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Repeated exposures to low-level chlorpyrifos results in impairments in sustained attention and increased impulsivity in rats

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Abstract

Organophosphates such as chlorpyrifos (CPF) are among the most commonly used pesticides in the world. Therefore, it is not surprising that measurable levels of organophosphates (including CPF) are found in over 50% of fresh fruits, vegetables and grains that we consume and that approximately 80% of adults in the US have detectable levels of CPF metabolites in their urine. It is well known that acute exposure to organophosphates can cause cognitive deficits; however, the effects of daily or intermittent contact with low levels of organophosphates (often reflective of environmental exposures) are not well understood. The objective of this study was to determine if repeated low-level exposures to CPF impaired performance of the 5-Choice Serial Reaction Time Task (5C-SRTT), an animal model of sustained attention. Adult rats were trained to stably perform the 5C-SRTT, then treated with vehicle or CPF 18.0 mg/kg daily for 14 consecutive days or every other day for 30 days. Behavioral testing occurred daily during the CPF-exposure period and throughout a 30 day washout period to assess recovery. All CPF-treated animals exhibited deficits in percent correct, an increase in omissions and premature responses without signs impaired motivation or overt toxicity. Deficits in 5-CSRTT accuracy were apparent well into the 30 day washout period despite significant recovery of cholinesterase activity. These results indicate that repeated exposures to relatively low levels of chlorpyrifos lead to protracted impairments of sustained attention and an increase in impulsive behaviors in rats.

Keywords

Organophosphate; Pesticide; Cholinesterase inhibitor; Cognition; Memory; Chronic

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1. Introduction

Organophosphates are used as pesticides not only in the agricultural industry but also in households, public buildings, on lawns, and by direct application to stagnant water [1]. Uses in the agricultural industry include pest control on grain crops, vegetables, and fruits culminating in approximately 1.2 billion pounds of pesticides per year, 10 million pounds of which is chlorpyrifos (CPF) [33,22,9]. In 2006 the USDA reported that approximately 73% of fresh fruits and vegetables, 61% of processed foods and 66% of drinking water in the US contained detectable levels of organophosphates [32]. Not surprisingly, Barr *et al.* [2] reported that 96% of individuals in the US have measurable levels of CPF metabolites in their urine. The FDA has instituted restrictions on the use of many organophosphates in an attempt to prevent exposure to levels that may be detrimental to human health. However these restrictions do not adequately consider the effects of long-term low-level exposure [20,9]. Moreover, the availability of human data is limited due to the fact that subtle symptoms of low-level organophosphate exposure can go unrecognized and therefore untreated.

Organophosphates such as CPF are known to irreversibly inhibit cholinesterase (ChE) activity by phosphorylating the serine residue within the active site of ChE thus inhibiting the degradation and inactivation of acetylcholine. The resulting accumulation of acetylcholine in the synaptic cleft increases the interaction between acetylcholine and its receptors on both the pre- and post-synaptic terminals, resulting in over-stimulation of the cholinergic system [21, 25]. Unfortunately these effects are non-selective for insects, and as a result acute exposure to organophosphates can lead to a number of toxicological symptoms including seizures, hypersecretory activity, and even death in humans and other non-target organisms [20]. Cognitive symptoms of organophosphate toxicity can include deficits in short-term memory, learning, attention, information processing, reaction time and depression [7]. Using a variety of tasks including digit span, the continuous performance task (CPT), finger tapping, match to sample, reversal learning, etc., deficits in processing speed, attention, learning and memory have been reported in humans after acute and/or repeated exposure to organophosphates and other pesticides [16,25,29,6]. These symptoms can linger for months to years after exposure despite the recovery of cholinesterase activity.

It should be noted, however, that the majority of the published human literature related to organophosphate exposure and cognition describe the consequences of relatively high-level (acute) exposures that also result in overt symptoms of cholinergic toxicity. Relatively little attention has been given to the subject of chronic, “low-level” exposures to organophosphates that are not associated with acute cholinergic symptoms [23]. Furthermore, most of the data in humans were obtained via retrospective analyses and/or individual case studies. Hence, the objective of this study was to prospectively evaluate the persistent behavioral effects (i.e., during and after an extended drug-free washout) of repeated, intermittent, and subthreshold exposures to CPF (i.e., studies that for ethical reasons can only be conducted in animal models). This dosing approach was used to model the types of exposure that may be experienced by agricultural and industrial workers, as well as pest control specialists. We have operationally defined “subthreshold exposures” as doses that do not produce overt signs of cholinergic toxicity (e.g., fasciculations, seizures, diarrhea, excessive urination, salivation, etc., see reviews, [26,30]). The behavioral analyses specifically focused on the effects of CPF on sustained attention using the 5 choice serial reaction time task (5C-SRTT), a rodent analog of the CPT in humans [24].

