Long-Lasting Reductions of Ethanol Drinking, Enhanced Ethanol-Induced Sedation, and Decreased c-fos Expression in the Edinger-Westphal Nucleus in Wistar Rats Exposed to the Organophosphate Chlorpyrifos

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Intermittent or continuous exposure to a wide variety of chemically unrelated environmental pollutants might result in the development of multiple chemical intolerance and increased sensitivity to drugs of abuse. Interestingly, clinical evidence suggests that exposure to organophosphates might be linked to increased ethanol sensitivity and reduced voluntary consumption of ethanol-containing beverages in humans. The growing body of clinical and experimental evidence emerging in this new scientific field that bridges environmental health sciences, toxicology, and drug research calls for well-controlled studies aimed to analyze the nature of the neurobiological interactions of drugs and pollutants. Present study specifically evaluated neurobiological and behavioral responses to ethanol in Wistar rats that were previously exposed to the pesticide organophosphate chlorpyrifos (CPF). In agreement with clinical data, animals pretreated with a single injection of CPF showed long-lasting ethanol avoidance that was not secondary to altered gustatory processing or enhancement of the aversive properties of ethanol. Furthermore, CPF pretreatment increased ethanol-induced sedation without altering blood ethanol levels. An immunocytochemical assay revealed reduced c-fos expression in the Edinger-Westphal nucleus following CPF treatment, a critical brain area that has been implicated in ethanol intake and aversive properties of ethanol. Long-lasting ethanol-avoidance which was not secondary to altered gustatory processing or enhancement of the aversive properties of ethanol. Present study specifically evaluated neurobiological and behavioral responses to ethanol in Wistar rats that were previously exposed to the pesticide organophosphate chlorpyrifos (CPF). In agreement with clinical data, animals pretreated with a single injection of CPF showed long-lasting ethanol avoidance that was not secondary to altered gustatory processing or enhancement of the aversive properties of ethanol. Furthermore, CPF pretreatment increased ethanol-induced sedation without altering blood ethanol levels. An immunocytochemical assay revealed reduced c-fos expression in the Edinger-Westphal nucleus following CPF treatment, a critical brain area that has been implicated in ethanol intake and sedation. We hypothesize that CPF might modulate cellular mechanisms (decreased intracellular cAMP signaling, alpha-7-nicotinic receptors, and/or cerebral acetylcholinesterase inhibition) in neuronal pathways critically involved in neurobiological responses to ethanol.

Key Words: chlorpyrifos; ethanol avoidance; chemical intolerance; Edinger-Westphal nucleus; cholinesterase inhibition; organophosphates.

During recent years, a growing body of clinical evidence has revealed that acute, intermittent or continuous exposure to a wide variety of chemically unrelated environmental pollutants (such as volatile organic chemicals, woods preservatives, solvents, or organophosphates pesticides) might result in the development of multiple chemical intolerance (Miller, 2000) and increased sensitivity to drugs of abuse (Sorg and Hochstatter, 1999; Newlin, 1994). Animal studies focused on the adverse effects of formaldehyde on behavioral responses to cocaine revealed drug-pollutant cross-sensitization (Sorg et al., 2001). In addition, clinical reports showed that a significant percentage (60%) of Gulf War veterans (Miller, 2000) as well as agricultural workers suffering acute organophosphate intoxication develops intolerance to nicotine- and ethanol-containing beverages (Tabershaw and Cooper, 1966). In agreement with clinical data, Overstreet and his colleagues reported that Flinder rats, which have been bred for increased sensitivity to organophosphate poisoning, showed enhanced responses to ethanol or nicotine (Overstreet et al., 1996). Thus, clinical and experimental evidence strongly points to the existence of important, but poorly understood, neurobiological interactions between environmental pollutants and drugs of abuse. This new scientific field that bridges environmental health sciences, toxicology, and drug research calls for well-controlled animal models aimed to characterize the neurobiological mechanisms of drug/pollutant interactions (Miller, 2000).

In the present study, we designed a series of experiments to specifically evaluate neurobiological and behavioral responses to ethanol in Wistar rats poisoned by an organophosphate. The main mechanism of organophosphate toxicity is cholinesterase (ChE) inhibition (Bushnell et al., 1993). However, levels of ChE inhibition below 40% do not seem to be correlated to any overt sign of cholinergic toxicity (Abou-Donia, 1992). Chlorpyrifos (CPF) is an organophosphate compound (OP) used worldwide as a pesticide in agriculture (Pope, 1999; Richardson, 1995). We selected CPF to study ethanol-organophosphate interactions for two main reasons: first, CPF is an unusual organophosphate that keeps ChE activity mildly inhibited for weeks after one single sc injection (Bushnell et al., 1993; Pope et al., 1992). Second, the temporal course of CPF-induced ChE inhibition has been...
previously described, being below 40% from the 4th to the 12th week after poisoning (Pope et al., 1992). Because this level of enzyme inhibition is not associated to overt cholinergic toxicity, exposure to a single injection of CPF would provide an animal model useful to conduct extensive neurobehavioral testing during an ample temporal window of approximately 8 weeks.

