell survival or release, respectively, compared to vehicle-treated controls. Each bar represents the mean  $\pm$  S.E.M from 2-3 independent experiments. “\*”,  $p < 0.05$  compared to control. Representative immunocytochemistry images (pseudo-colored) for contrast and to facilitate identifying stains in the figure and indicated structures) comparing the cellular morphology and structure after 24 hours exposure to 10 $\mu$ M COL or DFP exposure. (E) fluorophore-conjugated actin (Acti-stain 555, red) and nuclei (blue), (F) anti-doublecortin (DCX, axon, blue) and nuclei (yellow), (G) anti-microtubule associated protein 2 (MAP2, somato-dendritic, green) and nuclei (red). Scale Bar = 10  $\mu$ m.

**Fig 4. Diisopropylfluorophosphate (DFP) stability studies.** (A) 800 MHz  $^1\text{H}$ -NMR of DFP. (B)  $^1\text{H}$ -NMR spectra of DFP in deionized water ( $\text{D}_2\text{O}$ ) recorded over a period of 3 days. (C)  $^1\text{H}$ -NMR spectra of DFP recorded in PBS (pH 7.4) over a period of 20 hrs. A subsequently recorded spectrum at 14 days showed full hydrolysis.

**Fig 5. Colchicine impairs axonal transport in primary cortical neurons after 1hr of exposure.** (A) Velocities of APP containing membrane bound organelles (MBOs) in the anterograde and retrograde direction.  $n$  (moving events) = 116 . (B-D): Percentage of mobile (anterograde or retrograde) and stationary APP particles in segments of control and colchicine-

treated axons. n (axonal segments)=38. Each bar represents the mean  $\pm$  s.e.m from 4 independent experiments and 4-5 replicates per concentration.

**Fig 6. Colchicine impairs axonal transport in primary cortical neurons after 24hr of exposure.** (A) Velocities of APP containing MBOs in the anterograde and retrograde direction. n (moving events) = 91. (B-D): Percentage of mobile (anterograde or retrograde) and stationary APP particles in segments of control and colchicine-treated axons. n (axonal segments)=33. Each bar represents the mean  $\pm$  s.e.m from 3 independent experiments and 4-5 replicates per concentration.

**Fig 7. Diisopropylfluorophosphate (DFP) impairs axonal transport in primary cortical neurons after 1hr of exposure.** (A) Velocities of APP containing membrane bound organelles (MBOs) in the anterograde and retrograde direction. n(moving events)=235. (B-D) Percentage of mobile (anterograde or retrograde) and stationary APP particles in segments of control and colchicine-treated axons. n (axonal segments)=52. Each bar represents the mean  $\pm$  s.e.m from 4 independent experiments and 4-5 replicates per concentration.

**Fig 8. Diisopropylfluorophosphate (DFP) impairs axonal transport in primary cortical neurons after 24hr of exposure.** (A) Velocities of APP containing membrane bound organelles (MBOs) in the anterograde and retrograde direction. n(moving events)=90. (B-D) Percentage of mobile (anterograde or retrograde) and stationary APP particles in segments of control and colchicine-treated axons. n (axonal segments)=36. Each bar represents the mean  $\pm$  s.e.m from 3 independent experiments and 4-5 replicates per concentration.

**Fig 9. Muscarinic and nicotinic antagonists do not affect diisopropylfluorophosphate (DFP)-related impairments in axonal transport in primary cortical neurons after 24hr of exposure.** (A) Velocities of APP containing membrane bound organelles (MBOs) in the anterograde and retrograde direction. n (moving events)=90. (B-D): Percentage of mobile (anterograde or retrograde) and stationary APP particles in segments of control and colchicine-treated axons. n (axonal segments) = 36. Each bar represents the mean  $\pm$  s.e.m from 2 independent experiments and 4-5 replicates per concentration. ATR = atropine, MEC = mecamlamine

**Fig 10. Effects of Diisopropylfluorophosphate (DFP) on acetylcholinesterase (AChE) activity in vitro.** (A) The effects of one hr of exposure to DFP on AChE activity in neuronal cell lysates across the range of concentrations evaluated in the axonal transport studies. (B) Effects of DFP (100 nM) on AChE activity in neuronal cell lysates across a time course that ranged from 1-24 hrs. (C) Effects of DFP across a wide range of concentrations on purified eel AChE. Each symbol represents the mean  $\pm$  s.e.m from 2 independent experiments and 4 replicates per concentration.

**Supplemental Movie File #1:** Representative movie indicating the axonal transport of APP containing membrane bound organelles (MBOs) in rat cortical neurons after exposure to vehicle (culture media with deionized water 5.0% (v/v)) for 1 hour.

**Supplemental Movie File #2:** Representative movie indicating the axonal transport of APP containing membrane bound organelles (MBOs) in rat cortical neurons after exposure to COL 10 nM for 1 hour.

**Supplemental Movie File #3:** Representative movie indicating the axonal transport of APP containing membrane bound organelles (MBOs) in rat cortical neurons after exposure to DFP 10 nM for 1 hour.

## References

- Amaratunga A, Morin PJ, Kosik KS and Fine RE (1993) Inhibition of kinesin synthesis and rapid anterograde axonal transport in vivo by an antisense oligonucleotide. *The Journal of biological chemistry* **268**:17427-17430.
- Banks CN and Lein PJ (2012) A review of experimental evidence linking neurotoxic organophosphorus compounds and inflammation. *Neurotoxicology* **33**:575-584.
- Brown MA and Brix KA (1998) Review of health consequences from high-, intermediate- and low-level exposure to organophosphorus nerve agents. *Journal of applied toxicology : JAT* **18**:393-408.
- Buxbaum JD, Thinakaran G, Koliatsos V, O'Callahan J, Slunt HH, Price DL and Sisodia SS (1998) Alzheimer amyloid protein precursor in the rat hippocampus: transport and processing through the perforant path. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**:9629-9637.
- Casida JE and Quistad GB (2005) Serine hydrolase targets of organophosphorus toxicants. *Chemico-biological interactions* **157**:277-283.
- Costa L (2006) Current issues in organophosphate toxicology Clin Chim Acta 366: 1–13. *Find this article online.*
- Duncan JE and Goldstein LS (2006) The genetics of axonal transport and axonal transport disorders. *PLoS Genet* **2**:e124.
- Duysen EG, Li B, Xie W, Schopfer LM, Anderson RS, Broomfield CA and Lockridge O (2001) Evidence for nonacetylcholinesterase targets of organophosphorus nerve agent: supersensitivity of acetylcholinesterase knockout mouse to VX lethality. *The Journal of pharmacology and experimental therapeutics* **299**:528-535.
- Ecobichon DJ (2001) Pesticide use in developing countries. *Toxicology* **160**:27-33.
- Eddleston M, Buckley NA, Eyer P and Dawson AH (2008) Management of acute organophosphorus pesticide poisoning. *Lancet* **371**:597-607.
- Ferreira A, Caceres A and Kosik KS (1993) Intraneuronal compartments of the amyloid precursor protein. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **13**:3112-3123.
- Gao J, Adam B-L and Terry AV (2014) Evaluation of nicotine and cotinine analogs as potential neuroprotective agents for Alzheimer's disease. *Bioorganic & medicinal chemistry letters* **24**:1472-1478.
- Gearhart DA, Sickles DW, Buccafusco JJ, Prendergast MA and Terry AV, Jr. (2007) Chlorpyrifos, chlorpyrifos-oxon, and diisopropylfluorophosphate inhibit kinesin-dependent microtubule motility. *Toxicol Appl Pharmacol* **218**:20-29.
- Grigoryan H, Li B, Anderson EK, Xue W, Nachon F, Lockridge O and Schopfer LM (2009) Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine. *Chemico-biological interactions* **180**:492-498.
- Grigoryan H and Lockridge O (2009) Nanoimages show disruption of tubulin polymerization by chlorpyrifos oxon: implications for neurotoxicity. *Toxicology and applied pharmacology* **240**:143-148.
- Han Y, Malak H, Chaudhary A, Chordia M, Kingston DG and Bane S (1998) Distances between the paclitaxel, colchicine, and exchangeable GTP binding sites on tubulin. *Biochemistry* **37**:6636-6644.

