Repeated Exposures to Subthreshold Doses of Chlorpyrifos in Rats: Hippocampal Damage, Impaired Axonal Transport, and Deficits in Spatial Learning

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Abstract

Organophosphorus (OP) compounds are detectible in the environment for years after use and endanger many of the world’s populations. Whereas the effects of acutely toxic doses of many OPs are well described, much less is known about repeated, low-level exposures. The purpose of these studies was to further evaluate potential toxicological effects of the extensively used OP pesticide, chlorpyrifos (CPF) in rats. CPF, across a range of subthreshold doses (i.e., for acute toxicity), reduced rearing and sniffing activity and the magnitude of weight gain over 14 days of repeated exposure. Performance in a spatial learning task was impaired after 14 days of exposure to CPF (18.0 and 25.0 mg/kg) when testing was initiated 24 hours after the last injection, but not after a 14-day washout. However, inhibition of both fast anterograde and retrograde axonal transport was observed for up to 20 days after exposure to 25.0 mg/kg CPF. Studies using hippocampal cultures indicated that eight days of continuous exposure to the parent compound, CPF (≥100 micromolar), resulted in cell toxicity and death. Furthermore, a dose (2.5 mg/kg) of CPF which had no effects on weight gain or memory performance administered 5 days per week over 38 days impaired forelimb grip strength in the later days of testing. Collectively, these results indicate that repeated exposures to subthreshold doses of CPF may lead to growth retardation, behavioral abnormalities, and muscle weakness. Some of these symptoms may be attributed to effects of the OP on axonal transport.
A dramatic increase in the agricultural, industrial, and household use of pesticides over the past several decades (US EPA, 1999) has paralleled impressive improvements in farming productivity (US General Accounting Office, 1995), the control of debilitating vector-borne diseases (e.g., malaria, yellow fever, viral encephalitis, typhus, etc.), and “nuisance” pests (e.g., flies, roaches, ants, mosquitoes, etc.) in households, schools, and office buildings (Casarett and Doull, 1991). A consequence of such widespread use, however, is that pesticide residues are now among the most ubiquitous synthetic chemicals in our environment, detectable in the tissues of humans worldwide (Weiss, 1997). Accordingly, inherent dangers to the public health and environment persist, since no pesticide is completely innocuous and all carry significant toxicological risks. In fact, the most recent statistical estimates indicate that a minimum of three million pesticide poisonings occur annually resulting in up to 220,000 deaths worldwide (Jeyaratnam, 1990).

Of the wide variety of pesticide agents available, organophosphate (OP) and carbamate insecticides are the chemicals most commonly used in the US (Aspelin, 1994). However, because of the relative frequency of use and level of toxicity of the former, approximately 80% of hospital admissions associated with pesticides are the result of OP exposure (Litovitz, 1991). Nevertheless, many exposures, especially if they do not require hospitalization, undoubtedly go unreported (Weiss, 1997) and as a consequence, little is known about the long term adverse effects of chronic or repeated (subthreshold) exposures to OPs.

The broad spectrum OP, chlorpyrifos, O,O-di O-3,5,6-trichloropyridinyl phosphorothioate (CPF), is one of the most extensively used agricultural pesticides in the world. A study published in 1995 (based on measurements in 1000 individuals) indicated that up to 82% of US adults had detectible levels of the CPF metabolite, (3,5, 6-tricloro-pyridinol) present in their
urine (Hill et al., 1995). It should be noted that due its wide-spread use and concerns regarding evidence of developmental and neurobehavioral anomalies in young animals exposed to CPF, recent restrictions on the use of the agent in households (and certain other environments) have been implemented in the US (EPA Administrator’s Announcement, 2000).

The extensive use of CPF over the years has been attributed to evidence that it exhibits only moderate acute toxicity in many mammalian species, and a greater inhibitory potency for acetylcholinesterase (AChE) than for neurotoxic esterase. Clinical and experimental data indicate that OP induced delayed neuropathies (OPIDN) resulting from acute exposures to CPF require doses well in excess of the LD$_{50}$ and studies in hens show that subchronic exposures at the maximum tolerated daily dose do not result in OPIDN (reviewed, Richardson, 1995). However, recovery from CPF toxicity when it does occur is unusually slow even when compared to other phosphorthioates. Further, CPF has been shown to produce learning deficits in rats after acute and repeated administration (Bushnell et al., 1994; Cohn and Macphail, 1997), and to produce delayed sensory neuropathies in humans (Kaplan et al., 1993). Moreover, while CPF is well known to inhibit AChE activity, the degree of inhibition does not correlate well with the onset of toxicity or the amount of exposure (ATSDR, 1997). These findings support new inquiries into additional mechanisms of CPF toxicity in mammalian systems.

Several lines of evidence suggest that one mechanism underlying the delayed neurotoxicity associated with some OPs is the inhibition of axonal transport (AXT). OPs that produce delayed neurotoxicity (at high doses) cause accumulations of tubulovesicular profiles within axons prior to degeneration (Abou-Donia and Lapadula, 1990), a pathology that is consistent with the stagnation of membrane traffic (Chretien et al. 1981; Souyri et al., 1981). In one study, fast anterograde AXT was reduced by phenylphosphonothioate esters and tri-o-cresyl phosphate, but
not by the non-neurotoxic agent, parathion (Reichart et al., 1980). A progressive deficit of retrograde AXT was associated with the neurotoxicity of di-n-butyl dichlorvos in another study (Moretto et al., 1987). It is important to note, however, that none of these studies evaluated the so-called “moderately toxic” or “non-neuropathic” Ops (such as CPF) in a repeated low-level exposure paradigm.