In the first study, we evaluated if preexposure to the organophosphate CPF causes long-lasting ethanol avoidance in Wistar rats. For that aim, voluntary ethanol consumption was assessed during 4 weeks, starting 8 weeks post-CPF administration, a time point in which ChE activity is inhibited by approximately 40% (Bushnell et al., 1993; Pope et al., 1992). From weeks 4 through 8, an additional set of neurobiological, physiological, and behavioral responses to ethanol were evaluated; experiment 2 tested gustatory-olfactory sensorial processing by a taste preference test. Experiment 3 analyzed the effects of CPF on sensitivity to ethanol-induced hypothermia (Cunningham et al., 1992) as well as sensitivity to the aversive effects of ethanol by studying ethanol-induced conditioned flavor aversion learning (Risinger and Boyce, 2002). Increased ethanol-induced sedation and/or altered peripheral ethanol metabolism have been consistently linked to reduced voluntary ethanol intake (Thiele et al., 1998, 2003). In experiment 4, we quantified the sedative/hypnotic properties of ethanol by assessing the latency to recover the righting reflex after administration of sedative doses of ethanol.

Immunostaining for c-fos expression, the protein derived from the immediate-early gene c-fos, has been successfully employed as a marker of regional neural activity (Thiele et al., 1996) in such a way that low c-fos baseline levels are found in nonactive neurons, whereas increased c-fos expression is indicative of neural activity. Previously, we reported that CPF causes increased c-fos expression in the sickness-behavior brain system (Carvajal et al., 2005) 24 h after poisoning. That data revealed two interesting facts: first, 24 h after been administered, CPF triggers (whether directly or indirectly) measurable alterations in neural activity. Second, c-fos immunostaining is a powerful experimental strategy sensitive to CPF-induced changes in cellular activity. Given that our main objective in the present study was to explore the neurobiological pathways modulating ethanol-organophosphate interactions, we quantified c-fos expression in response to CPF 24 h after poisoning in the Edinger-Westphal nucleus (EWN), a brain region recently implicated in modulating neurobiological responses to ethanol (Bachtell et al., 2002; Ryabinin et al., 2003). This experimental approach would reveal if CPF targeted the EWN resulting in an alteration of basal neural activity when the level of ChE activity is inhibited around 40%.

**MATERIALS AND METHODS**

**Animals**

Wistar male rats (Charles River Laboratories, Barcelona, Spain) weighting 300–350 g at the beginning of the experiments were housed individually and maintained in an environmentally controlled room (22°C temperature on a 12:12 h light-dark cycle). Food and water were provided ad libitum (except in those experimental protocols requiring water restriction, i.e., experiment 3), and all the manipulations were conducted during the light phase. Behavioral procedures and pharmacological techniques were in agreement with the animal care guidelines established by the Spanish Royal Decrees 223/1988 and 1025/2005 for reducing animal pain and discomfort and the guidelines for the use of animals in toxicology assumed by the Society of Toxicology.

**CPF Administration**

Animals were habituated to the laboratory conditions for 15 days. Then they were weighed and distributed into two groups based on body weight. Group CPF was given a sc injection of CPF (O,O-diethyl-O-[3,5,6-trichloro-2-pyridyl] phosphorothioate, 99.5%, Riedel-de Haén, Seelze, Germany, dissolved in olive oil, 250 mg/kg in 1 ml/kg). Group vehicle (VEH) was given a sc injection of olive oil (1 ml/kg) as the vehicle. Immediately after injections, animals were returned to their home cages where they remained until the beginning of the experiments.

**Temporal Profile of Cerebral ChE Inhibition**

As mentioned in the “Introduction” section, the main objective in this study was aimed to evaluate long-term voluntary ethanol consumption after CPF poisoning. A single dose of CPF induces ChE inhibition that peaks 5 days after administration and slowly recovers over a period of approximately 12 weeks (Pope et al., 1992; Richardson, 1995). It is expected that ChE inhibition would be around 40% 4 weeks after administration of the poison. We provided a temporal profile of cerebral acetylcholinesterase (AChE) inhibition at those time points in which neurobehavioral and physiological responses to ethanol were tested throughout the different experiments (Fig. 1). To this end, a group of CPF- and vehicle-treated animals were randomly selected at the beginning of experiments 1, 2, and 4, and cerebral levels of AChE were quantified. The animals in each group were anesthetized with sodium pentothal (80 mg/kg in 1 mg/kg volume) and then decapitated. The whole brain was removed and immediately homogenized with 1% Triton X-100 in 0.1M sodium phosphate buffer at pH 8 at a ratio of 1/10 (wt/vol). The homogenate was centrifuged at 1000 × g for 10 min; then the pellet was discarded, and the supernatant was kept for AChE assay. AChE activity was determined by the Ellman Method (Ellman et al., 1961), which is based on the measurement of the thiocholine production rate when acetylthiocholine is hydrolyzed. This was achieved by observing the continuous reaction of thiocholine with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) that produce the yellow anion of 5-thio-2-nitrobenzoate. The rate of color production was measured at 412 nm by using a DU 530 Beckman spectrophotometer. The reaction was carried out by using 0.2 ml of 0.33mM DTNB, 0.05 ml of 50µM tetaisopropyl pyrophosphoramide (is-OMPA), specific inhibitors for Butyrylcholinesterase, 0.03 ml of 0.5mM acetylcholine iodide (used as the substrate), and 0.7 ml of 0.1M phosphate, pH 8. Enzyme activity was calculated relative to protein concentration by the Bradford method (Bradford, 1976). For biochemical assays, acetylthiocholine iodide, is-OMPA, and DTNB were purchased from Sigma-Quimica, Madrid, Spain.

