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Title Page

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

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Running Title Page

Running Title: Diisopropylfluorophosphate impairs axonal transport

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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

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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 cytoskeletal damage. Moreover, the effects of DFP on axonal transport 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.

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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 (e.g., (muscle weakness and fasciculations, vomiting, seizures) 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)).

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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) (14 total exposures), an OP not associated with OPIDN except at doses well above the LD_{50} (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 to14 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

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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.

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Materials and Methods

Drugs

Atropine (ATR), colchicine (COL), mecamylamine (MEC), deuterium oxide (D_2O), 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,

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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 5×10^5 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 1.1×10^6 cell/mL media on 10 cm petri dishes. Cultures were maintained at 37°C in a 5.0% CO_2 humidified atmosphere in Neurobasal™ (#21103) supplemented with B27 (2.0 (v/v) %; #17504), Glutamax™ (0.5M,

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#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^2, 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_2 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

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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 (μm). Individual particle velocities were measured in μm 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 μM) or mecamylamine (10 μM) 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

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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 μM) 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

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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 *x 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

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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 ± 0.05) were as follows (in mM): 0.48 acetylthiocholine, 7000 tetraisopropyl pyrophosphoramide (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)/ (1.36×10^4), then normalized to the intra-experiment vehicle-treated control by exposure time (i.e., 1 or 24 hours).

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 ± 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

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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) (Fig 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 (10uM) 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

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Fig 2E-G) revealed no overt alterations in cellular morphology or structure after one hour of exposure to 10 μM 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 μM COL or DFP.

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 (0.1nM–10μM) that were evaluated (Fig. 4A). 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

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accompanied by a concentration-dependent decrease in the overall percentage of MBOs moving in the anterograde direction (Fig 4B) and an increase in the percentage of stationary MBOs (Fig 4D). Interestingly, COL did not significantly affect the overall percentage of APP particles moving in retrograde direction (Fig 4C). 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 5A), a decrease in the overall percentage of particles moving in the anterograde (but not retrograde) direction (Fig 5B and C respectively), and an increase in the overall percentage of stationary particles (Fig 5D). 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. 6A). 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 6B and C respectively), and an increase in the overall percentage of stationary particles (Fig 6D). Subsequent experiments were also conducted to determine whether the deficits in transport

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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 7A), 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).

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 8 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 8A). Likewise, neither atropine nor mecamylamine changed the DFP-related effects on the number of moving (Fig 8B and C) or stationery particles (Fig 8D). Moreover, neither atropine nor mecamylamine (when administered alone) affected anterograde or retrograde axonal transport (Fig 8A) or the number of moving or stationary particles (Fig 8B-D).

Effects of DFP on AChE Activity

The effects of DFP on AChE activity in vitro are shown in Fig 9. In Fig 9A, 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 9B we evaluated a concentration of DFP that was close to the IC50 (100 nM) across a time course that

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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 (Fig 9C) the IC_{50} for DFP was considerably higher (~ 1.75 uM) when compared to neuronal cell lysates. Likewise, the threshold for inhibition was considerably higher (i.e., between 0.1 and 0.3 uM). 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 1 and 24 hours of 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 comprised 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 nuclear

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magnetic resonance (NMR) studies (see supplemental text and figure file 4) 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

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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

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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

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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

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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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Authorship Contributions

Participated in research design: Hernandez, Singh, Terry, Thomas, Wulff

Conducted experiments: Beck, Gao, Hernandez, Kaidery, Naughton, Singh

Contributed new reagents or analytic tools: Magrane

Performed data analysis: Gao, Hernandez, Singh, Terry

Wrote or contributed to the writing of the manuscript: Gao, Hernandez, Terry, Wulff

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Footnotes:

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Legends for Figures

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 μm.

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 ± S.E.M from 2-3 independent experiments. "*", $p < 0.05$ compared to vehicle control conditions. 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 10uM 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 μm.

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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 ± s.e.m. (n = 2-3 independent experiments), "*", $p < 0.05$ compared vehicle control conditions. 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 10uM 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 µm.

Fig 4. 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 ± s.e.m from 4 independent experiments and 4-5 replicates per concentration. "*", $p < 0.05$ compared to vehicle control conditions.

Fig 5. Colchicine impairs axonal transport in primary cortical neurons after 24hr of exposure. (A) Velocities of APP containing MBOs in the anterograde and retrograde direction.

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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 ± s.e.m from 3 independent experiments and 4-5 replicates per concentration. "*", $p < 0.05$ compared to vehicle control conditions.

Fig 6. 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 ± s.e.m from 4 independent experiments and 4-5 replicates per concentration. "*", $p < 0.05$ compared to vehicle control conditions.

Fig 7. 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 ± s.e.m from 3 independent experiments and 4-5 replicates per concentration. "*", $p < 0.05$ compared to vehicle control conditions.

Fig 8. Muscarinic and nicotinic antagonists do not affect diisopropylfluorophosphate (DFP)-related impairments in axonal transport in primary cortical neurons after 24hr of

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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 ± s.e.m from 2 independent experiments and 4-5 replicates per concentration. ATR = atropine, MEC = mecamylamine. "*", $p < 0.05$ compared to vehicle control conditions; #, $p<0.05$ compared to DFP 10 nM + vehicle conditions.

Fig 9. 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 ± 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.

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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.

Supplemental Text and Figure: Diisopropylfluorophosphate (DFP) stability studies. Methods, Results, Figure legend and Figure.

