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Chronic, Intermittent Exposure to Chlorpyrifos in Rats: Protracted Effects on Axonal Transport, Neurotrophin Receptors, Cholinergic Markers, and Information Processing

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Abbreviations:

AChE, acetylcholinersterase
ChAT, choline acetyltransferase
ChE, cholinesterase
CHT, high affinity choline transporter
CPF, chlorpyrifos
NGF, Nerve Growth Factor
nAChR, nicotinic acetylcholine receptor
OP, organophosphate
TCP, 3,5,6-trichloro-2-pyridinol
TrkA, tropomyosin-receptor kinase A
phospo- TrkA, phosphorylated TrkA
$p75^{NTR}$, p75 neurotrophin receptor
VAChT, vesicular acetylcholine transporter

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Abstract

Persistent behavioral abnormalities have been commonly associated with acute organophosphate (OP) pesticide poisoning; however, relatively little is known about the consequences of chronic OP exposures that are not associated with acute cholinergic symptoms. In this study, the behavioral and neurochemical effects of chronic, intermittent, and subthreshold exposures to the OP pesticide, chlorpyrifos (CPF) were investigated. Rat were injected with CPF subcutaneously (dose range, 2.5-18.0 mg/kg) every other day over the course of 30 days, and then given a two week, CPF-free washout period. In behavioral experiments conducted during the washout period, dose dependent decrements in a water maze hidden platform task and a prepulse inhibition procedure were observed, without significant effects on open field activity, rotorod performance, grip strength, or a spontaneous novel object recognition task. After washout, levels of CPF and its metabolite 3,5,6-trichloro-2-pyridinol (TCP) were minimal in plasma and brain, however, cholinesterase inhibition was still detectible. Further, the 18.0 mg/kg dose of CPF was associated with (brain region-dependent) decreases in nerve growth factor receptors and cholinergic proteins including the vesicular acetylcholine transporter, the high affinity choline transporter, and the α_7 nicotinic acetylcholine receptor. These deficits were accompanied by decreases in anterograde and retrograde axonal transport measured in sciatic nerves *ex vivo*. Thus, low-level (intermittent) exposure to CPF has persistent effects on neurotrophin receptors and cholinergic proteins, possibly through inhibition of fast axonal transport. Such neurochemical changes may lead to deficits in information processing and cognitive function.

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Introduction

Neurobehavioral sequelae of acute and chronic organophosphate (OP) exposure have been described in the literature for decades (see Tabershaw and Cooper 1966; Gershon and Shaw 1961). Such effects have been reported in cases of exposure to military OP nerve agents, including sarin, soman and VX (Brown and Brix, 1998); however, most of the more recent human data come from studies of occupational poisonings from OP insecticides (see review, Roldán-Tapia et al., 2005). Persistent behavioral consequences of OP pesticide poisoning include the deterioration of intellectual functioning, reasoning, and academic ability, as well as impaired motor skills (Savage et al. 1988). It should be noted, however, that while a large number of human and animal studies have focused on the long-term consequences of acute OP exposure, relatively little attention has been given to the subject of chronic, "low-level" OP exposures that are not associated with acute cholinergic symptoms (see Ray and Richards, 2001). This may be a particular concern given the widespread use of OP insecticides (and consequent human exposure) in household, agricultural, and commercial environments worldwide.

One OP that has been used extensively as an agricultural and commercial pesticide since its introduction in 1965 (Hayes and Laws, 1990) is chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate). This broad spectrum OP insecticide has been described as "non-neurotoxic", "non-neuropathic" or "moderately toxic", based upon published evidence that it exhibits only moderate acute toxicity in mammalian species and a greater inhibitory potency for acetylcholinesterase (AChE) than for neurotoxic esterase (reviewed, Richardson, 1995). However, due to concerns over published evidence of developmental and neurobehavioral anomalies in young animals exposed to chlorpyrifos (CPF), its use (particularly for residential applications) has been restricted in the United States (US) by the Environmental Protection

Agency (US EPA, 2002). Despite such restrictions, the major metabolite of CPF, 3,5,6-trichloro-2-pyridinol (TCP), was recently detected in 96% of approximately 2,000 urine samples collected from individuals (ages 2-59 years) living in the US (Barr et al., 2005). Further, CPF continues to be used for residential applications (and for other pest control-related purposes) in the countries of the European Union and elsewhere in the world.

While the insecticidal actions of CPF and its acute toxicity in non-target organisms have been attributed to inhibition of AChE by the reactive CPF-oxon metabolite (Amitai et al., 1998), as in the case of other OPs, the consequences of chronic, low-level, exposures to CPF are poorly understood (i.e., a focus of our laboratories over the last several years). Previously we observed spatial learning deficits in rats after 14 days of daily exposure to CPF (18.0 and 25.0 mg/kg) when testing was initiated 24 h after the last injection, but not after a 14-day washout. We also observed that axonal transport was impaired in sciatic nerves that were isolated from these rats (Terry et al., 2003), a potentially important finding given the fundamental role of axonal transport in the function of neurons. Here we specifically focused on persistent effects (i.e., during and after an extended CPF-free washout) of repeated, intermittent, and subthreshold exposures to CPF on behavior, neurotrophin and cholinergic marker proteins, and axonal transport. We have operationally defined “subthreshold exposures” as doses that do not produce overt signs of cholinergic toxicity such as muscle fasciculations, respiratory muscle paralysis, seizures, diarrhea, urination, meiosis, salivation, and lacrimation (see reviews, Rusyniak and Nanagas, 2004; Sungurtekin et al., 2006). The intermittent dosing regimen was used to provide a model for the types of exposure that might be experienced by agricultural or industrial workers. Subsequent neurochemical studies focused on central cholinergic pathways (i.e., those originating from the basal forebrain) which are known to play important roles in many aspects of

cognition (reviewed, Bartus 2000). The expression and/or the intrinsic activities of protein markers residing in these cholinergic pathways (e.g., choline acetyltransferase, the high affinity choline transporter, and the vesicular acetylcholine transporter) have been used for decades to ascertain the consequence of disease or injury in these cells. We also assessed the effects of CPF on homo-oligomeric α_7 nicotinic acetylcholine receptors (nAChRs), which are important to cognitive function and highly expressed in the cortical and hippocampal neurons that receive innervation from the basal forebrain (Buccafusco, 2004; Danil and Bertrand, 2007). Finally, we evaluated CPF effects on the receptors for the neurotrophin, nerve growth factor, which, through its interactions with high affinity TrkA and p75 neurotrophin receptors ($p75^{NTR}$), is important for the maintenance and survival of basal forebrain cholinergic neurons (Li et al., 1995; Auld et al., 2001).

Methods

Test Subjects

Male albino Wistar rats (Harlan, Indianapolis, IN) 2-3 months old were doubly housed in a temperature controlled room ($25^{O}C$), maintained on a reversed 12-hour light/dark cycle with free access to food (Teklad Rodent Diet 8604 pellets, Harlan, Madison, WI). Table 1 provides the details for all study cohorts, the numbers of animals tested per group, and the experiments conducted in each group. All procedures employed during this study were reviewed and approved by the Medical College of Georgia Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain or discomfort in accordance with the National Institute of Health Guide for the Care and Use of Laboratory

Animals (NIH Publications No. 80-23) revised 1996. Significant efforts were also made to minimize the total number of animals used while maintaining statistically valid group numbers.

Drug Administration and Observational Studies

Each experimental group received subcutaneous injections of vehicle (3% dimethylsulfoxide, 97% v/v peanut oil) or CPF (Chem Service, Inc., West Chester, PA) dissolved in vehicle in a volume of 0.7 ml/kg body weight every other day over a 30 day treatment period. Individual rats were weighed and monitored (in their home cages for a period of approximately 5 minutes) for visible cholinergic symptoms (diarrhea, excessive salivation or lacrimation, respiratory difficulties, muscle fasciculations, etc) or other signs of distress throughout the study.

Behavioral Experiments

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

Motor Function Tests

Open Field Activity- Rat open field activity monitors (43.2 x 43.2 cm, Med Associates, St. Albans, VT) were used for these experiments. The following parameters were recorded for each 5 min test session: horizontal activity (horizontal photobeam breaks or counts), number of stereotypy movements, and vertical activity (vertical photobeam breaks). Thus, spontaneous locomotor activity, olfactory activity (rearing and sniffing movements) and stereotypical movements were assessed. We also recorded the time spent in the central and peripheral zones

of the apparatus (defined areas represented approximately 75% and 25% of the total floor area, respectively) as an anxiety-related behavioral assessment.

Accelerating Rotarod- Motor coordination, balance, and motor learning were evaluated with an accelerating rotarod (Rotor-Rod System®, San Diego Instruments, San Diego, CA). Individual rats were assessed for their ability to maintain balance on a rotating bar that accelerated from 4 to 40 rpm over a 5-minute period. The amount of time elapsed before each subject fell from the rod was recorded. Each test subject was given four trials per day for two consecutive days with an intertrial interval of 30 min.

Grip Strength- Forelimb grip strength was measured with a digital grip strength meter (Animal Grip Strength System®, San Diego Instruments, San Diego, CA) by holding the rat by the nape of the neck and by the base of the tail. The forelimbs were placed on the tension the bar and the rat was pulled back gently until it released the bar. Each animal was assessed three times and mean grip strength (measured in kg of resistance) ± S.E.M. calculated.

Memory-Related Tasks

Water Maze

Water maze experiments were conducted as described in detail previously (Terry et al., 2006). Briefly, for the hidden platform test, rats were given 2 trials per day for 6 consecutive days to locate and climb on to the hidden platform. Probe trials were conducted twenty-four hours following the last hidden platform trial to measure spatial bias for the previous platform location. Visible platform tests were subsequently conducted after probe trials (as a gross estimate of visual acuity) using a highly visible (white) cover fitted with a small white flag was attached to the platform.

Spontaneous Novel Object Recognition Test (OR)

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OR tests were conducted as described in detail previously (Terry et al., 2007). Briefly, habituation to the test apparatus consisted of two daily 10-min sessions in which the animals were allowed to freely explore the open field box. Video-recorded OR testing began on the third day and ended on day 5. Each test day began with a 3-minute information session (i.e., the A/A session with identical objects) followed by a 1.0, 15.0, or 60.0 min delay period (administered in a pseudorandom order), and a subsequent a 3-minute dissimilar stimuli (A/B) session. The objects discriminated were made of glass, ceramic, clay, or plastic. The proportion of the total exploration time that the animal spent investigating the novel object was the index of recognition memory. A recognition index calculated for each animal was expressed as the ratio TB/(TA+TB); [TA=time spent exploring object A (familiar object), TB = time spent exploring object B (novel object)].

Prepulse Inhibition (PPI)

To assess the effects of CPF exposure on sensorimotor gating, a PPI procedure was conducted as described in detail previously (Hohnadel et al., 2007). Briefly, four startle chambers (San Diego Instruments, San Diego, CA) were used, the background white noise was set at 70 dB, and the PPI trials consisted of a prepulse (20 ms burst of white noise with intensities of 75, 80, or 85 dB) followed, 100 ms later, by a startle stimulus (120 dB, 20 ms white noise). PPI was calculated according to the formula: [100 - (startle amplitude on prepulse-pulse trials ÷ startle amplitude on pulse alone trials) x 100]. The mean level of PPI (i.e., averaged across the 3 prepulse intensities) was also analyzed.

