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A Single High Dose of Chlorpyrifos Reduces Long-Term Basal C-Fos Expression in the Rat Arcuate Hypothalamic Nucleus

La Administración de una Dosis Aguda de Clorpirifós Reduce a Largo Plazo la Expresión Basal de C-Fos en el Núcleo Arqueado del Hipotálamo

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CARVAJAL, F.; LERMA-CABRERA, J. M.; SÁNCHEZ-AMATE, M. C. & CUBERO, I. A single high dose of chlorpyrifos reduces long-term basal c-fos expression in the rat arcuate hypothalamic nucleus. ***Int. J. Morphol., 32(1)***:90-96, 2014.

SUMMARY: Chlorpyrifos (CPF) is an organophosphate compound used worldwide as a pesticide in agriculture that, after subcutaneous injection, keeps acetylcholinesterase (AChE) activity inhibited within an organism for months. Ample clinical and experimental evidence shows that CPF exposure induces relevant and persistent neurobehavioral deficits in humans and animals, even after one single episode/injection. Additionally, clinical and epidemiological studies evidence that a significant percentage (60%) of Gulf War veterans as well as agricultural workers suffering from acute OP intoxication may have developed intolerance to nicotine and ethanol-containing beverages. Consistent with it, administration of a single high dose of CPF to adult Wistar rats elicited long-lasting reduced voluntary ethanol drinking and increased sedation to ethanol without evidence of altered ethanol metabolism, which indicates that CPF-ethanol neurobiological interactions may exist. In this study, we explore whether CPF exposure induces significant disturbances in basal and/or ethanol-evoked neural activity in a set of cholinoceptive brain regions critically involved with neurobiological responses to ethanol. For this aim, brain regional c-fos expression in response to acute ethanol (1.5 or 3.0 g/kg, i.p.) or saline was assessed in adult male Wistar rats previously injected with either a single high dose of CPF (250 mg/kg, sc) or vehicle. We found that CPF administration reduces long-term basal, but not ethanol-evoked, c-fos expression in the arcuate hypothalamic nucleus. Because independent brain pathways may modulate acute responses to ethanol and voluntary ethanol consumption, we do not rule out the contribution of basal neural disturbances observed in the Arc to CPF-elicited ethanol avoidance.

KEY WORDS: Chlorpyrifos; Organophosphates; C-fos expression; Arcuate hypothalamic nucleus; Ethanol consumption.

INTRODUCTION

Humans are continuously exposed to a variety of environmental neurotoxicants. However, despite to the fact that every day we are exposed simultaneous and/or sequentially to a large number of them via multiple exposure routes, the vast majority of toxicity studies and risk evaluations deal with single chemicals. Thus, further studies is needed to specifically evaluate neurobiological interaction between neurotoxic.

Organophosphates (OPs) are potent neurotoxic compounds widely employed in industry, households and agriculture. The main mechanism of OPs neurotoxicity is cholinesterase inhibition although alternative non-cholinergic targets have also been described (Casida & Quistad, 2004). Clinical and experimental research suggests that acute and chronic exposure to OPs may cause behavioral, emotional and cognitive impairments in humans and animals (Delgado *et al*., 2004; Lopez-Crespo *et al*., 2007; Sanchez-Amate *et al*., 2001). Additionally, 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 toxicant-induced loss of tolerance, multiple chemical intolerance and increased sensitivity to drugs of abuse (Miller, 2001; Sorg *et al*., 2001). Noteworthy, a significant percentage (60%) of Gulf War veterans as well as agricultural workers suffering from acute OP intoxication may have developed intolerance to nicotine and ethanol-containing beverages (Miller;

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Tabershaw & Cooper, 1966). Furthermore, experimental studies have shown that Flinder rats, which have been bred for increased sensitivity to OP poisoning, exhibit enhanced responses to ethanol and nicotine (Overstreet *et al*., 1996). Taking together, available evidence in this new scientific field that bridges environmental health sciences, toxicology, and drug research suggests the existence of uncharacterized drugs-environmental pollutants neurobiological interactions.