The purpose of this study was, therefore, to evaluate the behavioral and neurochemical effects of repeated, subthreshold doses of CPF in rats. We assessed the effects of CPF on spatial learning in a water maze task, and on both fast anterograde and retrograde axonal transport ascertained ex vivo by video enhanced-differential interference contrast microscopy. Additional in vitro experiments were designed to assess the effects of extended CPF exposure on the viability of cells in the hippocampus, a structure known to contribute significantly to spatial learning.
Materials and Methods

In Vivo Studies

Test Subjects. Male albino Wistar rats (Harlan Sprague-Dawley, Inc.) approximately 3 months old (weighing 250-350 grams) were housed individually in stainless steel mesh cages in a temperature controlled room (25°C) with free access to food (NIH-07 formula) and water, and maintained on a 12-hour light/dark cycle. 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.

Drug Regimens: Each experimental group received daily subcutaneous injections of peanut oil (vehicle) or CPF dissolved in peanut oil in a volume of 1.0 ml/kg body weight for the time period specified below. Please refer to Table 1 for a summary of the experimental manipulations in all test groups.

Plasma Cholinesterase Activity: Rats (see Table 1) were injected subcutaneously with CPF in doses ranging from 2.5 to 100 mg/kg dissolved in peanut oil. Twenty-four hours later the animals were killed by decapitation and trunk blood was immediately collected into heparinized tubes. Plasma was separated from erythrocytes by centrifugation (4000 rpm, 15 min, 4°C) and immediately assayed spectrophotometrically using a modification of a method described in detail previously (Prendergast et al., 1998). Briefly, 100 µl plasma samples were added to 24 well plates containing the reaction mixture (7.5 nM acetylthiocholine iodide substrate and 6.9 mM dithiobisnitrobenzoic acid in phosphate buffer at pH 7.9). Absorbance at 412 nm was recorded at 25°C for 4 min with a μQuant Universal Microplate spectrophotometer, BioTek Instruments, Inc., Winooski, VT. Protein concentrations were also measured spectrophotometrically using the Bio-Rad Protein Assay (Richmond. Calif., USA) system with bovine serum albumin as
standard. Data were expressed as µmol substrate hydrolyzed/min/mg protein and the levels of enzyme activity for each CPF dose relative to vehicle control levels was determined.

Observational Data: During all repeated exposure experiments, test subjects were placed in a clear polypropylene tub (25x45x25 cm) every 1-2 days and monitored for signs of OP toxicity in the morning before the daily drug injection (i.e., approximately 24 hr after the previous day’s injection). After a 5-min acclimation period, rearing and sniffing movements were recorded for 20 min. In addition, animals were weighed and the occurrence and/or frequency of other indicators of OP toxicity were recorded, including: tremor, salivation, diarrhea, lacrimation, and urination. At the end of the 20-min observation period, animals were injected with CPF or vehicle and returned to their home cages.

Water Maze Experiments: Testing Apparatus. Maze testing was performed beginning at either one day (24 hours) or 14 days after two weeks of repeated (daily) exposure to CPF. Testing was conducted in a circular pool (diameter: 180 cm, height: 76 cm) made of plastic (Bonar Plastics, Noonan, GA) with the inner surface painted black as described in detail previously (Terry, 2000). The pool was located in a large room with a number of extra-maze visual cues including highly reflective geometric images (squares, triangles circles etc.) hung on the wall. Diffuse lighting, and black curtains were used to visually isolate the test rat from the experimenter and resting rats. The pool was filled with water (maintained at 25.0 ± 1.0°C) to a depth of 35 cm. An invisible (black) 10 cm square platform was submerged approximately 1.0 cm below the surface of the water and placed in the center of the northeast quadrant. Swimming activity of each rat was monitored with a video camera mounted overhead, which relayed information including latency to find the platform, total distance traveled, time and distance spent in each quadrant etc. to a video tracking system (Poly-Track, San Diego Instruments, San
Tracking was accomplished via visualization and monitoring the movements of a white rat on a black background.

Hidden Platform Test: Each rat was given four trials per day for four consecutive days. On days 1-4, a trial was initiated by placing the rat in the water facing the pool wall in one of the four quadrants (designated NE, NW, SE, SW). The daily order of placement into individual quadrants was pseudo-randomized such that all 4 quadrants were used once every day. For each trial, the rat was allowed to swim a maximum of 90 seconds, in order to find the hidden platform. When successful, the rat was given a 30 second rest period on the platform. If unsuccessful, the rat was given a score of 90 seconds and then placed on the platform for 30 seconds. In either case, the rat was given the next trial approximately one minute (intertrial interval = 60 sec) after the rest period.

Probe Trials- On day 5, probe trials (transfer tests) were conducted in which the platform was removed from the pool to measure “spatial bias” (Morris 1984) for the previous platform location. This was accomplished by measuring the time spent and the distance traveled in each of the 4 quadrants and expressing each as the percent of the total. The number of crossings over the previous 10 cm square platform location was also determined.