Blood Collection for Plasma assays

Blood sampling occurred weekly throughout the CPF treatment regimen and two-week washout. Rats were anesthetized by intraperitoneal injection (1 ml/kg body weight) of a cocktail

containing ketamine (40 mg/ml) and xylazine (8 mg/ml). Blood was collected from the jugular vein using a 1.0 cc syringe fitted with a 25 G needle; 0.7 ml of blood was immediately divided into two separate Microtainer® tubes as follows. Blood (300 µl) was added to an EDTA Microtainer® Tube (BD catalog #365973) that contained 25 µl of trichloroacetic acid (22.5 mg) in ultrapure water. The tube was vortexed, snap frozen in liquid nitrogen, and stored at -70°C until analyzed for CPF and its metabolites. The remaining blood (400 µl) was added to a Microtainer® Plasma Separator Tube containing lithium heparin (BD catalog t#365958). This tube was inverted eight times, and then centrifuged according to the BD protocol. The resulting plasma was aliquoted into 0.5 ml tubes, snap frozen in liquid nitrogen, and stored at −70° C until analyzed for cholinesterase activity.

At the end of the washout period, rats used in the blood sampling study were anesthetized with isoflurane; brains were harvested and snap frozen in dry ice-chilled isopentane, and then cut in half (sagittally) before storage at −70°C. One-half of each brain was used in enzyme activity assays (choline acetyltransferase and cholinesterase), and the other half of the brain was analyzed for levels of CPF and its metabolites.

Plasma and Brain CPF and TCP levels

CPF and TCP were measured in samples of brain tissue using coupled-column liquid chromatography/electrospray ionization tandem mass spectrometry as we previously described (Williamson et al., 2006). Acidified blood samples (previously collected in EDTA Microtainer® Tubes) were extracted by liquid-liquid extraction. Ethyl acetate/acetonitrile (200 µl, 60:40 v/v) was added to 50 µl of acidified rat blood. The samples were then vortexed for 5 min and centrifuged at 16,000 × g for 10 min. The upper organic layer was dried under a gentle stream of nitrogen, and the resulting residue was reconstituted in acetonitrile. The samples were

briefly sonicated, centrifuged for 10 min at 16,000 × g; and then the supernatant (10 µl) was analyzed by liquid chromatography (LC)/electrospray ionization tandem mass spectrometry.

The sample extraction and LC method used for blood analysis was different from the LC method used for brain (Williamson et al., 2006). The column used was an Agilent (Palo Alto, CA, USA) C8 column (2.0×150 mm, 5µm) equipped with a 4.0 × 2.0 mm Phenomenex (Torrance, CA, USA) Security Guard C8 guard column. The compounds were separated by gradient elution using 0.0025% formic acid in acetonitrile (mobile phase A) and 0.0025% formic acid deionized water (mobile phase B). The flow rate was 0.3 ml/min and the column temperature was 25°C. Mobile phase A was linearly increased from 60% to 80% over 2 minutes, and held at 80% for 3 min. The column was returned to the initial mobile phase conditions and re-equilibrated for 4 minutes. The total run time for each injection was 10 min. TCP eluted first at 3.5 minutes, followed by CPF-oxon and CPF at 4.2 and 8.0 minutes, respectively. Column effluent was analyzed by electrospray ionization tandem mass spectrometry (Williamson et al., 2007).

Homogenization of Brain for Enzyme assays

The dissection protocol described by Gearhart et al. (2007) was used to isolate six brain regions — basal forebrain, hippocampus (anterior and posterior), striatum, prefrontal cortex, and cortex — from frozen half brains (collected above). Brain regions were manually homogenized using a polypropylene pestle in a 1.5 ml microcentrifuge tube (Scienceware® catalog #19923-0000; Bel-Art Products, Pequannock, NJ). Homogenation buffer contained sucrose (0.25 M), EDTA (10 mM), Triton-X 100 (0.5% v/v), and 0.01 M sodium phosphate (pH 7.4). After manual homogenation in five volumes (5 µl of buffer per mg tissue) of ice-cold homogenation buffer, the crude homogenate was briefly sonicated (on ice) using a Sonic Dismemberator™

(Model #100; set at level 1; Fisher Scientific). Sonicated homogenates were aliquoted into 0.5 ml tubes (20 µl/tube), and stored at −20°C. Within two weeks of freezing, the homogenates from all six brain regions were analyzed for total protein (Coomassie Plus Assay; catalog #23236; Pierce Biotechnology, Rockford, IL), cholinesterase activity, and choline acetyltransferase activity.

Plasma and Brain Cholinesterase Activities

Cholinesterase activity in plasma samples and brain homogenates were done according to Ellman et al. (1961) in a 96-well plate format at room temperature. Five microliters (5 µl) of plasma (100-130 µg protein/µl) or brain homogenate (20-50 µg protein/µl) were dispensed into the bottom of the wells of the 96-well plate (Fisher Scientific #12-565-501). An 8- or 12-channel pipeter was used to quickly add 310 µl of reaction mixture to the wells. The reaction mixture contained acetylthiocholine (0.48 mM; Sigma # D-8130)) and dithiobisnitrobenzoic acid (0.52 mM; Acros # 102710050) in 0.1 M sodium phosphate buffer (pH 8.0). The microplate was shaken for ~30 seconds using a Jitterbug™ plate shaker (Boekel Scientific; Feasterville, PA), before placing the microplate in a µQuant™ Microplate Spectrophotometer (BioTek Instruments Inc.; Winooski, VT). The formation of reaction product (yellow color) was monitored by measuring absorbance at 412 nm every 2 min for 16 min. The cholinesterase-mediated reaction rate (moles/liter per min) was calculated by dividing the change in absorbance per minute by 13,600 (for details, see Ellman et al., 1961). Each plasma sample or brain homogenate was assayed in triplicate.

Choline Acetyltransferase Activity

Brain homogenates (prepared above) were analyzed for choline acetyltransferase activity using a modification of method described by Fonnum (1969). Typically a set of 24-30 samples

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were processed at once—for example, cortex homogenates from the four treatment groups (N=5-6 samples per group). Each assay (20 µl total volume) was prepared in a 0.5 ml tube. For each set of assays, brain homogenate (8 µl; 20-50 µg protein/µl) was added to every tube, and then 12 µl of "reagent master mix" were quickly added to start the reaction. The "reagent master mix" contained (per assay tube): 0.2 µCi of tritium-labeled acetyl coenzyme A (Perkin Elmer #NET290); non-tritiated acetyl coenzyme A 0.4 mM; choline chloride 10 mM; eserine 0.2 mM; EDTA 10 mM; sodium chloride 0.3 mM; and Triton X-100 0.5% v/v in sodium phosphate 50 mM, pH 7.4. Tubes were maintained at 37°C for 30 min. The reaction was quenched by placing the assay tubes in a pre-chilled (−20°C) microcentrifuge rack and adding 100 µl of cold ultrapure water to each tube. Liquid-liquid extraction was used to isolate the acetylcholine formed during the reaction: 300 µl of an organic solution containing 20 mg/ml sodium tetraphenylborate in 3-heptanone was added to the quenched reaction. The mixture was vortexed for 30 sec, and then centrifuged for 5 min at 7,000*xg*. Two-hundred microliters (200 µl) of the upper organic layer was carefully pipeted out of the assay tube, and then transferred to a 20 ml glass scintillation vial that contained 10 ml of Scintiverse BD™ (Fisher Scientific; Waltham, MA) liquid scintillation cocktail. A Beckman LS6000TA (Beckman Instruments, Inc; Fullerton, CA) was used for liquid scintillation counting.

ELISA Methods for Cholinergic Proteins

At the end of the 14-day washout, relative levels of six specific brain proteins ($p75^{NTR}$, TrkA, phospho-TrkA, VAChT, CHT, and α7-nAChR) were measured in tissue lysates from four brain regions (basal forebrain, hippocampus, prefrontal cortex, and cortex) prepared from vehicle- and CPF-treated rats. We previously published detailed methods for the dissection of brain regions, preparation of brain lysates, and ELISA (Gearhart et al. 2006).

Axonal Transport

The effects of the CPF treatment regimen on fast anterograde and retrograde axonal transport were evaluated in single axons of sciatic nerves (ex vivo) by direct visualization of vesicle movement using video-enhanced differential interference contrast microscopy (AVEC-DIC). This procedure has been described in detail previously (Terry et al., 2003). Briefly, rats were anesthetized with 4% chloral hydrate (10 ml/kg, IP), the mid-thigh sciatic nerve was exposed, and 6-0 silk ligatures were tied at the proximal and distal ends. Exceptional care was taken to prevent stretching and trauma to the nerve during excision. The proximal-to-distal orientation of the nerve was maintained throughout the experiment. The sample was placed between two coverslips in a custom-designed aluminum chamber that was sealed in place with 1:1:1 Vaseline petroleum jelly/lanolin/paraffin. Before sealing the chamber, the nerve was extended to its original length, and the ligatures were attached to the bottom of the chamber using 1:1:1 Vaseline petroleum jelly/lanolin/paraffin. All procedures were accomplished with the nerve continually submersed in oxygenated physiologic buffer (94 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 2.0 mM Na_2HPO_4, 24 mM $NaHCO_3$, and 11 mM glucose, pH 7.4). Axons were viewed through a Zeiss Axiovert 10 microscope with DIC optics (Lehman Scientific, Red Lion, PA) equipped with a Hamamatsu C2400-07 camera (Hamamatsu Corp., Bridgewater, NJ), Argus-20 image processor (Spectra Services, Inc., Webster, NY), and Hamamatsu high-resolution monitor), in an observation chamber on a 37°C heated stage (Zeiss TRZ model 3700). Video enhancement of the axons was achieved with analog contrast enhancement (camera controller) and digital contrast enhancement (video computer) with background subtraction. The number of vesicles moving in the anterograde and retrograde

directions that completely traversed a 2-cm square window (drawn directly on the screen of the video monitor) was counted for a 10-min interval.

Statistical Analyses

Comparisons between treatment groups were made using analysis of variance (with repeated measures when indicated) followed by the Student-Newman-Keuls method for post hoc analysis. Statistical significance was assessed at an alpha level of 0.05. In all studies, the investigator performing the experiments was blind to the treatment group.

Results

Body Weight and Observational Studies

The effect of repeated (i.e., every other day) exposures to CPF on body weight over a 30 day period are illustrated in Fig 1. As indicated, the rats in all treatment groups progressively gained weight over the 30 day period, day effect, $F(11,33)=184.2$, $p<0.001$. There were no significant differences between any of the CPF-treated groups and vehicle controls, i.e., the dose effect and the dose x day interaction were not significant, $p>0.05$. There were no observations of acute cholinergic side effects associated with any of these CPF doses at any point in the study.

Assessments of Exploratory Activity and Motor Function

In these experiments we were interested in determining whether CPF exposure had significant effects on exploratory activity or motor function (i.e., effects that might have influenced performance in the memory-related tests, particularly the water maze experiments). The results of these experiments are provided in Figs 2-4.

Open Field Activity- Fig 2A illustrates the effects of CPF on open field locomotor activity at various points during exposure and during the drug free washout period. Horizontal and

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vertical locomotor activity, stereotypical movements are depicted. There were no significant treatment-related effects ($p>0.05$ for all differences) on any of these measures. The time spent in the peripheral versus central zones of the test apparatus was also assessed. Again, there were no significant CPF-related effects observed (data not shown).

Grip Strength- The effects of CPF exposure on forelimb grip strength at various points during exposure and during the drug free washout period are illustrated in Fig 2B. There was a statistically significant trial effect ($p<0.05$), indicating that grip strength increased in all groups over the course of repeated testing; however, no significant treatment-related differences ($p>0.05$ for all comparisons) were observed.