2. Materials and Methods

2.1. Compound Formulation and Administration

The organophosphate compound chlorpyrifos (CPF) was used in this study. CPF was obtained from ChemService Inc. (Cat# PS-674, West Chester, PA, USA) and was dissolved in 3% DMSO and 97% peanut oil. CPF and/or Vehicle (3% DMSO + 97% peanut oil (v/v)) were administered by subcutaneous (s.c.) injection in a volume of 0.7ml/kg approximately 5 hours after testing in the 5C-SRTT (see below). CPF and vehicle treated animals were given 18mg/kg CPF (s.c.) and vehicle, respectively, every day for 14 days or every other day for 30 days. A second cohort of animals was exposed to 18mg/kg CPF or vehicle for 14 days everyday or 30 days every other day and euthanized 24 hours after the last day of administration for analysis of ChE activity. This dosing procedure was selected based on previous studies by Terry, Jr. *et al.* [31] and was defined as subthreshold according to the definition provided above. All other chemicals were reagent grade or better and purchased from Fisher Scientific and Sigma Aldrich (see Gearhart *et al.* [11] for further details).

2.2. Test Subjects

Male albino Wistar rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN, USA) approximately 2 months old were housed individually in a temperature controlled room (25°C), maintained on a 12:12h reverse light-dark cycle (lights off at 6 am) with free access to water and food during the first week. From week 2 until the end of the study animals were food restricted to approximately 85% of their age-dependent, free-feeding weights based upon Harlan Laboratories growth rate curves. All testing began 2 hours after the initiation of the dark cycle with a minimum of 30 minutes habituation to the light environment prior to testing. 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 and discomfort in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

2.3. 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/m²). Animals in the behavioral cohort were transferred (in their home cages) to the behavioral testing rooms each morning approximately 30 min before the beginning of experiments.

2.3.1. Test Apparatus—Training and testing was conducted using six ventilated, sound attenuated operant chambers (Med Associates, St. Albans, VT, USA). Each operant chamber consisted of 9 nose pokes/apertures, 4 of which were closed off with metal inserts, leaving every other nose poke available (2.5 cm wide, 4 cm deep). The apertures were arranged on a curved panel 2 cm above the floor and were equipped with a photocell beam to detect nose pokes. There was a lamp (2.8W) on the rear wall of each aperture that could be illuminated randomly and for varying durations. Food pellets were delivered automatically to a magazine on the opposite wall to the nose pokes. A light inside the food magazine was also turned on to indicate that a pellet (45mg chow pellet, BioServ, Frenchtown, NJ, USA) had been dispensed. The food magazine was equidistant from all nose poke apertures. There was a house light that remained on for the entire session unless an error or omission occurred; the light was located towards the roof of the operant chamber above the magazine. The apparatus was controlled using MedPC software (Med Associates, St. Albans, VT, USA).

2.3.2. Behavioral Procedures—Week one consisted of handling animals to reduce stress and anxiety in preparation for training and testing. Week 2 consisted of handling and food restriction. Week 3 consisted of food restriction, habituation to the apparatus and preparation for training. Specifically, on days 1 and 2 of week 3 subjects were placed in the operant chambers for 15 minutes with the house light and magazine light on and 10 pellets in the magazine dispenser. On days 3–5 of week 3 animals performed a non-spatial training program. Briefly, the animals were placed in the 5C-SRTT operant chamber with the house light illuminated. A pellet was released into the food magazine which was simultaneously illuminated for a maximum of 5 seconds or until the animal collected the pellet. After a 10 second interval another pellet was released from the simultaneously illuminated food magazine. This continued until the 15 minute habituation period expired.

Spatial training in the task began on day 1 of week 4. Animals began with the stimulus duration of 10 seconds, each session being 100 trials or 30 minutes in duration. An initial pellet was delivered to the magazine to facilitate the start of each session. One of the 5 nose poke apertures was illuminated randomly for 10 seconds after which the light was extinguished. The animal was then required to respond correctly by nose poking the previously illuminated aperture within 5 seconds of the light being extinguished. A correct response in the previously established time frame (5 seconds) resulted in a pellet being dispensed into the magazine that was simultaneously illuminated for a maximum of 5 seconds or until the animal retrieved the pellet. Collection of this pellet initiated the intertrial interval, a delay of 5 seconds, before the next trial began. An incorrect response, premature response, or failure to respond (omission) resulted in a 5 second timeout, marked by the extinction of the house light for 5 seconds and no food reward, after which the animal initiated the next trial by a nose poke into the magazine. Animals were trained 7 days a week until they reached stable performance levels at the 10 second stimulus duration (stable performance criterion defined as 2 consecutive days at >80% accuracy, <20% omissions and completion of all 100 trials). Once this criterion was achieved the animals were moved to the next, more challenging stimulus duration and the same performance criterion was applied before the next progression. The following stimulus durations were employed: 5 → 2.5 → 1.25 → 1.0 → 0.8 → 0.6 → 0.5 seconds.

The animals were required to meet criterion for a minimum of 3 days at the 0.5 second stimulus duration prior to being moved to the testing groups. Reaching criterion at the 0.5 second stimulus duration required on average 42 ± 1.36 sessions. Once the animals met criterion they were randomly divided into 1 of 4 groups; every other day 30 day vehicle, every other day 30 day 18mg/kg CPF, everyday 14 day vehicle or everyday 14 day 18mg/kg CPF. Testing began approximately 15 hours after the first injection of CPF or vehicle.