Chlorpyrifos (CPF) is an OP used worldwide in agriculture, industry and household settings as a pesticide (United States Environmental Protection Agency, EPA, 2011) which keeps acetylcholinesterase (AChE) activity inhibited for months (Bushnell *et al*., 1993; Pope *et al*., 1992). Moreover, in agreement with previous clinical and epidemiological reports suggesting enhanced ethanol sensitivity and reduced voluntary ethanol consumption following OP exposure, preliminary evidence in our lab has revealed that adult rats treated with a single high dose of CPF show enhanced ethanol sedation and long-term avoidance of ethanol solutions, four and eight weeks post-exposure, respectively, without evidence of altered ethanol metabolism disturbances (Carvajal *et al*., 2007). Considering these later findings, there is the interesting possibility that some OPs such as CPF might induce long-lasting neural disturbances in brain systems critically involved in neurobehavioral responses to ethanol.

Immunostaining for c-fos expression, the protein derived from the immediate-early gene c-fos, has been extensively employed as a marker of regional neural activity (Thiele *et al*., 1997). Additionally, c-fos expression has been shown following OP administration (Kaufer *et al*., 1998), and we have successfully used regional assessment of c-fos activity within the brain in an animal model of CPF exposure to characterize the neuroanatomical targets of this compound (Carvajal *et al*., 2005, 2007). These data suggests that c-fos immunostaining is a valid experimental strategy to identify brain regions in which CPF significantly disturbs neural activity.

The aim of the present study was to explore whether a single high dose of CPF (250 mg/kg) that keeps cerebral AChE activity inhibited for weeks (Bushnell *et al*.; Pope *et al*.) elicits long-term alterations in basal and/or ethanol induced neural activity of cholinoceptive brain regions critically involved in ethanol intake, as assessed by regional c-fos expression. To this end, eight week after CPF treatment, at a time when CPF treatment has been shown to trigger avoidance of ethanol solutions in rats (Carvajal *et al*., 2007), regional c-fos expression in response to acute ethanol (1.5 or 3.0 g/kg) or saline i.p. was quantitatively assessed in a set of cholinoceptive brain regions which express ethanol-induced c-fos immunoreactivity (Chang *et al*., 1995; Thiele *et al*., 1997); namely, the central nucleus of the amygdala (CeA), the Edinger-Westphal nucleus (EW), the lateral parabrachial area (lPB), and the locus coeruleus (LC).

Finally, given the key role of the hypothalamic feeding neuropeptides produced in the arcuate nucleus (Arc) in ethanol consumption (for review, Thiele *et al*., 2003), our second aim was to assessed the impact of CPF administration on basal and ethanol-induced c-fos expression in these cerebral area.

MATERIAL AND METHOD

Animals. Wistar male rats (Charles River Laboratories, Spain) weighing 300-350 g at the beginning of the experiments were housed individually and maintained in an environmentally controlled room (22°C; 12:12-h light-dark cycle). Experimental procedures were in agreement with the animal care guidelines established by the Spanish Royal Decree 1025/2005 for reducing animal pain and discomfort, and all of the procedures were approved by the Bioethical Committee for Animal Research at the University of Almeria.

CPF administration. Animals were habituated to the lab conditions for 15 days. Then they were weighed and equally distributed into two groups based on body weight. Group CPF (n=16) was given a s.c. injection of CPF (O,O´-diethyl-O-[3,5,6–trichloro-2-pyridyl] phosphorothioate, 99.5%, Riedel-de Haën, Germany, dissolved in olive oil, 250 mg/kg in 1 ml/kg). Group vehicle (VEH; n= 14) was given a sc injection of olive oil (1 ml/kg) as the vehicle. The dose and route of administration of CPF has been previously shown to elicit neurobehavioral disturbances without classical sign of cholinergic toxicity in adult rats (Lopez-Crespo *et al*.; Cardona *et al*., 2006). Immediately after injection, the animals were returned to their home cages where they remained until the beginning of the experiment.