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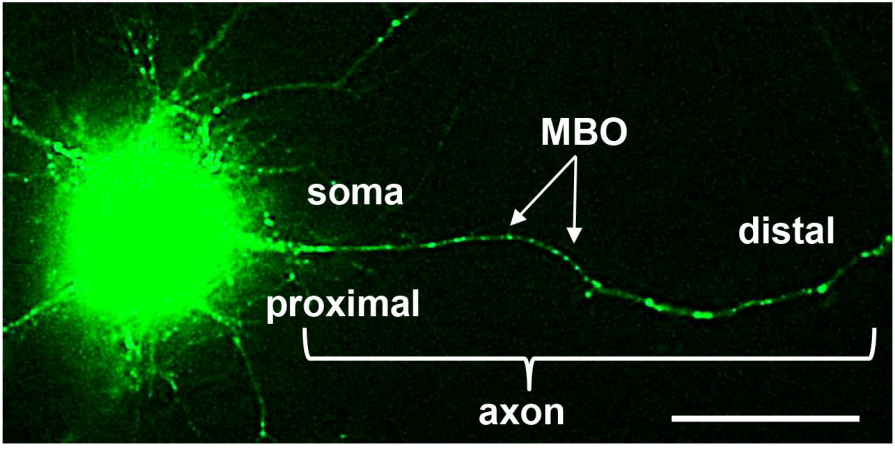
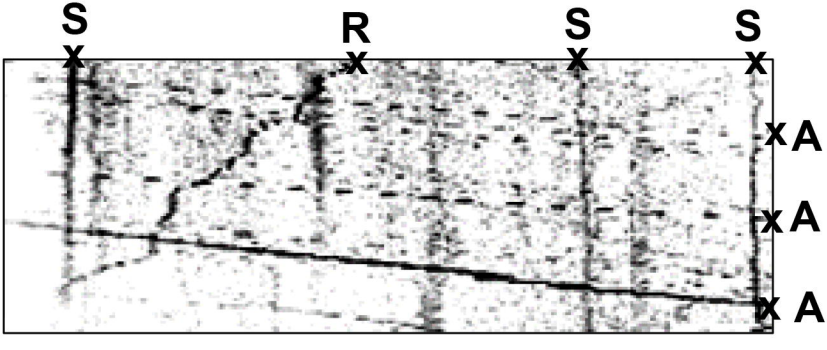
A. APPDendra2 transfected neuron



