

Neuroanatomical Targets of the Organophosphate Chlorpyrifos by c-fos immunolabeling

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Abstract

Chlorpyrifos (CPF) is an Organophosphate widely used as insecticide in agriculture (Pope, 1999, Richardson, 1995) which elicits short- and long-term neurobehavioral deficits after acute administration (Abou-Donia, 2003a; Richardson, 1995). Since little is known about the specific brain areas targeted by CPF, investigating for the location of its neuroanatomical targets could help to describe the brain systems involved in the neurobehavioral toxicity developed in CPF-exposed organisms. To meet this objective, in the present study we evaluated CPF-induced c-fos expression. In addition, locomotor behaviour and cerebral cholinesterase level was evaluated. We found two main sets of results. First, no significant c-fos expression was found in cholinceptive regions in CPF-treated rats 2h or 24h post-administration, despite the fact that 41% and 62% acetylcholinesterase inhibition were respectively present in brain homogenates. These results are consistent with previous reports showing CPF induced activation of adaptive neural mechanisms re-establishing cholinergic tone. Second, 24h post-intoxication CPF elicited c-fos expression in cytokine-related areas. Cytokines have been involved in anxiety-like responses and psychiatric stress syndromes (Anisman and Merali, 2003; Dantzer, 2001; Kronfol and Remick, 2000). Taking into account that CPF triggers the synthesis of peripheral cytokines the present data stress the need to further clarify functional relations between Organophosphates-triggered peripheral cytokines and emotional disturbances reported in intoxicated organisms.

Key words: Chlorpyrifos, cytokines, c-fos, Lithium Chloride, Cholinceptive areas.

1. Introduction

Chlorpyrifos (CPF) is an Organophosphate compound widely used as insecticide in agriculture with both cholinergic and non-cholinergic activity (Pope, 1999, Richardson, 1995). Because it is slowly delivered in the organism when administered subcutaneously (Richardson, 1995), a single dose of CPF induces acetylcholinesterase (AChE) inhibition that peaks 5 days post-intoxication, followed by a progressive and slow enzymatic recovery rate that keeps AChE activity mildly inhibited for weeks (Pope, 1999).

Despite the fact that CPF exerts acute cholinergic activity, surprisingly, no overt toxicity signs are found after administration of high doses of the compound (Richardson, 1995). However, several reports have shown short- and long-term neurobehavioral and emotional deficits in animals (Abou-Donia, 2003a; Richardson, 1995). In our lab, we have previously reported CPF-induced anxiogenic-like responses as measured by the plus maze at 48 h post-intoxication in rats (Sanchez-Amate et al, 2001). In addition, a long lasting CPF generalization to PTZ, an anxiogenic compound, was found in a drug-discrimination task (Sanchez-Amate et al, 2002). Finally, 6 months after CPF intoxication, impaired spatial acquisition and disrupted amphetamine-induced place preference responses were observed (Sanchez-Santed et al, 2004).

Most of the basic research aimed to describe cerebral mechanisms involved with organophosphate-induced neurotoxicity has centered its efforts at the molecular and neurochemical level (Abou-Donia et al, 2003; Bushnell et al, 1994; Chaudhuri et al, 1993; Gupta, 2004; Huff et al, 2001; Huff and Abou-Donia, 1995; Katz et al, 1997; Nostrandt et al, 1997; Ward et al, 1993) and little is known about the specific brain areas or neural circuits upon which organophosphates exert their action. Investigating for specific brain targets has been recently proposed as the main tool for deeper

understanding and/or prevention of emotional and cognitive impairments caused by organophosphate compounds (Gupta, 2004).