Rotarod Performance- The effects of CPF exposure on the performance of the rotarod task at various points during exposure and during the drug free washout period are illustrated in Fig 2C. As in the case of grip strength testing, there was a statistically significant trial effect ($p<0.001$), indicating that rotarod performance improved in all groups over the course of repeated testing. There was no significant overall dose effect, but a nearly significant CPF dose x trial interaction ($p<0.07$). There were also no significant drug effects observed in post hoc analysis, although there were some cases where trends toward inferior rotarod performance in CPF-treated rats was evident particularly later in the study during the drug free washout period.

Water Maze Testing

Hidden Platform Test- The latencies required to locate a hidden platform in the water maze beginning on day 7 of a drug free washout (i.e., after the 30 day regimen of CPF exposure) are illustrated in Fig 3A. Statistical comparisons of latencies across the 4 groups revealed the following results: dose effect, $F(3,55)=2.9$, $p<0.05$; day effect, $F(5,15)=46.8$, $p<0.001$; dose x day interaction, $F(274,352)=1.2$, $p=0.3$. Similar results were evident when swim distances were

analyzed. Thus, after exposure to vehicle or CPF, the rats learned to locate the hidden platform with progressively shorter latencies (and swim distances) across the 6 days of training. Post hoc analyses indicated that the 18.0 mg/kg dose of CPF was associated with modest, but significant ($p<0.05$) impairment in performance of the task (i.e., indicated by higher mean latencies to locate the hidden platform) on days 1 and 4 of testing.

Swim Speeds- Swim speeds (Fig 3B) , i.e., the distance swam (cm) divided by the latency to find the platform (sec) were compared daily across the treatment groups for all 6 days of water maze testing. Statistical comparisons revealed the following results: dose effect, $F(3,55)=0.5$, $p=0.7$; day effect, $F(5,15)=8.8$, $p<0.001$; dose x day interaction, $F(274,352)=2.6$, $p<0.001$. Interestingly, post hoc analyses indicated that the animals previously exposed to CPF at 10.0 mg/kg, swam significantly ($p<0.05$) faster than did controls on day 6 of testing.

Probe Trials- Fig 3C illustrates the performance of probe trials by the various treatment groups conducted on day 7 of water maze testing. There was a clear trend toward inferior performance in all of the CPF groups (as indicated by a reduced number of crossings over the previous 10 cm x 10 cm target area); however, the effects did not reach the required level of statistical significance (main effect, $p=0.1$).

Visible Platform Test-the average times required to reach a highly visible (reflective) platform (data not shown) ranged between 8.4 and 13.6 seconds across all groups in the study and were not significantly different (i.e., all p values were >0.05), indicating that differences in performance of the previous hidden platform tests or probe trials were unlikely to be a result of gross impairments in visual acuity associated with CPF.

Spontaneous Novel Object Recognition Test (OR)

Fig 4 illustrates the effects of CPF exposure on performance in the OR task (only the A/B sessions are illustrated). There was a clear preference noted for all treatment groups for the novel object at each delay interval in the A/B sessions ($p<0.01$). All other factors including dose, delay and the interactions between dose, delay, and trial type were not statistically significant.

PPI Experiments

The effects of prior exposure to CPF on PPI testing assessed on day 12 of the drug-free washout are presented in Figs 5A-C. As indicated in Fig 5A, there were significant dose-related differences, $F(3,51)=3.9$, $p=<0.02$; there was a highly significant difference in response to the different prepulse levels, $F(2,6)=66.3$, $p=<0.001$, but the dose x prepulse level interaction was not significant ($p>0.05$). Post hoc analyses indicated that prior exposure to CPF at 10.0 and 18.0 mg/kg significantly ($p<0.05$) diminished PPI at the 80 dB prepulse level (compared to vehicle controls) and that CPF 18.0 mg/kg significantly ($p<0.05$) diminished PPI at the 85 dB prepulse level. The PPI-impairing effects of the 10.0 and 18.0 mg/kg doses of CPF were also apparent when the data were averaged across the prepulse levels (see Fig 5C). There were no significant effects of any of the doses of CPF on startle amplitude (Fig 5B).

CPF Levels

Alternate day administration of CPF over 30 days produced a concentration-dependent increase in the plasma levels of CPF (Fig. 6A). Maximal levels were attained by experimental day 21. After the final injection on experimental day 30, plasma levels dropped precipitously such that minimally detectable levels were present after the 2-week washout. For the non-toxic metabolite TCP, plasma levels rose more rapidly than the parent compound, peaking on experimental day 7 (Fig. 6B). Despite the chronic regimen, TCP levels gradually decreased

through experimental day 30, becoming minimally detectable during the discontinuation period. Whole brain levels of CPF and TCP were only measured on experimental day 44 since the primary objective was to determine whether significant amounts of CPF or TCP remained after washout. The data are presented in Table 2. CPF was detectible in the brains of all of the animals treated with the 10.0 and 18.0 mg/kg doses, whereas TCP was only present in 1 of 12 animals.

Cholinesterase Activity

Alternate day administration of CPF produced a concentration-dependent decrease in plasma cholinesterase activity which was maximal at experimental day 7 (Fig. 7A). Enzyme activity remained at about 10% of control during the 18 mg/kg CPF regimen until experimental day 30. During the 2-week washout period, cholinesterase activity began to increase, however, 14 days after the final 18 mg/kg dose, plasma cholinesterase activity was still decreased to 44% of control. Figure 7B shows the data for CPF (18 mg/kg) and TCP levels and for cholinesterase activity over time normalized with respect to the respective % maximal level in each parameter respectively. Thus cholinesterase activity was maximally inhibited at a time when CPF levels were at about 50% of maximal attained levels. During washout cholinesterase activity continued to be inhibited when CPF and TCP levels were only minimally detectable.

Cholinesterase activity was measured in six brain regions 14 days after the final administration of CPF (Fig. 8). For five of the six brain regions cholinesterase activity was significantly decreased for 14 days after the 10 and 18 mg/kg regimens $F(3,20)=5.0\text{-}24.0$, $p<0.01$. For the striatum, cholinesterase inhibition was significant 14 days after completing all three dose regimens $F(3,20)=10.0$, $p<0.001$. Across the six brain regions, 14 days after the final 18 mg/kg dose of CPF, cholinesterase activity was still inhibited by 55%. After discontinuation

of the 18 mg/kg CPF regimen the brain:plasma ratio for cholinesterase activity varied from a low of 0.67 in the striatum to a high of 1.04 in the anterior hippocampus. Across all 3 dose regimens and across all 6 brain regions the ratio averaged 0.82 ± 0.033.

Cholinergic Marker Proteins

Six proteins that are important for cholinergic function were measured by ELISA in four brain regions derived from tissues harvested on experimental day 44 of the vehicle or 18 mg/kg CPF treatment regimen (Fig. 9). In the prefrontal cortex there were significant decreases in TrkA, phosphorylated-TrkA, and α_7 nAChRs. In the remaining cortical tissues, phosphorylated-TrkA was the only marker protein to show a significant decrease relative to control. In the hippocampus, the high affinity choline transporter and the vesicular acetylcholine transporter were decreased as was the expression of α_7 nAChRs. The basal forebrain was the only region to show a decrease in the expression of p75 neurotropin receptor. This was accompanied by a decrease in the expression of the high affinity choline transporter and the α_7 nAChR. Thus, the most consistent finding was the decrease in the expression of α7 nAChRs.

Choline acetyltransferase (ChAT) activities were measured in the same brain regions as were the cholinergic protein markers. ChAT activity was measured 14 days after the last administration of 18 mg/kg CPF. None of the brain regions showed significant changes relative to control ($P>0.21$; data not shown).

Axonal Transport

Anterograde and retrograde axonal transport were assessed *ex vivo* in sciatic nerve fibers obtained from rats on experimental days 30 and 43 of the 18 mg/kg CPF regimen (Fig. 10A and B). In vehicle-treated rats, the number of vesicles moving in the anterograde direction was about twice that of those moving in the retrograde direction. On experimental day 30 (the day after the

last CPF injection) anterograde and retrograde transport was significantly decreased by approximately 20%, anterograde, $F(3,16)=33.3$, $p<0.0001$ and retrograde, $F(3,16=10.9$, $p<0.001$. Two weeks after the regimen was discontinued anterograde transport was still depressed by 20% and retrograde transport was depressed by approximately 30%. Fast anterograde and retrograde axonal transport were also assessed *ex vivo* in sciatic nerves obtained from rats after a single injection of 18 mg/kg CPF (Fig. 10C and D). In general, the number of vesicles moving in the anterograde direction was about twice that of those moving in the retrograde direction. In both cases, treatment with CPF resulted in a significant time-dependent decrease in the number of vesicles moving, up to about 20% in the anterograde direction $F(4,13)=25.9$, $p<0.0001$, and up to about 33% for the retrograde direction $F(4,13)=22.8$, $p<0.0001$. The time point after injection for maximal inhibition of transport was 24 hr. There was some recovery in axonal transport by 48 hr, but it was still significantly decreased relative to control means.

Discussion

In this study there was no evidence of overt cholinergic side effects or CPF-related effects on weight gain confirming our premise that the doses evaluated were “subthreshold” for acute toxicity. Further, CPF treatment had no effect in the open field, rotarod, or grip strength analyses, or on swim speeds or visible platform tests in the water maze. These observations argue that deficits in memory-related task performance were not due to residual OP effects on locomotor activity, anxiety levels, or visual acuity. The highest dose of CPF evaluated (18.0 mg/kg) was associated with a 90% inhibition of cholinesterase by day 6 of treatment, a level of inhibition that would normally be expected to produce overt cholinergic symptoms. The lack of

symptoms is suggestive of some type of adaptive mechanism or tolerance that protected the animals against the more severe toxic effects of CPF after repeated exposure.

The water maze procedure was utilized since it is a visuospatial learning task that is sensitive to cholinergic dysfunction (McNamara and Skelton, 1993), and importantly, deficits in visuospatial processing have been identified as one of the negative outcomes in patients previously exposed to OPs for chronic periods (Rolda´n-Tapia et al., 2005). In the hidden platform task, rats exposed to the 18.0 mg/kg dose of CPF were impaired on days 1 and 4; however, all treatment groups reached an asymptotic level of performance by day 5, indicating a modest impairment of task acquisition that could be overcome with repeated testing. Further, there was some (albeit not statistically significant) evidence of CPF-related impairment of task retention in subsequent probe trials. In our earlier study (Terry et al., 2003) daily treatment with CPF (dose range 2.5-25.0 mg/kg) for two weeks resulted in a dose-dependent decrement in the water maze that was evident from 1-5 days after CPF discontinuation. However, within 2 weeks after discontinuation, performance was similar to vehicle-treated controls. The results reported here support the findings of the prior study; however, is the present study, exposure was intermittent, and performance deficits were more prolonged (i.e., evident up to two weeks after CPF discontinuation). The protracted effect of CPF in this study might be expected in view of the longer treatment period (30 days vs. 14 days), although the total number of CPF injections was similar in the two studies (15 vs. 14).