Visible Platform Test- Immediately following probe trials on day 5, the platform was reintroduced to the pool in the quadrant diametrically opposite the original position (SW quadrant) with a highly visible (i.e., light reflective) cover attached to the platform which was raised above the surface of the water (approximately 1.5 cm). Room lighting was changed such that extramaze cues were no longer visible and a spotlight illuminated the visible platform. Each rat was given one trial in order to acclimate to the new set of conditions and locate the platform visually for this trial. A 180 second time limit was used and the trial was repeated if necessary.
until the rat located the platform. The rat was then immediately given a second visible platform trial and the latency to find the platform was recorded as a measure of visual acuity.

**Grip Strength Measurements** - Both forelimb and hindlimb grip strength (measured in kg of resistance) was assessed for each subject in groups 13 and 14 (see Table 1). During this series, 2.5 mg/kg of CPF or vehicle was administered once each weekday for 38 days. Grip strength was measured by using a Digital Grip Strength Meter (Columbus Instruments, Columbus, Ohio). Forelimb strength was measured 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. For measuring hindlimb grip strength, the hind limbs were placed on the tension bar and the rat was pulled toward the meter until it released.

**Ex Vivo and In Vitro Studies**

**Axonal Transport Studies** - The effects of repeated doses of CPF on fast anterograde and retrograde axonal transport were evaluated in single axons of sciatic nerves removed from animals previously exposed to CPF (or vehicle) by direct visualization of vesicle movement using video enhanced-differential interference contrast (AVEC-DIC) microscopy. This procedure has been described in detail previously (Stone et al., 1999). Briefly, rats were anesthetized with 4% chloral hydrate (10 ml/kg), 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 in a custom designed aluminum chamber, between two cover slips, which were sealed in place with VALAP (1:1:1 vaseline, lanolin, and paraffin). Prior to sealing the chamber, the nerve was extended to its original length and the ligatures attached to the bottom of the chamber using VALAP. All
procedures were accomplished with the nerve continually submersed in oxygenated physiologic buffer (in mM: 94 NaCl, 5 KCl, 1.5 CaCl₂, 1.0 MgSO₄, 2.0 Na₂HPO₄, 24 NaHCO₃, 11 glucose, pH 7.4). Axons were viewed through a DIC microscope (Zeiss Axiovert 10 microscope with DIC optics, Hamamatsu C2400-07 camera, Argus 20 video computer, and Hamamatsu high-resolution monitor) with the observation chamber on a 37°C heated stage (Zeis TRZ Model 3700). Video enhancement of the axons was achieved with analogue contrast enhancement (camera controller) and digital contrast enhancement (video computer) with background subtraction. The number of vesicles moving in the anterograde and retrograde directions, which completely traversed a 2 cm square window (drawn directly on the screen of the video monitor), were counted for a 10 min interval.

**Preparation of Hippocampal Cultures** Organotypic hippocampal cultures were prepared according to methods described previously (Prendergast et al., 2001). Briefly, whole brains from 8 day old rat pups were aseptically removed and placed in dissection medium (4°C) made of Minimum Essential Medium (MEM) plus glutamine, 25 mM Hanks buffered salt solution (HBSS) and 25 mM HEPES, 200 mM glutamine, and 50 μM penicillin/streptomycin solution. Bilateral hippocampi were dissected out and placed into culture medium at room temperature. Culture medium was made of dissection medium with the addition of 36 mM glucose, 25% (v/v) HBSS and 25% Heat-Inactivated Horse Serum (HIHS). Each hippocampus was sectioned coronally at 400 μM using a McIlwain tissue chopper and placed into fresh culture medium. Each unilateral hippocampus yielded approximately 12 slices (24 per animal). Three slices were then transferred onto each Millicell-CM 0.4 μM biopore membrane insert in pre-incubated culture medium. Inserts were then placed in 35 mm six-well culture plates and excess medium on top of the slices was aspirated so that explants remained exposed to the atmosphere of 5%
CO₂/95% air. Cultures were kept constantly at 37°C and medium was changed every five days. All culture medium solutions were supplied by Gibco BRL (Gaithersburg, MD) with the exception of HIHS (Sigma, St. Louis, MO).

**Assessment of cytotoxicity**- Hippocampal sections were allowed to acclimate to *ex vivo* conditions for five days then were transferred to wells containing culture medium and the non-vital stain propidium iodide (PI; 2.5 mg/ml) or medium containing PI and one of three concentrations of CPF (10, 100, or 500 µM). Slices were exposed to their respective treatments for eight continuous days. Uptake of propidium iodide was visualized using a 4X objective on a Nikon TE200 microscope (Nikon, Melville, NY) fitted for fluorescence detection (mercury-arc lamp) connected to a personal computer via a CCD (charge-coupled device) camera. Propidium iodide has a peak excitation wavelength of 536 nM and was excited using a band-pass filter exciting the wavelengths between 510-560 nM. The emission wavelength of propidium iodide is 620 nM in the visual range. Intensity of propidium iodide fluorescence was analyzed by densitometry using the image analysis program NIH Image. Optical intensity (in arbitrary optical units) was determined in the CA1 region of each individual slice culture. Comparisons were made between relative intensity of fluorescence in control slices and those exposed to CPF.