Several studies have successfully employed c-fos activity as a marker of neural activity (Thiele et al, 1996; Yamamoto et al, 1992) in such a way that low c-fos baseline levels are found in non-active neurons, whereas increased c-fos expression is indicative of neural activity. Moreover, c-fos expression has been involved with organophosphate administration (Nitsch et al, 1998; Kaufer et al, 1998). In the present study, regional c-fos expression in CPF exposed rats was quantified in order to search for specific neuroanatomical targets, with a double objective. First, recent reports have shown that organophosphates such as sarin (Henderson et al, 2002), soman (Svensson et al, 2001) or CPF (Gordon and Rowsey, 1999; Rowsey and Gordon, 1999) acutely stimulate cytokine synthesis, molecules that relay the inflammatory and immune message to the brain. Moreover, cytokines are involved with anxiety-like responses and psychiatric stress syndromes (Anisman and Merali, 2003; Dantzer, 2001; Kronfol and Remick, 2000) and it has been recently proposed that organophosphates-exposed soldiers in the Gulf War developed persistent psychological symptoms that closely correspond to the physiological and behavioral sequelae of a cytokine-mediated sickness response (Ferguson and Cassaday, 1999). Experimental evidence has shown a consistent pattern of regional c-fos expression in response to chemical compounds such as Lithium Chloride (LiCl), known to induce cytokine synthesis (Thiele et al, 1996; Yamamoto et al, 1992). Thus, the first goal in this study was to evaluate CPF-induced c-fos expression in brain regions targeted by cytokines (Konsman et al, 2002).

Second, unlikely other Organophosphates, acute administration of high doses of CPF, inducing profound AChE inhibition, is not immediately followed by the neurobehavioral “cholinergic syndrome” classically associated with acetylcholinesterase

inhibitors (Richardson, 1995). Several fast compensatory molecular mechanisms involving cholinergic receptors as well as pharmacodynamic properties intrinsic to the organophosphate CPF (Pope, 1999) seem to compensate for the increase in cholinergic tone. Moreover, a fast but long-lasting increase in AchE mRNA levels has been found in cellular neurites in response to 3 days of exposure to very low doses of the organophosphate diisopropylfluorophosphonate (Meshorer et al, 2002). Taken together, previous data strongly point to fast feedback cellular reactions re-establishing cholinergic activity in response to anticholinesterase compounds. Thus, in order to indirectly evaluate cholinergic tone in CPF-treated rats, the present study will quantify c-fos expression in cholinceptive areas as well as levels of cerebral AchE inhibition in response to CPF.

The CPF-induced c-fos regional pattern will be compared with that which emerged in response to the toxin Lithium Chloride (LiCl) for several reasons. First, LiCl is a cytokine inductor (Maier et al, 1993, Nemeth et al, 2002) that activates transcriptional factors, and immunohistochemistry procedures have consistently shown a well-defined pattern of regional c-fos expression in cytokine-related areas (Thiele et al, 1996, Yamamoto et al, 1992), which provides a helpful comparative frame to discuss CPF-induced c-fos expression data. Second, LiCl-induced c-fos was used as a positive control in our immunohistochemistry procedure.

In addition to the labeling procedure, we assessed locomotor activity together with the biochemical acetylcholinesterase profile resulting from toxin administration.

2. Materials and methods

2.1. Animals

Wistar male rats (Charles River Laboratories, Spain) weighting 300-350 g at the beginning of the experiments were housed 4/cage and maintained in an environmentally controlled room (22 °C temperature on a 12:12h light-dark cycle). Food and water were provided *ad libitum* 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 Decree 223/1988 for reducing animal pain and discomfort.

2.2. Behavioral Procedure

After 15 days of habituation to the laboratory conditions, the animals were weighted, homogenously distributed in three groups ((n= 6), CPF, LiCl and Veh) and then injected with Chlorpyrifos *sc* (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 volume), or Lithium Chloride *ip* (LiCl, 0.15 M, Sigma, Spain, dissolved in isotonic saline (0.9%), 20 ml/kg), or olive oil *sc* as vehicle, respectively. Immediately after the injections, the animals were put back in their home cages. Half of them remained there for 2h and the rest remained there for 24h. Once these pre-established temporal intervals were completed, a small group of animals (n=4) belonging to each treatment group were decapitated to assess brain acetylcholinesterase activity (see procedural details below). The rest of animals received a locomotor activity test for 5 minutes. The locomotor activity test was conducted in an open field chamber, 100cm × 100cm × 50cm, painted white. The testing was done in a dim room illuminated by four lights of 60W and locomotor behavior was monitored by a small video camera located 150cm above the open-field connected to a video-tracking recording system (Ethovision, Noldus, The Netherlands). For testing, the animals were placed in a corner

of the open field arena and the total distance travelled, mean velocity and total number of rearings were recorded for 5 minutes. The open field chamber was carefully wiped down between animals. Locomotor activity was evaluated in two possible periods of time, 2h or 24h after drug injections. Then animals were overdosed with pentothal sodium (80 mg/kg in 1 mg/kg volume), their brains were removed and the immunostaining protocol for c-fos was initiated (see below for procedure specifications).