Cerebral c-fos expression induced by acute intraperitoneal ethanol. Eight weeks post CPF-poisoning, animals in both groups were injected with saline or ethanol i.p. (25% w/v, mixed in isotonic saline) in one of two possible doses: 1.5 or 3.0 g/kg, which trigger a significant increase in c-fos expression in the LC, lPB, CeA and EW (Chang *et al*.; Thiele *et al*., 1997). Following injection, their brains were processed to quantify ethanol, and saline-induced c-fos expression. The animals were euthanized 2 h post-ethanol administration with an overdose of sodium pentothal (80 mg/kg in 1 ml/kg volume) and transcardially perfused with phosphate buffered saline (PBS) followed by 0.1 M

phosphate buffered paraformaldehyde 4% (ph 7.4). The brains were removed and immersed in PBS for 48 h at 4ºC. A motorized vibratome was used to cut 50-µm-thick coronal sections. Brain regions were identified based on Paxinos and Watson stereotaxic atlas coordinates (Paxinos & Watson, 1998): the arcuate hypothalamic nucleus (Arc), bregma -2.12 to -2.8 mm; the central nucleus of the amygdala (CeA), bregma -3.14 to -2.30 mm; the Edinger-Westphal nucleus (EW), bregma -5.8 mm; the lateral parabrachial area (lPB), bregma -9.16 mm and the Locus Coeruleus (LC), bregma -10.04 to -9.68 mm.

Immunostaining for c-fos. Brain slices were rinsed (3X, PBS), incubated for 30 min in 0.3% H_2O_2 in absolute methanol to quench endogenous peroxidase, rinsed again (3X, 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 of 1:10000 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 4ºC, slices were rinsed (10X, 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 (10X, PBS), transferred to avidin-biotin peroxidase for 1 h (5X, PBS; 30 min; then 5X, PB; 30 min) and developed with nickel-intensified diaminobenzidine substrate (6 min). Following proper development, slices were rinsed (PBS, 10 min), mounted on slides and coverslipped with Permount. Stained sections were examined through a microscope (Olympus, BX50) with 40X magnification; c-fos–positive cells in selected brain regions (area of approximately 0.38 mm2) were scored through an attached camera lucida by an observer blind to the experimental conditions. For analysis, great care was taken so that structures were scored in the same level using anatomic landmarks with the aid of a rat stereotaxic atlas (Paxinos & Watson). Anatomically matched pictures of the left and right sides of the brain were used to produce an average value for each brain region from each slice. In all cases, quantification of immunohistochemistry data was conducted by an experimenter unaware of group treatment.

Statistics. Regional c-fos expression data were analyzed by a 2 x 3 (treatment x dose) analysis of variance (ANOVA). The treatment factor examined differences in c-fos expression in the CPF-exposed versus the vehicle group, and dose factor evaluated regional differences in c-fos expression following i.p. administration of saline (CPF n= 5; VEH n=4) or ethanol [1.5 g/kg (CPF n= 5; VEH n=5) or 3.0 g/kg CPF n= 6; VEH n=5)]. Whenever the main factors or the interaction attained statistical significance, additional post-hoc Newman-Keuls (NK) tests were performed ($p<0.05$).

RESULTS

Central nucleus of the amygdala. Figure 1A represents average c-fos positive cells in the CeA of CPF and VEH treated rats given an i.p. injection of saline or ethanol (1.5 or 3.0 g/kg). The ANOVA showed a significant main effect of the dose ($F(2, 21) = 8.289$; $p<0.05$), and additional NK tests indicated that both doses of ethanol similarly increased c-fos expression in the CeA; no differences were observed between the high and low dose of ethanol administered. There was no statistical significance for the treatment main effect ($F(1,21) = 0.22$; $p > 0.05$) or the interaction effect ($F(2,21) = 0.91$; $p > 0.05$).

Edinger-Westphal nucleus. Figure 1B represents average c-fos positive cells in the EW of CPF and VEH treated rats given an i.p. injection of saline or ethanol (1.5 or 3.0 g/kg). The ANOVA showed a significant dose main effect ($F(2, 20) = 8.89$; $p < 0.05$), and additional NK tests indicated that acute ethanol administration elicited a dose-dependent increase of c-fos immunoreactivity compared to c-fos expression induced by saline injection. Both doses of ethanol increased c-fos expression compared to saline administration and significant differences were observed between the high and low dose of ethanol administered. There was no statistical significance for the treatment main effect ($F(1,20) = 0.02$; $p > 0.05$) or the interaction effect ($F(2,20) = 0.10$; $p > 0.05$).