- Hancock DB, Martin ER, Mayhew GM, Stajich JM, Jewett R, Stacy MA, Scott BL, Vance JM and Scott WK (2008) Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. *BMC neurology* **8**:6.
- Harkany T, Varga C, Grosche J, Mulder J, Luiten PG, Hortobagyi T, Penke B and Hartig W (2002) Distinct subsets of nucleus basalis neurons exhibit similar sensitivity to excitotoxicity. *Neuroreport* **13**:767-772.
- Hastie SB (1991) Interactions of colchicine with tubulin. *Pharmacology & therapeutics* **51**:377-401.
- Hayden KM, Norton MC, Darcey D, Ostbye T, Zandi PP, Breitner JC and Welsh-Bohmer KA (2010) Occupational exposure to pesticides increases the risk of incident AD: the Cache County study. *Neurology* **74**:1524-1530.
- Hernandez CM, Beck WD, Naughton SX, Poddar I, Adam BL, Yanasak N, Middleton C and Terry AV, Jr. (2015) Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain. *Neurotoxicology* **47**:17-26.
- Hobbiger F (1972) Chemotherapy in pesticide poisoning. *Toxicology, Biodegradation and Efficacy of Livestock Pesticides*.
- Jiang W, Duysen EG, Hansen H, Shlyakhtenko L, Schopfer LM and Lockridge O (2010) Mice treated with chlorpyrifos or chlorpyrifos oxon have organophosphorylated tubulin in the brain and disrupted microtubule structures, suggesting a role for tubulin in neurotoxicity associated with exposure to organophosphorus agents. *Toxicological sciences : an official journal of the Society of Toxicology* **115**:183-193.
- Koo EH, Sisodia SS, Archer DR, Martin LJ, Weidemann A, Beyreuther K, Fischer P, Masters CL and Price DL (1990) Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. *Proc Natl Acad Sci U S A* **87**:1561-1565.
- LoPachin RM and DeCaprio AP (2005) Protein adduct formation as a molecular mechanism in neurotoxicity. *Toxicological Sciences* **86**:214-225.
- Lotti M and Moretto A (2005) Organophosphate-induced delayed polyneuropathy. *Toxicological reviews* **24**:37-49.
- Macilwain C (1993) Study proves Iraq used nerve gas. *Nature* **363**:3.
- Magrane J, Sahawneh MA, Przedborski S, Estevez AG and Manfredi G (2012) Mitochondrial dynamics and bioenergetic dysfunction is associated with synaptic alterations in mutant SOD1 motor neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**:229-242.
- Middlemore-Risher ML, Adam BL, Lambert NA and Terry AV, Jr. (2011) Effects of chlorpyrifos and chlorpyrifos-oxon on the dynamics and movement of mitochondria in rat cortical neurons. *The Journal of pharmacology and experimental therapeutics* **339**:341-349.
- Miyaki K, Nishiwaki Y, Maekawa K, Ogawa Y, Asukai N, Yoshimura K, Etoh N, Matsumoto Y, Kikuchi Y, Kumagai N and Omae K (2005) Effects of sarin on the nervous system of subway workers seven years after the Tokyo subway sarin attack. *Journal of occupational health* **47**:299-304.
- Nagao M, Takatori T, Matsuda Y, Nakajima M, Iwase H and Iwadata K (1997) Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. *Toxicology and applied pharmacology* **144**:198-203.
- National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals., Institute for Laboratory Animal Research (U.S.) and National

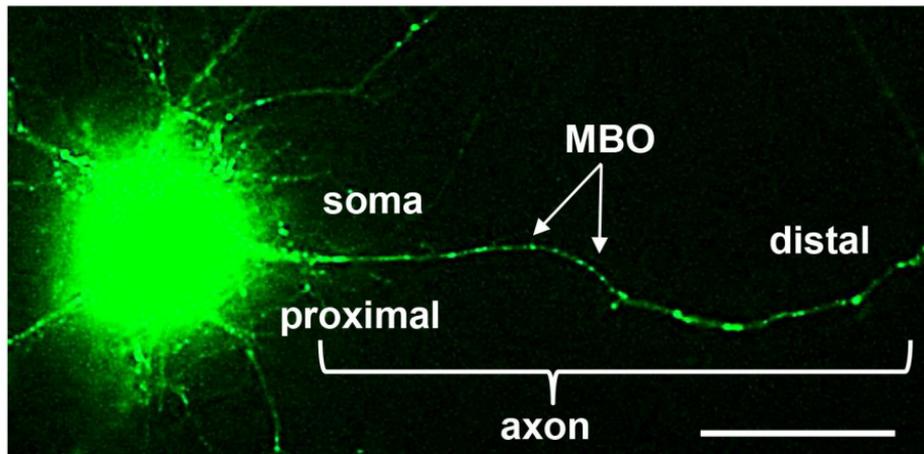
- Academies Press (U.S.) (2011) *Guide for the care and use of laboratory animals*. National Academies Press, Washington, D.C.
- Pereira EF, Aracava Y, DeTolla LJ, Jr., Beecham EJ, Basinger GW, Jr., Wakayama EJ and Albuquerque EX (2014) Animal models that best reproduce the clinical manifestations of human intoxication with organophosphorus compounds. *The Journal of pharmacology and experimental therapeutics* **350**:313-321.
- Pope C, Karanth S and Liu J (2005) Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. *Environmental Toxicology and Pharmacology* **19**:433-446.
- Prendergast MA, Self RL, Smith KJ, Ghayoumi L, Mullins MM, Butler TR, Buccafusco JJ, Gearhart DA and Terry AV, Jr. (2007) Microtubule-associated targets in chlorpyrifos oxon hippocampal neurotoxicity. *Neuroscience* **146**:330-339.
- RAC (2014) Gulf War Illness and the Health of Gulf War Veterans: Research Update and Recommendations, in pp 1-123, U.S. Department of Veterans Affairs, Washington, D.C.
- Reichert BL and Abou-Donia MB (1980) Inhibition of fast axoplasmic transport by delayed neurotoxic organophosphorus esters: a possible mode of action. *Molecular pharmacology* **17**:56-60.
- Richardson RJ (1995) Assessment of the neurotoxic potential of chlorpyrifos relative to other organophosphorus compounds: a critical review of the literature. *Journal of toxicology and environmental health* **44**:135-165.
- Rohan de Silva HA, Jen A, Wickenden C, Jen LS, Wilkinson SL and Patel AJ (1997) Cell-specific expression of beta-amyloid precursor protein isoform mRNAs and proteins in neurons and astrocytes. *Brain research Molecular brain research* **47**:147-156.
- Ross SM, McManus IC, Harrison V and Mason O (2013) Neurobehavioral problems following low-level exposure to organophosphate pesticides: a systematic and meta-analytic review. *Crit Rev Toxicol* **43**:21-44.
- Sellström A, Cairns S and Barbeschi M (2013) United nations mission to investigate allegations of the use of chemical weapons in the Syrian Arab Republic. *Final Report* **12**.
- Sisodia SS, Koo EH, Hoffman PN, Perry G and Price DL (1993) Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. *J Neurosci* **13**:3136-3142.
- Soltaninejad K and Abdollahi M (2009) Current opinion on the science of organophosphate pesticides and toxic stress: a systematic review. *Medical science monitor : international medical journal of experimental and clinical research* **15**:RA75-90.
- Stecher PG (1968) *The Merck index; an encyclopedia of chemicals and drugs*. Merck, Rahway, N.J.,.
- Stokin GB and Goldstein LS (2006) Axonal transport and Alzheimer's disease. *Annu Rev Biochem* **75**:607-627.
- Terry AV, Jr. (2012) Functional consequences of repeated organophosphate exposure: potential non-cholinergic mechanisms. *Pharmacology & therapeutics* **134**:355-365.
- Terry AV, Jr., Gearhart DA, Beck WD, Jr., Truan JN, Middlemore ML, Williamson LN, Bartlett MG, Prendergast MA, Sickles DW and Buccafusco JJ (2007) Chronic, intermittent exposure to chlorpyrifos in rats: protracted effects on axonal transport, neurotrophin receptors, cholinergic markers, and information processing. *J Pharmacol Exp Ther* **322**:1117-1128.

- Terry AV, Jr., Stone JD, Buccafusco JJ, Sickles DW, Sood A and Prendergast MA (2003) Repeated exposures to subthreshold doses of chlorpyrifos in rats: hippocampal damage, impaired axonal transport, and deficits in spatial learning. *J Pharmacol Exp Ther* **305**:375-384.
- Uppuluri S, Knipling L, Sackett DL and Wolff J (1993) Localization of the colchicine-binding site of tubulin. *Proceedings of the National Academy of Sciences* **90**:11598-11602.
- Zaganas I, Kapetanaki S, Mastorodemos V, Kanavouras K, Colosio C, Wilks MF and Tsatsakis AM (2013) Linking pesticide exposure and dementia: what is the evidence? *Toxicology* **307**:3-11.