Thus, this experimental procedure would enable us to comparatively evaluate cerebral c-fos expression and locomotor activity as well as acetylcholinesterase profile, in rats pre-treated with CPF, LiCl or vehicle, in two different intervals, 2h and 24h, post-injection.

2.3. *Acetylcholinesterase Assay*

For Acetylcholinesterase assays, a group of animals (n=4/pre-treated group) randomly selected from pre-treated rats were anesthetized 2h post-injection with pentothal sodium (80 mg/ kg in 1 mg/kg volumen) and then decapitated. The same protocol was followed 24h later for a second group of pre-treated rats. The whole brain was removed and immediately homogenized with 1% Triton X-100 in 0.1 M Na 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. Acetylcholinesterase activity was determined by spectrophotometer (DU 530 Beckman spectrophotometer) by the Ellman method (Ellman et al, 1961) using tetraisopropyl pyrophosphoramidate (iso-OMPA, specific inhibitors for BuChE) (50µl; final concentration 50µM), acetylthiocholine iodide (30µl; final concentration 0.5 mM) as substrate and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (200µl; final concentration

0.33 mM). Assay tubes were completed to 1 ml with Na phosphate buffer pH 8. Enzyme activity was calculated relative to protein concentration by the Bradford method (Bradford, 1976). For biochemical assays, acetylthiocholine iodide, tetraisopropyl pyrophosphoramidate (iso-OMPA) and 5,5'-dithio-bis-nitrobenzoic acid (DTNB) were purchased from Sigma-Quimica, Madrid, Spain

2.4. Immunostaining for c-fos procedure

Immediately upon completion of the open field test the animals were euthanized with an overdose of pentothal sodium (80 mg/kg in 1 mg/kg volumen) and transcardially perfused with PBS followed by 0.1M phosphate buffered paraformaldehyde 4% (pH 7.4). The brains were removed and immersed in PBS for 48h at 4 °C. Cerebral sections were cut in coronal section 50 μ m thick with a motorized vibratome. Following our experimental objectives, we were focussed in two different sets of brain regions: Cytokine-related areas and Cholinoceptive regions. Thus, a total of 11 different brain regions were collected based on Paxinos and Watson stereotaxic atlas coordinates (Paxinos and Watson, 1998): nucleus of the Solitary Tract (NTS), bregma -13.3 mm; Area Postrema (AP) bregma -14.08 to -13.68 mm; central nucleus of the Amygdala (CeA), bregma -3.14 to -2.30 mm; lateral Parabrachial area (IPB), bregma -9.16 mm; Hippocampus (HC), bregma -3.6 mm to -3.14 mm; Globus pallidus (GP), bregma -2.3 mm to -3.14 mm; dorsomedial nucleus of the Thalamus (DMT), bregma -2.3 mm to -3.14 mm; Interpeduncular nucleus (IP), bregma -5.8 mm; posterior Hypothalamus (PH), bregma -3mm to -3.8; Paraventricular nucleus of the Hypothalamus (Pa), bregma -3.3mm to -3.8mm; and locus coeruleus (LC), bregma -10.04 mm to -9.68 mm. Slices were rinsed (3 \times , PBS), incubated for 20 min in 0.3% H₂O₂ in absolute methanol to quench endogenous peroxidase, rinsed (3 \times , 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 4°C, slices were rinsed (10×, PBS, 2h) and processed using the ABC method (Vector Laboratories, Burlingame, CA). Briefly, slices were transferred to a solution containing biotinylated anti-rabbit IgG for 1 h, rinsed (10× PBS 1 h), transferred to avidin–biotin peroxidase for 1 h, rinsed (5× PBS 30 min, then 5× PB 30 min), and developed with nickel-intensified diaminobenzidine substrate (6 min). Following proper development, slices were rinsed (PBS, 30 min), mounted on slides, and coverslipped with Permount.

Stained sections were examined through a microscope (Olympus, B250) using 40X magnification; c-fos positive cells were scored through an attached camera lucida in selected brain region (area 100 x 100 microns) by an observer blind to the experimental conditions.