Lateral parabrachial area. Figure 1C represents average c-fos positive cells in the lPB of CPF and VEH treated rats given an i.p. injection of saline or ethanol (1.5 or 3.0 g/kg). The ANOVA showed a significant main effect of the dose ($F(2, 21) = 16.19$; $p < 0.05$), and additional NK tests showed that both doses of ethanol increased c-fos expression compared to saline administration; no differences were observed between the high and low dose of ethanol administered. There was no statistical significance for the treatment main effect ($F(1,21) = 0.72$; $p > 0.05$) and the interaction effect ($F(2,21) = 0.55$; $p>0.05$).

Locus coeruleus. Figure 1D represents average c-fos positive cells in the LC of CPF and VEH treated rats given an i.p. injection of saline or ethanol (1.5 or 3.0 g/kg). The ANOVA showed a significant dose main effect ($F(2, 17) = 9.528$ $p < 0.05$), and additional NK tests showed that the high dose of ethanol significantly increased c-fos expression in the LC compared to c-fos expression induced by saline administration and the low dose of ethanol; no differences were observed between the low dose of ethanol administered and saline administration. There was no statistical significance for the treatment main effect ($F(1,17) = 0.16$; $p > 0.05$) and the interaction effect ($F(2,17) = 1.03$; $p>0.05$) were not statistically significant.