**Experiment 1. Voluntary Ethanol Consumption**

In humans, organophosphate exposure has been associated with reduced intake of ethanol-containing beverages (Tabershov and Cooper, 1966). In the present experiment, we assessed long-term voluntary ethanol consumption in two groups of Wistar rats, one exposed to a single dose of CPF (n = 16) and a second one exposed to VEH (n = 14) (see “CPF Administration” section for procedural details). Once CPF or VEH were administered, the animals rested in their home cages with no experimental manipulations for 8 weeks. During this time, water and food intake and body weight were taken every 3 days. Eight weeks post-CPF poisoning, a group of animals from the CPF (n = 5) and VEH (n = 5) groups were randomly selected for cerebral AChE assays as mentioned above. The rest of the animals were then exposed to an ethanol-drinking schedule. Animals were given 24 h access to two bottles, one containing plain water and the other containing a solution of ethanol in plain water.
The positions of the bottles were changed every 3 days to control for position preferences, and the concentration of the ethanol solution offered to the animals was progressively increased (8, 15, and 20%) every 10 days. In order to obtain a measure of ethanol consumption that corrected for individual differences in the rat size, the index "g ethanol/kg/24 h" was calculated for each ethanol concentration. In addition, food intake (g/kg/24 h), water intake (ml/kg/24 h), and body weight were recorded. Finally, an ethanol preference ratio independent of the rat size was calculated at each ethanol concentration (ml ethanol/ml ethanol + ml water).

Experiment 2. Taste Preference Test for Sucrose, Saccharin, and Quinine

Ethanol is a bitter-sweet complex stimulus (Kiefer et al., 1990), and altered voluntary ethanol consumption could be the result of altered gustatory processing. To evaluate this possibility, we conducted a taste preference study to saccharin, sucrose, and quinine in CPF and VEH groups through a discriminative procedure described elsewhere (Thiele et al., 1998). A new group of animals received 15 days of habituation to the laboratory conditions. Then, the animals were weighed and distributed to groups based on body weight. Groups CPF (n = 20) and VEH (n = 20) were injected with CPF sc or olive oil, respectively. Immediately after injections, the animals were returned to their home cages, and food and water were provided ad libitum. The animals were tested for daily consumption of sucrose, saccharin, and quinine, (sucrose, saccharin, quinine hydrobromide [Sigma]) given in two different concentrations 4 weeks after injections (see Fig. 1). The order of the compounds was as follows: sucrose solutions (1.70 and 4.25%) followed by saccharin solutions (0.05 and 0.15%), and then quinine solutions (0.03 and 0.10mM). The rats had 24 h access to each solution during 48 h, and the position of the solution was counterbalanced between animals. The preference ratio for each solution was calculated by dividing the volume of the taste solution consumed by the total volume of fluid (ml taste solution/ml water + ml taste solution). At the end of the experiment, 6 weeks post-CPF administration, a group of animals in CPF (n = 4) and VEH (n = 4) groups were randomly selected for biochemical AChE assays. The rest of the animals were trained in an ethanol-induced conditioned flavor aversion task 1 week later.

Experiment 3. Ethanol-Induced Conditioned Flavor Avoidance: A Dose-Response Study

In the experiment 1, we tested voluntary ethanol consumption in CPF versus vehicle pretreated rats. Taking into account that there is an inverse relationship between sensitivity to the aversive properties of ethanol and voluntary ethanol intake (Caillol and Mormede, 2002), in the next study we determined if CPF administration would cause an increase in the aversive properties of ethanol. For that purpose, ethanol-induced conditioned flavor avoidance and body weight were recorded. Finally, an ethanol preference ratio independent of the rat size was calculated at each ethanol concentration (ml ethanol/ml ethanol + ml water).

FIG. 1. Schematic representation of time points of experimental testing.

TABLE 1

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<th>Balanced Experimental Conditions during the Flavor Avoidance Task</th>
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<td>Days 1–3</td>
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Note. On day 1 and 3, flavor 1 + ethanol represents, for half of the animals, the paired session in which the flavor is associated to the aversive stimulus ethanol. Flavor 1 + saline represents the unpaired session for the rest of the animals. On Day 2 and 4, there is a reversal of the experimental conditions and a second flavor is presented. Day 5 is a two-bottle free-choice test, in which both flavored solutions 1 and 2 are simultaneously offered for 10 min.
Body temperature. Body hypothermia in response to ethanol or vehicle was measured during flavor aversion training (Cunningham et al., 1992). Rectal temperature was measured 30 min after ethanol or vehicle injection by a lubricated flexible probe connected to an electronic Cybertec device (Barcelona, Spain).