**Footnotes:**

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## A. APPDendra2 transfected neuron



## B. Sample Kymograph

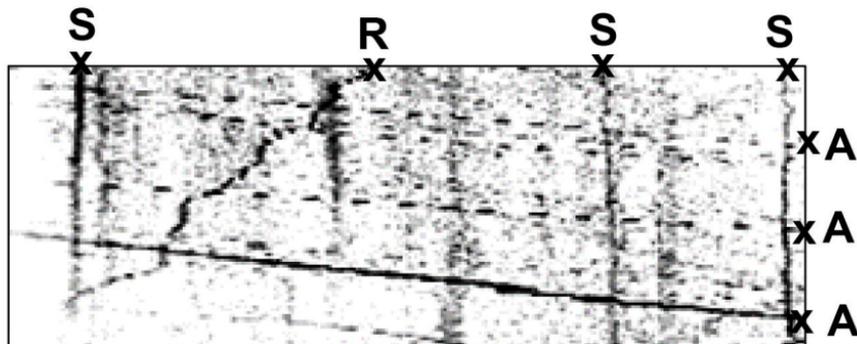
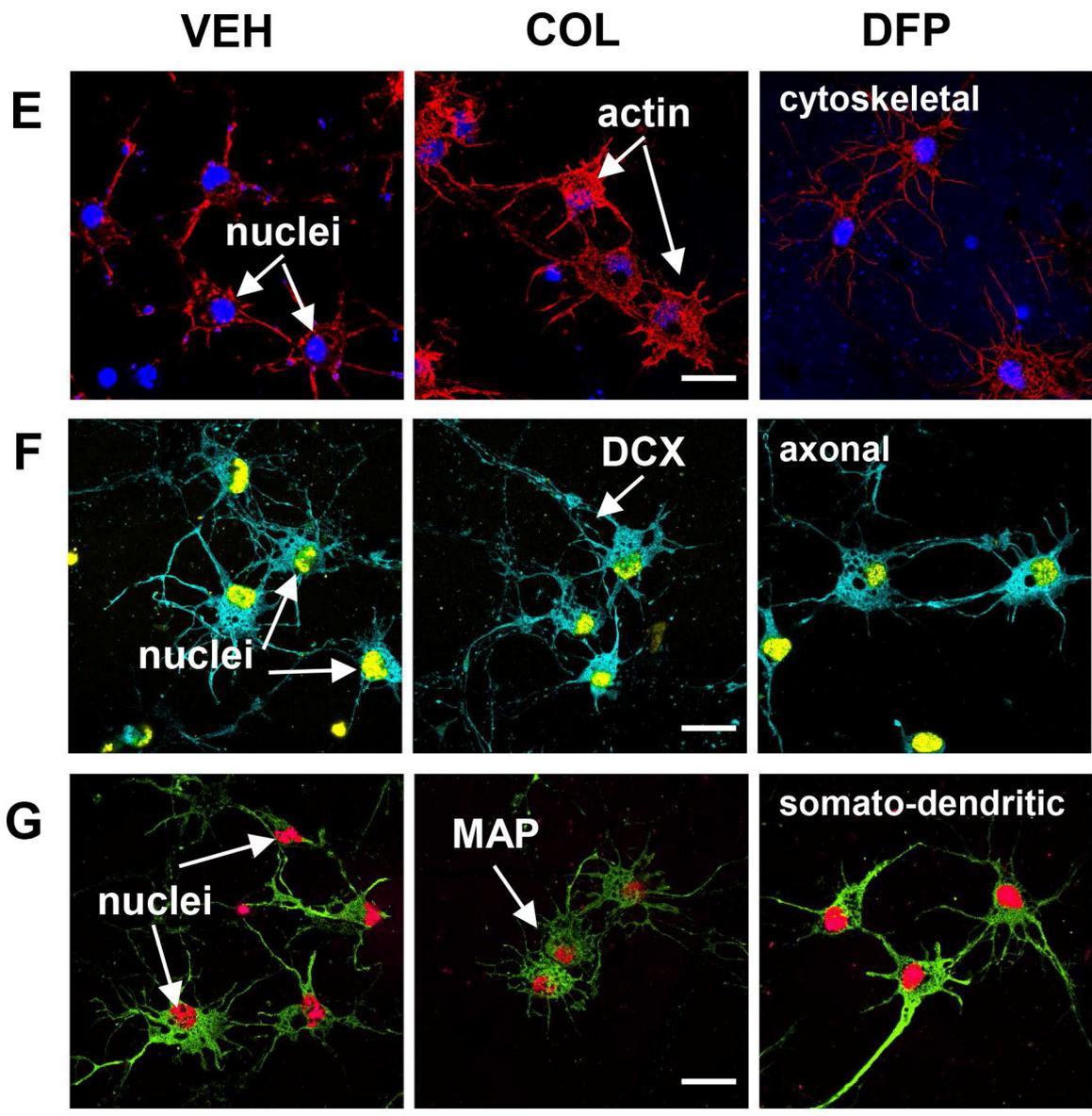
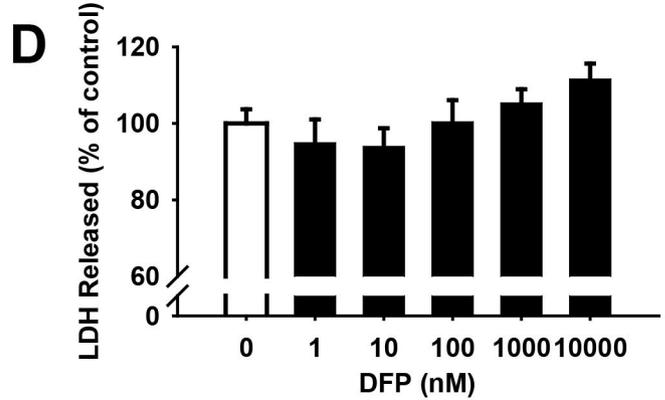
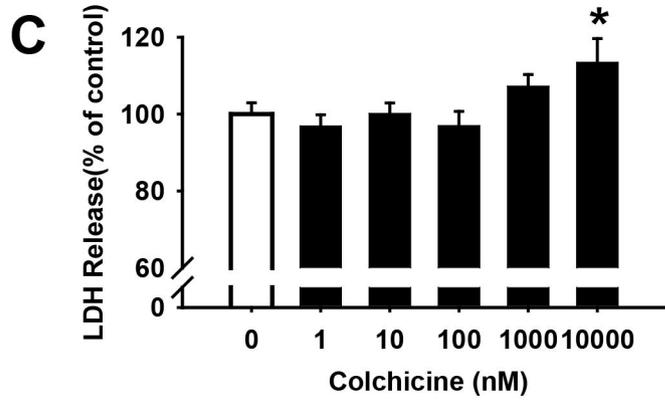
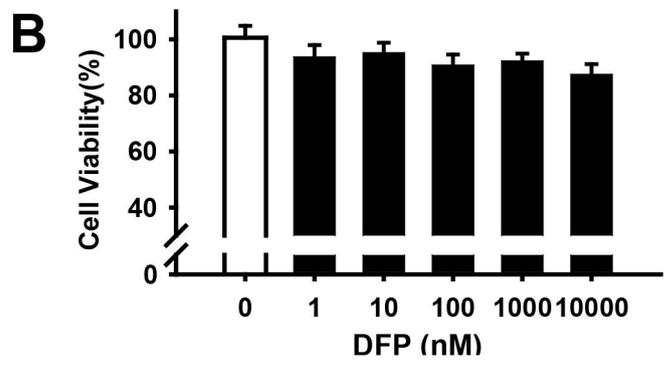