Since all cardiac perfusions were performed 2 or 24h far apart from drug administration but only 5 minutes separated them from locomotor testing, this procedure will allow us to specifically correlate c-fos expression with drug injection.

3. Results

3.1. Locomotor activity in Open-field

Total distance traveled, mean velocity and total rearing recorded in the open field chamber over a 5 minutes measurement period are represented in Fig 1. Data obtained 2h and 24h post-injections were analysed through independents one-way ANOVAs with a single between-subject factor, “Drug”, which compared locomotor activity in CPF, LiCl and vehicle administered rats. The statistical analysis performed

for each dependent variable showed no significant effects for the factor “Drug”, at 2h or 24h post-intoxication, ($F \leq 1$, $p > 0.05$). Thus, present data suggest that, under the experimental conditions and doses we employed, neither LiCl nor CPF significantly altered locomotor activity.

INSERT FIGURE 1 ABOUT HERE

3.2. Brain Acetylcholinesterase Profile

Data obtained 2h and 24h post-injections were analyzed through independent one-way ANOVAs with a single between-subject factor “Drug”, which compared acetylcholinesterase activity in CPF, LiCl and vehicle administered rats. 2h post-administration, the analysis revealed a statistically significant effect for the main factor, “Drug”, ($F(2, 9) = 26.70$, $P < 0.05$) in cholinesterase activity (CPF 0.028 ± 0.0033 $\mu\text{M}/\text{mg}/\text{min}$, LiCl 0.048 ± 0.0018 $\mu\text{M}/\text{mg}/\text{min}$, Veh 0.048 ± 0.0006 $\mu\text{M}/\text{mg}/\text{min}$). Posterior post-hoc Newman-Keuls test showed decreased acetylcholinesterase activity in CPF-treated rats ($p < 0.0005$), being 41% inhibited when compared with acetylcholinesterase level in control group (Fig.2).

In addition, the ANOVA conducted on data obtained 24h post-intoxication, revealed a significant effect for the factor “Drug”, ($F(2,9) = 60.70$, $P < 0.01$) in cholinesterase activity (CPF 0.014 ± 0.0020 $\mu\text{M}/\text{mg}/\text{min}$, LiCl 0.043 ± 0.0013 $\mu\text{M}/\text{mg}/\text{min}$, Veh 0.038 ± 0.0025 $\mu\text{M}/\text{mg}/\text{min}$), and posterior Newman-Keuls tests showed that CPF-treated rats had a reduced Ache activity ($p < 0.0002$), being 62% inhibited when compared to vehicle administered rats (Fig.2). No differences were found at any evaluated interval, when cholinesterase activity in LiCl and vehicle treated rats were compared.

INSERT FIGURE 2 ABOUT HERE

In summary, AchE activity was similar in vehicle and LiCl injected rats. By contrast and as expected, CPF-treated rats showed a time-dependent decrease in AchE activity, being 41% and 62% inhibited when it was compared with the level of enzymatic activity in the control group at 2 and 24h post-administration, respectively.

3.3. Immunostaining data: Regional c-fos brain expression

Data from c-fos expression obtained in brain regions after drug treatment, 2h or 24h post-injections, were analysed through independent one-way ANOVAs with a single between-subject factor, “Drug”, which compared, in each scored region, total c-fos expression in CPF, LiCl and vehicle administered rats.

3.3.1. C-fos expression in cytokine-related regions: Area Postrema, nucleus of the Solitary Tract, lateral Parabrachial Area, Paraventricular nucleus of the Hypothalamus and central nucleus of the Amygdala

ANOVAs conducted on c-fos data obtained 2h post-administration revealed a significant main effect for the factor “Drug” in the Area Postrema, AP ($F(2, 9) = 16.84$, $p < 0.05$), nucleus of the Solitary Tract, NTS ($F(2, 13) = 9.25$; $P < 0.05$), lateral Parabrachial area, IPB ($F(2, 12) = 4.99$, $P < 0.05$) and the central nucleus of the Amygdala, CeA ($F(2, 13) = 9.83$, $P < 0.05$). No differences were found in the number of c-fos positive cells in the Pa, ($p > 0.05$). Consistent with previous reports (Konsman et al, 2002; Thiele et al, 1996; Yamamoto et al, 1992), subsequent post hoc analyses with Newman-Keuls test showed that 2h post-administration LiCl administration led to significant increases in c-fos expression in the AP ($p < 0.001$), NTS ($p < 0.004$), IPB

($p < 0.05$) and the CeA ($p < 0.005$). By contrast, no significant increases in regional c-fos expression were found in response to CPF or vehicle administrations (Table 1).