**Statistical Analyses**

Comparisons between treatment groups were made using analysis of variance (with repeated measures when necessary) 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 experiments was blind to the treatment group.
Results

**In Vivo Studies**

**Plasma Cholinesterase Activity:** The dose related effects of CPF on plasma cholinesterase activity assayed 24 hours after single injections are illustrated in Fig 1. As indicated, there were dose-related decreases (i.e., dose-effect p<0.001) in cholinesterase activity ranging from approximately 30% inhibition with the 2.5 mg/kg dose to approximately 80% inhibition at 100.0 mg/kg dose.

**Body Weight, Rearing and Sniffing Activity:** The effects of repeated exposures (i.e., 14 days, one injection per day) to several doses of CPF or vehicle (see Table 1) on body weight and rearing and sniffing activity were assessed during 6 individual observation sessions (i.e, every 1-2 days) during dosing (see Fig 2). Rats exposed to the 50 or 100 mg/kg daily dose of CPF developed significant tremors and other signs of OP toxicity (salivation, diarrhea) by the third to fourth observation session, and were thus removed from the study and not evaluated further. These doses had initially been included for the higher end of the dose-effect range based on levels of plasma cholinesterase inhibition of roughly 60 and 80% (respectively) observed 24 hours after injection (Fig 1). The effects of these doses on body weight and rearing and sniffing activity prior to the appearance of overt signs of OP toxicity are presented in Fig 2 and 3, but were not included in the statistical analysis. As indicated in Fig 2, all animals exposed to CPF or vehicle (excluding the 50 and 100 mg doses) gained significant weight over the course of the 14 days of injections [i.e., observation-day effect, F(5,20)=277.5, p<0.001]. There was a highly significant dose-related effect of CPF on body weight over the course of the injections, however [i.e., all doses except 2.5 mg/kg decreased the overall magnitude of weight gain when compared to vehicle, dose effect F(4,57)=3.9, p<0.01, dose by observation day interaction, F(20,283)=3.0,
All doses including the 2.5 mg/kg dose of CPF resulted in significant decreases in rearing and sniffing activity across the 14 days of injections (Fig 3), dose effect, $F(4,57)=26.7$, $p<0.001$, observation day effect, $F(5,20)=10.6$, $p<0.001$, dose by observation day interaction, $F(20,285)=4.0$, $p<0.001$.

**Water Maze Testing - 24 Hour Washout**

**Hidden Platform Test**- The latencies and swim distances required to locate a hidden platform in the water maze beginning 24 hours after a 14 day regimen of CPF exposure are illustrated in Fig 4. Statistical comparisons of latencies across the 5 groups revealed the following results: dose effect, $F(4,48)=21.7$, $p<0.001$; day effect, $F(3,12)=43.2$, $p<0.001$; dose x day interaction, $F(12,144)=3.1$, $p<0.001$. Similar results were evident when swim distances were analyzed. Thus, after exposure to vehicle or doses of 2.5 or 10 mg/kg of CPF for 14 days, the rats learned to locate the hidden platform with progressively shorter latencies (and swim distances) across the 4 days of training. However, both the 18.0 and 25 mg/kg doses were associated with significant (p<0.05) impairments in performance of the task (i.e., indicated by higher mean latencies and distances swam to locate the hidden platform).

**Transfer Test (Probe Trials)**- The effects of CPF on “spatial bias” are presented in Fig 5 as the percent of time spent and distance swam in the quadrant where the platform had been located during the first 4 days of testing, and the number of crossing over the previous platform location (i.e., the 10 cm square area). All treatment groups preferred the previous target quadrant (as opposed to the other 3 quadrants) as indicated by a greater than chance (i.e., 25%) percentage of time spent and distance swam in this region of the pool (p<0.05, one-tailed t tests). The higher doses of CPF (18.0 and 25.0 mg/kg) were associated with inferior performance of probe trials,
however, as indicated by the reduced number of platform area crossings compared to vehicle controls, F(4,49)=22.1, p<0.02 (Fig 5C). For the percent time and distance measurements the 18.0 mg/kg dose was associated with impaired performance, whereas impairment associated with the 25.0 mg/kg dose did not reach statistical significance (i.e., p>0.05) possibly due to the level of variance.

**Swim Speeds**- Swim speeds (i.e., distance swam in cm/latency to find the platform in sec = cm/sec) were compared daily across the treatment groups for all 5 days of water maze testing and appear in Fig 6. Statistical comparisons revealed significant dose related differences in swim speeds among the treatment groups after 14 days of exposure, dose effect, F(4,48)=4.8, p<0.01; day effect, F(4,16)=18.5, p<0.001; with out a significant dose x day interaction, F(16,192)=1.5, p<0.1. Post hoc analyses indicated that the animals previously exposed to CPF at 18.0 or 25.0 mg/kg swam significantly (p<0.05) slower than did controls.

**Visible platform studies**-the average times required to reach a highly visible (reflective) platform ranged between 12 and 30 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 impaired visual acuity associated with CPF.

**Water Maze Testing-Two week Washout**

Following water maze studies conducted after a 1-day (24 hour) washout from the various treatment groups were completed, we were interested to learn whether residual effects associated with the higher dose of CPF persisted after a longer washout interval. Additional rats (see Table 1) were administered either vehicle or CPF, 25.0 mg/kg daily for 14 days, then given a two week washout and tested in the various water maze procedures. These results of these tests are
presented in a composite figure (Fig 7). There were no statistically significant differences (i.e., all p values were > 0.05) in the hidden platform test, in probe trials, or on swim speeds, indicating that the animals previously treated with 25.0 mg/kg CPF for 14 days had fully recovered (i.e., memory-related performance) after a two-week washout.