2.3.3. 5C-SRTT Performance Assessment—When administration of vehicle or CPF began, rats were assessed daily for signs of acute toxicity. Signs included muscle fasciculations, ataxia, tremors, increased salivation, lacrimation, diarrhea and rhinorrhea. Testing at the 0.5 second stimulus duration continued 7 days a week throughout the administration of CPF/vehicle and for 30 days after the last injection (30 day, CPF-free washout). On day 31 of washout the animals were tested using a randomized, varying stimulus duration of 0.1, 0.25 and 0.5 seconds to increase demands of the task. The following parameters were measured to assess performance before, during and after exposure to CPF/vehicle:

$$\% \text{ correct} = [\# \text{ correct} / (\# \text{ correct} + \# \text{ incorrect})] \times 100$$

$$\% \text{ omissions} = [\# \text{ omissions} / (\# \text{ trials completed})] \times 100$$

% Premature responses (Impulsivity) = [(premature responses/trials initiated)] $\times$ 100 (specifically, the % of responses made after the trial began, but before onset of the light stimulus, i.e., during the 5 second intertrial interval).

% Perseverative responses (Compulsivity) = [(perseverative responses/correct responses)] $\times$ 100 (specifically, the % of nose pokes made after the correct response has been made but before collecting the reward). Responding can occur in the aperture where the responding has just earned a food reward or at another location.

Trials completed = (# correct + # incorrect + # omissions)

Latency to correct = time elapsed from the onset of the light stimulus to making the correct nose poke response.

Latency to incorrect = time elapsed from the onset of the light stimulus to making the incorrect nose poke response.

Latency to reward = time elapsed from making a correct nose poke response to retrieving the food reward from the magazine.

2.4. Measurement of Cholinesterase (ChE) Activity

On day 31 of washout (behavioral cohort) or 24 hours after the final CPF/vehicle administration (cholinesterase cohort) the animals were anesthetized with isoflurane. The blood was then drawn directly from the left ventricle, collected in EDTA 4ml Vacutainers (BD, Franklin Lakes, NJ, USA) and centrifuged for 15 min in a clinical centrifuge. The plasma was then removed and quick frozen with liquid nitrogen and stored at -70°C . After the blood draw the animals were quickly decapitated, brains were removed within 3 minutes and quick frozen in liquid nitrogen for storage at -70°C until use.

Preparation of brain homogenates for the ChE activity assay was described in detail in Gearhart *et al.* [10]. Briefly, brains were thawed and the basal forebrain, hippocampus, cortex (excluding prefrontal cortex), prefrontal cortex and striatum were dissected and homogenized in a modified RIPA buffer. Samples were sonicated, filtered and stored at -70°C until needed. ChE activity was assessed using the Ellman method with modifications [31]. Briefly, tissue homogenate or plasma was added to 96 well plates with 300uL of reaction mixture (4.8uM acetylthiocholine iodide and 321uM dithiobisnitrobenzoate in 0.1 M Na_2HPO_4 buffer, pH 8.0; sigma) and shaken for 30 seconds on a Jitterbug, Boekel Scientific (Feasterville, PA, USA). Plates were then placed in Biotek uQuant plate reader (Philadelphia, PA, USA) and read at 412nm every 2 minutes for 16 minutes. The rate of ChE activity was then calculated using the formula rate (moles/liter per min) = $(\Delta \text{ absorbance/min}) / (1.36 \times 10^4)$.

2.5. Statistics

Statistical comparisons of treatment groups were conducted using a two way repeated measures ANOVA followed by post hoc analysis using the Student-Newman-Keuls multiple comparison method (SigmaStat 2.03, SPSS Inc., Chicago, IL, USA). Statistical significance was assessed using an alpha level of 0.05. Data are shown as Mean $\pm$ SEM.

3. Results

3.1. 5C-SRTT - 14 Day Everyday Exposure: CPF vs. VEH

There was a significant decrease in accuracy during exposure to CPF when compared to vehicle matched controls (Fig 1) and this deficit persisted well into the 30 day washout period, main effect for treatment ($F_{(1,22)} = 16.12, p < 0.001$), session effect ($F_{(43,946)} = 4.01 p < 0.001$),