B. Sample Kymograph



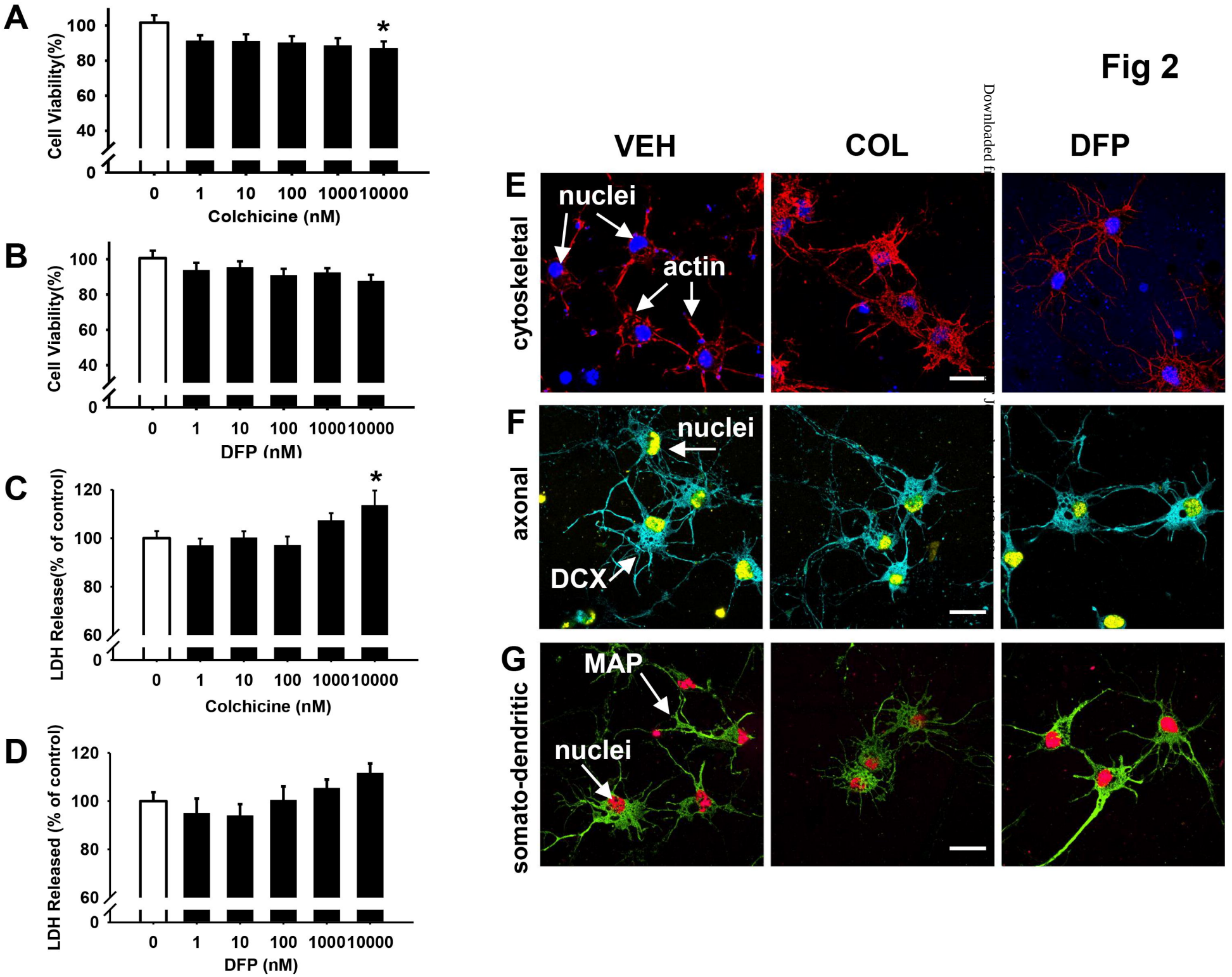


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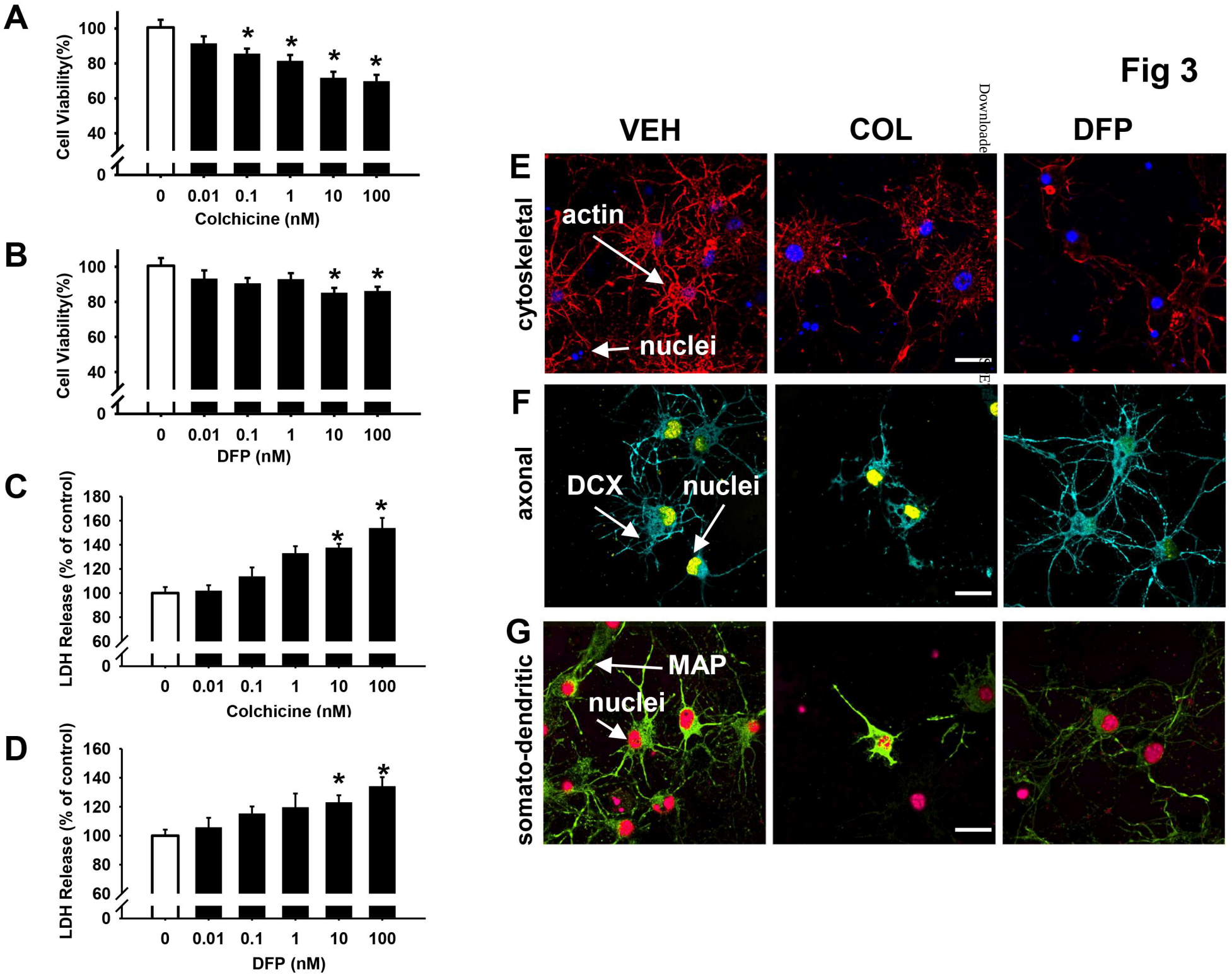