**Ex Vivo and In Vitro Studies**

**Axonal Transport Studies**

24 hour washout- The effects of repeated exposures to several doses of CPF on bi-directional, fast axonal transport (*ex vivo*) in peripheral (sciatic) nerve axons after the one day washout and water maze testing (i.e., axons evaluated 6 days after the last CPF injection) are illustrated in Fig 8. Deficits in both anterograde and retrograde transport were observed in the animals given the 10.0, 18.0, and 25.0 mg/kg doses compared to controls [anterograde transport-dose effect, F(4,12)=18.8, p<0.001, retrograde transport-dose effect, F(4,12)=14.4, p<0.001].

14 Day Washout- The effects of CPF 25.0 mg/kg (14 day chronic regimen), after water maze testing and an additional 14 day washout (i.e., 20 days after the last CPF injection), are illustrated in Fig 9. As illustrated, subtle, but significant, deficits in bi-directional fast axonal transport were still observed for up to 20 days after the last CPF injection [anterograde transport-treatment effect, p=0.05, retrograde transport-treatment effect, p=0.03 (t-tests)].

Organotypic Hippocampal Culture Experiments- The cytotoxic effects of CPF on organotypic hippocampal slices are illustrated in Fig 10. At one day and eight days after the initiation of CPF exposure, relative uptake of PI was quantified via fluorescent microscopy as described in the Methods section. Fig 10A provides representative examples of slices (and the level of propidium iodide uptake) under control conditions (media only) compared to slices that
had been exposed to 500 µM CPF (parent compound) for 24 hours or eight days. Fig 10B illustrates the concentration related effects of CPF exposure after one and eight days of exposure. CPF did not markedly alter neuronal viability in slices when examined 24 hrs after the beginning of exposure, indicating the absence of acute toxicity. However, eight days of continuous exposure to CPF (at 100 and 500 µM) produced significant cell death in the CA1 region of the hippocampus. (p<0.05 vs control) as indicated by enhanced uptake of propidium iodide.

Grip Strength - The effects of repeated administration of a subthreshold dose (2.5 mg/kg) of CPF administered 5 days/week over 38 days on grip strength appear in Fig 11. The purpose of this experiment was to determine whether such an extended regimen could lead to impairment of motor function in the absence of other overt symptoms of toxicity. After 4 weeks of treatment, hind limb grip strength declined, [treatment by day interaction, F(140,175)=2.8, p<0.01], (significant on experimental day 39, post hoc analysis, p<0.05) even though weight gain was not different from controls. A similar trend was observed for the forelimb measurements although the treatment by day interaction did not reach statistical significance (i.e., p>0.05). The reductions in grip strength appeared to be reversible after discontinuation (arrow) of the CPF regimen.
Discussion

The initial experiments outlined in this report indicated that all of the doses of CPF examined (even doses as low as 2.5 mg/kg which had only mild effects on plasma cholinesterase and no effects on weight gain) significantly decreased typical exploratory behaviors in rats. These behaviors, namely rearing (i.e., vertical activation) and sniffing activity reflect responses to environmental novelty as well as the emotional state (e.g., stress level) in rodents (Gironi-Carnevale et al., 1990; Sadile, 1996). Rearing responses observed upon repeated exposures to the same environment are strongly influenced by inter-individual differences in habituation learning, a variety of pharmacologic and toxicologic agents, and, interestingly, cholinergic activity in the forebrain and hippocampus (Theil et al., 1998).

Daily doses of 2.5–25.0 mg/kg of CPF for 14 days did not elicit any other overt signs of OP toxicity, however, the two higher doses (18.0 and 25.0 mg/kg) impaired water maze performance and swim speeds for up to 5 days after discontinuation of dosing. The dose-effect relationship for CPF in altering maze performance was very steep with less than one log unit of dosing separating an absence of effect from a near maximal effect. While swims speeds were reduced at the higher doses of CPF, differences in task performance could not be entirely attributed to motor deficits as indicated by the fact that all groups clearly preferred the target quadrant (over the other three quadrants) in probe trials on day 5 and that all groups were able to perform the visible platform task equally well. At 2 weeks after discontinuation of CPF 25.0 mg/kg (in a separate group of animals) all aspects of water maze performance (i.e., latencies to find the hidden platform, swim speeds, and performance of probe trials) were indistinguishable from vehicle controls, indicating a full recovery of memory-related behavioral function.
The next series of experiments were performed in order to assess the effects of CPF on axonal transport. This impetus for these experiments came from the results of previous studies in which repeated, subthreshold exposures to the OP acetylcholinesterase inhibitor, diisopropylfluorophosphate (DFP) impaired working memory in non-human primates as well as spatial learning in rats (Prendergast, et al., 1997; Prendergast, et al., 1998) that persisted for up to 21 days after drug discontinuation. The DFP regimen also resulted in a protracted decrease in the expression of central nicotinic and muscarinic-acetylcholine receptors (Stone et al., 2000). The reason for this delayed recovery of cholinergic receptors was unclear, but was hypothesized to result from direct or indirect interactions of the OP with microtubular transport proteins. We formulated this hypothesis based on previous reports of accumulations of tubulovesicular profiles within axons exposed to neurotoxic doses of OPs (Abou-Donia and Lapadula, 1990). We predicted that similar processes might occur with repeated exposures to lower OP doses or agents considered non-neuropathic such as CPF.