Independent one-way ANOVAs conducted on c-fos data collected 24h after experimental treatments, revealed statistical significance for the factor “Drug” in the nucleus of the Solitary Tract, NTS ($F(2, 13) = 11.50, P < 0.05$), lateral Parabrachial Area, LPB ($F(2, 14) = 6.84, P < 0.05$) and the central nucleus of the Amygdala, CeA ($F(2, 13) = 15.06, P < 0.05$). No differences were found in the number of c-fos positive cells in the AP, ($p > 0.05$). Post-hoc Newman-Keuls tests showed that c-fos expression in these areas in response to LiCl were similar to that induced by vehicle administration. However, Chlorpyrifos-treated rats showed a significant increase, different to that exhibited by LiCl and vehicle treatments, in the number of c-fos positive cells in the NTS ($p < 0.003$), the LPB ($p < 0.01$) and the CeA ($p < 0.001$). In addition, because a reduced number of subjects were finally scored in the Pa ($n = 4$), a non-parametric U-Mann-Whitney test was conducted, revealing that CPF group also expressed a higher level of c-fos positive cells when compared to vehicle treated rats ($p < 0.05$), (Table 1).

	2h post-administration			24h post-administration		
	VEH	CPF	LiCl	VEH	CPF	LiCl
AP	26.25 ± 9.41	25 ± 7.88	110.33 ± 18.3*	18.75 ± 6.26	23.4 ± 3.78	10.25 ± 1.93
NTS	16.4 ± 3.45	26.66 ± 13.16	104 ± 23.44*	17 ± 5.81	33 ± 7.92*	13.8 ± 1.98
LPB	27.75 ± 5.51	42.33 ± 7.01	81 ± 18.2*	19.66 ± 6.17	47.66 ± 9.94*	11 ± 11.2
CeA	27.2 ± 6.55	51.16 ± 9.03	131 ± 28.66*	13.6 ± 2.83	31.16 ± 2.74*	12.6 ± 2.65
Pa	55.8 ± 10.08	54.33 ± 6.18	60.25 ± 9.76	30.33 ± 6.02	42.33 ± 5.86*	27 ± 7.06

Table 1. Mean (\pm SEM) of c-fos positive cells in cytokine-related regions in response to vehicle, chlorpyrifos and Lithium chloride administration, at two intervals post-intoxication (2 or 24h). AP, Area Postrema; NTS nucleus of the Solitary Tract; LPB lateral parabrachial area; Pa, Paraventricular nucleus of the Hypothalamus; CeA, central nucleus of the Amygdala. Significant differences from vehicle group * < 0.05

Thus, a similar but delayed pattern of increased regional c-fos expression emerged in LiCl- and CPF-treated rats, 2h and 24h post-administration respectively, suggesting cellular activity in brain regions known to be targeted by cytokines.

3.3.2. *c-fos* expression in cholinceptive areas: posterior Hypothalamus, central nucleus of the Amygdala, Interpeduncular nucleus, Globus pallidus, dorsomedial nucleus of Thalamus, Hippocampus, Locus Coeruleus.

Regional *c-fos* data obtained in cholinceptive regions 2h and 24h post-intoxication, were analysed through independents one-way ANOVAs, with a between-subject factor, “Drug”. No statistically significant differences were found in any cholinceptive area scored ($p>0.05$), but the CeA (see previous results 3.3.1 for CeA analyses), (table 2).

Thus, the present preliminary data obtained in cholinceptive areas suggests that, despite significant levels of AchE inhibition in CPF-treated rats, surprisingly, no correlatives significant increases in CPF-induced cholinergic neural activity were detected as measured by *c-fos* immunostaining.