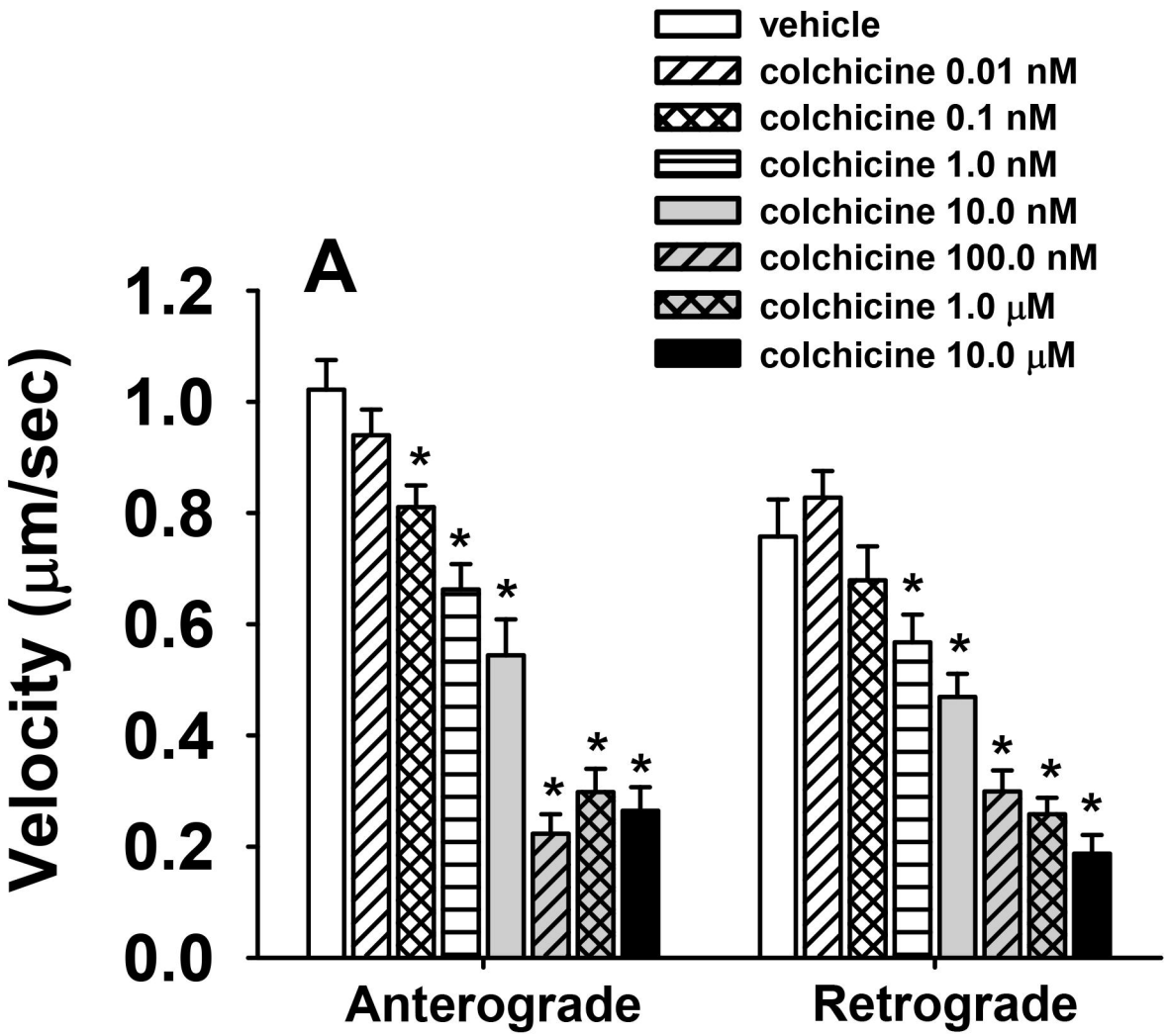
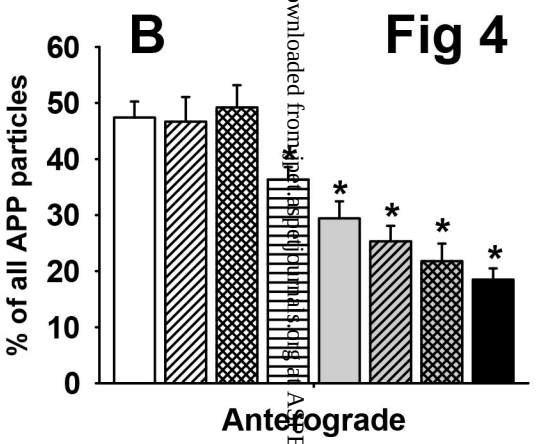
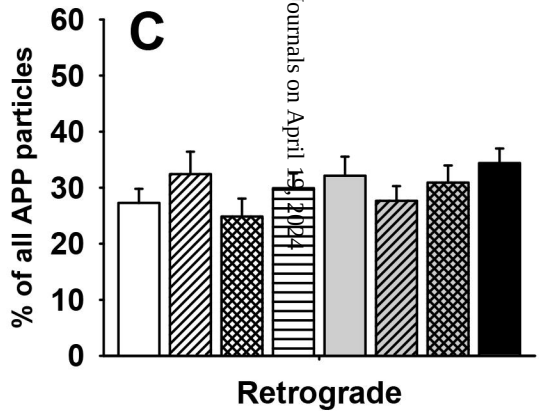
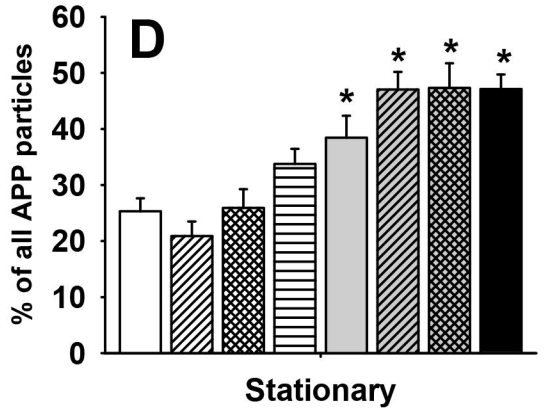
Fig 3