treatment x session interaction ($F_{(43,946)} = 3.57, p < 0.001$). Post hoc analysis indicated that CPF treated rats were impaired relative to control rats on all sessions from session 3 until session 33 of the study (see the asterisks in Fig 1). Further analysis revealed that CPF exposure resulted in a significant increase in % omissions during the exposure period and that this effect persisted throughout the 30 day washout period, main effect for treatment ($F_{(1,22)} = 18.23, p < 0.001$). The averaged data for the baseline, drug exposure period, and during the washout period are provided in Table 1. There were no significant differences in the total number of trials completed (Table 1). The percentage of premature and perseverative responses is shown in Fig 2A and 2B, respectively. The figure insets illustrate the data averaged across the sessions during the exposure period and washout periods, respectively. Statistical analysis of premature responses revealed the following results: main effect for treatment ($F_{(1,18)} = 4.1, p = 0.05$), session effect ($F_{(43,774)} = 1.98, p < 0.001$), treatment x session interaction ($F_{(43,774)} = 1.01, p = 0.42$). Post hoc analysis indicated a significantly higher number of premature responses ($p < 0.05$) in the CPF-treated subjects on sessions 6, 18, 19, 20, and 21. There was also a significant CPF-related decrease in the percentage of perseverative responses primarily associated with the drug exposure period, main effect for treatment ($F_{(1,18)} = 0.14, p = 0.71$), session effect ($F_{(43,774)} = 1.54, p = 0.03$), treatment x session interaction ($F_{(43,774)} = 2.29, p < 0.001$). Post hoc analysis indicated a significant CPF-related decrease in perseverative responses on sessions 6, 8, 9, 10, 11, and 12 of exposure, an effect that abated during the 30 day washout period (Fig. 2B). The mean latencies associated with correct responses, incorrect responses, and reward collection (magazine latency) at baseline (i.e., before drug exposure), during the drug exposure period, and during the washout period are provided in Table 1. There were statistically significant CPF-related increases in the latency to correct and incorrect responses, main effect of treatment ($F_{(1,22)} = 7.78, p = 0.01$ and incorrect ($F_{(1,22)} = 5.58, p < 0.03$), respectively. Post hoc analyses indicated that CPF was associated with an increased latency to correct response during the exposure period and that this effect persisted during the washout period, while the latency to incorrect response was increased during the exposure period only.

3.1.1. Varying Stimulus Duration (14 day everyday exposure): Day 31 of washout

—The effects of a pseudorandom presentation of three different stimulus durations during session 31 of washout from the every day drug exposure regimen are shown in Fig 3 and Table 2. For the percent correct assessment statistical analysis revealed the following results, main effect of treatment ($F_{(1,14)} = 1.32, p = 0.27$), stimulus duration ($F_{(2,28)} = 32.17, p < 0.001$), treatment by stimulus duration interaction ($F_{(2,28)} = 0.10, p = 0.90$). Post hoc analysis indicated (as expected) that accuracy in all subjects was increased with an increase in stimulus duration. While visual inspection of Fig 3 indicates that accuracy was slightly lower in the subjects previously exposed to CPF at each stimulus duration, the differences were not statistically significant ($p > 0.05$). There were also non-significant trends ($p < 0.1$) toward increased omissions (Table 2) in CPF treated subjects at each of the three stimulus durations (0.1, 0.25, 0.5 seconds). There were no significant differences in any of the latency measures (Table 2) or measures of impulsive or compulsive behaviors (Fig. 3B, 3C).

3.2. 30 Day Every Other Day Exposure: CPF vs. VEH

As in the case of the daily CPF exposure method, subjects exposed to CPF every other day also demonstrated a significant decrease in accuracy (during the drug exposure period, see Fig 4) and this deficit persisted well into the 30 day washout period, main effect for treatment ($F_{(1,22)} = 13.47, p < 0.001$), session effect ($F_{(59,1298)} = 4.69, p < 0.001$), treatment x session interaction ($F_{(59,1298)} = 4.17, p < 0.001$). Post hoc analysis indicated that CPF treated rats were significantly ($p < 0.05$) impaired relative to control rats during session 7, all sessions from 11 through 42, as well as during sessions 44, 47, and 57 (see the asterisks in Fig 4). CPF exposure (every other day) also resulted in a significant increase in % omissions during the exposure period and this effect persisted throughout the 30 day washout period, main effect for treatment

($F_{(1,21)} = 18.26, p < 0.001$). The averaged data for the baseline, drug exposure period, and during the washout period are provided in Table 1. There were no significant differences in the total number of trials completed (Table 1). The percentage of premature and perseverative responses is shown in Fig 5A and 5B, respectively. The figure insets illustrate the data averaged across the sessions during the exposure period and washout periods, respectively. Statistical analysis of premature responses revealed the following results: main effect for treatment ($F_{(1,22)} = 13.64, p = 0.001$), session effect ($F_{(59,1298)} = 1.10, p = 0.28$), treatment x session interaction $F_{(59,1298)} = 1.60, p = 0.003$. Post hoc analysis indicated a significantly higher number of premature responses ($p < 0.05$) in the CPF-treated subjects on nearly all the sessions from session 21 until the end of the study. There were also significant CPF-related alterations in the percentage of perseverative responses ($F_{(1,21)} = 0.22, p = 0.65$), session effect ($F_{(59,1239)} = 1.22, p = 0.13$), treatment x session interaction $F_{(59,1239)} = 4.76, p < 0.001$. Post hoc analysis indicated a significant biphasic effect of CPF exposure (i.e., perseverative responses were significantly or nearly significantly lower in CPF-treated subjects during sessions 6, 10, 11, 12) during the exposure period, but actually increased relative to vehicle controls during the washout period (i.e., during multiple sessions). The mean latencies associated with correct responses, incorrect responses, and reward collection at baseline, during the drug exposure period and during the washout period are provided in Table 1. There were statistically significant CPF-related increases in the latency to correct and incorrect responses. In the case of the latency to correct responses the main effect of treatment was not significant, ($F_{(1,21)} = 2.30, p = 0.14$), however, the treatment x exposure period interaction was highly significant, ($F_{(2,42)} = 14.85, p < 0.001$). There was a significant main effect of treatment on incorrect response latencies ($F_{(1,21)} = 7.64, p = 0.01$). Post hoc analyses indicated that CPF was associated with an increased latency to correct and incorrect responses during the exposure period, but this effect abated to control levels during the washout period.