	2h post-administration			24h post-administration		
	Veh	CPF	LiCl	Veh	CPF	LiCl
PH	42.2 ± 9.31	26 ± 4.56	26.66 ± 4.84	25.66 ± 4.71	29.4 ± 4.76	14.5 ± 2.46
CeA	27.2 ± 6.55	51.16 ± 9.03	131 ± 28.66*	13.6 ± 2.83	31.16 ± 2.74*	12.6 ± 2.65
IP	20.50 ± 3.47	22 ± 3.24	22 ± 0	11.80 ± 1.06	19.16 ± 3.22	12 ± 0
GP	21.8 ± 3.27	14.2 ± 3.76	11.5 ± 1.55	8.66 ± 0.66	10.16 ± 2.44	7.33 ± 0.66
DMT	28.6 ± 6.24	25 ± 4.33	23.33 ± 7.51	19 ± 2.42	16.5 ± 3.73	8.75 ± 6.10
HC	9.40 ± 1.46	8.33 ± 0.71	6 ± 1.48	2.5 ± 0.34	3.66 ± 0.33	3.8 ± 1.11
LC	17.25 ± 7.71	17.33 ± 3.48	21.75 ± 8.36	10.50 ± 1.80	10.83 ± 1.88	4.75 ± 1.49

Table 2. Mean (±SEM) *c-fos* positive cells in cholinceptive brain areas in vehicle, chlorpyrifos and Lithium chloride treated rats, at two intervals post-intoxication (2h or 24h). PH, posterior Hypothalamus; CeA, central nucleus of the Amygdala; IP, Interpeduncular nucleus; GP, Globus pallidus; DMT, dorsomedial nucleus of Thalamus; HC, Hippocampus; LC, Locus Coeruleus. Significant differences respect the vehicle group * <0.05

4. Discussion

c-fos expression in cytokine-related regions: Area Postrema, nucleus of the Solitary Tract, lateral Parabrachial Area, Paraventricular nucleus of the Hypothalamus and central nucleus of the Amygdala

Consistent with previous evidence (Thiele et al, 1996; Yamamoto et al, 1992), the present results show that 2h after LiCl is delivered in the organism, an acute response is elicited in cytokine-related brain regions such as the AP, the NTS or the IPB (Konsman et al, 2002) as well as in brain areas organizing sickness behaviors, such as the CeA (Buller and Day, 2002). As expected, no *c-fos* expression was found 24h post-treatment in the LiCl group in any scored region, probably due to LiCl metabolism. In contrast, CPF administration did not seem to induce significant *c-fos* activity 2h post-administration in any scored cerebral region.

However, 24h after CPF injection an interesting pattern of *c-fos* expression emerged, partially matching that elicited by LiCl 2h post-injection. The analysis of the pattern of regional *c-fos* expression induced by LiCl 2h post-administration and that evoked by CPF 24h after exposure, revealed some interesting similarities suggesting, in both cases, cellular activity in cytokine related areas involved with the “sickness behavior” cerebral system (Konsman et al, 2002). It is known that this system triggers an “alert response” when potentially dangerous chemicals gain access to the organism (Dantzer, 2001) enabling neural adaptive responses to be properly organized. The toxicity message is mediated by peripheral cytokine synthesis represented by Interleukin 1 (IL1) and TNF-alpha, which activate their target structures via humoral and neural pathways projecting to specific brain areas (Konsman et al, 2002). In this context, several potentially dangerous stimuli such as aversive and sickness-inducing chemical stimuli (Maier et al, 1993; Nemeth et al, 2002), proteins from virus

membranes (Lipopolisaccarids, LPP) and some organophosphate compounds (Gordon and Rowsey, 1999; Henderson et al, 2002; Rowsey and Gordon, 1999; Svensson et al, 2001) induce a cytokine relay acute signal that conveys the immune and inflammatory message toward the brain sickness-system.

Interestingly, behavioral studies have suggested a strong relationship between cytokines and some emotion-based psychiatric syndromes (Anisman and Merali, 2003; Kronfol and Remick, 2000; Pollmacher et al, 2002). I.c.v. administration of the cytokine TNF-alpha elicits anxiety-like responses as measured by the Plus maze test, even without otherwise noticeable behavioral or physiological overt symptoms of sickness (Connor et al, 1998). On the other hand, immunostaining data has revealed that TNF-alpha influences stressor-reactive brain regions and also induces expression of c-fos in the CeA (Buller and Day, 2002). In light of these results, it has been proposed that increased activity in the sickness-behavior system due to stimuli triggering peripheral cytokines could also sustain disease-associated hyper-reactivity, emotional disturbances and anxiety-like responses (Anisman and Merali, 2003; Kronfol and Remick, 2000; Pollmacher et al, 2002).