A
Cell Viability(%)
100
80
60
40
0
0
0.01
0.1
1
10
100
Colchicine (nM)
B
Cell Viability(%)
DFP (nM)
C
LDH Release (% of control)
180
160
140
120
100
80
60
Colchicine (nM)
D
LDH Release (% of control)
DFP (nM)
VEH
COL
DFP
E
cytoskeletal
actin
nuclei
F
axonal
DCX
nuclei
G
somato-dendritic
MAP
nuclei









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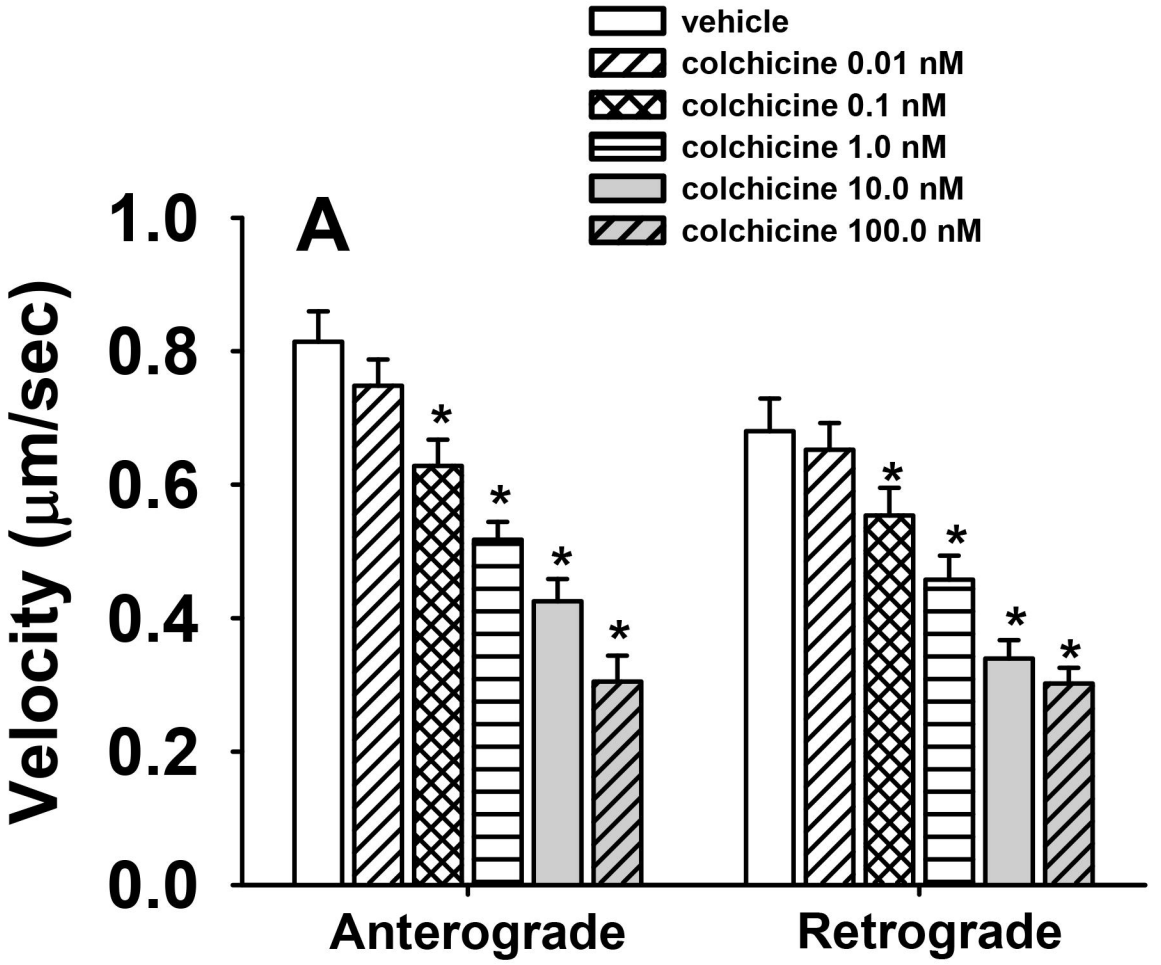