3.2.1. Varying Stimulus Duration (30 day every other day exposure): Day 31 of washout—The effects of a pseudorandom presentation of three different stimulus durations during session 31 of washout from the every other day drug exposure regimen are shown in Fig 6 and Table 2. For the percent correct assessment statistical analysis revealed the following results, main effect of treatment ($F_{(1,14)} = 0.64, p = 0.44$), stimulus duration ($F_{(2,28)} = 43.2, p < 0.001$), treatment by stimulus duration interaction ($F_{(2,28)} = 3.85, p = 0.03$). Post hoc analysis indicated (again, as expected) that accuracy in all subjects was increased with an increase in stimulus duration. However, accuracy in subjects previously exposed to CPF was lower (compared to vehicle controls) at the 0.5 sec stimulus duration. Interestingly, there was a significant CPF-related reduction in the mean latency to collect the reward associated with the 0.25 second stimulus duration ($p < 0.05$). There were no other statistically significant differences in the latencies, omissions or in the measures of impulsive or compulsive behaviors (see Fig. 6B, 6C, Table 2).

3.3. ChE Activity

Acetylcholinesterase activity was assessed in plasma and selected brain regions in the cholinesterase cohort (i.e., in rats not behaviorally tested) 24 hours after the 14 day everyday CPF-exposure period or after the 30 day every other day exposure period. The behavioral cohort (i.e., animals evaluated in the 5C-SRTT) was used to assess acetylcholinesterase activity at the end of the washout period. The prefrontal cortex, cortex (i.e., the remaining cortical areas), hippocampus, striatum, and basal forebrain were selected for analysis due to their function in the basal forebrain cholinergic system (and the well documented roles of this system in attention and general cognition) and/or their specific roles in performance of the 5C-SRTT (see [23] for review). Immediately after exposure to CPF, plasma ChE activity was significantly reduced ($F_{(5,60)} = 1.748, p < 0.05$) to 21% and 31% of control for everyday and every other day exposed animals respectively (see Fig. 7 and 8). However, after the 31 day washout period the

plasma ChE activity levels recovered to 80% and 89% of control for their respective groups resulting in no significant difference in activity after the 30 day washout period when compared to vehicle matched controls ($p=0.782$, $p=0.62$ respectively). ChE activity in brain homogenates immediately after exposure to CPF was significantly decreased in all brain regions when compared to vehicle matched control groups. Specifically, ChE activity in animals exposed to CPF for 14 days (every day) was decreased to 46.6%, 59.4%, 63.3%, 55.9%, and 23.5% of control for; cortex, prefrontal cortex, hippocampus, striatum, and basal forebrain, respectively, ($F_{(5,60)}=1.748$, $p<0.05$) (Fig. 7). Similar results were found in the animals exposed to CPF for 30 days every other day, with ChE activities of 54.5%, 44.6%, 50.9%, 48.7%, and 25.9% of control for the same respective brain regions ($F_{(5,60)}=3.572$, $p<0.05$) (Fig. 8). Incorporation of the 31 day washout period resulted in near full recovery of ChE activity in four of the six brain regions analyzed. The striatum and basal forebrain continued to have a small, but significant reduction in ChE activity when compared to control animals after the 31 day washout period for both every day ($F_{(5,60)}=3.572$, $p<0.05$) and every other day ($F_{(5,60)}=0.750$, $p<0.05$) treatment groups.

4. Discussion

The results of this study utilizing the 5C-SRTT indicate that repeated exposures to a subthreshold dose of CPF in rats results in protracted impairments in sustained attention and a decrease of inhibitory control (i.e., elevated impulsivity). These observations were evident in animals administered CPF on a daily basis for 14 days or every other day for 30 days so that the cumulative number of exposures was similar. Deficits in sustained attention were indicated by a decrease in accuracy (% correct) and a modest increase in the number of omissions; and increases in impulsivity were suggested by the elevated number of premature responses. It is also interesting to note that the percentage of perseverative responses (surprisingly) was diminished in CPF-treated subjects during the drug exposure period in both the daily exposed and every other day exposed subjects. This effect combined with the increase in response latencies (i.e., for correct and incorrect nose poke selections) observed in both CPF treatment groups might be suggestive of a general motor slowing or performance deficit. However, the effect on perseverative responding abated in the every-day exposed subjects during the washout period, and the effect was actually reversed during washout in the subjects previously treated every other day with CPF. Specifically, in these subjects, there was a clear increase in perseverative responses during the washout period (i.e., possibly indicative of an increase in compulsive behaviors).

