Developmental Neurotoxicity of Organophosphorous Pesticides: Fetal and Neonatal Exposure to Chlorpyrifos Alters Sex-Specific Behaviors at Adulthood in Mice

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Developmental exposure to the organophosphorous insecticide chlorpyrifos (CPF) induces long-term effects on brain and behavior in laboratory rodents. We evaluated in adult mice the behavioral effects of either fetal and/or neonatal CPF exposure at doses not inhibiting fetal and neonatal brain cholinesterase. CPF (3 or 6 mg/kg) was given by oral treatment to pregnant females on gestational days 15–18 and offspring were treated sc (1 or 3 mg/kg) on postnatal days (PNDs) 11–14. Serum and brain acetylcholinesterase (AChE) activity was evaluated at birth and 24 h from termination of postnatal treatments. On PND 70, male mice were assessed for spontaneous motor activity in an open-field test and in a socioagonistic encounter with an unfamiliar conspecific. Virgin females underwent a maternal induction test following presentation of foster pups. Both sexes were subjected to a plus-maze test to evaluate exploration and anxiety levels. Gestational and postnatal CPF exposure (higher doses) affected motor activity in the open field and enhanced synergistically agonistic behavior. Postnatal CPF exposure increased maternal responsiveness toward pups in females. Mice of both sexes exposed to postnatal CPF showed reduced anxiety response in the plus-maze, an effect greater in females. Altogether, developmental exposure to CPF at doses that do not cause brain AChE inhibition induces long-term alterations in sex-specific behavior patterns of the mouse species. Late neonatal exposure on PNDs 11–14 was the most effective in causing behavioral changes. These findings support the hypothesis that developmental CPF may represent a risk factor for increased vulnerability to neurodevelopmental disorders in humans.

Key Words: chlorpyrifos; hyperactivity; aggressive behavior; maternal behavior; plus-maze rodents.

Prolonged exposure to environmental contaminants at apparently nontoxic doses might represent a major risk factor for children’s health. In this respect, chlorpyrifos (CPF), one of the most widely used organophosphorous pesticide, represents a paradigmatic example, as it elicits developmental neurotoxicity at exposure levels below the threshold for systemic toxicity, such that adverse effects can occur in pregnant women and children in the absence of symptoms. Evidence of the selective vulnerability of embryos, fetuses, neonates, and adolescents to organophosphates has been well documented (Chakraborti et al., 1993; Greenlee et al., 2004; Moser 2000). Furthermore, infants and children might be exposed to CPF doses well above the established “not observed effect level” due to the persistent accumulation of CPF on residential surfaces and toys after household application (Gurunathan et al., 1998).

CPF was originally thought to interfere with brain development through its inhibitory action on cholinesterase, but it has become evident that other noncholinergic mechanisms are involved in CPF developmental neurotoxicity. CPF exposure at doses below the threshold for systemic toxicity and inhibition of brain cholinesterase exerts disruptive effects on neural cell development, with respect to DNA synthesis, gene transcription, cell differentiation, and synaptogenesis (Crumpton et al., 1998; Levin et al., 1998). In addition, several rat studies have indicated that CPF targets neurotransmitter systems further to the cholinergic one, as the monoamines, norepinephrine, dopamine, and serotonin (Aldridge et al., 2004; Dam et al., 1999). Interference with brain maturation is associated with behavioral disturbances in exposed rodents (Aldridge et al., 2005; Carr et al., 2001; Dam et al., 2000; Levin et al., 2001). Behavioral alterations not associated to brain acetylcholinesterase (AChE) inhibition have been also characterized in the mouse species, where either early or late neonatal exposure to CPF affected responses to environmental and social cues in adolescent male mice (Ricceri et al., 2003). Altogether, CPF appears to have a wide window of maturational vulnerability, as interference with brain and behavior development is reported for exposures ranging from embryonic stages through the preweaning phase (Garcia et al., 2003). In addition, most of CPF neurobehavioral effects appear to be gender selective and dependent on time of...
administration, with the two sexes showing different periods of critical sensitivity and in some cases even opposite behavioral outcomes (Dam et al., 2000; Garcia et al., 2003).

The present study is aimed at characterizing in the mouse the long-term behavioral effects of both gestational and postnatal exposure to CPF, considering two complex behavioral patterns that can be easily elicited in this rodent species, namely, intermale socioagonistic behavior and induction of maternal responsiveness in virgin females. Analysis of sex-specific behavior patterns may contribute to verify the hypothesis that CPF interferes with sexual differentiation of the brain, by blunting normal sex differences in neural function and behavior (Aldridge et al., 2005). Furthermore, intermale socioagonistic behavior and pup-induced maternal response in mice, further to being under the control of sex hormones, are largely modulated by serotonergic and dopaminergic neurotransmissions (Olazabal et al., 2004), which are the neural systems found to be mostly affected by developmental CPF exposure in rats (Aldridge et al., 2004, 2005).