Experiment 4. Ethanol-induced Sedation

Decreased voluntary ethanol intake might be linked to enhanced sensitivity to the sedative properties of ethanol and/or altered peripheral ethanol metabolism (Thiele et al., 1998, 2003). We evaluated ethanol-induced sedation 4 weeks post-CPF intoxication, a time point in which cerebral ChE activity is moderately inhibited around 40% (Pope et al., 1992).

A new group of animals (n = 24) were habituated to the laboratory conditions for 15 days. The animals were weighed and distributed to groups based on body weight. Groups CPF (n = 12) and VEH (n = 12) were given sc injection of CPF or olive oil, respectively. Immediately after injections, the animals were returned to their home cages where they remained for 4 weeks until the sedation study began.

On the test day, randomly selected rats in CPF group (n = 4) and VEH group (n = 4) were decapitated, and their brains were extracted for biochemical AChE assays. The rest of the animals received a sedative dose of ethanol in an ip injection (3.0 g/kg, 25% [wt/vol] mixed in isotonic saline), and the latency to regain the righting reflex was recorded. At the onset of ethanol-induced sedation, each rat was placed on its back. The time that elapsed between ethanol administration and when the rat could complete turn over onto all four paws, three times within a 30-s interval, was employed as the latency index to regain the righting reflex.

Blood ethanol levels. In order to evaluate if blood ethanol levels differed in CPF- versus vehicle-treated rats in the ethanol-induced sedation test, a tail blood sample was collected 1 h after ethanol administration in all the animals in the experiment 4. Blood samples were collected in Eppendorf vials containing heparin (Byk Elmu S.A., Arganda del Rey, Spain). The samples were centrifuged at 1000 g for 10 min; then the pellet was discarded, and the supernatant was kept for the assay. Ten microliter of plasma was mixed with 3 ml nicotinamide adenine dinucleotide-alcohol dehydrogenase reagent in glycine buffer (NAD, 9.6 mmol; ADH, 800U in 16 ml glycine buffer, Sigma, St Louis, MO). Plasma ethanol levels were determined by spectrophotometric methods (Enzymatic Determination of Alcohol Test; Sigma) and calculated as mg/dl.

Experiment 5. Quantification of c-fos Expression in the EWN in CPF-Treated Rats: An Immunocytochemical Study

It has been recently reported that the EWN is an important neural target for ethanol (Bachtell et al., 2002; Ryabinin et al., 2003). Chronic ethanol intake or acute ethanol administration elevates c-fos expression in the EWN (Chang et al., 1995). Because CPF alters neural activity in several brain regions 24 h after administration (Carvajal et al., 2005), we determined if CPF modifies neural activity in the EWN by quantifying c-fos expression.

A new group of animals received 15 days of habituation to the laboratory conditions. Then, they were weighed, distributed in two groups, and given sc injection of CPF (n = 6) (250 mg/kg/ml) or VEH (n = 6) (olive oil sc), respectively. The animals were euthanized 24 h later with an overdose of sodium pentothal (80 mg/kg in 1 mg/kg volume) and transcardially perfused with phosphate-buffered saline (PBS) followed by 0.1M phosphate-buffered paraformaldehyde 4% (pH 7.4). The brains were removed and immersed in PBS for 48 h at 4°C. Brains were cut in coronal sections 50 mm thick with a motorized vibratome. Slices were rinsed (3× PBS), incubated for 30 min in 0.3% H2O2 in absolute methanol to quench endogenous peroxidase, and rinsed (3× PBS) and incubated for 1 h in 3% goat serum in PBS. Slices were then transferred, without rinsing, to the primary antibody solution; which consisted in 1:10,000 c-fos polyclonal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) that recognizes residues 3–16 of the c-fos protein. After 36 h incubation at 4°C, the slices were rinsed (10× PBS) and processed with ABC method (Vector Laboratories, Burlingame, CA). Briefly, the slices were transferred to a solution containing biotinylated anti-rabbit IgG for 1 h, rinsed (10× PBS), transferred to avidin-biotin peroxidase for 1 h (5× PBS, 30 min, then 5× PBS, 30 min), and developed with nickel-intensified diaminobenzidine substrate (6 min). Following proper development, slices were rinsed (PBS, 10 min), mounted on slides, and coveslipped with Permoun.

Stained sections were examined through a microscope (Olympus, B250) with ×40 magnification; c-fos–positive cells in EWN (area 100 × 100 microns) were scored through an attached camera lucida by an observer blind to the experimental conditions.