There are several additional observations that lead us to conclude that the protracted effects of CPF on performance of the 5C-SRTT are related to deficits of information processing and/or attention as opposed to general deficits in motivation, locomotor activity, malaise etc. For example, we did not observe any alterations in the magazine latency (i.e., the latency to collect food rewards) which has been described as an index of motivation in the 5C-SRTT (see review, [24]). In addition, we did not observe any significant alterations in the total number of trials completed. In the 5C-SRTT, subjects must nose poke the food magazine after an incorrect response (i.e., after an incorrect nose poke into one of the stimulus apertures) or after an omission (i.e., when 5 seconds expired after the stimulus presentation without a nose poke) in order to initiate the next trial. Therefore, significant delays in initiation would be exemplified by a decrease in the total number of trials completed (which, as noted above, we did not observe). As reviewed by Robbins, 2002 [24], changes in the response latencies in the absence of changes in latencies to collect earned food reinforcers (as observed in the present study) likely reflect alterations of information processing and/or decisional processes. In addition, increases in omissions in the absence of changes in magazine latencies (which we also observed) are most likely due to gross impairments in attention. Finally, it is also important to note that on day 31 of washout, when a CPF-related sensitivity to the presentation of the

variable stimulus durations was observed (i.e., accuracy was decreased), there was a clear trend toward decreases in response latencies in CPF treated animals (statistically significant for the magazine latency associated with the 0.25 sec stimulus duration).

The data described in this report complement previous studies in our laboratory [31] where a series of behavioral tests were conducted during a washout period after CPF was injected (dose range, 2.5–18.0 mg/kg) every other day over the course of 30 days. In that study, dose-dependent decrements in a water maze hidden platform task and a prepulse inhibition (PPI) procedure were observed, without significant effects on open field activity, rotorod performance, or grip strength. Those results further support the argument presented here that repeated exposure to 18.0 mg/kg of CPF is not associated with deleterious motivational or locomotor effects that might confound the interpretation of (cognition-related) behavioral results. The study also suggested that repeated exposures to CPF results in prolonged impairments of other domains of cognition. The deficits in PPI observed in that study may be particularly relevant to the 5C-SRTT results presented here given that PPI has been widely used as a neurophysiological measure of the early pre-attentive stages of information processing [3]. Further, PPI has been shown in animals to be sensitive to cholinergic manipulations [14]. Our observations of prolonged elevations in impulsivity in animals previously exposed to CPF (in the current study) would appear to agree with the previous report of Cardona *et al.* [5] who demonstrated that one acute dose of CPF is associated with increases in impulsivity in rats 6 months after exposure.

The biochemical basis for the effects on 5C-SRTT performance noted here may be multifactorial. As observed in Figures 1 and 4, there was a relatively rapid decrease in accuracy of the task associated with both CPF-exposure methods, an effect that appears to correlate with previous publications of CPF-related time courses of ChE inhibition. For example, we [31] previously observed that the 18mg/kg CPF every other exposure method was associated with maximal plasma ChE inhibition beginning around day 7 of dosing. This level of ChE inhibition correlates reasonably well with the performance deficits in the 5C-SRTT in the current study that begin around day 10 of dosing (see Fig 4). In the every day CPF exposure approach where the cumulative dose is higher at an earlier time point, the decrease in 5C-SRTT accuracy occurred earlier (see Fig 1). This behavioral effect (i.e., performance deficits beginning around day 4 of dosing) would appear to correlate with Bushnell et al., 1993 [4] who reported that maximal inhibition of whole blood cholinesterase activity occurred four days after a single subcutaneous injection of CPF (albeit at considerably higher doses than used in the present study). It should be noted here that the CPF-related effects in the 5C-SRTT described above were evident when cholinesterase inhibition was maximal as well as when it had significantly recovered toward baseline levels of activity in both plasma and brain (i.e., during the CPF-free washout period). Conversely, in the Bushnell study cited above [4], functional deficits in working memory and motor function in rats administered single (relatively high) doses of CPF were apparent within two days of administration, but recovered within three weeks, long before ChE activity had returned to normal. Collectively, the results of that study and ours suggest that the behavioral deficits associated with CPF may not be fully explained by ChE inhibition alone.

A variety of studies employing cholinergic agonists and antagonists as well as cholinergic lesions (i.e., of the nucleus basalis magnocellularis, NBM) have clearly documented an important role of the cholinergic system in performance in the 5C-SRTT [12,24]. We [31] have previously observed protracted decreases in several cholinergic proteins in multiple brain regions after chronic subthreshold exposure to CPF (i.e., effects that may contribute to the deficits in 5C-SRTT in the present study). Our previous observations of reduced expression of the α_7 nicotinic acetylcholine receptor (nAChR) in multiple brain regions including the basal forebrain and prefrontal cortex may be of particular importance in light of findings in other

laboratories. For example, Hoyle *et al.* [15] previously found that α_7 nAChR knockout mice exhibited decreases in accuracy in the 5C-SRTT and an increase in impulsivity. Similar findings (i.e., performance deficits and elevations in impulsivity) have been reported in α_7 nAChR mutant mice in a signaled nose poke task designed to measure appetitive learning and behavioral impulsivity [17]. Collectively, such studies support a role of α_7 nAChRs in sustained attention and behavioral disinhibition. Thus, it is interesting to speculate that the mechanism for the CPF-related attentional deficits and elevations in impulsivity (observed in our study) are related to α_7 nAChR deficits. Interestingly, Smulders *et al.* [27] have also made the argument (as we have, see above) that persistent neurobehavioral effects of organophosphates (including CPF) cannot be explained by acetylcholinesterase inhibition alone, and, that such effects may involve direct actions at nAChRs.