In the present study, both anterograde and retrograde axonal transport (measured in sciatic nerves derived from study animals) was impaired at 6 days after discontinuation of the 14-day, 25.0 mg/kg CPF regimen thus supporting the water maze deficits in the first series of experiments. Surprisingly, measurable deficits in axonal transport persisted for up to 20 days after discontinuation of the 14 day, 25.0 mg/kg regimen even though water maze task performance appeared normal. These findings suggest that there is a threshold level of axonal transport impairment before water maze deficits are detectible, but also suggest that longer times of exposure to CPF (i.e., greater than 14 days) could, in fact, result in more prolonged (and adverse) cellular and perhaps behavioral effects.
As indicated above, the residual effect (through 5 days after CPF discontinuation) of CPF was associated with substantial impairments of the water maze task, a learning paradigm dependent upon intact hippocampal function (Morris et al., 1982). This finding, and similar findings after DFP administration, led to the next experimental series to determine whether CPF could induce cytotoxic effects directly on hippocampal cells (see below). Although the oxone metabolite of CPF is considered the active moiety in vivo due its inhibitory effects on acetylcholinesterase activity, we were interested to determine whether the parent compound itself might also be cytotoxic. Few studies have been designed to study the direct neurotoxic properties of CPF even though there are a number of published reports that would support such experiments. For example, while both the CPF parent compound and its principal metabolites are eliminated relatively rapidly in humans, (i.e., metabolized and then eliminated primarily through the kidneys-U.S. National Library of Medicine, 1995), studies in rats (National Academy of Sciences, 1982) and other mammalian species (Gallo and Lawryk, 1991) indicate that CPF is redistributed to adipose tissue forming a depot for slow release. Thus, the OP may be retained for longer periods than is evident using plasma measurements. Furthermore, when CPF was fed to cows, unchanged pesticide was found in the feces, but not in the urine or milk (U.S. EPA, 1984). However, it was detected in the milk of cows for 4 days following spray dipping (Gallo and Lawryk, 1991).

Organotypic hippocampal cultures were selected for experiments designed to investigate the direct effects of CPF on the hippocampus for several reasons. The preparations retain the connective neuronal organization found in situ, but yet are readily accessible to experimental manipulation, and are quite sensitive to pathological insults (reviewed, Bahr, 1995). Cellular damage/death in hippocampal slices can be assessed by incubation of slices with the nucleic acid
stain propidium iodide (PI) since uptake by hippocampal cells of PI is observed only after lysis of cells. The use of this method for detecting cytotoxicity has wide acceptance, and the intensity of fluorescence has been shown to correlate very well with other measures of cell death, such as release of lactate dehydrogenase (Abdel-Hamid and Tymianski, 1997). As indicated in the Results, eight days of continuous exposure to CPF (100 or 500 µM) produced significant cell death in the CA1 region of the hippocampus. These data are physiologically significant, since they provide direct evidence that CPF may be neurotoxic (especially with extended exposure) irrespective of its indirect effects on acetylcholinesterase. Whole body molar concentrations associated with the doses of CPF (i.e., 2.5-25.0 mg/kg) used in behavioral experiments were calculated as ranging between approximately 7.0-8.0 to 70-80.0 µM. Since these doses were administered for 14 consecutive days, these concentrations certainly appear relevant to the lower in vitro concentration (100 µM) that was associated with significant hippocampal cytotoxicity.

The final experiments performed in this study were designed to determine whether a low dose of CPF might have measurable effects on motor strength if administered for longer time periods. Low level (2.5 mg/kg), intermittent (5 days/week) administration of CPF for 38 days impaired grip strength in rats without causing any other discernable side effects. Although the effect partially abated toward control values within 6 days of the exposure regimen, the data show that the toxicant does have the potential to produce neuropathic changes with long-term exposure. These data are consistent with our findings of prolonged decreases in axonal transport after repeated CPF exposure.

The observations described in this report are, therefore, important because they begin to address the cellular and behavioral consequences of repeated exposures to doses of OPs that produce no overt signs of acute toxicity. Previously, the interactions of OPs with the enzymes
Acetylcholinesterase and neurotoxic esterase have been the major focus of most mechanistic studies used to explain both acute and long-term effects of overtly toxic doses of a variety of OPs. The issue of repeated, subthreshold exposures to OPs is very important since detectible levels of OP’s (including CPF) can remain in the environment (particularly indoor environments) for extended periods after application (Krieger et al., 2001), thereby posing an ongoing risk for low-level exposure. Furthermore, a variety of non-specific symptoms of OP exposure have been suspected (but difficult to attribute directly to OPs) or have been unrecognized as symptoms of OP toxicity for many years. These symptoms include fatigue, anorexia, headache, chest tightness, dizziness, and sweating, (Abou-Donia and Lapadula, 1990), as well as psychiatric sequelae including anxiety, depression, apathy, irritability, and even schizophreniform manifestations (Metcalf and Holmes, 1969; Karczmar, 1984). Information such as that described in this report may lead to a better understanding of the mechanisms that underlie such symptoms.