RESULTS

Experiment 1. Voluntary Ethanol Consumption

Food, water, and ethanol intake data obtained 8 weeks post-CPF poisoning were analyzed for each ethanol concentration (8, 15, and 20% vol/wt) by independent two-way 2 × 3 (treatment × concentration) repeated-measures ANOVAs. This analysis allowed us to compare consumption of water, food, and ethanol in CPF- versus vehicle-administered rats. The ANOVA performed on ethanol intake data showed a significant treatment effect (F1,20 = 9.086; p < 0.01), a significant concentration effect (F2,40 = 3.14; p < .05), and a significant treatment × concentration interaction (F2,40 = 4.50; p < .05). Newman-Keuls post hoc tests revealed that CPF pretreated rats showed lower ethanol consumption than control group at 8, 15, and 20% concentration (Fig. 2a). Moreover, ethanol avoidance observed in CPF-treated rats seem to be unrelated to nonspecific alterations in fluid regulation given that no statistical differences in the total fluid consumption (F1,20 = 0.252; p > .05) were observed in CPF versus VEH rats (Fig. 2c). In addition, statistical analysis aimed to compare food intake in CPF and VEH group yielded a significant treatment effect (F1,20 = 4.608; p < .05), a significant concentration effect (F2,40 = 63.26; p < .001), and a significant treatment × concentration interaction (F2,40 = 22.077; p < .001); Newman-Keuls post hoc test revealed decreased food consumption in VEH-treated rats relative to food intake in CPF animals, which might be the result of the physiological regulatory process aimed to properly equilibrate the final caloric balance in VEH rats that drink higher amounts of ethanol than CPF rats. This hypothesis is consistent with the absence of statistical differences in averaged body weight in CPF versus VEH rats all over the study (F1,20 = 0.026; p > .05).

Ethanol preference data confirmed previous results by revealing an ethanol avoidance in CPF-poisoned animals. The ANOVA conducted showed a statistically significant treatment effect (F1,20 = 12.56; p < .01), a significant concentration effect (F2,40 = 38.39; p < .001), and a significant treatment × concentration interaction (F2,40 = 11.91; p < .001); subsequent Newman-Keuls tests revealed that CPF-treated rats showed a lower ethanol preference when compared to control animals at 8 (p < .001) and 15% (p < .001) (Fig. 2b) and a nonstatistically significant trend at 20%. Thus, CPF administration reduces ethanol preference in Wistar rats even 8 weeks after poisoning.
Considering the water, food, and ethanol consumption/preference data, it is tempting to suggest that CPF administration elicited neurobiological changes that lead to ethanol avoidance in experiment 1, an effect that was not secondary to nonspecific disturbances in fluid homeostasis or caloric unbalance.

Experiment 2. Taste Preference Test: A Dose-Response Study

To determine if CPF elicits nonspecific alterations in taste processing that might underlie a secondary decrease in voluntary ethanol intake in experiment 1, we tested consumption of several tastes: sucrose, saccharin, and quinine, in CPF versus VEH-treated rats. The preference ratio (volume of the taste solution consumed/the total volume of fluid [water + solution]) of each taste solution was independently analyzed by a student t-test for independent samples. The analyses revealed no significant differences in CPF and VEH rats in taste preferences for sucrose 1.70%: $t = -1.338, p > .05$; sucrose 4.25%: $t = -1.383, p > .05$; saccharin, 0.05%: $t = 0.598, p > .05$; saccharin 0.15%: $t = 0.889, p > .05$ or quinine 0.03mM: $t = 1.061, p > .05$; quinine 0.10mM: $t = 1.833, p > .05$ (Fig. 3). In addition, consistent with proper fluid homeostasis, CPF and VEH groups showed a similar amount of total fluid consumption during the taste preference study ($p > .05$). Thus, ethanol avoidance after CPF administration is not the result of alterations of taste preference.

Experiment 3. Ethanol-Induced Conditioned Flavor Avoidance: A Dose-Response Study

Consumption data obtained in the two-bottle choice test after conditioned flavor avoidance training were analyzed by a three-way, $2 \times 2 \times 3$ (treatment $\times$ drug $\times$ dose) ANOVA. The treatment factor examined differences in total liquid intake between CPF and vehicle group; the drug factor compared the total intake of the flavored solution specifically paired with ethanol with the total intake of the flavored solution paired with saline; the dose factor assessed if different doses of ethanol induce different rate of liquid consumption. The ANOVA conducted on flavor avoidance data revealed a statistically significant effect for the main factor, dose ($F_{2,24} = 5.986; p < .05$), and drug ($F_{1,24} = 21.041; p < .01$). Neither the treatment factor nor any of the interactions assessed attained statistical significance.

Thus, according to previous data (Cubero et al., 2001), present results suggest that the experimental procedure employed in the study to induce flavor avoidance was successful; so the animals trained in the conditioning task mostly avoided the flavored solution specifically paired to ethanol on the test day. That is, both groups, VEH and CPF, acquired an ethanol-induced conditioned avoidance response to the paired flavor.

In order to assess if the strength of the flavor avoidance developed by CPF versus VEH animals was comparable, an aversion index (ml unpaired flavor/total ml) was calculated.