From a translational perspective there are several points that should be considered. Substantial evidence suggests that a variety of OPs (including CPF) can persist in the environment for extended periods after use and as a result they represent a threat to numerous people, especially from a low-level chronic or intermittent exposure standpoint. Many insecticides were (by design) manufactured to persist in soil to eradicate pests such as termites. Thus, (as noted in the Introduction) the short term (daily) and intermittent (every other day) dosing regimens used in this study provided a model for the types of exposures that might be experienced by agricultural and industrial workers, as well as pest control specialists. Our dosing approach in rats was subthreshold for acute signs of toxicity; however, it resulted in plasma and brain inhibition of cholinesterase ranging between 40–80%. This level of inhibition is likely to be significantly higher than that associated with most environmental exposures in the general population or that encountered by most agricultural workers. However, Dyer *et al.* [8] previously reported that termiticide applicators in Australia chronically exposed to organophosphates had serum cholinesterase inhibition levels of approximately 50% with no signs of acute toxicity, suggesting that (like in our animals studies) physiological adaptations may take place over time such that acute symptoms are not manifested. Further, in a study of termiticide applicators in North Carolina, levels of the CPF metabolite, TCP (3,5,6-trichloro-pyridinol) were found to exceed that of the general population by an average factor of 70 [13], suggestive of relatively high levels of CPF exposure. It is interesting to note that termiticide applicators (i.e., specifically exposed to chlorpyrifos) more commonly complain of attention and memory-related difficulties compared to non-exposed controls [28]. It should be noted, however, that (with a few exceptions) such deficits have not been objectively confirmed when termiticide applicators have been clinically evaluated using quantitative tasks designed to evaluate specific domains of cognition.

5. Conclusion

In conclusion, the results of this rodent study support the premise that repeated, low-level exposures to commercial organophosphate pesticides like CPF may lead to protracted deficits in sustained attention and to increases in impulsivity in the absence of acute (cholinergic) side effects or motivational deficits. The observations are significant given the widespread use of OP insecticides in household, agricultural, and commercial environments worldwide and the importance of attention and impulse control to human cognition and general function in society. The results also emphasize the critical need for additional (prospective) investigations to determine more definitively how specific OPs at low-levels (over time) affect specific domains of cognition (as well as the neurobiological substrates of the cognitive effects, i.e., experiments that can only be conducted in animal models).

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Fig 1.

The effects of CPF (18.0 mg/kg) exposure on accuracy (% correct) in the 5C-SRTT during the 14 day every day exposure period and during the 30 day (drug-free) washout period. All data are presented as mean $\pm$ SEM. * = Statistically significant difference ($p < 0.05$) between CPF-treated and vehicle-treated controls. (N=8–10).

Fig 2.

A. The effects of CPF (18.0 mg/kg) exposure on the percentage of premature responses (A) and perseverative responses (B) in the 5C-SRTT during the 14 day every day exposure period and during the 30 day (drug-free) washout period. The histogram insets illustrate the overall effect (i.e., responses averaged across the sessions) during the drug exposure and washout periods. * = Statistically significant difference ($p < 0.05$) between CPF-treated and vehicle-treated controls. (N=8–10).

Fig 3.

The residual effects of CPF (18.0 mg/kg) exposure (14 day every day exposure method) in a variable stimulus duration version of the 5C-SRTT on day 31 of a drug-free washout period. Dependent measures include: % correct (A), percentage of premature responses (B) and the percentage of perseverative responses (C). Data are presented as mean $\pm$ SEM for each stimulus duration. (N=6–8).

Fig 4.

The effects of CPF (18.0 mg/kg) exposure on accuracy (% correct) in the 5C-SRTT during the 30 day every other day exposure period and during the 30 day (drug-free) washout period. All data are presented as mean $\pm$ SEM. * = Statistically significant difference ($p < 0.05$) between CPF-treated and vehicle-treated controls. (N=8–10).

Fig 5.

The effects of CPF (18.0 mg/kg) exposure on the percentage of premature responses (A) and perseverative responses (B) in the 5C-SRTT during the 30 day every other day exposure period and during the 30 day (drug-free) washout period. The histogram insets illustrate the overall effect (i.e., responses averaged across the sessions) during the drug exposure and washout periods. * = Statistically significant difference ($p < 0.05$) between CPF-treated and vehicle-treated controls. ** = $p < 0.01$; † = $p < 0.06$. (N=8–10).

Fig 6.

The residual effects of CPF (18.0 mg/kg) exposure (30 day every other day exposure method) in a variable stimulus duration version of the 5C-SRTT on day 31 of a drug-free washout period. Dependent measures include: % correct (A), percentage of premature responses (B) and the percentage of perseverative responses (C). Data are presented as mean ± SEM for each stimulus duration. * = Statistically significant difference ($p < 0.05$) between CPF-treated and vehicle-treated controls. (N=6–8).