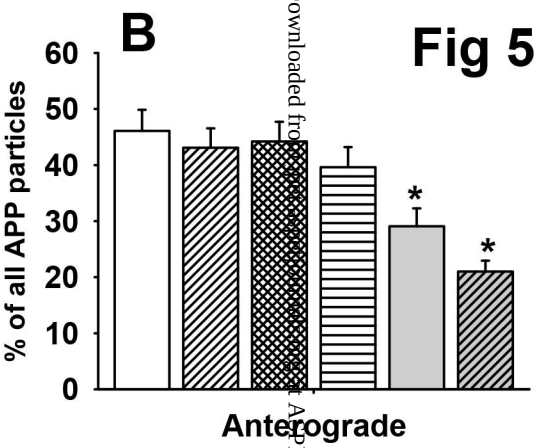

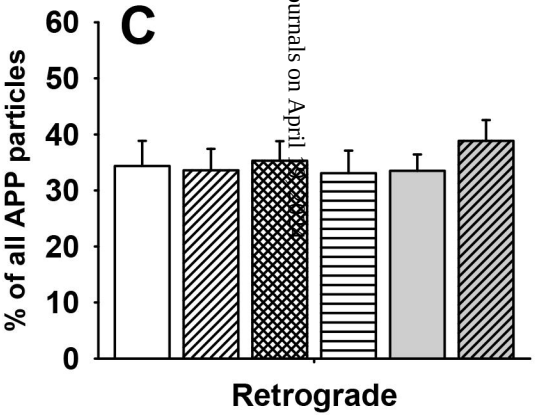

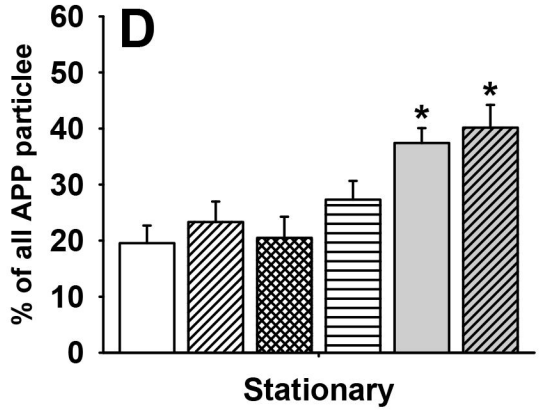

Fig 5

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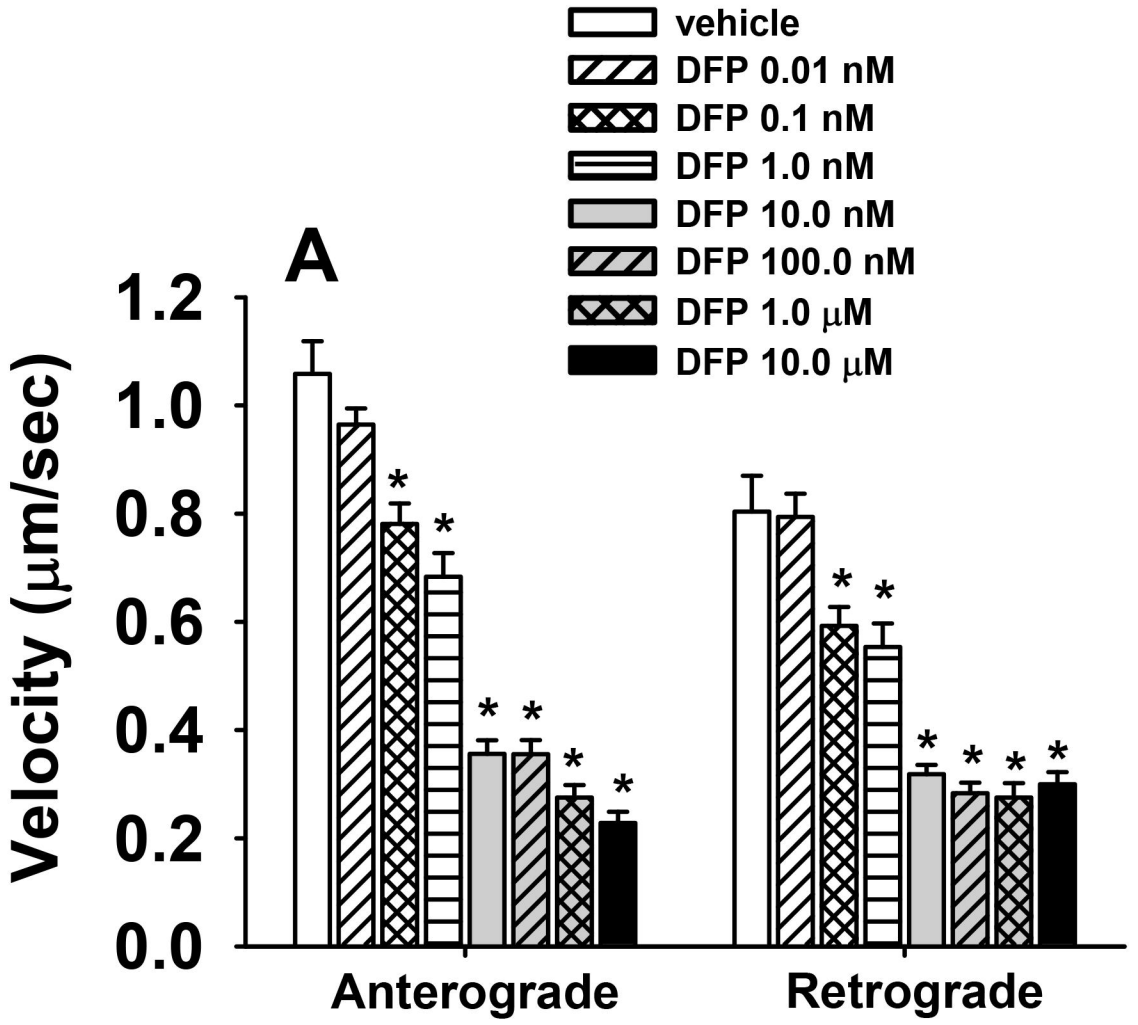