The CPF-related impairments in prepulse inhibition (PPI) were interesting in light of reports of sensorimotor abnormalities (see Kamel and Hoppin, 2004) and attentional impairments in humans previously exposed to OPs. PPI, defined as the reduction in startle response produced by a low-intensity stimulus presented before a high-intensity, startle-producing stimulus has been

widely used as a neurophysiological measure of the early pre-attentive stages of information processing (Braff and Geyer, 1990), Further, it has been shown in animals to be sensitive to cholinergic manipulations (Hohnadel et al., 2007). PPI impairments are evident in a variety of conditions where deficits in attention and information processing are observed, such as Asperger's syndrome (McAlonan et al., 2002), Huntington's disease (Swerdlow et al., 1995), and schizophrenia (Braff et al., 2001) leading to the logical question of whether OP exposure might be a risk factor for such illnesses. There have been several reports in which pesticide exposures were linked to a higher incidence of Parkinson's disease (and other movement disorders); however, most of these studies have not implicated specific compounds (reviewed, Kamel and Hoppin, 2004).

Given the water maze and PPI results, we were surprised that there were no significant effects of CPF in the novel object recognition task. This test for rodents was selected since it is a non-spatial procedure that models some components of episodic and recognition memory (Ennaceur and Delacour 1988) and since it is sensitive to cholinergic alterations (Bartolini et al., 1996). The lack of CPF effect may simply indicate that residual effects of the OP are task (i.e., cognitive domain) specific. Since we did not evaluate delays longer than 60 min, it is certainly feasible that negative effects of CPF might emerge as the cognitive demands of the procedure increase.

In neurochemical studies, we sought to determine whether the protracted behavioral deficits could be related to alterations in proteins that have important roles in cholinergic function. Protein levels (or the activity) of molecules selective for cholinergic neurons (VAChT, CHT, and ChAT) were measured along with molecules related to, but not specific for, cholinergic cells ($p75^{NTR}$, TrkA, phosphorylated TrkA, and α_7 nAChRs). Decrements in one or

more of these proteins were evident for each of four brain regions analyzed. For example, α_7 nAChRS were decreased in basal forebrain, hippocampus, and prefrontal cortex, while VAChT and CHT were decreased in the hippocampus. The decreases in α_7 nAChRs and the CHT in the basal forebrain were interesting since cholinergic neurons arising from the basal forebrain nucleus basalis innervate sensory regions of the cerebral cortex and play a role in attentional processes (Sarter et al., 2006). Further, the decrease in TrkA and phosphorylated-TrkA (i.e., the activated form of the receptor) in the prefrontal cortex was interesting given that NGF-mediated TrkA activation is required for the survival and maintenance of basal forebrain cholinergic neurons (Li et al., 1995; Auld et al., 2001). The PPI impairments and α_7 nAChR decrements in the hippocampus and prefrontal cortex were interesting since α_7 nAChR deficits in these same brain regions are thought to contribute to impairments in sensory gating and attention in schizophrenics (reviewed Martin et al., 2004). Finally, the notion that the VAChT and CHT deficits observed in the hippocampus contributed to the water maze learning impairments is consistent with the well-documented role of cholinergic neurons and hippocampal function to place learning (reviewed, McNamara and Skelton, 1993).

The decreased protein markers (described above) could reflect neuronal loss, altered protein turnover, or perhaps changes in axonal transport. We reported previously that after rats completed a treatment regimen of 14 daily (25 mg/kg) injections of CPF, bidirectional fast axonal transport measured in peripheral nerves was significantly decreased up to 20 days after CPF discontinuation (Terry et al., 2003). The results of the present study support these earlier findings and indicate that CPF can decrease axonal transport for up to at least 48 hr after a single injection. This decrease was still evident two weeks after the intermittent 30 day regimen, coinciding with the decreased cholinergic markers. Though we have no direct evidence that

axonal transport was inhibited in brain neurons, it has been reported that the development of delayed neuropathies following OP exposure in susceptible species often results in alterations in axonal transport in peripheral nerves (Gupta et al., 1997; Damodaran et al., 2001). Further, elements of axonal transport such as tubulin and tau proteins are excessively phosphorylated after animals are exposed to OP agents (Gupta and Abou-Donia, 1998; Damodaran et al., 2001). More recently, we demonstrated that the OPs CPF, CPF-oxon, and diisopropylfluorophosphate directly affect the motor protein kinesin, thereby disrupting kinesin-dependent transport on microtubules (Gearhart et al., 2007).

The contribution of residual levels of CPF or its metabolite TCP to the protracted effects on neurotrophin receptors and cholinergic proteins appears minimal since the levels of CPF and TCP were low or nearly undetectable at the time of neurochemical assessments. Conversely, plasma and brain cholinesterase continued to be inhibited by at least 50% two weeks after the final CPF injection. Although it is not yet possible to rule out the effects of residual cholinesterase inhibition, it is unlikely that the differential effects on specific neuronal markers in different brain regions would be explained by cholinesterase inhibition. Alternately, axonal transport deficits might be expected to have differential effects in different brain regions depending upon the length of neuronal projections and the specific motor proteins present in those projections.

As noted previously, the CPF dosing regimen was selected to provide a model for the types of exposure that might be experienced by agricultural or industrial workers. From an environmental toxicology perspective, the exposure levels would be considerably higher than that expected to occur in the general population. Since even the lower dose (2.5 mg/kg/day) caused cholinesterase inhibition (and no behavioral effects), this represents the Lowest Observed

Effects Level (LOEL) of the study. By standard correction for safety factors (10x10x10), this provides a reference dose (RfD) of 2.5 μg/kg/day, in close agreement with the reported RfD of 3.0 μg/kg/day derived from a No Observed Effect Level (NOEL) of 0.3 mg/kg/day (US EPA, 1991). On the basis of TCP urinary data, Barr et al. 2005 estimated that the median exposure of adults to CPF is 0.008 μg/kg/day, i.e. over 300-fold less than the RfD, 300,000-fold less than the LOEL in this study, and over $2x10^6$-fold less than the dose level that caused behavioral and biochemical effects reported here. However, occupational exposures to CPF such as that found in pesticide applicators is considerably higher than the general population. In a study of termiticide applicators in North Carolina, TCP levels were found to exceed that of the general population by an average factor of 70 (Hines and Deddens, 2001). Even more germane to our results is a study of termiticide applicators in Australia where, in the absence of acute cholinergic symptoms, serum cholinesterase activity was found to be only 52% of control levels (Dyer et al., 2001).

In conclusion, the results of this study are consistent with the possibility that low-level intermittent exposures to commercial OP pesticides like CPF have the potential to induce protracted deficits in information processing and cognitive function. Such deficits could be subsequent to functional changes in brain cholinergic pathways resulting from alterations in bidirectional fast axonal transport.

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Footnotes:

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Legends for Figures

Fig 1. Effect of repeated exposure (injections every other day) to subthreshold doses of CPF on body weight over a 30 day period. Each point represents the mean ± S.E.M. N=12-18 rats/group

Fig 2. A. Open Field Activity**,** on day 8 and day 22 of CPF exposure and on day 7 of a drug free washout period (WO). Top to bottom**:** horizontal activity measured as the number of photobeam breaks/5 min; vertical activity measured as the number of photobeam breaks/5 min; stereotypical movements (repetitive photobeam breaks/5 min). **B.** Forelimb grip strength measured in kg of force. Day 7, day 14, day 21, and day 28 of CPF exposure are depicted as well day 6 and day 13 of a drug free washout period (WO). **C.** Accelerating rotarod performance expressed as time maintained on a rotating bar that accelerated from 4 to 40 rpm over a 5-min period. Day 16, and day 30 of CPF exposure are depicted as well as day 4 and day 14 of a drug free washout period (WO). The bars in each figure represent the mean ± S.E.M. N=12-18 rats/group.

Fig 3. Effects of prior chronic exposure to CPF (i.e., testing beginning day 7 of a drug-free washout period) on a water maze spatial learning procedure. **A**: Hidden platform test (mean ± S.E.M), 2 trials/day over 6 consecutive days of testing. **B**: Daily swim speeds (mean ± S.E.M. cm/sec) during water maze hidden platform trials **C**: Water maze probe trials (mean platform area crossings ± S.E.M.) conducted on day 14 after the last day of a 30 day exposure period to CPF or vehicle. * = significantly different from vehicle controls ($p<0.05$). N=12-18 rats/group.

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Fig 4. Effects of prior chronic exposure to CPF (i.e., testing on days 2-5 of a drug free washout) on the performance of a spontaneous novel object recognition task. The illustrations at the left indicate the preference for the novel object compared with the familiar object (*= $p<0.05$) at each of the 3 delays. The insets at right illustrate drug effects on the "Recognition Index" which refers to the proportion of the total exploration time the animal spent investigating the novel object (see Methods); $^{+}$ = significantly different ($p<0.01$) from vehicle control performance. Data are expressed as the mean ± S.E.M. N=12-18 rats/group

Fig 5. **A**: Effects of prior chronic exposure to CPF (i.e., testing on day 13 of a drug free washout) on the percentage of prepulse inhibition (PPI) in rats for three prepulse intensities (5, 10, and 15 dB above background). **B**: CPF effects on the mean startle amplitude to 120-dB, 20-ms noise burst. **C**: CPF effects on the percentage of prepulse inhibition averaged across the three prepulse intensities. Bars represent mean ± S.E.M. for each treatment. * = significantly different from vehicle controls ($p<0.05$). N=12-18 rats/group.

Fig 6. Plasma levels of chlorpyrifos (**A**) and its non-toxic metabolite TCP (**B**) during a treatment regimen in which chlorpyrifos was administered s.c. on alternate days over a 30 day period. The regimen was followed by a 14 day wash-out period. The arrows indicate days during which chlorpyrifos was administered prior to removing the blood sample for analysis. Each value represents the mean ± S.E.M. derived from 6 rats.

Fig 7. A. Plasma cholinesterase (ChE) activity as a % of control during a treatment regimen in which chlorpyrifos (CPF) was administered s.c. on alternate days over a 30 day period. The

regimen was followed by a 14 day wash-out period. The arrows indicate days during which chlorpyrifos was administered prior to removing the blood sample for analysis. Each value represents the mean ± S.E.M. derived from 5-6 rats. **B**. The data for chlorpyrifos, TCP levels, and cholinesterase activity (over time) is normalized with respect to the respective maximal change for each parameter. The data are derived from animals that received the 18 mg/kg alternate day regimen of chlorpyrifos plus the two-week washout period.

Fig 8. Brain cholinesterase (ChE) activities measured in 6 brain regions 14 days after the last CPF administration. Each value represents the mean ± S.E.M. derived from 5-6 rats. *$p<0.05$ with respect to vehicle control mean.

Fig 9. The expression of neural protein markers measured in 6 brain regions derived from rats that had completed the 18 mg/kg alternate day regimen of chlorpyrifos plus the two-week washout period. Each value represents the mean ± S.E.M. derived from 5-6 rats. *$p<0.05$ with respect to vehicle control mean. Tropomyosin-receptor kinase A (TrkA); phosphorylated-TrkA (p-TrkA); vesicular acetylcholine transporter (VAChT); high affinity choline transporter (CHT); α_7 nicotinic acetylcholine receptor (α_7 nAChR); p75 neurotrophin receptor ($p75^{NTR}$)

Fig 10. A. Anterograde and retrograde axonal transport assessed *ex vivo* in sciatic nerves obtained from rats on experimental day 30 (one day after the last administration) of the 18 mg/kg chlorpyrifos regimen. **B.** Anterograde and retrograde axonal transport assessed *ex vivo* in sciatic nerves obtained from rats on experimental day 43 (last day of a 14 day drug-free washout) of the 18 mg/kg chlorpyrifos (CPF) regimen. **C.** Temporal effect of a single chlorpyrifos (18.0 mg/kg)

injection on fast anterograde transport. **D.** Temporal effect of a single chlorpyrifos (18.0 mg/kg) injection on fast retrograde transport Each value represents the mean ± S.E.M. derived from 3-5 rats. In Figs A and B, *$p<0.05$ with respect to vehicle control mean; in Figs C and D, *$p<0.05$ with respect to vehicle injected controls at 24 hours post injection.