This is the first study, to our knowledge, showing delayed cellular activity after CPF administration in cytokine-related brain areas, as measured by regional c-fos expression. Given the fact that CPF triggers TNF-alpha synthesis (Gordon and Rowsey, 1999; Rowsey and Gordon, 1999) and that CPF induces anxiety-like responses in the plus maze at 48h, the present data extends previous results and provides c-fos evidence of CPF-induced activity in cytokine related brain areas. Future studies aimed to co-localize TNF-receptors and c-fos expression in the brain of CPF treated rats will further characterize the phenotype of activated cells. Our study stresses the need for future research aimed to understand potential interactions between CPF-induced cytokines,

chronic activation in the brain sickness-behavior system and delayed development of emotional disturbances.

Nonetheless, the present results cannot rule out direct toxic actions of the organophosphate on the reported c-fos-expressing areas. Whether our data are demonstrative of indirect actions of CPF in the “sickness behavior” cerebral system modulated by peripheral cytokine synthesis or rather they represent a direct toxic action of that compound, needs further investigation.

c-fos expression in cholinceptive areas: posterior Hypothalamus, central nucleus of the Amygdala, Interpeduncular nucleus, Globus pallidus, dorsomedial nucleus of Thalamus, Hippocampus, Locus Coeruleus

In order to indirectly assess CPF-induced cholinergic activity, the second objective in the study was to evaluate c-fos expression in cholinceptive regions (Zhu et al, 2001). Despite measures obtained in brain homogenates revealed a significant level of AchE inhibition, both 2h and 24h post-intoxication, no significant CPF-induced neural activity appeared in any (but the CeA) cholinceptive region scored. Moreover, although decreased locomotor activity due to cholinesterase inhibition is a consistent effect reported in the literature (Moser, 2000; Nostrandt et al, 1997; Timofeeva and Gordon, 2002), the present preliminary data, together with previous studies conducted in our lab (Sanchez-Amate et al, 2001; Sanchez-Amate et al, 2002; Sanchez-Santed et al, 2004), suggest that no significant changes would be observed in this behavior after CPF exposure unless 70% in AchE inhibition is reached in brain homogenates.

Several compensatory neural mechanisms could help to explain these apparently contradictory results. It has been proposed that adaptive presynaptic and postsynaptic mechanisms triggered by Organophosphate intoxication successfully prevent acute

increases in cholinergic tone (Chaudhuri et al, 1993; Huff et al, 2001; Huff and Abou-Donia, 1995; Pope, 1999; Ward et al, 1993; Meshorer et al, 2002). CPF acts as muscarinic agonist at m2 and m4 receptors, where Ach release is decreased through adenilcyclase inhibition (Huff et al, 2001; Huff and Abou-Donia, 1995). In addition, more recently it is been reported that chronic treatment with low doses of CPF or Nicotine can induce increased Ach levels at the synaptic cleft. This process is immediately followed by Nicotinic-receptor desensitization (Abou-Donia et al, 2003; Fenster et al, 1999; Katz et al, 1997), a cellular mechanism not associated to transcriptional factors and c-fos expression. Finally, new synthesis of the rare AchE-R has been described in cellular neurites as a mechanism re-establishing cholinergic communication in response to Organophosphate insults (Meshorer et al, 2002). Thus, present behavioral and molecular data are consistent with active CPF-triggered presynaptic and postsynaptic adaptive mechanisms preventing acute increases in cholinergic tone.

CeA is a cholinoceptive region, although, in this study a different pattern of c-fos expression to that observed in other cholinoceptive scored regions emerged, with significant increases at 24h post-CPF intoxication. Some explanations could account for the obtained data. First of all, because receptor densities differ in brain cholinergic areas (Chaudhuri et al, 1993; Gupta, 2004; Mesulam, 1995; Nostrandt et al, 1997), regulatory molecular mechanisms successfully modulating the cholinergic tone in some brain regions could be missed at the CeA. Second, CPF could be directly interacting with specific amygdala cells. Finally, the hypothesis holding amygdala activity as the final neural relay in the cytokine-induced activation of the sickness behavior circuit is also consistent with present data.