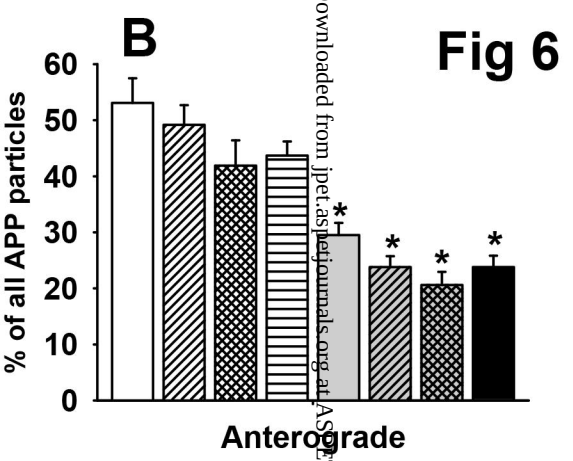

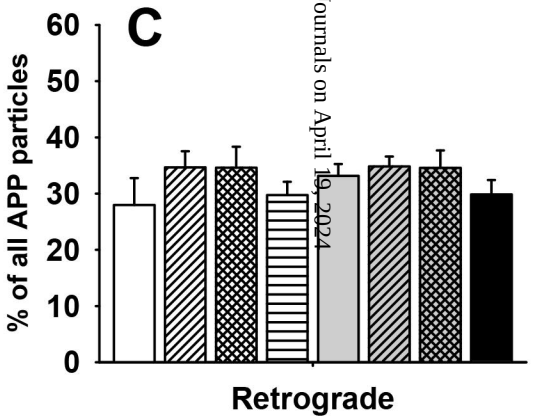

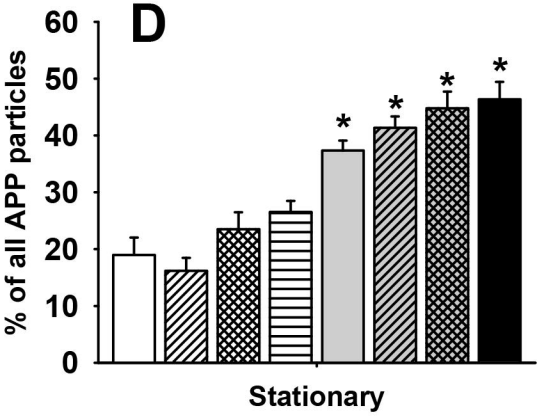

Fig 6

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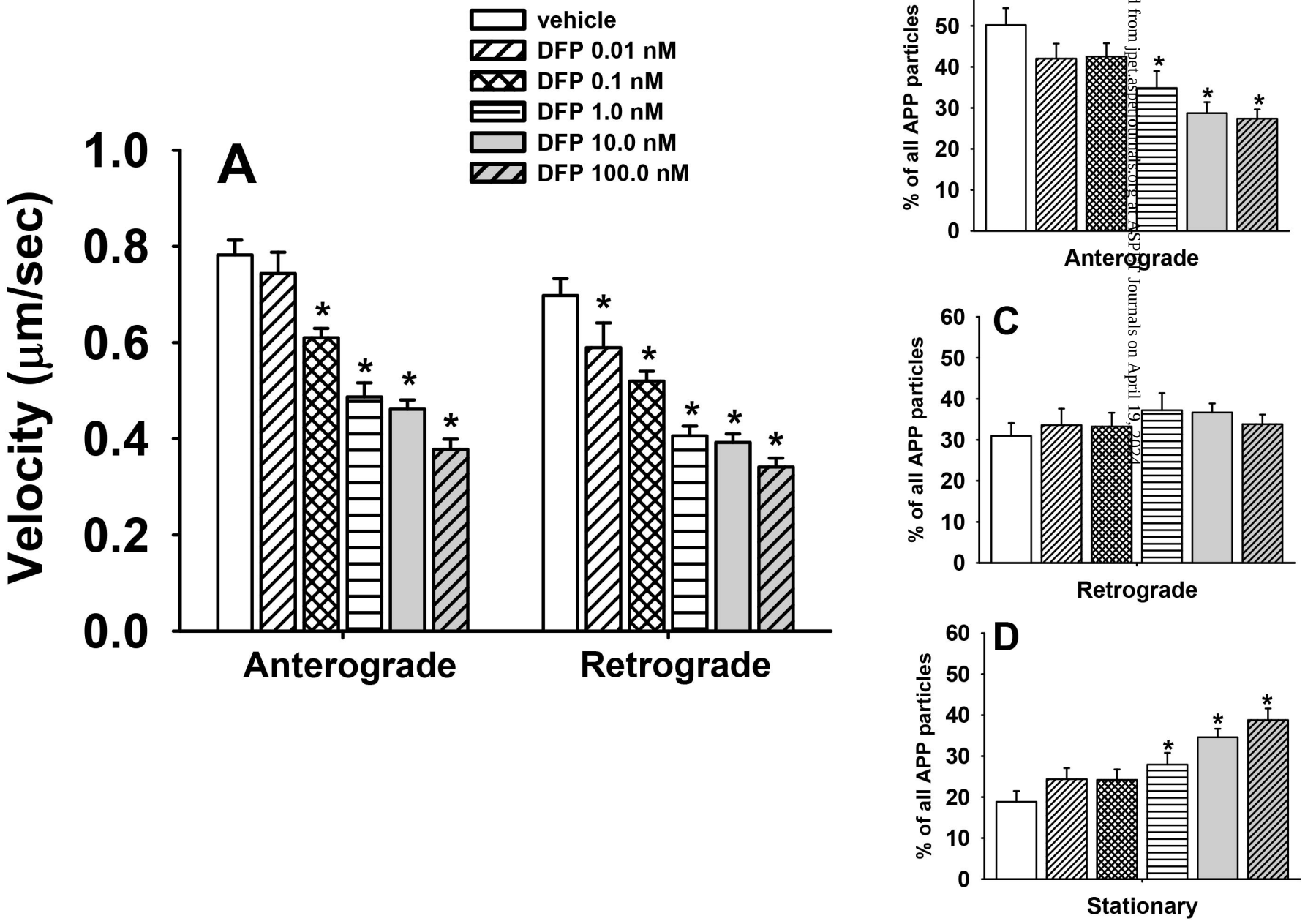

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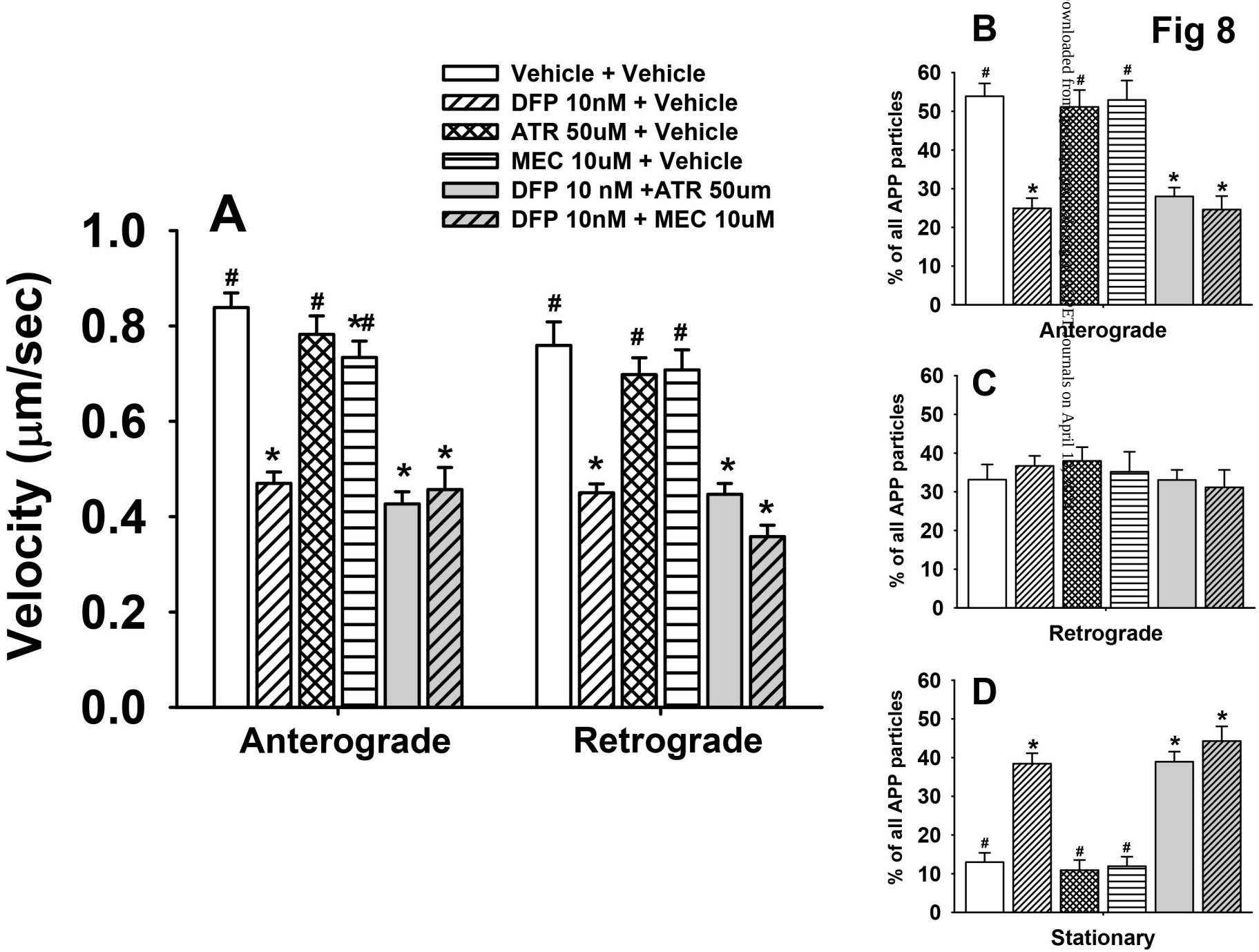