Table 1. Rat Testing Protocol

Cohort	Group	N	Treatment	Procedures Conducted
1	A	12	VEH	Motor Function, OR, WM, PPI
	B	12	CPF 2.5	Motor Function, OR, WM, PPI
	C	12	CPF 10.0	Motor Function, OR, WM, PPI
	D	12	CPF 18.0	Motor Function, OR, WM, PPI
2	E	6	VEH	Motor Function, OR, WM, PPI, ELISA
	F	6	CPF 18.0	Motor Function, OR, WM, PPI, ELISA
3	G	6	VEH	Plasma and Brain ChAT and ChE; plasma and brain CPF and TCP
	H	6	CPF 2.5	Plasma and Brain ChAT and ChE; plasma and brain CPF and TCP
	I	6	CPF 10.0	Plasma and Brain ChAT and ChE; plasma and brain CPF and TCP
	J	6	CPF 18.0	Plasma and Brain ChAT and ChE; plasma and brain CPF and TCP
4	K	11	VEH	AXT Studies
	L	22	CPF 18.0	AXT Studies

VEH, vehicle; CPF, chlorpyrifos; Motor Function (open field, rotarod, and grip strength); OR, object recognition, WM, water maze, PPI, prepulse inhibition; ELISA (enzyme linked immunosorbant assays for brain neurotrophin and cholinergic proteins) ChAT, choline acetyltransferase activity, ChE, cholinesterase activity; TCP, 3,5,6-trichloro-2-pyridinol; AXT = axonal transport

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Table 2. Brain chlorpyrifos (CPF) and TCP levels two weeks after discontinuation of the chronic CPF regimen.

	ng/g Brain Tissue			
	CPF	**TCP**	**CPF**	**TCP**
	Vehicle		**2.5 mg/kg**	
	ND	ND	1.06	ND
	ND	ND	ND	ND
	ND	ND	ND	ND
	ND	ND	ND	ND
	ND	ND	ND	ND
	ND	ND	ND	ND
	10 mg/kg		**18 mg/kg**	
	1.67	ND	3.49	ND
	3.86	ND	5.83	1.13
	2.66	ND	7.99	ND
	3.94	ND	6.09	ND
	1.93	ND	2.03	ND
	2.18	ND	4.46	ND
Average	**2.71**		**4.98**	
SEM	**0.40**		**0.86**	

ND= Not Detected

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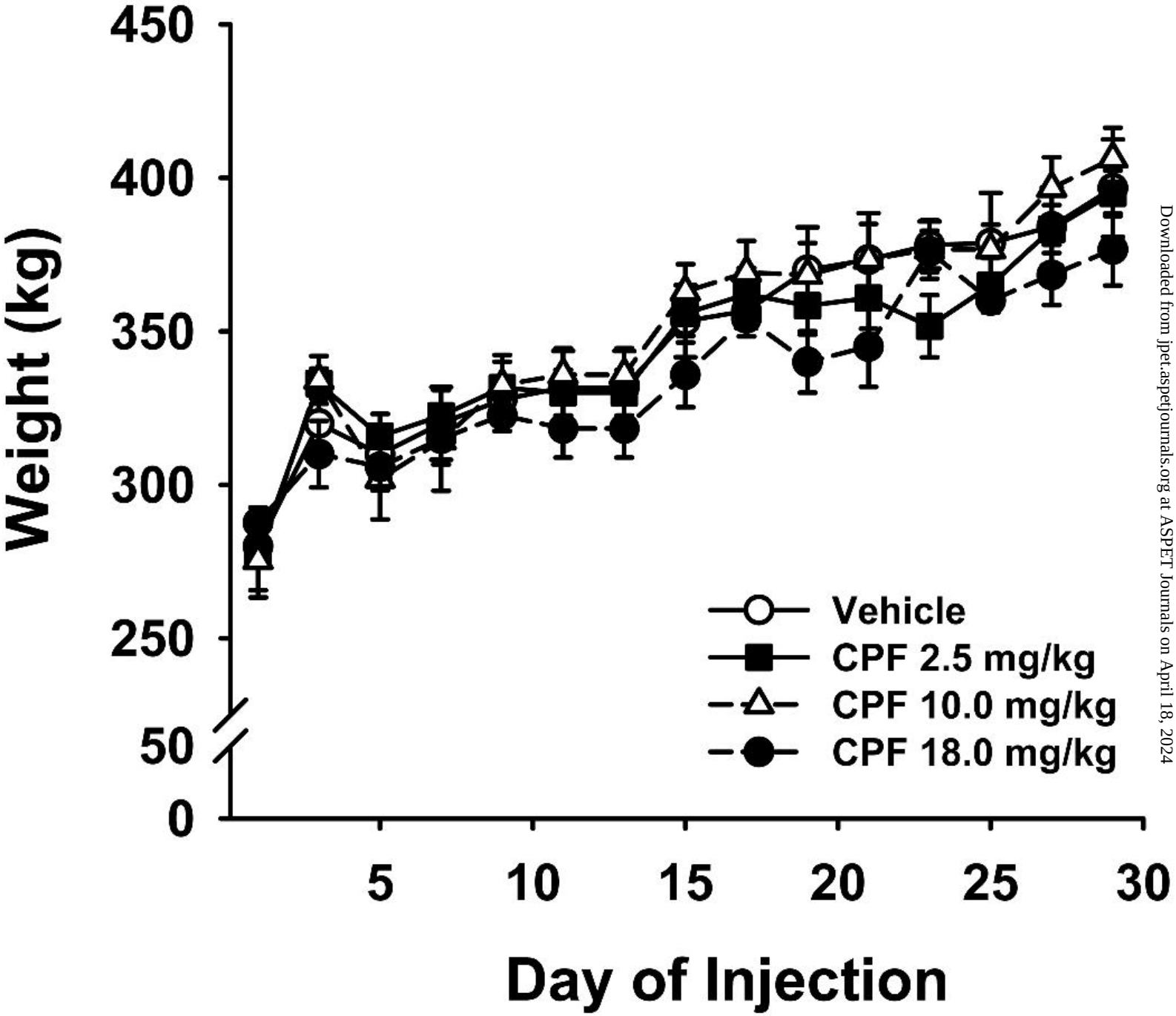
Fig 1



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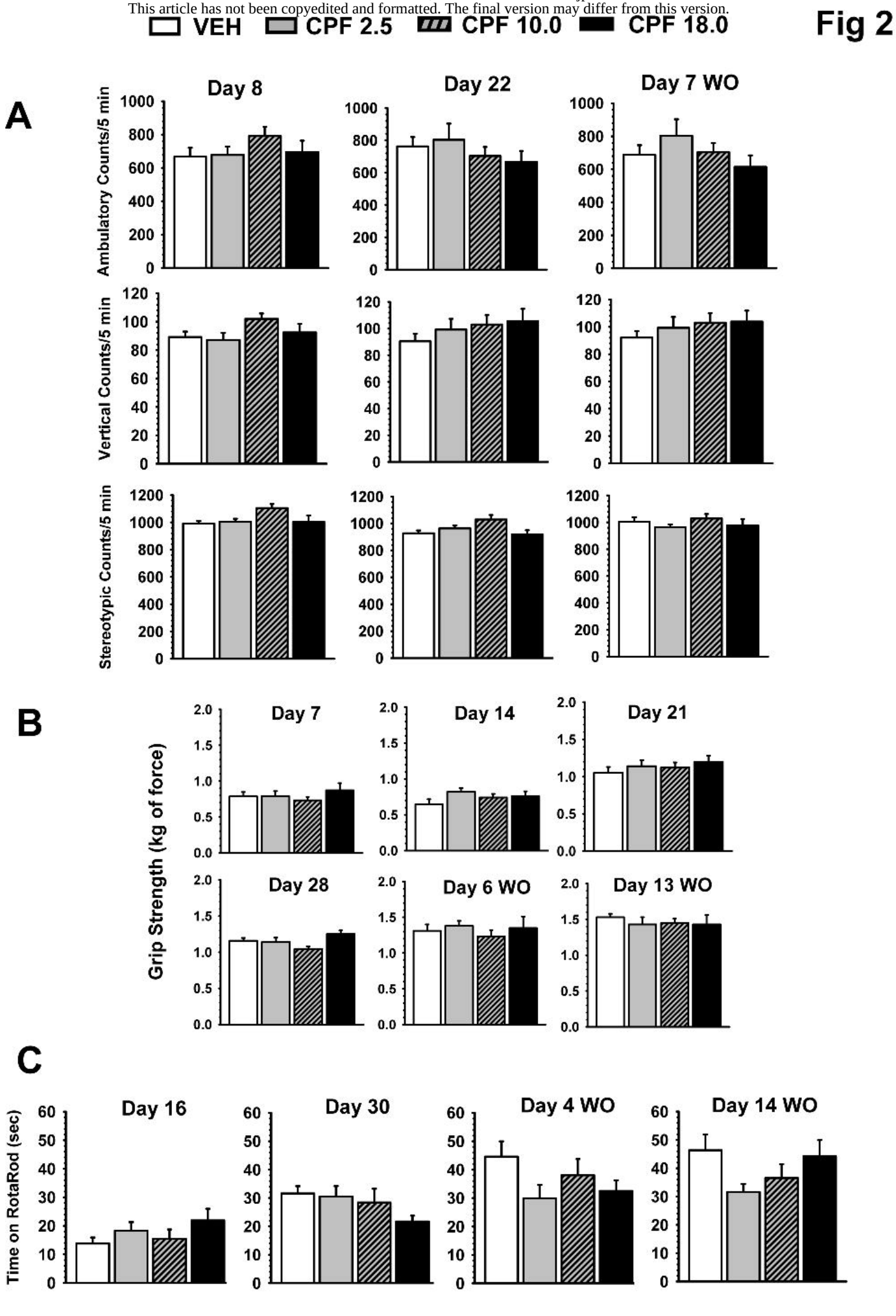