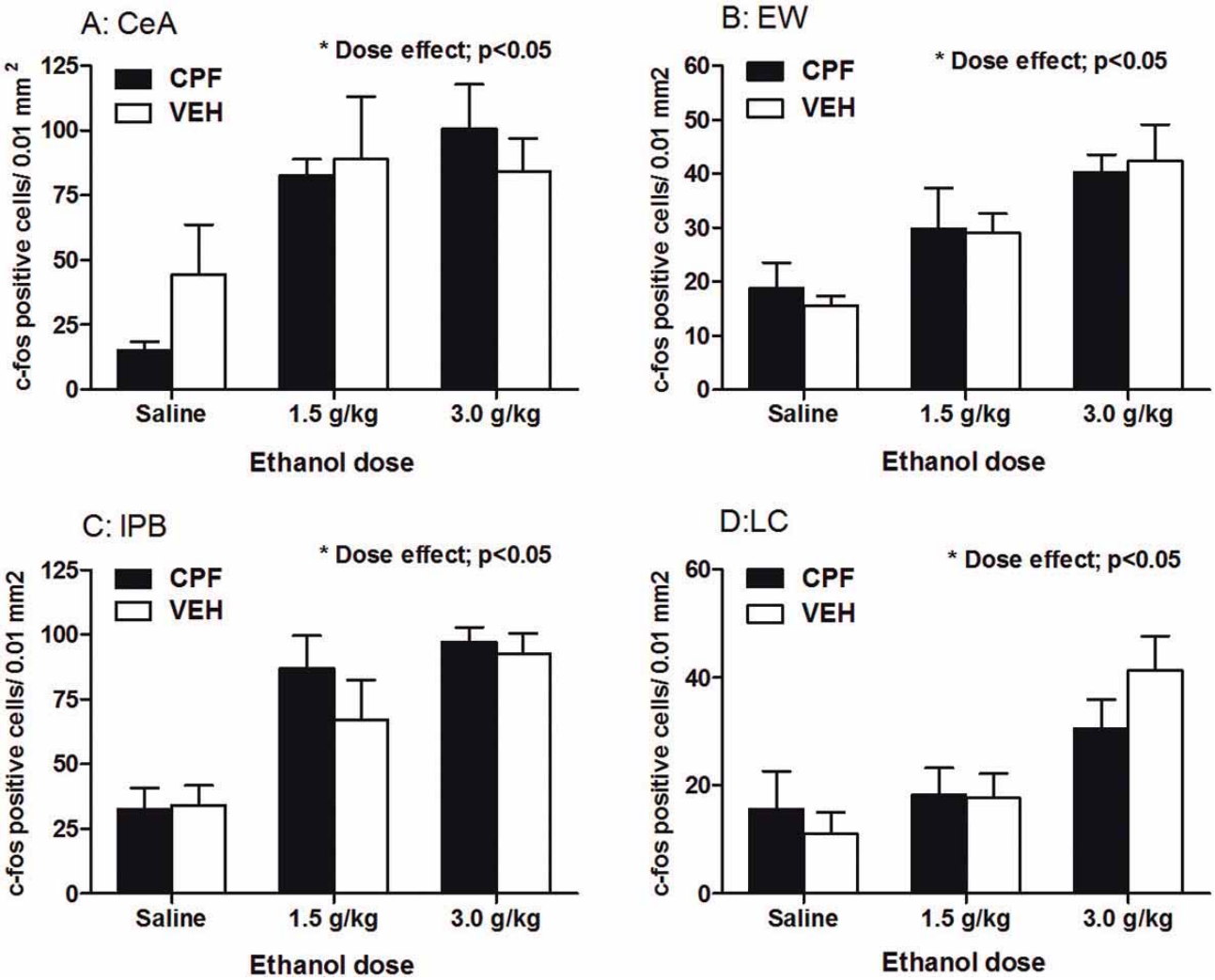

Fig. 1. Mean (±SEM) of c-fos positive cells in the central nucleus of the amygdala (A), the Edinger-Westphal nucleus (B), the lateral parabrachial area (C) and the locus coeruleus (D). Eight weeks following CPF or VEH administration, rats were given saline or ethanol, 1.5 g/kg or 3.0 g/kg i.p., and brains were collected 2 hours after injections.

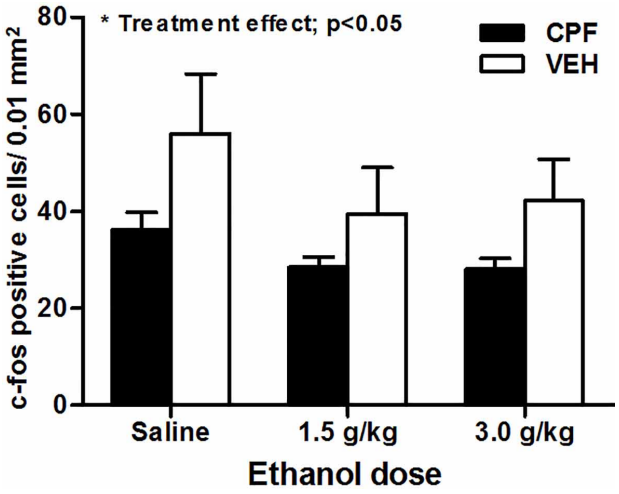

Fig. 2. Mean (±SEM) of c-fos positive cells in the arcuate hypothalamic nucleus. Eight weeks after CPF or VEH administration, rats were given saline or ethanol, 1.5 g/kg or 3.0 g/kg i.p., and brains were collected 2 hours after injections.