![FIG. 2. Values shown here represent voluntary consumption (mean ± SEM) of 8, 15, and 20% vol/wt ethanol solutions (g/kg/24 h) (a); preference ratio (ml ethanol/total ml) (b); total fluid consumed (ethanol + water, ml/kg/24 h) (c). The analysis indicated that CPF group drinks less alcohol but more water (data no shown) than vehicle group. The total volume of liquid daily consumed by experimental and control animals did not differ. Also, the ethanol preference index was lower in CPF-treated animals than in vehicle-treated rats. Significant difference from vehicle group *$p < .05$.](https://academic.oup.com/toxsci/article-abstract/96/2/310/1661089/3)
A version index data were analyzed by a two-way, 2 × 3 (treatment × dose) ANOVA. The ANOVA showed that neither the treatment factor (F1,24 = 0.454; p > .05), the dose factor (F2,24 = 2.933; p > .05), or the interaction treatment × dose (F2,24 = 0.102; p > .05) attained significance. Thus, present analysis suggests that the strength of conditioned aversion was strong and similar at all doses of ethanol employed, both in CPF and vehicle pretreated rats (Fig. 4). A petición del reviewer 1 apartado 6. Therefore, a ceiling effect cannot be ruled out. Testing lower doses of ethanol in the conditioned flavor aversion task inducing a weaker conditioned aversion would, potentially, reveal enhanced sensitivity to the aversive properties of ethanol in CPF-treated rats.

**Body temperature.** We measured ethanol-induced hypothermia during the flavor avoidance training, as a sensitive physiological parameter indicative of ethanol toxicity (Cunningham et al., 1992). A two-way (treatment × dose) ANOVA, which compared body hypothermia induced by different doses of ethanol in CPF and VEH rats, revealed no significant treatment (F1,24 = 0.039; p > .05) or dose (F2,24 = 2.147; p > .05) effects. Thus, 7 weeks after poisoning, CPF treatment did not alter ethanol-induced hypothermia. Together, the present data suggest that CPF does not increase the hypothermic or aversive properties of ethanol as assessed by conditioned flavor avoidance and ethanol-elicted hypothermia, at least when tested 7 weeks after CPF administration (Fig. 5).

**Experiment 4. Ethanol-Induced Sedation**

Sedation data were analyzed using a one-way ANOVA, with a between-subject factor that compared the sedation time elicited by ethanol injections in CPF- and VEH-treated rats, as measured by the latency to recover the righting reflex, 4 weeks after poisoning. The ANOVA showed a statistically significant effect of treatment (F1,13 = 7.79; p < .05). That is, 4 weeks post-CPF treatment, poisoned rats were more sensitive to the sedative effects of 3.0 g/kg dose of ethanol revealed by a longer latency to regain the righting reflex (Fig. 6). Thus, present data suggest long-term increases in the sedative/hypnotic effects of ethanol as a result of a single dose of CPF.

**Blood ethanol levels.** During the sedation test, 1 h after ethanol administration, a tail blood sample was collected to evaluate blood ethanol level. Data were analyzed using a one-way ANOVA with treatment as the main between-subject factor. The analysis conducted revealed that blood ethanol levels did not differ significantly between CPF-treated rats (109.93 ± 33.03 mg/dl) and vehicle-treated rats (78.71 ± 29.14 mg/dl) 1 h post-ethanol administration. Therefore, the different pattern of sedation in response to ethanol in CPF versus VEH rats found in experiment 4 is not due to increased blood ethanol levels in CPF-poisoned rats.

**Temporal Profile of ChE Inhibition**

Given that CPF inhibits AChE over weeks (Bushnell et al., 1993; Pope et al., 1992), we assessed the cerebral temporal profile of AChE activity in three parallel groups of rats randomly selected from the experiments 1, 2, 3, and 4. Cerebral AChE data were analyzed by independent one-way ANOVAs...
with a single between-subject factor, treatment, which compared AChE activity in CPF- and vehicle-administered rats at relevant time points, 4, 6, and 8 weeks post-CPF poisoning. The ANOVA conducted in brain AChE data obtained 4 weeks after poisoning revealed a statistically significant effect of treatment ($F_{1,6} = 42.00, p < .01$) in ChE activity (CPF $0.02683 \pm 0.00052 \mu$mol/min/mg, VEH $0.04830 \pm 0.00327 \mu$mol/min/mg). Newman-Keuls post hoc test showed decreased AChE activity in CPF-treated rats ($p < .001$), being 44.6% inhibited, when compared with AChE activity in the control group (Fig. 7).

The analysis conducted on AChE data obtained 6 weeks post-CPF poisoning revealed a statistically significant effect of treatment ($F_{1,6} = 10.35; p < .05$) in AChE activity (CPF $0.05295 \pm 0.0036, \mu$mol/min/mg; VEH $0.06922 \pm 0.0035 \mu$mol/min/mg). Newman-Keuls post hoc tests showed decreased AChE activity in CPF-treated rats ($p < .001$), being 23.6% inhibited when compared with cerebral AChE inhibition in the control group (Fig. 7).

Finally, 8 weeks after injection, the analysis conducted to cerebral AChE data revealed a statistically significant effect of treatment ($F_{1,8} = 6.42; p < .01$) in AChE activity (CPF $0.05646 \pm 0.0033 \mu$mol/min/mg, VEH $0.06564 \pm 0.0014 \mu$mol/min/mg). Newman-Keuls post hoc tests showed a slight inhibition (14%) of AChE activity in CPF-treated rats ($p < .001$) compared to AChE activity in the control group (Fig. 7).