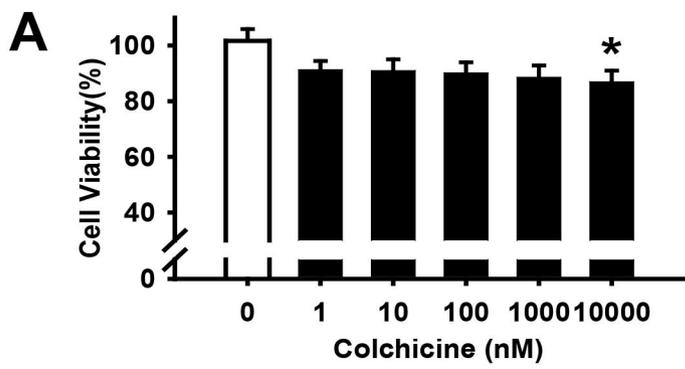
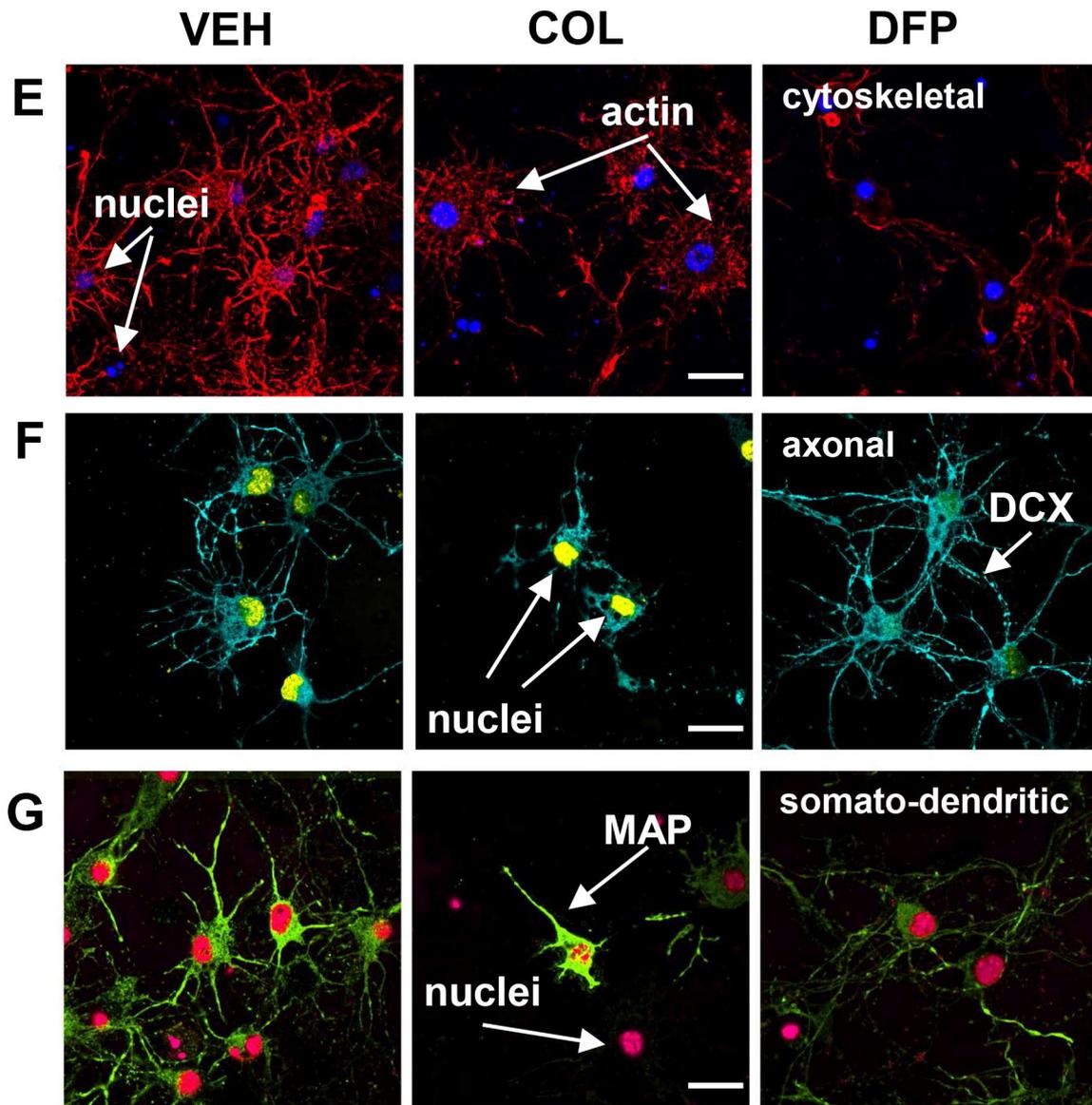
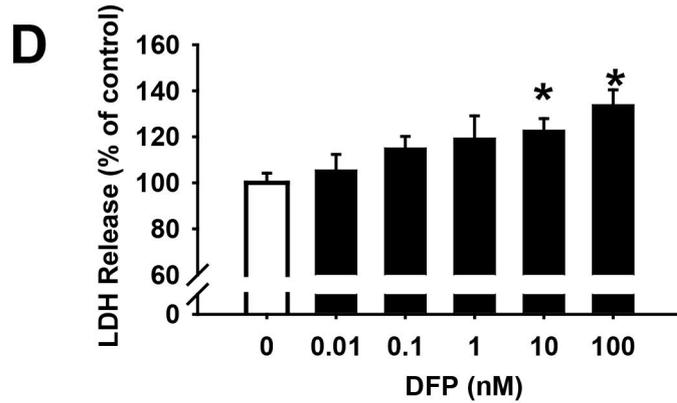
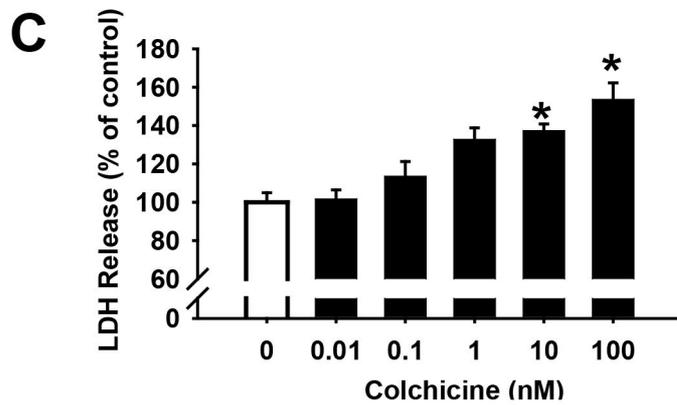
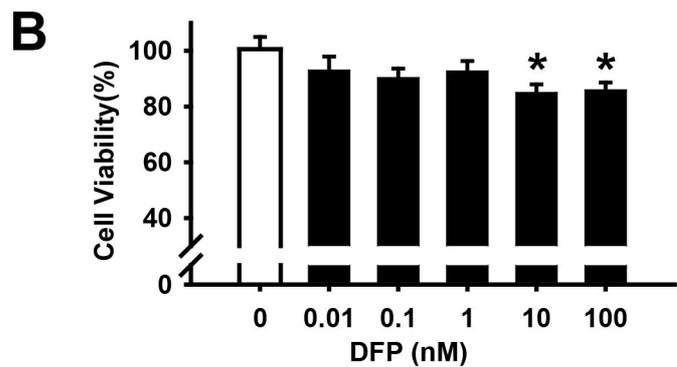
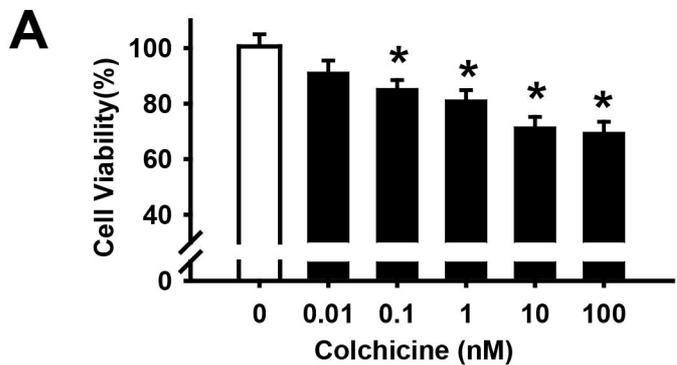
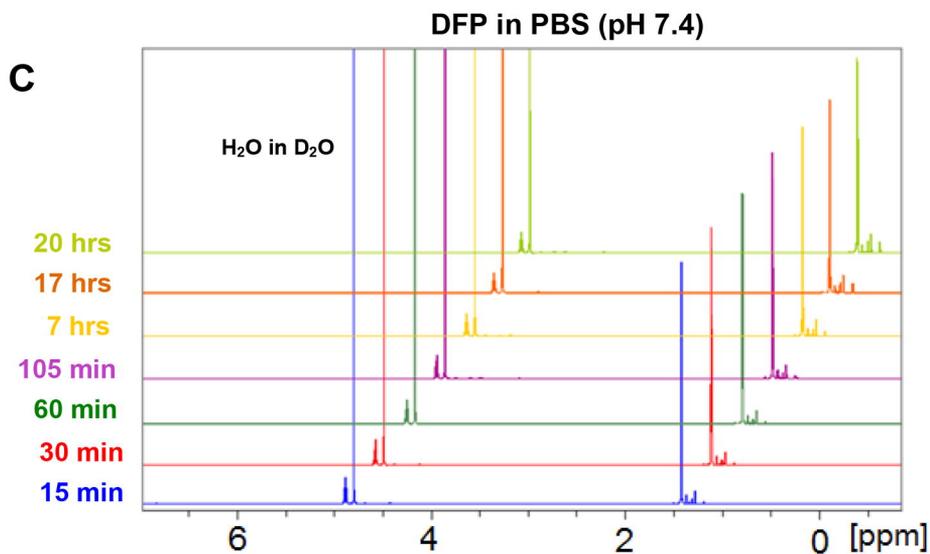
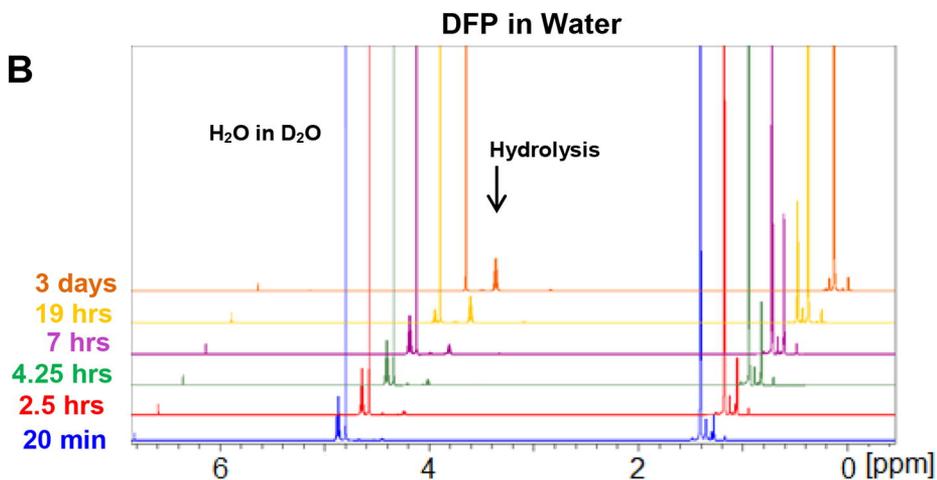
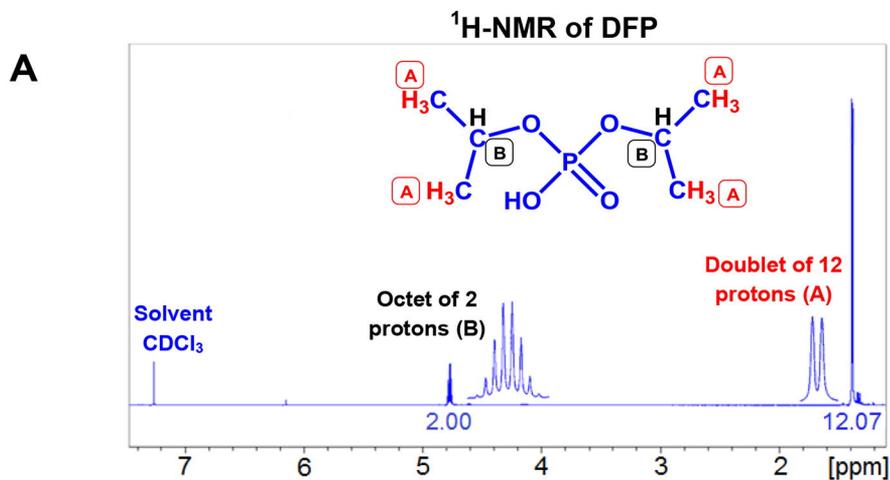