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Fig 3

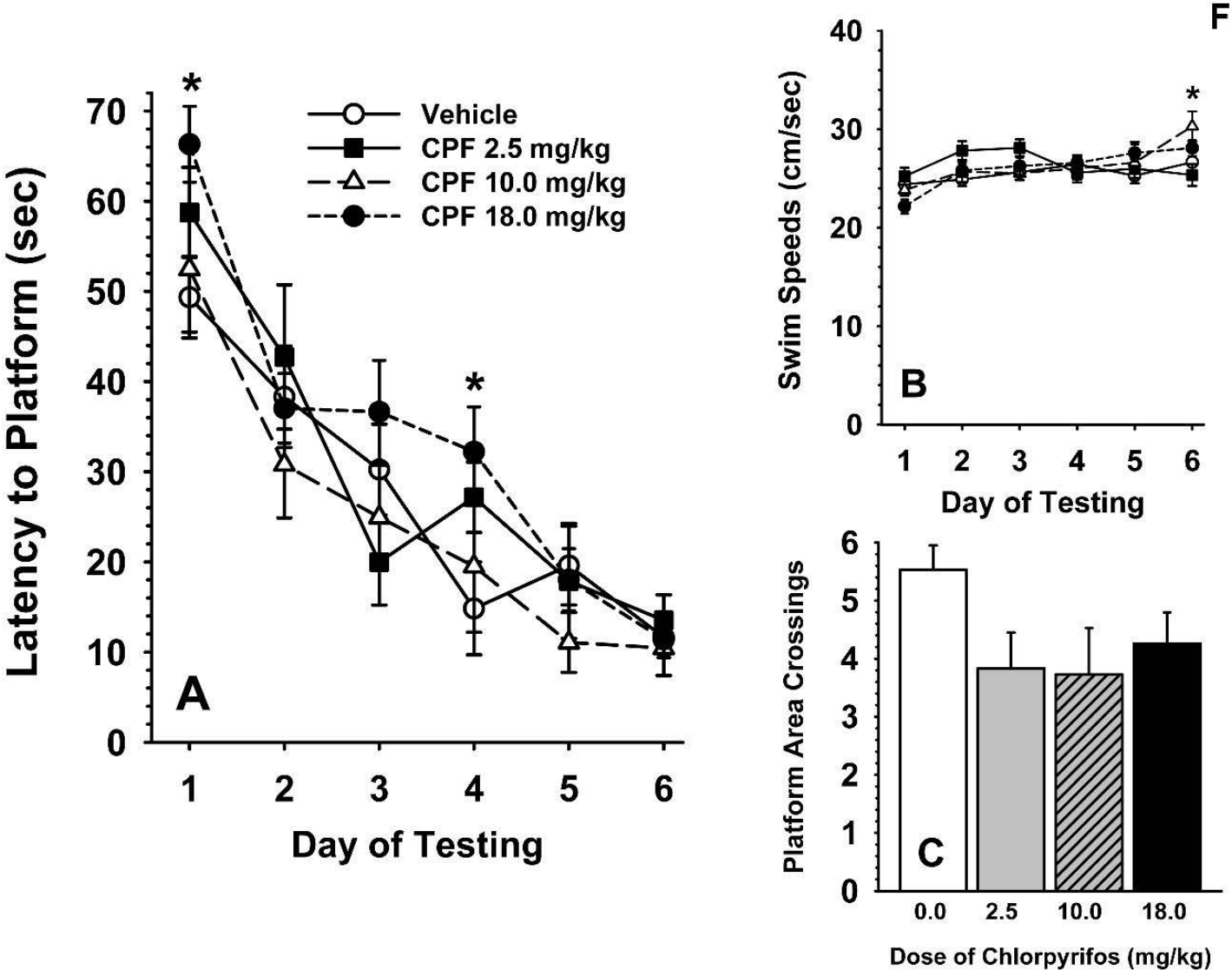

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Fig 4

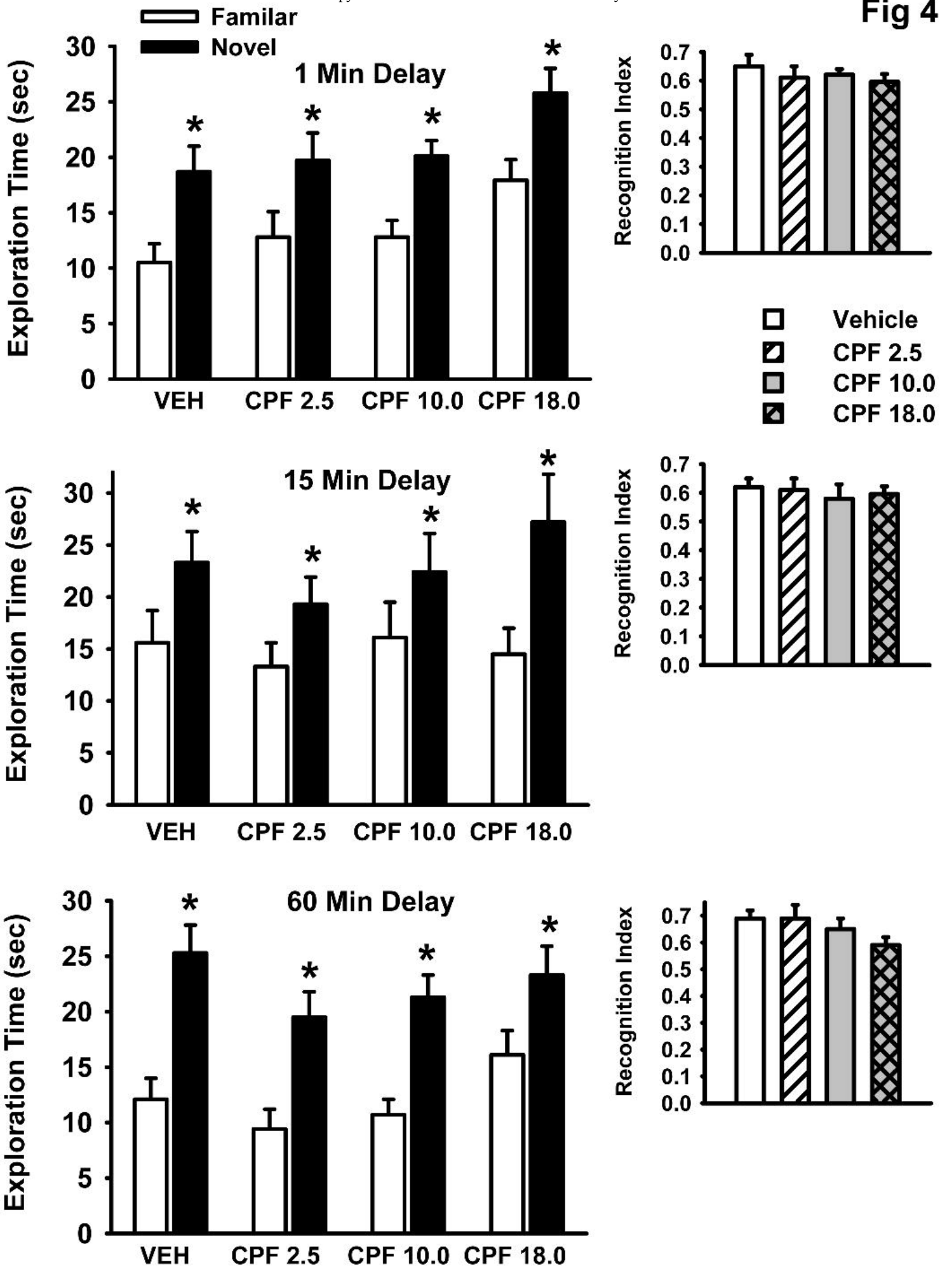

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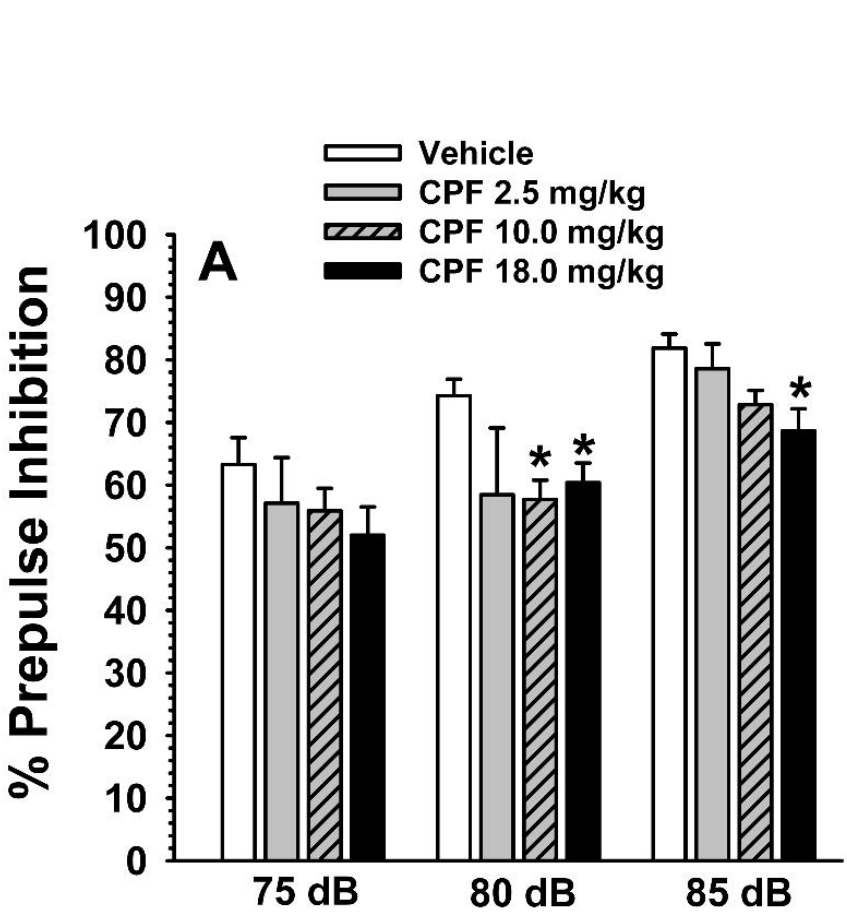
Fig 5