Fig 7.

The effects of CPF (18.0 mg/kg) exposure (14 day every day exposure method) on cholinesterase activity. Data (mean $\pm$ SEM) are presented as % of vehicle matched control levels immediately following 14 consecutive days of exposure to CPF (black bars) and 31 days after the last exposure to CPF (gray bars). Cholinesterase activity was obtained from: plasma (PL); CX (cortex); PFC (prefrontal cortex); HIPP (hippocampus); STR (striatum); BF (basal forebrain). *Statistically significant at $p < 0.05$. (N=6).

Fig 8.

The effects of CPF (18.0 mg/kg) exposure (30 day every other day exposure method) on cholinesterase activity. Data (mean $\pm$ SEM) are presented as % of vehicle matched control levels immediately following the last exposure to CPF (black bars) and 31 days after the last exposure (gray bars) Cholinesterase activity was obtained from: plasma (PL); CX (cortex); PFC (prefrontal cortex); HIPP (hippocampus); STR (striatum); BF (basal forebrain).

*Statistically significant at $p < 0.05$. (N=6).

Table 1
Effects of Chlorpyrifos on Latencies, Omissions, and Trials Completed in the Five Choice Serial Reaction Time Task

Group/Dosing Frequency	Treatment Period	Latency Correct (s)	Latency Incorrect (s)	Magazine Latency (s)	% Omissions	Trials Completed
Vehicle (Every Day)	Baseline	0.70±0.03	1.73±0.13	1.53±0.12	4.06±0.47	100.00±0.00
	Exposure	0.70±0.02	1.72±0.07	1.44±0.13	2.94±0.37	99.83±0.17
	Washout	0.69±0.03	1.72±0.07	1.55±0.10	2.85±0.62	100.00±0.00
Chlorpyrifos (Every Day)	Baseline	0.64±0.02	1.37±0.10	1.66±0.14	5.23±1.03	100.00±0.00
	Exposure	0.90±0.03*	2.24±0.07*	1.53±0.07	22.99±0.3,1.4*	88.73±2.58
	Washout	0.89±0.12*	1.87±0.05	1.53±0.10	15.24±3.60*	97.28±0.66
Vehicle (Every Other Day)	Baseline	0.69±0.03	1.72±0.16	1.62±0.07	3.59±1.37	100.00±0.00
	Exposure	0.70±0.04	1.72±0.08	1.70±0.07	3.28±0.59	99.99±0.01
	Washout	0.66±0.05	1.73±0.09	1.58±0.08	2.00±0.44	100.00±0.00
Chlorpyrifos (Every Other Day)	Baseline	0.67±0.02	2.13±0.08	1.39±0.11	3.62±0.69	100.00±0.00
	Exposure	0.83±0.03*	2.01±0.05*	1.65±0.12	12.01±1.51*	96.45±1.40
	Washout	0.73±0.03	1.75±0.07	1.54±0.11	5.18±0.67*	98.69±0.07

Data are presented as the mean ± SEM. Baseline = before the drug exposure period; exposure = during the drug exposure period; washout = during the 30 day drug-free washout period

* statistically significant difference ($p<0.05$) between vehicle and chlorpyrifos-treated group during the same treatment period. N=8–10

Table 2

Effects of Chlorpyrifos on Latencies, Omissions, and Trials Completed in the Variable Stimulus Duration Version of the Five Choice Serial Reaction Time Task

Group/Dosing Frequency	Stimulus Duration (s)	Latency Correct (s)	Latency Incorrect (s)	Magazine Latency (s)	% Omissions	Trials Completed
Vehicle (Every Day)	0.10	0.70±0.05	1.40±0.13	1.40±0.09	6.57±2.29	
	0.25	0.74±0.12	1.72±0.20	1.51±0.18	4.70±1.72	(total)
	0.5	0.75±0.06	1.62±0.29	1.37±0.11	6.79±2.39	98.75±1.25
Chlorpyrifos (Every Day)	0.10	0.77±0.12	1.51±0.18	1.42±0.13	11.57±5.33	
	0.25	0.71±0.06	1.46±0.19	1.35±0.09	8.34±5.42	(total)
	0.5	0.68±0.07	1.65±0.25	1.32±0.05	10.67±4.89	100.00±0.00
Vehicle (Every Other Day)	0.10	0.70±0.08	1.13±0.18	1.76±0.36	3.70±1.21	
	0.25	0.73±0.08	1.31±0.22	2.13±0.34	1.34±0.53	(total)
	0.5	0.69±0.06	1.29±0.25	1.62±0.30	4.10±1.17	100.00±0.00
Chlorpyrifos (Every Other Day)	0.10	0.46±0.04	0.96±0.20	1.33±0.13	2.60±1.35	
	0.25	0.52±0.07	1.19±0.38	1.06±0.09*	2.85±0.95	(total)
	0.5	0.52±0.06	1.29±0.35	1.23±0.17	5.73±4.03	96.63±3.38

Data shown here were collected on day 31 of the drug free washout period. All data are presented as the mean ± SEM.

* statistically significant difference ($p<0.05$) between vehicle and chlorpyrifos-treated group at the same stimulus duration. N=6-8