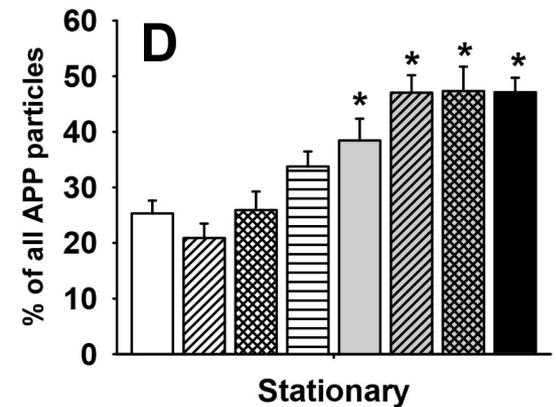
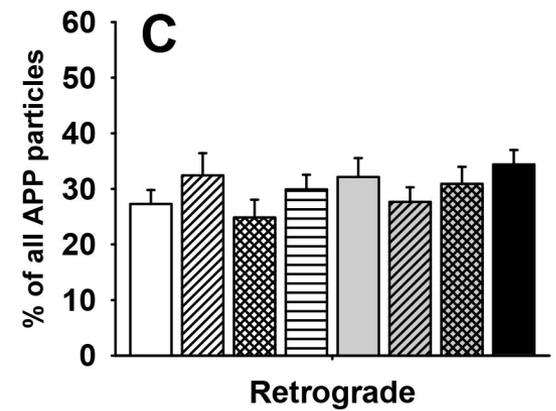
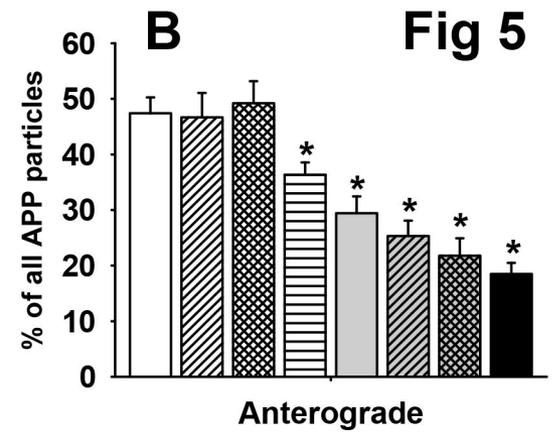
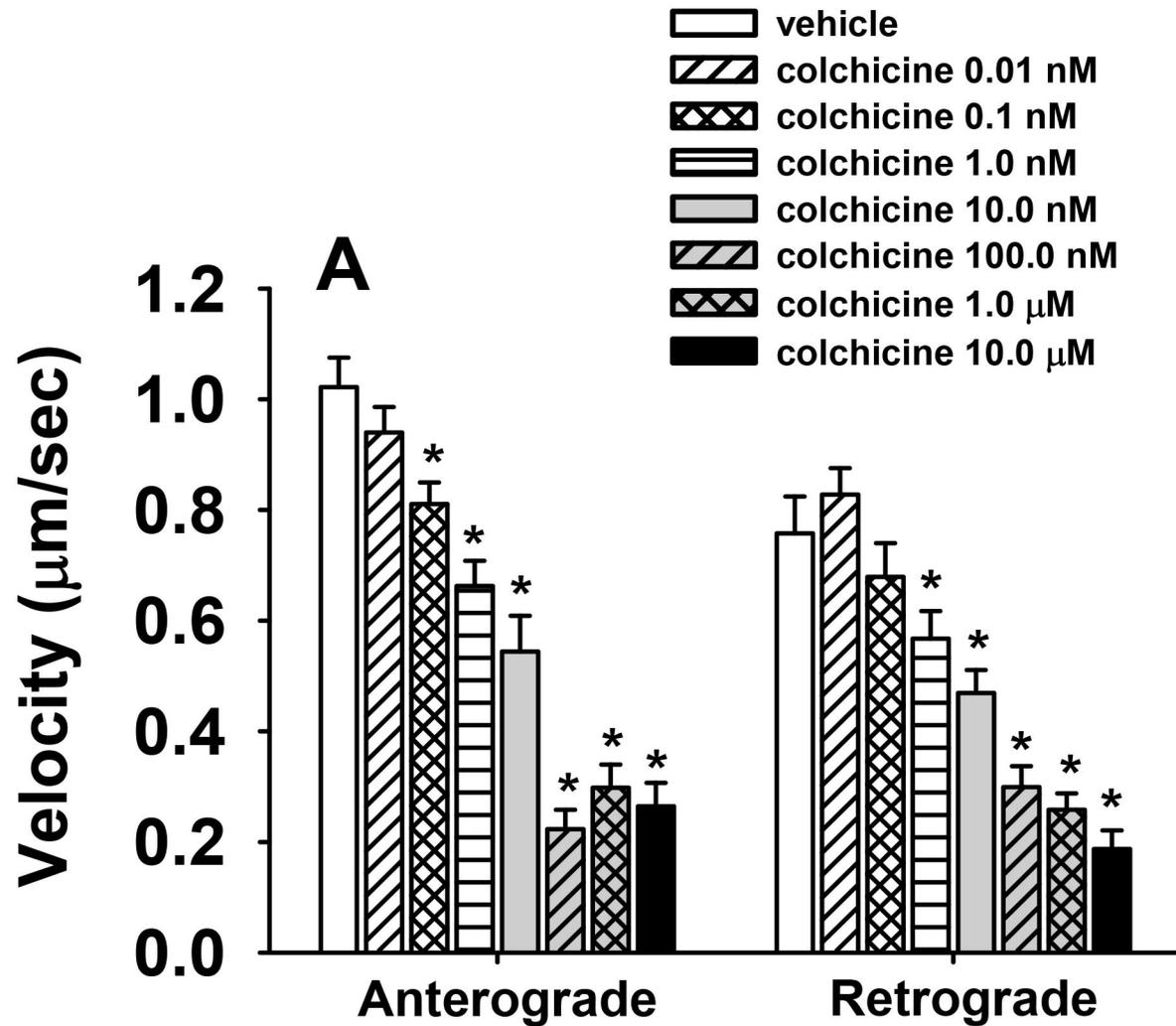
Fig 2