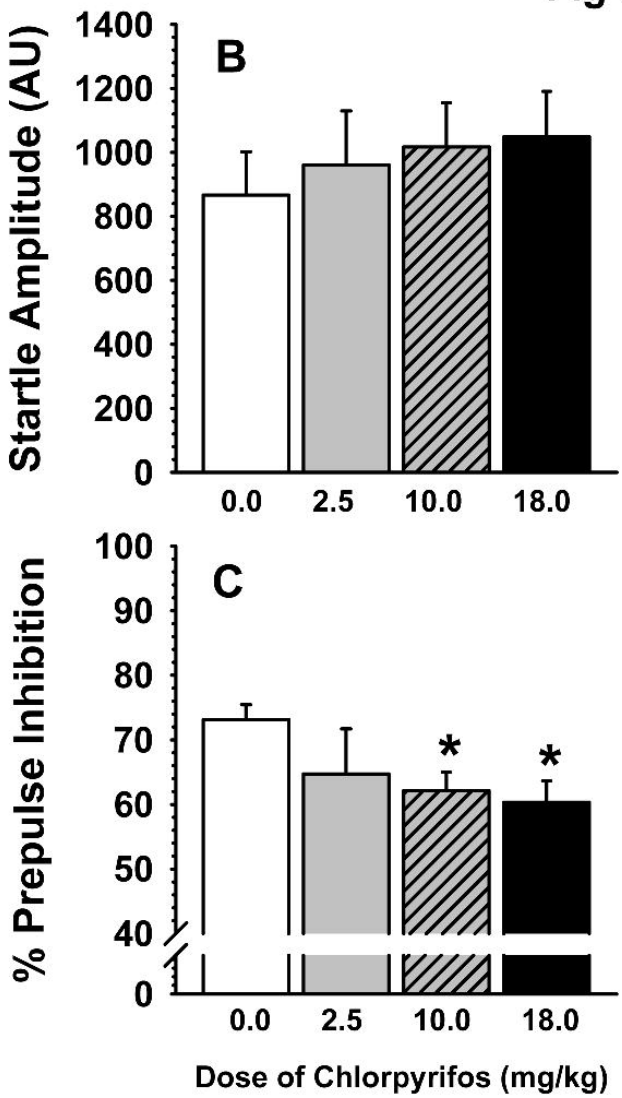


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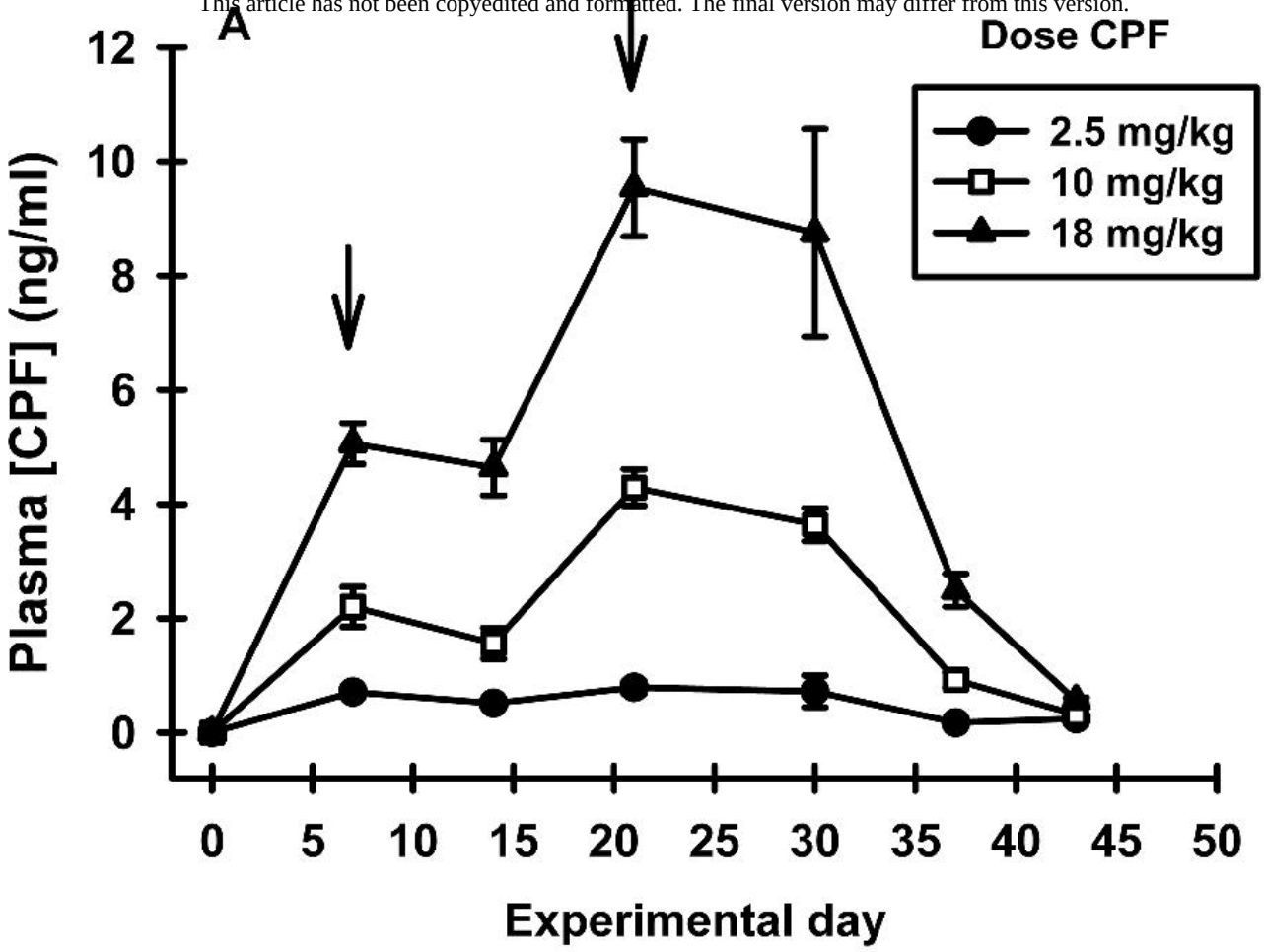
Fig 6



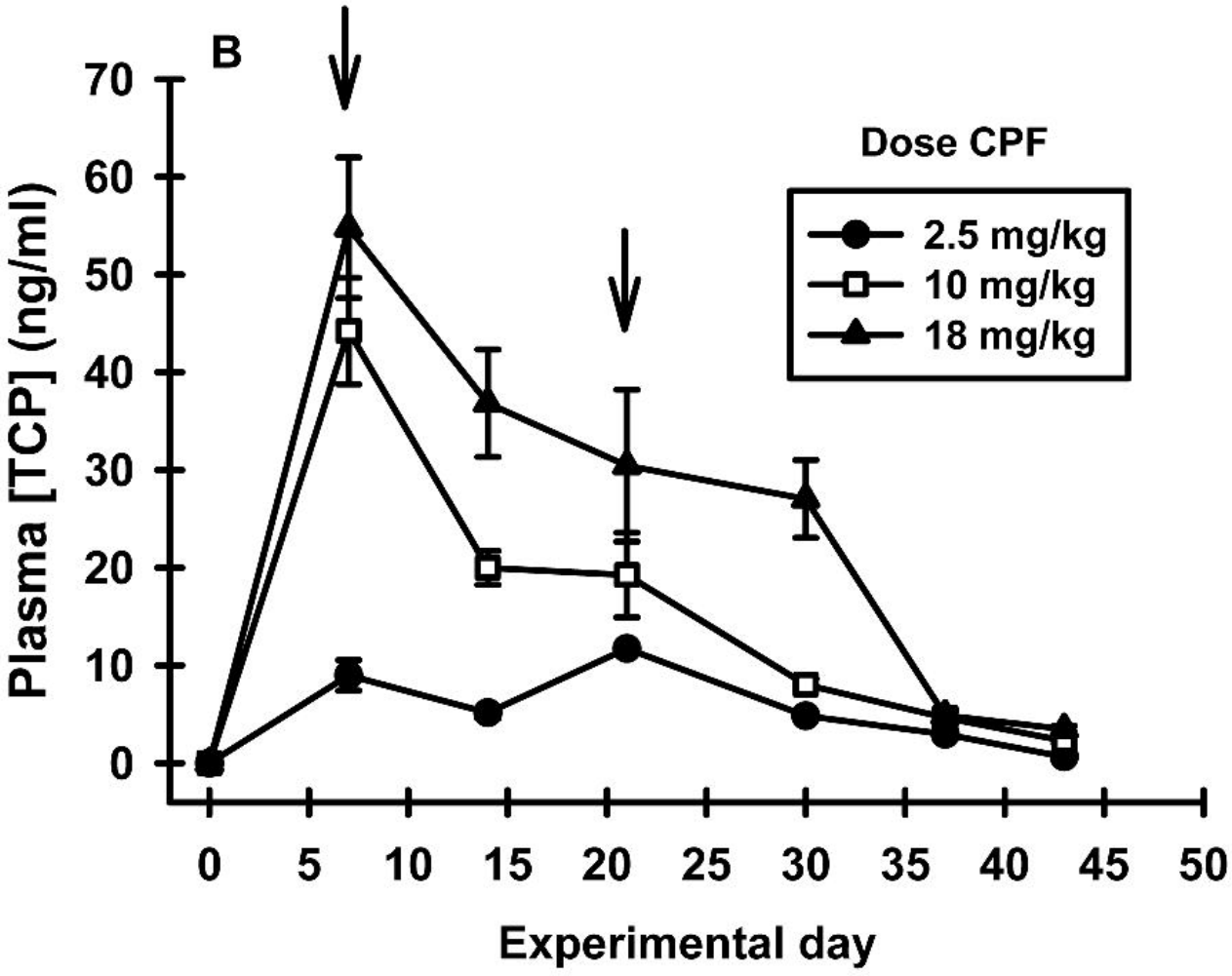


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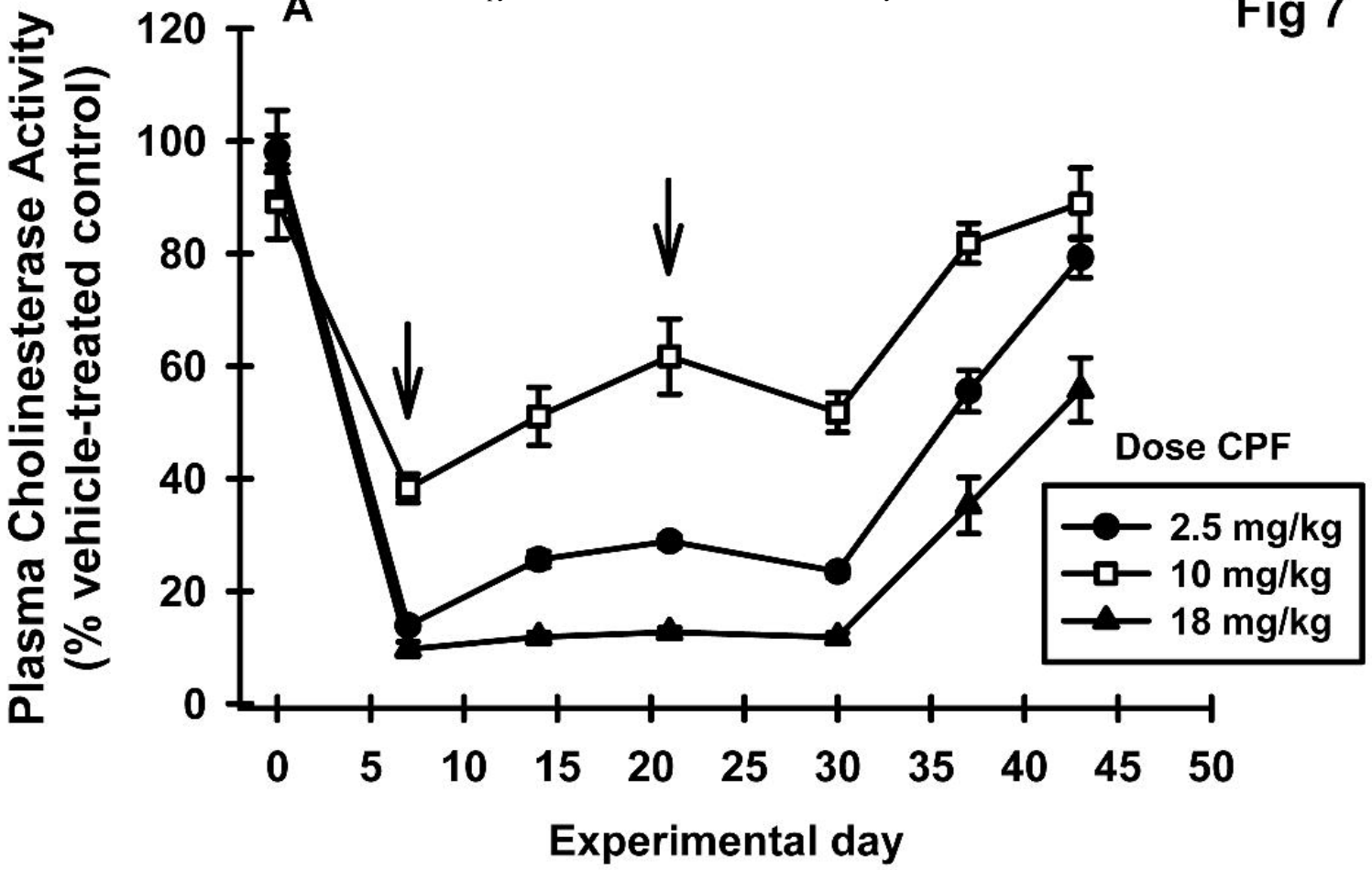
Fig 7