In conclusion, the results of this study indicate that the threshold for neurotoxic consequences (usually associated with higher doses of OPs) may be exceeded during repeated exposure to subthreshold doses of OPs, even for agents like CPF which have been considered “moderately toxic” or non-neuropathic. One potential mechanism for these observations is the prolonged inhibition of fast axonal transport. Finally, it is also important to note that CPF itself may have neurotoxic properties in the absence of conversion to its oxone or other metabolites. Although many of the toxic consequences of low dose CPF administration were shown to be reversible after discontinuation, the cytotoxic action observed ex vivo suggests that under certain conditions of exposure, some actions may prove to be longer lasting.
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Figure Legends

Fig 1. Dose related inhibition of plasma cholinesterase activity by chlorpyrifos (CPF) assayed 24 hours after a single subcutaneous injection. Each bar represents the mean (expressed as percent of vehicle control) ± S.E.M. N= 3-12 rats per group. * = significantly different (p<0.05, one-way ANOVA, Student-Newman-Keuls post hoc analysis) from vehicle control; # = significantly different from the 2.5 mg/kg CPF dose; + = significantly different from the 10.0 mg/kg CPF dose; δ = significantly different from the 50.0 mg/kg CPF dose.

Fig 2. Effects of repeated exposures to chlorpyrifos (CPF) on body weight in male, Wistar rats (N=10-12). Body weight measurements were made during six observation sessions conducted throughout a series of daily subcutaneous injections of CPF (at the indicated doses) or vehicle for 14 days. Each point represents the mean ± S.E.M. * = significantly (p<0.05) different from vehicle control, two-way repeated measures AVOVA, Student-Newman-Keuls post hoc analysis. *** indicates that the 10.0, 18.0, and 25.0 mg/kg doses were significantly different from vehicle controls during the observation sessions indicated. The 50.0 mg/kg and 100.0 mg/kg doses were not included in the statistical analysis.

Fig 3. Effects of repeated exposures to chlorpyrifos (CPF) on rearing and sniffing behavior in male, Wistar rats (N=10-12). Rearing and sniffing movements (i.e., total movements per animal per minute) were recorded over a 20 min period during six observation sessions conducted throughout a series of daily subcutaneous injections of CPF (at the indicated doses) or vehicle for 14 days. Each point represents the mean ± S.E.M. * = significantly (p<0.05) different from vehicle control, two-way repeated measures ANOVA, Student-Newman-Keuls post hoc analysis.
*** indicates that the 10.0, 18.0, and 25.0 mg/kg doses were significantly different from vehicle controls during the observation session indicated. **** indicates that the 2.5, 10.0, 18.0, and 25.0 mg/kg doses were significantly different from vehicle controls during the observation sessions indicated. The 50.0 mg/kg and 100.0 mg/kg doses were not included in the statistical analysis.

Fig 4. Residual effects of repeated exposures to chlorpyrifos (CPF) on the water maze hidden platform test. Male Wistar rats (N=8-16) were given daily subcutaneous injections of CPF (at the indicated doses) or vehicle for 14 days, then tested 4 times daily for 4 days beginning 24 hours after the last injection. (A). Latency (sec) to locate the hidden platform. (B). Distance swam (cm) to locate the hidden platform. Each point represents the mean ± S.E.M of 4 trials per day. * = significantly (p<0.05) different from vehicle control, two-way repeated measures AVOVA, Student-Newman-Keuls post hoc analysis.

Fig 5. Residual effects of repeated exposures to chlorpyrifos (CPF) on water maze probe trials. Male Wistar rats (N=8-16) were given daily subcutaneous injections of CPF or vehicle for 14 days then tested in the hidden platform procedure for 4 days beginning 24 hours after the last injection. Probe trials were subsequently conducted on the following day (i.e. on day 5 after the discontinuation of CPF). Each bar represents the mean (± S.E.M) of (A) percentage of the total time spent in the previous target quadrant; (B) percentage of total distance swam in the previous target quadrant or (C) number of crossings over the previous 10 square cm platform area. * = significantly different (p<0.05) from vehicle control value, one-way AVOVA, Student-Newman-Keuls post hoc analysis.
Fig 6. Residual effects of repeated exposures to chlorpyrifos (CPF) on swim speeds in the water maze over 5 consecutive days of testing. Male Wistar rats (N=8-16) were given daily subcutaneous injections of CPF (at the indicated doses) or vehicle for 14 days, then tested beginning 24 hours after the last injection. Each point represents the mean (cm traveled/sec ± S.E.M) of for all trials conducted each day. There was a significant dose effect, (p<0.01); without a significant dose x day interaction (p>0.05).

Fig 7. The lack of residual effects of repeated exposures to chlorpyrifos (CPF) on all aspects of water maze testing after an extended washout period. Male Wistar rats (N=10) were given daily subcutaneous injections of CPF (25/mg/kg/day) or vehicle for 14 days, then tested beginning 14 days after the last injection. (A) Hidden platform test-latencies (sec) to locate the platform. (B). Hidden platform test-distances swam (cm) to locate the platform. (C) Swim speeds (cm/sec) over 5 consecutive days of testing. (D) Probe trials- % of time spent in the previous target quadrant Probe trials- (E) Probe trials- % of total distance swam in the previous target quadrant (F) Probe trials -the mean number of crossings over the previous 10 square cm platform area. Each point or bar represents the mean ± S.E.M.