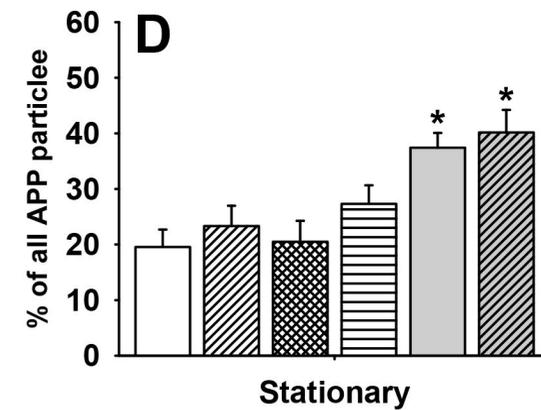
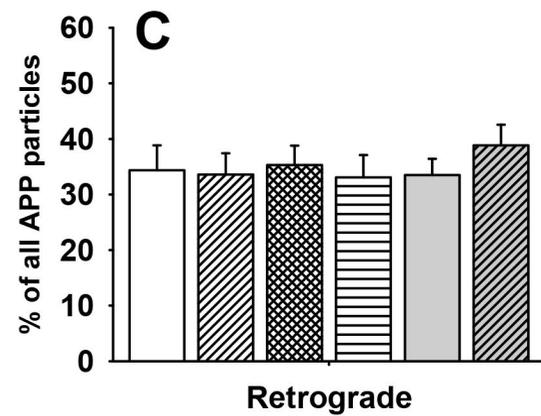
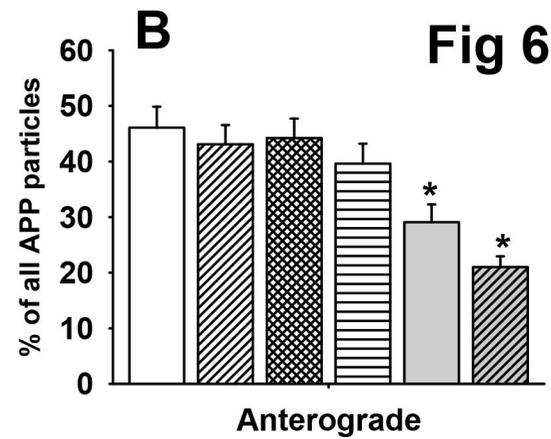
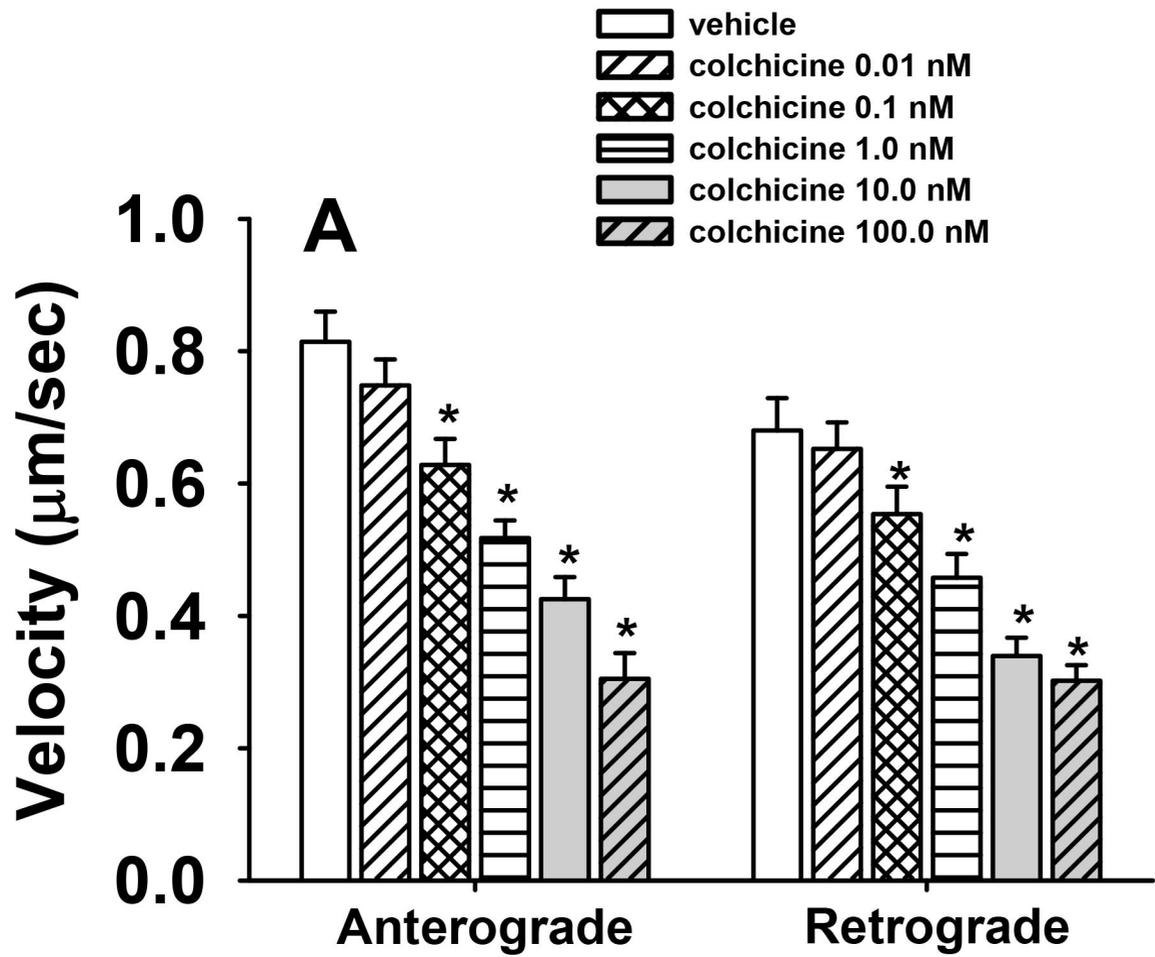