In summary, the obtained results reveal delayed c-fos activity in the brain 24h post-CPF intoxication and strongly suggest that a single dose of this Organophosphate whether directly or indirectly, can target specific brain regions. Moreover, our data clearly shows that c-fos immunostaining may be a useful experimental approach to evaluate whether CPF exerts delayed actions targeting specific cerebral circuits. However, because loss of c-fos expression is not definitively demonstrative of reduced neural activity, with the present data we cannot rule out other early-immediate genes as involved in cellular activation induced by CPF. Further research is needed to clarify functional relations between CPF exposition, subsequent cholinergic activity, cytokines synthesis and development of behavioral/emotional disturbances.

5. References

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FIGURE LEGENDS

Fig 1. Data showing Mean (\pm SEM) of the total distance travelled (A), velocity (B) and number of rearing counts (C), during a 5 min open field test, 2h and 24h after Vehicle, CPF or LiCl administration. No altered behaviors were observed

Fig. 2. Data showing the percentage (\pm SEM) of brain AchE activity in CPF and LiCl groups respect the vehicle group enzymatic activity. CPF group showed a time-dependent decrease in AchE activity, being 59% and 38%, 2 and 24h post-injection, respectively. Significant differences respect vehicle-group * < 0.05

Figure 1

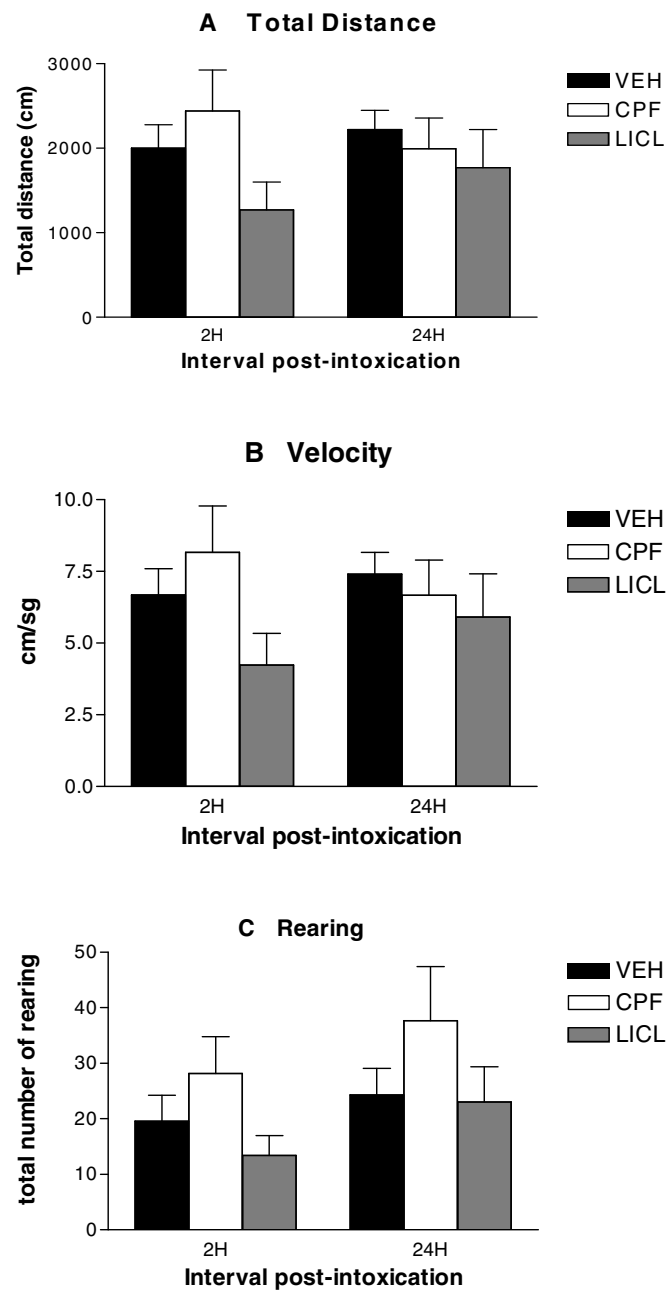


Figure 2