Fig 8. Residual effects of repeated exposures to chlorpyrifos (CPF) on fast bidirectional axonal transport ex vivo. Male Wistar rats (N=8-16) were given daily subcutaneous injections of CPF (at the indicated doses) for 14 days, tested in the water maze task for 5 days, and then used in axonal transport studies. Axonal transport was thus measured in peripheral nerve axons 6 days after the last CPF injection by video enhanced-differential interference contrast microscopy. (A)
anterograde axonal transport (B) Retrograde axonal transport. * = significantly (p<0.05) different from vehicle control, one way AVOVA, Student-Newman-Keuls post hoc analysis.

Fig 9: Residual effects of chlorpyrifos (CPF) 25.0 mg/kg (14 day repeated dose regimen, N=10) or vehicle on fast bidirectional axonal transport *ex vivo* after water maze testing and an additional 14-day washout. Axonal transport was thus measured in peripheral nerve axons 20 days after the last CPF injection by video enhanced-differential interference contrast microscopy. (A) anterograde axonal transport (B) Retrograde axonal transport. * = significantly (p<0.05) different from vehicle control, one way AVOVA, Student-Newman-Keuls post hoc analysis.

Fig 10. Hippocampal cytotoxicity produced by prolonged exposure to chlorpyrifos (CPF) at the indicated concentrations. Organotypic hippocampal cultures from eight day old rat pups were exposed to control (medium) or CPF (dissolved in medium) for either 24 hours or eight days. (A) Representative hippocampal slices exposed to control conditions or CPF 500 µM for the time periods indicated. (B) Concentration dependent effects of CPF for the time periods indicated. Relative uptake of propidium iodide was quantified via fluorescent microscopy * = significantly (p<0.05) different from vehicle control, one-way AVOVA, Student-Newman-Keuls post hoc analysis.

Fig 11. Effect of repeated administration of a subthreshold dose (2.5 mg/kg) of chlorpyrifos (CPF) administered to male Wistar rats (N=6-10), 5 days/week over 38 days on (A) body weight, (B) hindlimb grip strength, (C) forelimb grip strength. Each point represents the mean ± S.E.M. * = significantly (p<0.05) different from vehicle control, two-way repeated measures AVOVA,
Student-Newman-Keuls post hoc analysis. The dotted line and arrow indicate the day of the last exposure to CPF.
Table 1: Chlorpyrifos Dosing and Testing Protocols

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>Exposure Period</th>
<th>Measurements/Testing Procedures</th>
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<tr>
<td>1</td>
<td>0.0</td>
<td>12</td>
<td>24 hours</td>
<td>plasma cholinesterase</td>
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<td>Weight, grip strength</td>
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<td>2.5</td>
<td>10</td>
<td>38 days</td>
<td>Weight, grip strength</td>
</tr>
</tbody>
</table>

- 1 day drug washout
- Water maze
- Axonal Transport
Plasma Cholinesterase Activity (percent of Control)

Dose of Chlorpyrifos (mg/kg)

- 2.5
- 10.0
- 50.0
- 100.0

Fig 1
Fig 2

Observation Session

Body Weight (grams)

Vehicle
CPF 2.5 mg/kg
CPF 10.0 mg/kg
CPF 18.0 mg/kg
CPF 25.0 mg/kg
CPF 50.0 mg/kg
CPF 100.0 mg/kg

Fig 2
Fig 3

Rearing/sniffing movements/min

Observation Session

Vehicle
CPF 2.5 mg/kg
CPF 10.0 mg/kg
CPF 18.0 mg/kg
CPF 25.0 mg/kg
CPF 50.0 mg/kg
CPF 100.0 mg/kg
Training Day

Time to Platform (sec)

Distance to Platform (cm)

Training Day

Vehicle
- Chlorpyrifos 2.5 mg/kg
- Chlorpyrifos 10 mg/kg
- Chlorpyrifos 18 mg/kg
- Chlorpyrifos 25 mg/kg
Swim Speed (cm/sec) vs Training Day

- Vehicle
- Chlorpyrifos 2.5 mg/kg
- Chlorpyrifos 10 mg/kg
- Chlorpyrifos 18 mg/kg
- Chlorpyrifos 25 mg/kg

Fig 6
Fig 7

A) Time to Platform (sec) vs Training Day

B) Distance to Platform (cm) vs Training Day

C) Swim Speed (cm/sec) vs Training Day

D) % Time in Target Quadrant vs Dose of Chlorpyrifos (mg/kg)

E) % Distance in Target Quadrant vs Dose of Chlorpyrifos (mg/kg)

F) Platform Area Crossings vs Dose of Chlorpyrifos (mg/kg)

Vehicle vs Chlorpyrifos 25.0 mg/kg
Fig 8

A

Average Number of Vesicles Moving

0  50  100  150  200  250  300

0.0  2.5  10.0  18.0  25.0

B

Average Number of Vesicles Moving

0  20  40  60  80  100  120

0.0  2.5  10.0  18.0  25.0

Dose of Chlorpyrifos (mg/kg)

* * *

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Fig 9

**A**

![Graph A showing average number of vesicles moving vs. dose of Chlorpyrifos.](image)

**B**

![Graph B showing average number of vesicles moving vs. dose of Chlorpyrifos.](image)
Fig 10

A

Control

Chlorpyrifos
500 µM

24 Hours

8 Days

B

Hippocampal Damage (propidium iodide uptake)

Days of Chlorpyrifos Exposure

control
10 µM
100 µM
500 µM

CA1

*