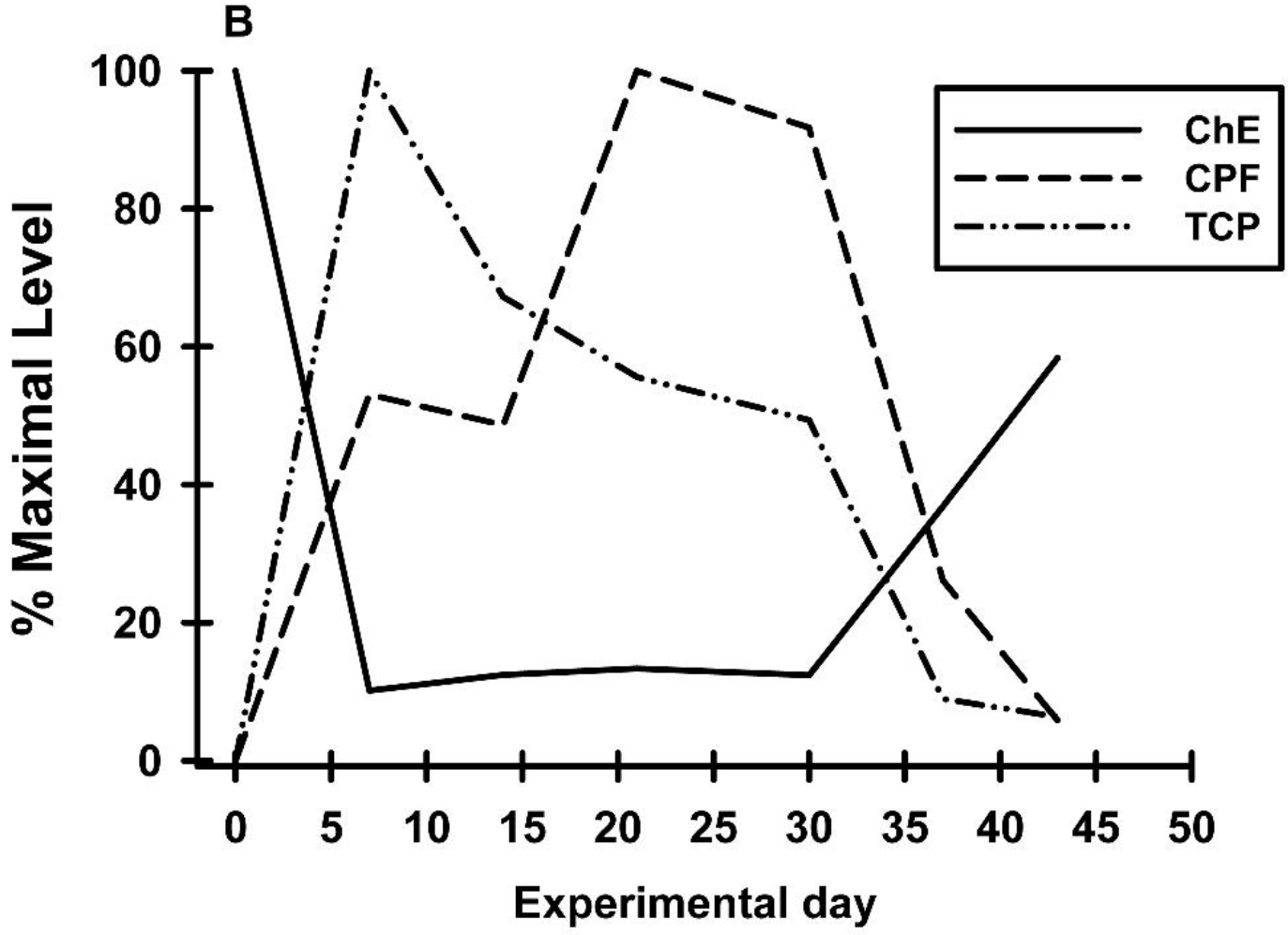


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Fig 8

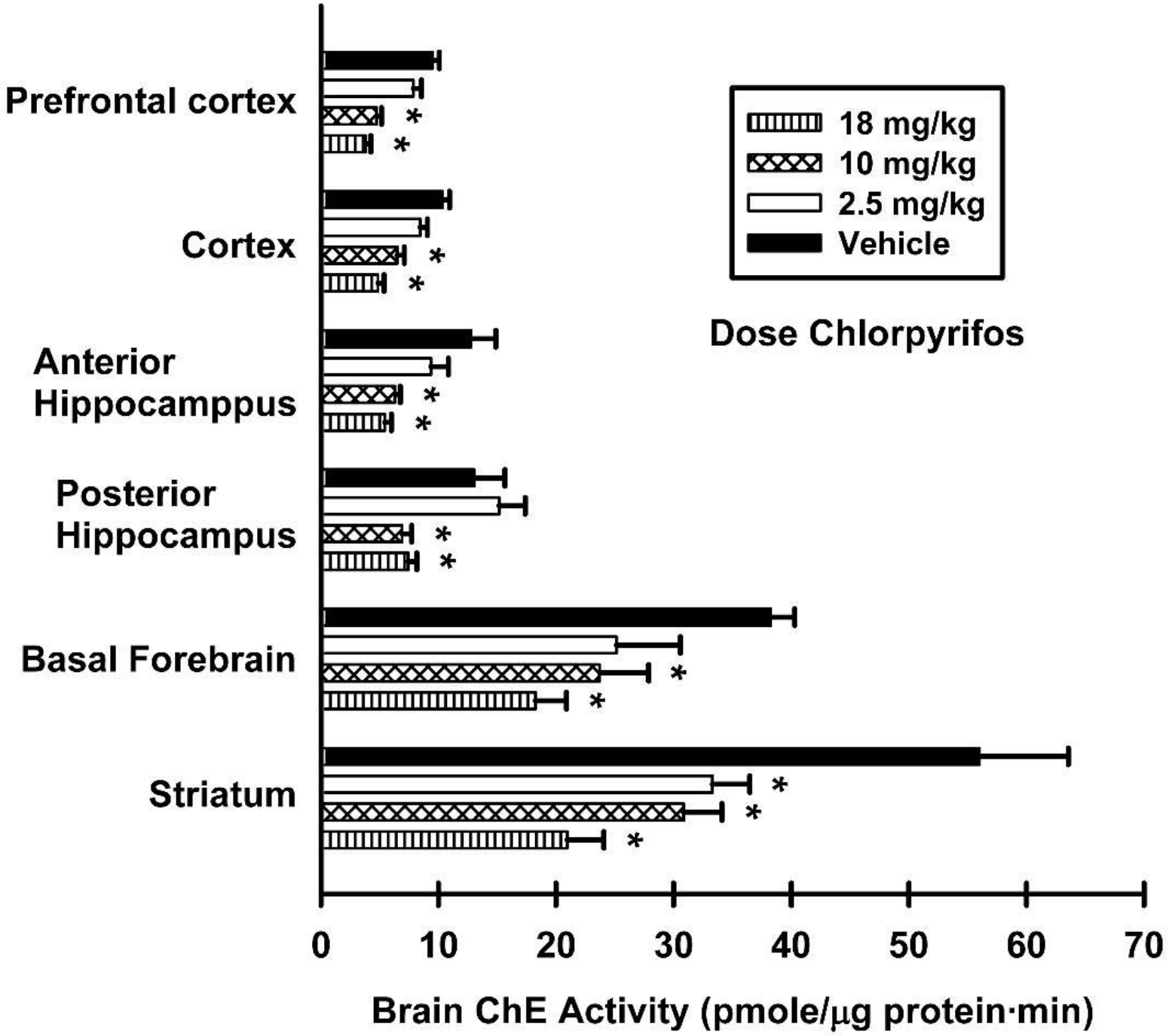

Fig 9

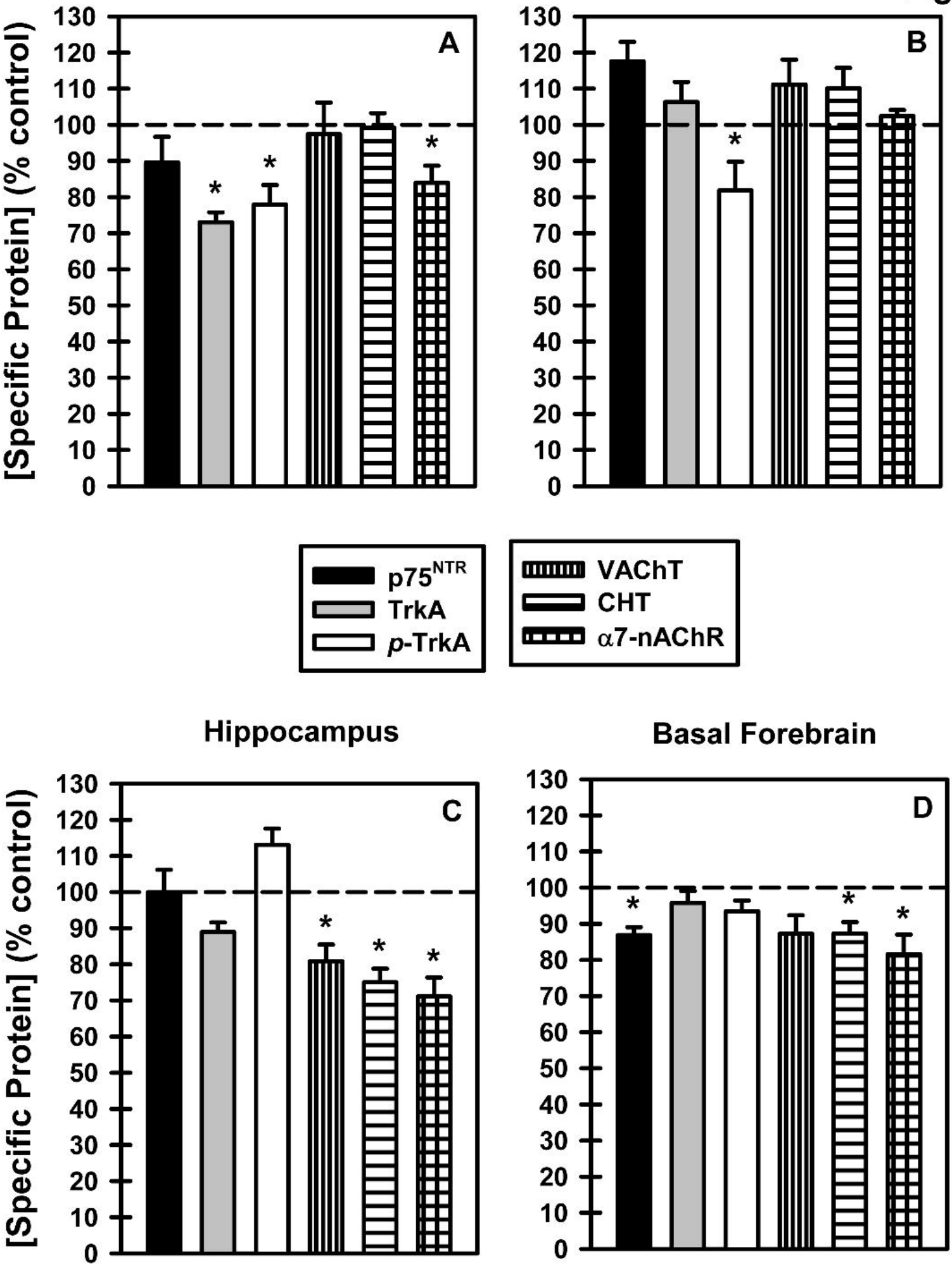

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Fig 10