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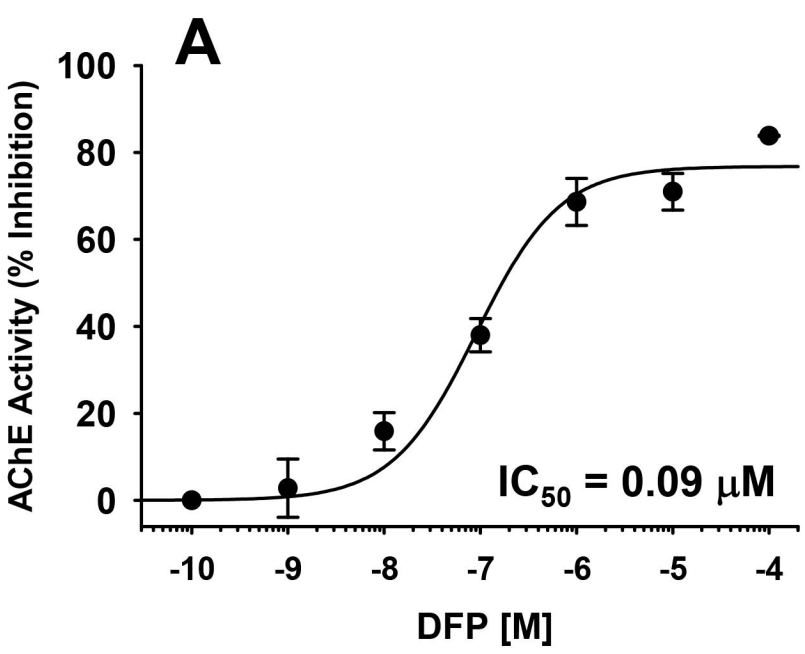
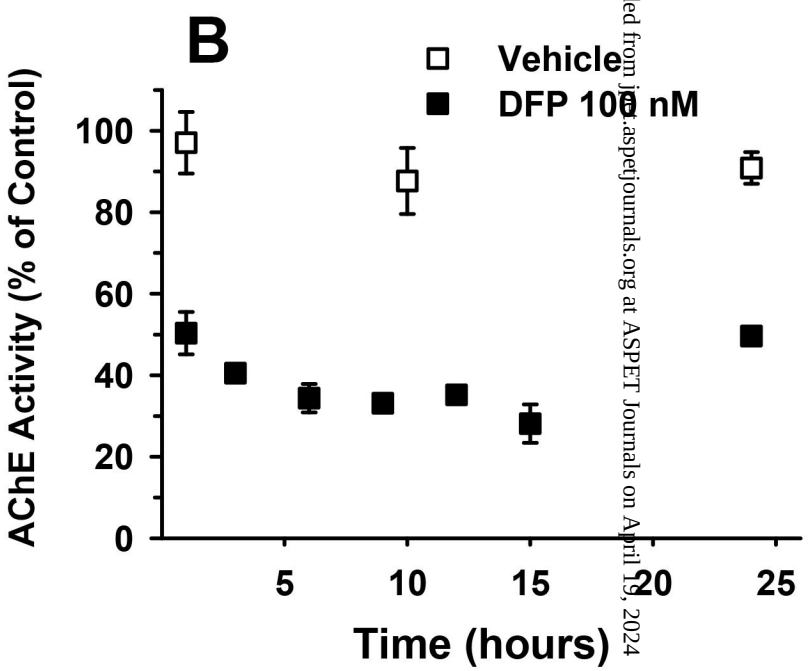
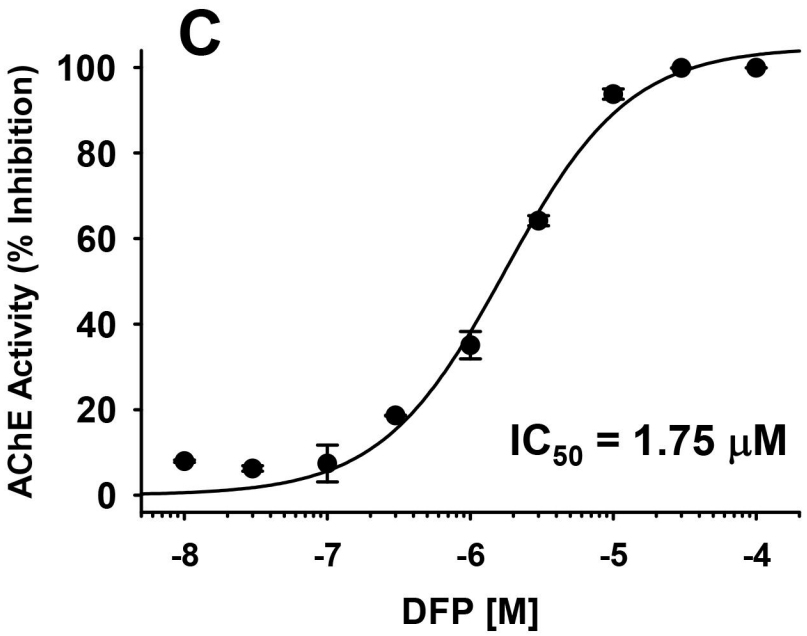
Fig 9







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