Thus, consistent with previous work reporting a temporal profile of AChE inhibition lasting for 12 weeks (Pope et al., 1992), the present study showed that a single dose of CPF keeps AChE activity moderately inhibited for 10 weeks, which suggests a long-lasting active presence of the toxic compound in the organism (Richardson, 1995).

**Experiment 5. c-fos Expression in EWN**

Data of c-fos–positive cells in the EWN (Fig. 8) in response to CPF versus vehicle administration were analyzed with a one-way ANOVA that revealed a significant effect of treatment, $F_{1,9} = 15.196; p < .05$. The present results provided evidence of a significant decrease in the number of c-fos–positive cells in the EWN 24 h after CPF administration when compared to
c-fos–positive cells after a vehicle injection (Fig. 9). This reduced c-fos expression might be indicative of reduced basal neural activity as a result of CPF administration.

**DISCUSSION**

Clinical reports have described intolerance to nicotine and ethanol-containing beverages in organophosphate-exposed Gulf War veterans (Miller, 2000) and agricultural workers (Tabershow and Cooper, 1966). In addition, Flinder rats, which have been bred for increased sensitivity to organophosphate poisoning, showed enhanced responses to ethanol and nicotine (Overstreet et al., 1996). In agreement with clinical and experimental data, the present study provides evidence of enhanced sensitivity to ethanol in animals exposed to the organophosphate CPF. Animals showed reduced voluntary ethanol consumption 8 weeks after poisoning unrelated to primary disturbances in fluid homeostasis or caloric unbalance. Moreover, ethanol avoidance was not secondary to sensorial gustatory disturbances since both CPF and VEH rats showed the same pattern of taste preference for sweet and bitter stimuli in a taste preference test (experiment 2). In the present study, we tested if CPF modifies the sickness/toxicant properties of ethanol. To this end, ethanol-induced flavor aversion and hypothermia were evaluated 7 weeks after poisoning, a time point in which AchE activity was 23% inhibited. Animals pretreated with CPF and trained in an ethanol-induced flavor avoidance task showed a similar pattern of flavor avoidance and body hypothermia to that showed by VEH rats in response to ethanol. However, given that the results obtained in that study did not provide a clear dose-response effect, a ceiling effect that might have overshadowed a potential enhancement of sensitivity to the aversive properties of ethanol cannot be ruled out.

Finally, data obtained in experiment 4 revealed that 4 weeks after poisoning, CPF-treated rats showed enhanced sensitivity to the sedative properties of ethanol that were not associated with altered blood ethanol levels. In addition, AchE data obtained at that time point of experimental testing showed a 40% reduction of AchE in CPF-treated animals. Previous research has demonstrated that low voluntary ethanol intake is strongly correlated with increased ethanol-induced sedation (Thiele et al., 1998, 2003). Thus, it is tempting to postulate that the reduction in ethanol intake described in the first experiment, 8 weeks after intoxication, might be due to CPF-induced enhancement in ethanol sedation. However, given that experiments 1 and 4 were conducted at different time points, further testing is needed to specifically evaluate this hypothesis.
The present study demonstrates that administration of a single dose of CPF elicits increased sensitivity to ethanol, as revealed by long-lasting ethanol avoidance and increased sensitivity to ethanol-induced sedation. The fact that one single injection of an organophosphate caused long-term and long-lasting enhanced sensitivity to ethanol might be surprising; however, the temporal biochemical profile provided in this study showing cerebral AChE mildly inhibited for weeks (see Fig. 6) is indicative, as previously suggested, of a prolonged active presence of the organophosphate and/or the oxon metabolite in the brain (Bushnell et al., 1993; Richardson, 1995).

Taking all results together, it is tempting to suggest that CPF exposure modulates cellular activity in neuronal pathways critically involved in ethanol processing. Several mechanisms of CPF toxicity might account for that increased ethanol sensitivity. First, ChE inhibition is the main mechanism of organophosphate toxicity (Pope, 1999; Bushnell et al., 1993) and the present data have provided evidence of long-lasting AChE inhibition correlated with disturbances in neurobehavioral responses to ethanol. Interestingly, experimental studies that have explored the effect of AChE upon ethanol drinking in alcohol-prefering Alko-alcohol rats report that administration of the ChE inhibitors desoxypegaine and galanthamine reduce ethanol intake and preference dose dependently without altering total fluid intake and food consumption (Doetkotte et al., 2005). Thus, long-lasting CPF-induced inhibition of cerebral AChE might cause ethanol avoidance.