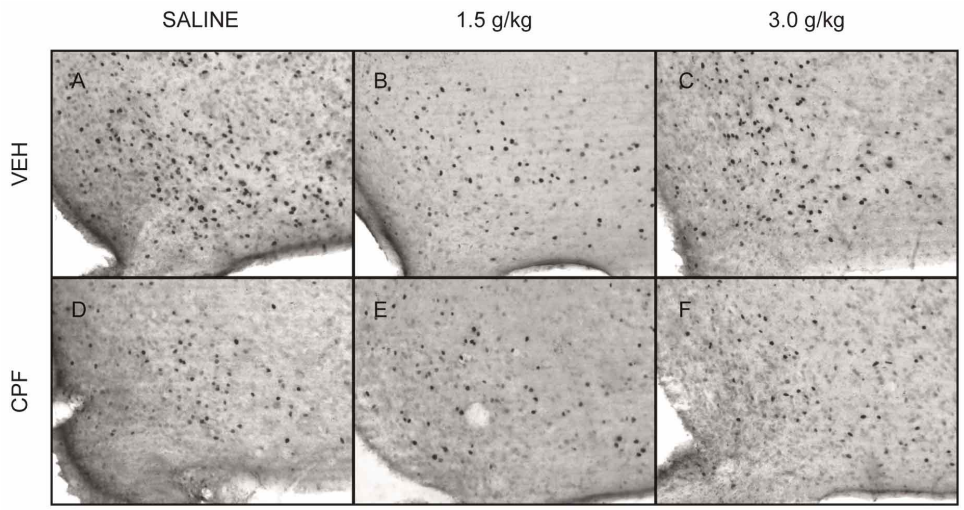

Fig. 3. Representative photomicrographs of 50 μm coronal sections showing c-fos immunoreactivity through the arcuate nucleus of the hypothalamus (Arc) in VEH (A-C) and CPF-treated rats (D-F) that were given intraperitoneal injection of isotonic saline or a 1.5 g/kg or 3.0 g/kg dose of ethanol. Images were photographed at -2.12 to -2.8 mm relative to Bregma and quantified at a magnification of 40x. Scale bar = 50 μm.

Arcuate hypothalamic nucleus. Figure 2 represents average c-fos positive cells in the Arc of CPF and VEH treated rats given an i.p. injection of saline or ethanol (1.5 or 3.0 g/kg). The ANOVA showed a significant treatment main effect ($F(1, 16) = 6.30$; $p<0.05$) indicating that CPF treatment decreased basal c-fos expression. Representative photomicrographs of c-fos immunoreactivity in both groups are presented in Figure 3. There was no statistical significance for the dose main effect ($F(2,16) = 1.65$; $p>0.05$) and the interaction effect ($F(2,16) = 0.19$; $p>0.05$).

DISCUSSION

Previous epidemiological and clinical evidence have suggested that OP exposure may cause enhanced sensitivity to ethanol and avoidance of ethanol-containing beverages. Consistent with these findings, we reported reduced voluntary ethanol consumption 8 weeks post-CPF exposure without evidence of altered ethanol metabolism (Carvajal *et al*., 2007). These results raise the possibility of the existence of uncharacterized ethanol-CPF neurobiological interactions. In the present study, we assessed basal and ethanol-evoked c-fos expression in the CeA, EW, lPB, LC and the Arc, eight weeks post-CPF exposure to test the hypothesis that this OP, either directly or indirectly through adaptive neural processes, induces long-term disturbances in cholinoceptive brain regions critically involved with neurobehavioral responses to ethanol and ethanol intake.

The main finding was that administration of a single high dose of CPF (250 mg/kg) reduced basal c-fos expression in the Arc eight weeks post-exposure. Because at this time-point there were no BW differences between groups (data not shown), absolute ethanol/saline dosing volume cannot explain differential c-fos expression in CPF-treated animals. The fact that a single injection of CPF caused long-term disturbances in neural activity might be surprising; however, in animals, it is well established that administration of 250 mg/kg of CPF triggers a strong AChE inhibition that peaks 5 days post-intoxication and slowly recovers over a period of approximately 12 weeks (Bushnell *et al*., 1993). The time-course of the biochemical profile of CPF-induced AChE inhibition together with neural adaptive processes in cholinergic and/or non-cholinergic systems (Gupta, 2004), might explain the existence of long-term neurobiological disturbances, even after one single dose of CPF.

Ethanol and CPF are both metabolized by cytochromes P450 (Ma & Chambers, 1994; Zakhari, 2006). Thus, there is the pharmacokinetic possibility of an interaction by which CPF modifies the metabolism of ethanol. However, the observation that acute ethanol administration similarly increased c-fos expression in the CeA, EW and lPB at 1.5 g/kg and 3 g/kg, and in the LC at 3 g/kg, of CPF- and saline-treated rats indicates no relevant disturbances in ethanol metabolism as a result of CPF administration, which is supported by a previous study from our lab showing similar levels of blood ethanol concentration in CPF and saline pre-treated rats in response to intraperitoneal administration of ethanol (Carvajal *et al*., 2007).

Because the Arc is a pivotal brain region contributing to energy homeostasis (Sainsbury & Zhang, 2010) one caveat that must be considered when discussing the meaning of present molecular c-fos data is the possibility that CPF triggered energy imbalance. Nonetheless, because BW is the result of the equilibrium between energy intake and energy expenditure, and we did not observe group differences in BW measures, it is unlikely that energy imbalance in CPF-treated rats accounts for present c-fos data.