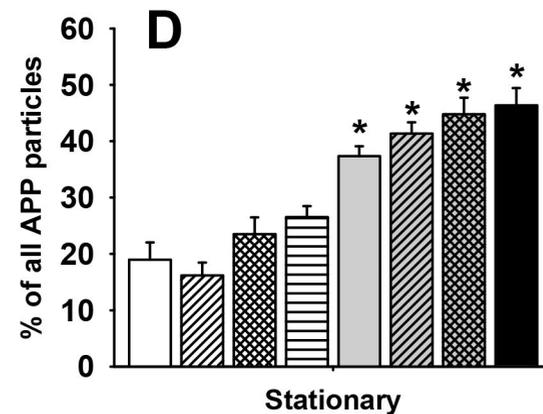
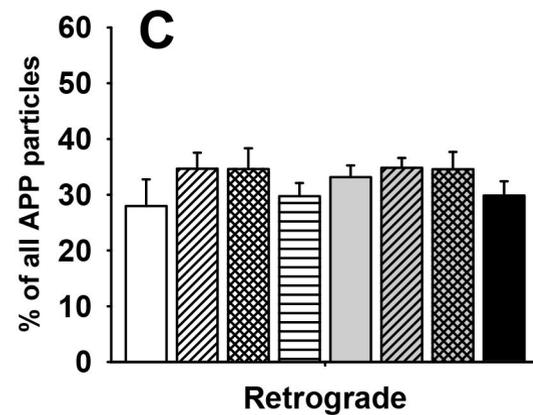
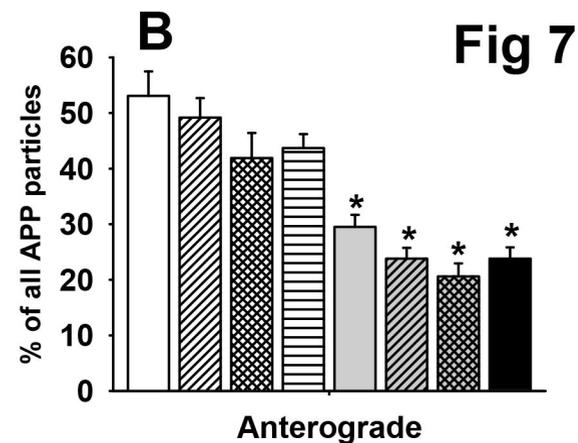
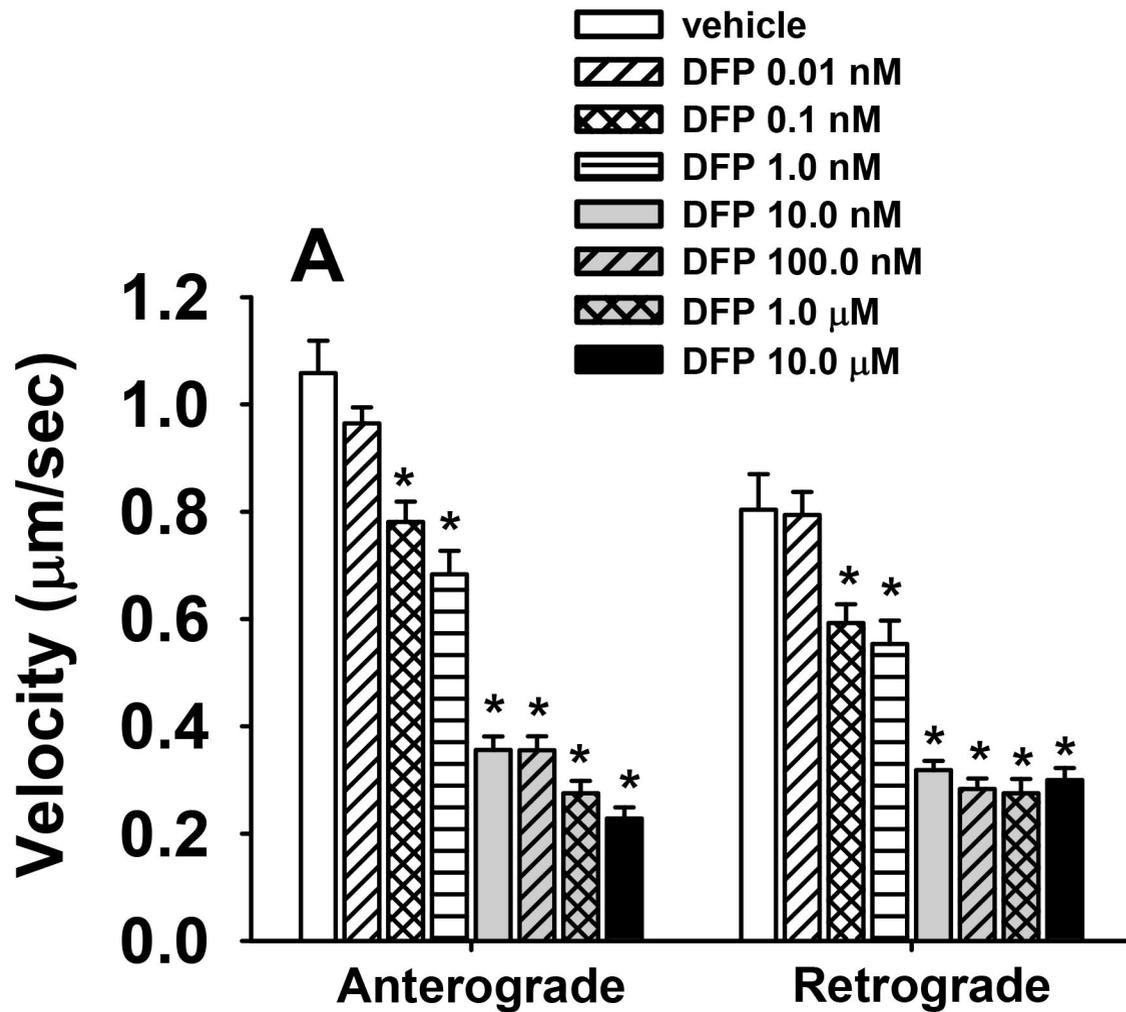
**Fig 3**



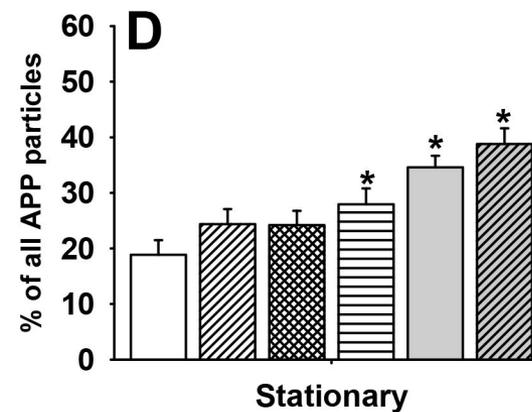
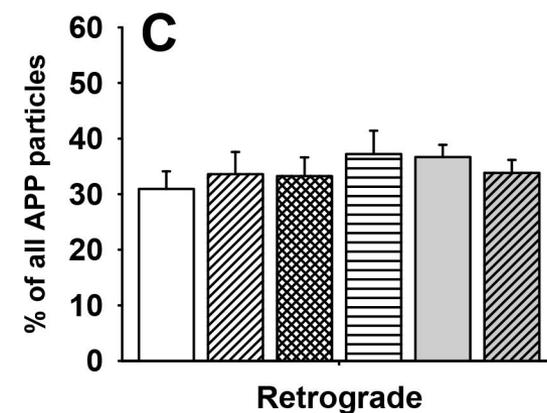
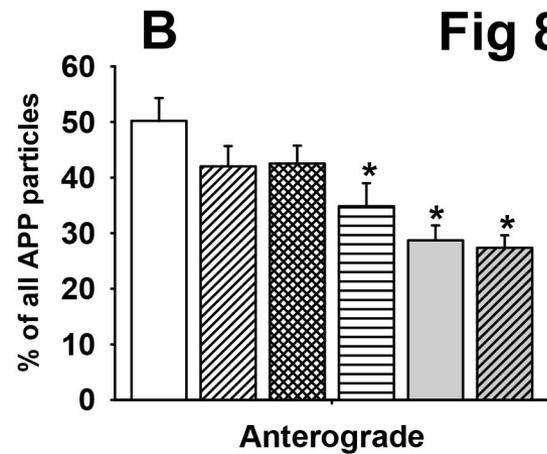
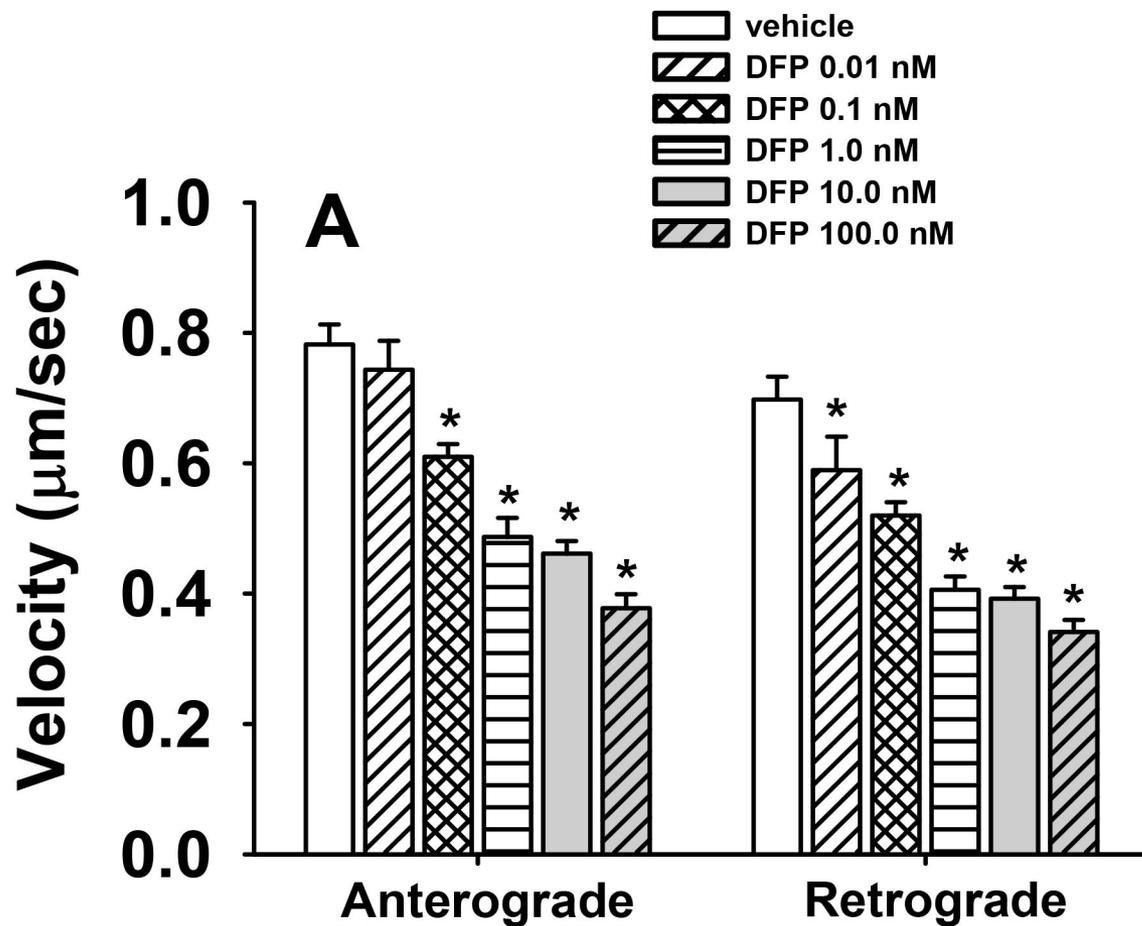