Second, alternative noncholinesterasic mechanisms of CPF neurotoxicity (Casida and Quistad, 2004) might also be involved. It has been consistently reported that CPF-oxon inhibits intracellular adenylate cyclase activity and cAMP signaling both directly, by muscarinic receptor–independent mechanisms, and indirectly, through agonistic interaction with m2/m4 subtypes of muscarinic acetylcholine receptors (Huff and Abou-Donia, 1995; Ward and Mundy, 1996). Interestingly, it has been reported that genetic alteration of the neuronal cAMP-protein kinase A (PKA) signaling pathway modulates alcohol drinking behavior as well as sensitivity to the sedative effects of alcohol. Mice with the targeted disruption of one Galpha allele as well as mice with reduced neuronal PKA activity show decreased alcohol consumption compared with their wild-type littermates and increased sensitivity to the sedative effects of ethanol (Wand et al., 2001; Chandler et al., 2004). In addition, genetic studies employing altered adenylate cyclase isoforms (AC1/AC8) show that AC1 knockout (KO) mice and double knockout (DKO) mice with genetic deletion of AC1, a specific isoform of adenylate cyclase, display substantially increased sensitivity to ethanol-induced sedation compared with wild-type mice. Moreover, AC8 KO and DKO mice demonstrate decreased voluntary alcohol consumption compared with wild-type mice. Thus, one may predict that CPF modulation of adenylate cyclases activity and cAMP signaling in critical neuronal pathways might elicit increased sensitivity to the sedative effects of ethanol and reduced alcohol consumption.

Third, it was recently reported that CPF targets and decreases nicotinic alpha-7nAch receptor density (Slotkin et al., 2004). Interestingly, knockout mice lacking alpha-7nAchR receptor specifically show reduced ethanol intake and increased sedation to ethanol with no evidence of altered peripheral ethanol metabolism or primary alterations in gustatory sensorial processing (Bowers et al., 2005; de Fiebre and de Fiebre, 2005). Therefore, long-lasting alpha-7nAchR modulation by CPF might also represent a critical molecular substrate underlying enhanced sensitivity to ethanol in poisoned organisms.

Given that CPF exerts multiple forms of cellular toxicity, the present results cannot clarify whether alterations in neurobehavioral responses to ethanol are the result of one single biochemical mechanism or rather they are the final expression of a complex cellular dysfunction involving interactions among several intracellular biochemical mechanisms. Most of the basic research aimed to describe cerebral mechanisms involved with organophosphate-induced neurotoxicity has focused on the molecular and neurochemical level (Abou-Donia et al., 2003; Huff and Abou-Donia, 1995; Katz et al., 1997).

However, identifying specific anatomical targets has recently been proposed as a valuable tool for a more complete understanding of the neurobehavioral impairments caused by OPs (Gupta, 2004). Since organophosphate exposure has been associated to increased sensitivity to ethanol in humans and animals, determining if CPF targets specific brain systems critically involved in neurobiological responses to ethanol might open new research lines. In this regard, we have recently described a pattern of c-fos expression in the “sickness-behavior” cerebral system after CPF administration (Carvajal et al., 2005) that resembles the well-known regional pattern of neural activity elicited by an ample range of toxins such as ethanol (Chang et al., 1995; Thiele et al., 1996). It is known that this neuroanatomical system triggers an “alert response” when dangerous chemicals gain access to the brain enabling neurobehavioral adaptive responses to be properly organized (Dantzer, 2001). In this regard, one may predict that CPF might target the sickness-behavior brain system, inducing a persistent increase in neural activity. If the sickness-behavior brain system is exposed to ethanol in conditions of neural overstimulation, then one might predict an enhancement in the sickness-responses usually elicited by ethanol. Our present data did not detect significant enhancement of sensitivity to the sickness properties of ethanol; however, the presence of certain trends in our data and potential ceiling effects in the flavor aversion study as well might explain lacking of experimental results in that direction. Thus, the hypothesis claiming neural sensitization of the sickness-response brain system cannot be ruled out.

The experiment 5 revealed decreased c-fos expression in response to CPF in the EWN, which is a brain area recently implicated as an important neural target for voluntary ethanol intake and sedative responses to ethanol (Bachtell et al., 2002,
2004; Chang et al., 1995; Ryabinin et al., 2003). Moreover, among all the brain areas known to be involved in ethanol intake, the EWN is the only one that has been related both to voluntary or forced ethanol administration. Lesions of the EWN are followed by disrupted ethanol consumption and enhanced sedative responses in C57BL/6 mice (Bachtell et al., 2004). Interestingly, EWN cells express nicotinic receptors in their membranes which might act as cellular gates through which CPF agonistic activity regulates intracellular signaling and neural activity in a brain region critically involved in ethanol. Thus, the present c-fos data suggest that CPF administration elicits inhibition of basal neural activity in the EWN, which is in agreement with neurobehavioral data showing reduced ethanol intake and increased sedation in CPF-treated animals.

Taken together, the present results suggest that CPF administration might be a valuable tool to further explore the neurobiological substrates of ethanol sensitivity in organisms exposed to organophosphates. Future experimental research is required to more extensively test neurobiological responses to ethanol at 8 weeks after intoxication. The search for specific brain systems and biochemical mechanisms of CPF toxicity underlying ethanol-organophosphates neurobehavioral interactions would open new perspectives to that promising and exciting scientific field that tries to bridge environmental health sciences, toxicology, and drug research.

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