A second caveat that must additionally be considered is the fact that other muscarinic-expressing regions in the study, the CeA, LC and lPB, did not show altered c-fos expression in CPF-treated rats. Present data cannot provide a conclusive explanation, but differential region-dependent muscarinic receptor density, sensitivity to CPF or regional heterogeneity for CPF-induced neural adaptations (Gupta) might account, among other, for this anatomical divergence.

Our study failed to show significant dose x treatment interactions indicating that CPF exposure did not modify the regional c-fos expression pattern emerging in response to acute ethanol administration in brain regions involved in ethanol consumption. However, because independent brain pathways may modulate acute responses to ethanol and voluntary ethanol consumption (for a review see Vilpoux *et al*., 2009), we do not rule out the contribution of basal neural disturbances observed in the Arc to CPF-elicited ethanol avoidance (Carvajal *et al*., 2007).

Investigating specific brain targets has been proposed as an important tool for developing our understanding of behavioral, emotional and cognitive impairments caused by OP compounds (Gupta). Supporting this experimental approach, we have provided molecular evidence that administration of a single high dose of CPF reduces basal neural activity, at two different time-points, in two different brain regions involved in ethanol intake: short-term in the EW (Carvajal *et al*., 2007) and long-term in the arcuate hypothalamic nucleus (present results). Taken together, our studies reinforce the notion that c-fos immunolabeling is a valuable experimental approach to identify neuroanatomical sites in which CPF either directly or indirectly contributes to long-term neural disturbances in the brain (Carvajal *et al*., 2005). Future behavioral and molecular studies will help our understanding on the role of Arc neural disturbances in long-term and long-lasting CPF-induced ethanol avoidance.

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CARVAJAL, F.; LERMA-CABRERA, J. M.; SÁNCHEZ-AMATE, M. C. & CUBERO, I. La administración de una dosis aguda de clorpirifós reduce a largo plazo la expresión basal de c-fos en el núcleo arqueado del hipotálamo. ***Int. J. Morphol., 32(1)***:90-96, 2014.

RESUMEN: El clorpirifós (CPF) es un compuesto organofosforado utilizado como plaguicida en todo el mundo. Después de ser inyectado de manera subcutánea, mantiene la actividad de la enzima acetilcolinesterasa (AChE) inhibida durante meses. Estudios clínicos y experimentales muestran que la exposición al CPF induce déficits neuroconductuales persistentes en seres humanos y animales, incluso después de un solo episodio/inyección. Además, estudios epidemiológicos evidencian que un porcentaje significativo (60%) de los veteranos de la Guerra del Golfo, así como los agricultores que sufren una intoxicación aguda por organofosforados, desarrollan intolerancia a la nicotina y las bebidas que contienen etanol. Datos experimentales mostraron que la administración de una sola dosis alta de CPF en ratas Wistar adultas provoca una reducción a largo plazo del consumo voluntario de etanol y un incremento en la sedación provocada por etanol sin evidencias de alteración del metabolismo de esta sustancia, lo que indica que pueden existir interacciones neurobiológicas entre CPF-etanol. En este estudio, se explora si la exposición a CPF induce alteraciones significativas en la actividad neuronal basal o evocada por el etanol en un conjunto de regiones colinoceptivas del cerebro involucradas en las respuestas neurobiológicas al etanol. Para ello, se evaluó la expresión de c-fos en respuesta a una dosis de etanol aguda (1.5 o 3.0 g/kg, ip) o solución salina en ratas Wistar macho adultas previamente inyectados con dosis aguda de CPF (250 mg/kg, sc) o un vehículo. Encontramos que la administración de CPF redujo la expresión basal de c-fos a largo plazo, pero no la evocada por el etanol en el núcleo arqueado del hipotalámo. Debido a que vías cerebrales independientes podrían modular las respuestas agudas al etanol y el consumo voluntario del mismo, no se descarta la contribución de las alteraciones neuronales basales observadas en el Arc en la evitación del consumo de etanol provocado por CPF.

PALABRAS CLAVE: Clorpirifós; Organofosforados; Expresión de c-Fos ; Núcleo arqueado del hipotálamo; Consumo de etanol .

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