**Fig 8**



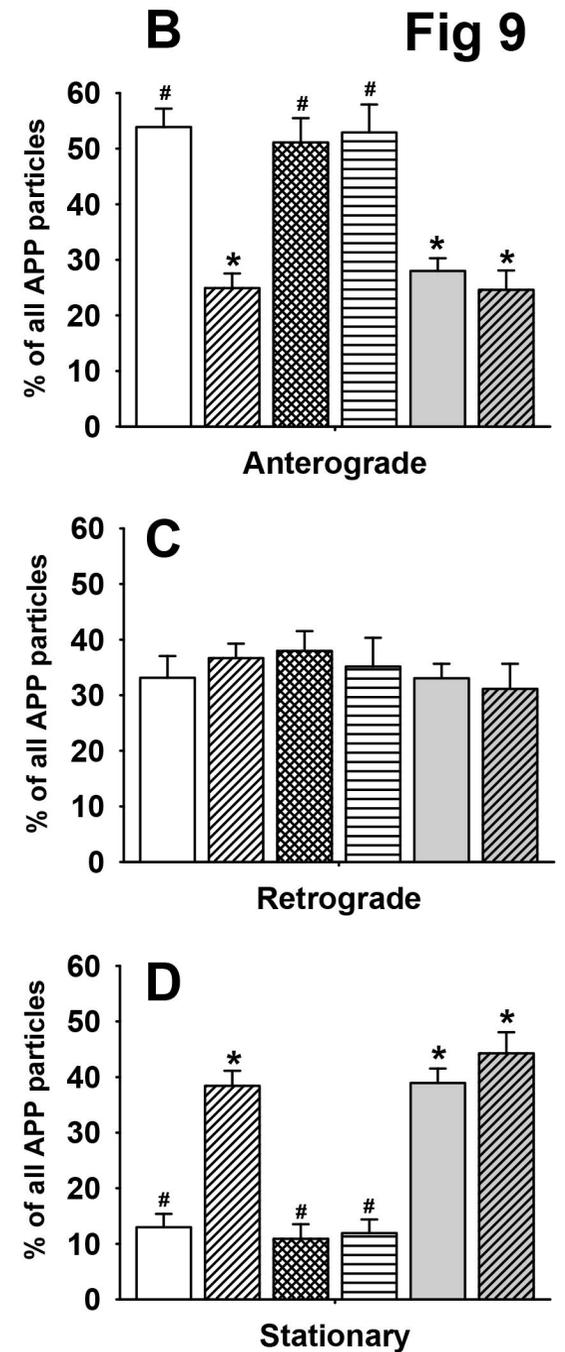
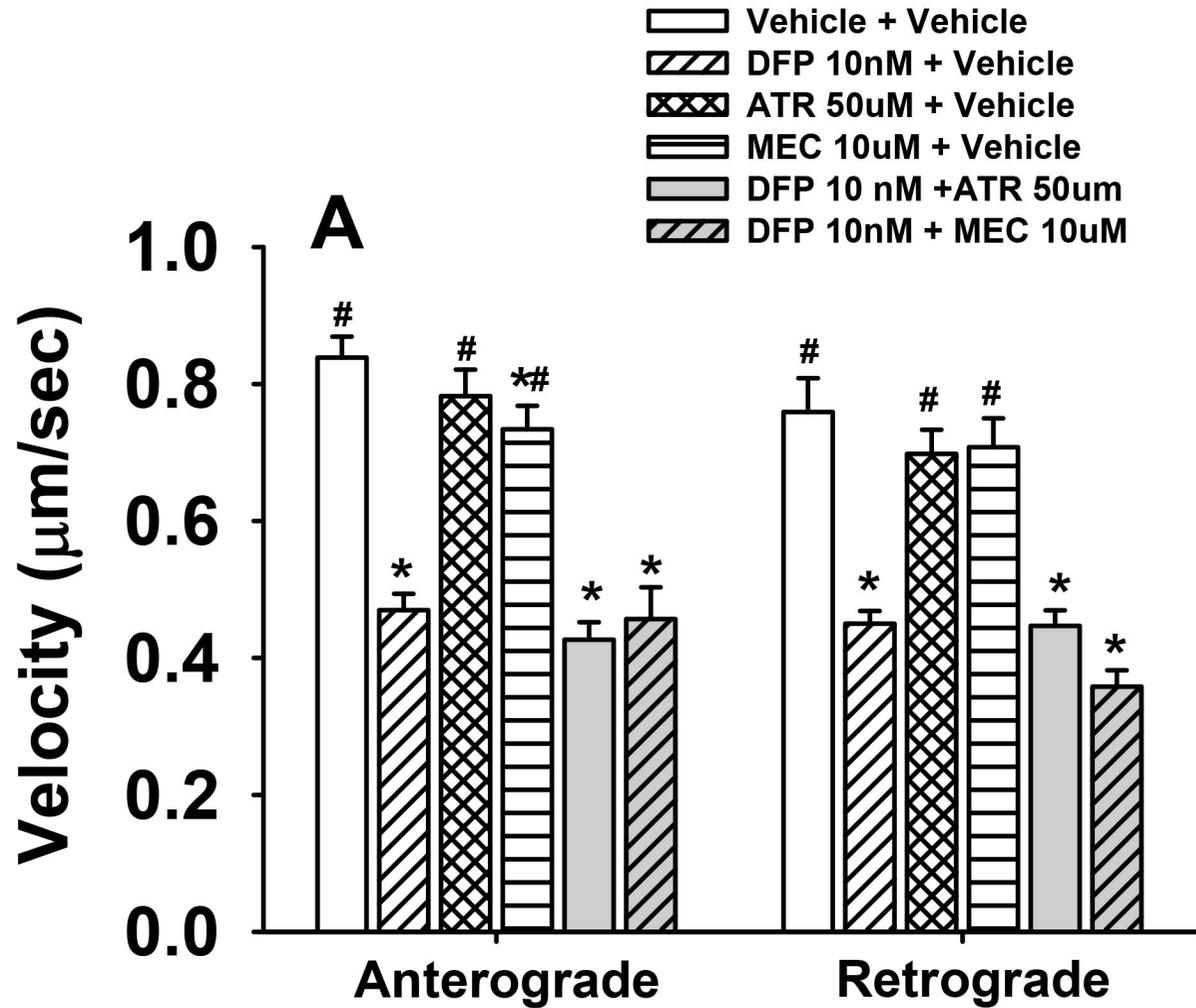


Fig 10