We evaluated two different treatment windows, namely, the late gestational phase (gestational days [GDs] 15–18) and the late neonatal stage (postnatal days [PNDs] 11–14), characterized by different CNS maturational events, and they have been found to represent critical phases of susceptibility to CPF action in rodents (Garcia et al., 2003). Our study was designed to compare the effects of prenatal, postnatal, and prenatal + postnatal CPF exposure to verify if the repeated exposure affected the behavioral effects brought about by either single exposure per se. Behavioral analysis was carried out in both sexes at adulthood. Further to assessment of intermale socioagonistic behavior and of pup-induced maternal behavior in virgin females, spontaneous behavior and activity levels were also evaluated in an open-field arena and in an elevated plus-maze in order to evidence potential alterations in response to novel environmental cues.

**MATERIALS AND METHODS**

*Animals and treatments.* All experiments on animals were performed according to the European Community Council Directive 86/609/EEC. Male and female mice of an outbreed Swiss-derived strain (CD-1, Harlan, S. Pietro al Natisone, Italy) were housed in breeding cages with a 12-h light/dark cycle and with free access to food and water. Females were inspected daily for the presence of the vaginal plug (GD 0). Thirty females were randomly assigned to one of the three prenatal treatments and litters culled at birth to four males and four females. On the day of birth, pups were tattooed with blue ink on their limbs for individual identification and randomly assigned to one of the three postnatal treatments.

CPF (Chem Service, West Chester, PA) was dissolved in peanut oil (vehicle) to provide rapid and complete absorption CPF (in a volume of 0.1 ml/kg at a dose of either 3 or 6 mg/kg), or its vehicle was administered to pregnant females from GDs 15 to 18 by intraoral gavage. As for postnatal treatment, CPF (at a dose of either 1 or 3 mg/kg) or its vehicle was administered sc in the nape of the neck to prenatally treated pups from PNDs 11 to 14. Within each litter assigned to one prenatal treatment (vehicle, 3 or 6 mg/kg), one male and one female were randomly assigned to vehicle (Veh), one male and one female to CPF 1 mg/kg (CPF1), and one male and one female to CPF 3 mg/kg (CPF3) treatment (split-litter design). Eventually, nine treatment groups were established: preVeh-postVeh, preCPF3-postVeh, preCPF6-postVeh, preVeh-postCPF1, preCPF3-postCPF1, preCPF6-postCPF1, preVeh-postCPF3, preCPF3-postCPF3, and preCPF6-postCPF3.

CPF doses for gestational exposure were selected on the basis of a pilot study showing 50 and 75% of serum AChE inhibition by 3 and 6 mg/kg dose, respectively, 24 h after termination of a 4-day treatment in pregnant females while brain AChE activity was reduced (60% of control activity) in females treated with CPF6. Such doses did not elicit systemic toxicity and weight loss in pregnant females, and they did not affect fetuses’ viability and general maternal care. Doses selected for postnatal exposure were the same as in our previous mouse study (Ricceri et al., 2003). Developing mice treated with CPF were monitored for signs of cholinergic intoxication (tremors etc.) at 1 and 6 h following injection. Litters were weaned on PND 23, housed in cages containing littersmates of the same sex, and left undisturbed until the start of the behavioral studies (PND 60). Additional animals were used to control for CPF effects on serum and brain levels of AChE at birth (pups discarded at litter culling) and on PND 15 (two pups in each litter).

**AChE assay.** Mice were killed by decapitation 24 h after the last gestational or postnatal CPF treatment (only pups born on GD 19 were used for AChE determination), and their whole brain minus cerebellum and blood were immediately taken. Brain was weighed and homogenized, using a Polytron apparatus (Kinematica Gmbh, Littan-Luzern, CH) for 1 min, in 20 volumes of cold 0.038M Tris-HCl buffer, pH 7.6. Aliquots of brain homogenates (10 μl diluted 1:10) were used for enzymatic analyses.

Blood samples were centrifuged at 1000 × g at 4°C for 5 min in a Sorwall RC-5B centrifuge to separate serum from red blood cells. Aliquots of serum (25 μl diluted 1:20) were used for enzymatic analyses.

AChE activity was determined in each preparation by Ellman’s spectrophotometric method (Ellman et al., 1961) as previously described (Meneguz et al., 1992; Volpe et al., 1990). The samples were incubated with the substrate in 0.05M sodium phosphate buffer, pH 7.2, containing 0.25mM 5,5'-dithiobis-2-nitrobenzoic acid in a final volume of 1.4 ml, for 30 min at 25°C (in duplicate). Acetyl thioclohexile (AcThCh), 0.56mM/tube, was used as substrate. The enzymatic activity was expressed as micromoles of AcThCh hydrolyzed per minute per gram of wet tissue or per milliliter of serum. The protein content of the enzyme preparation was determined using bovine serum albumin as standard (Lowry et al., 1953).

**Open field.** On PND 70, male mice from each prenatal and postnatal combination (n = 10 in each group) were tested in an open-field test 24 h before the agonistic encounter (see below). Individual mice were transferred from the home cage to an open-field arena (40 × 40 × 36 cm) made of black Plexiglas with a white bottom subdivided by black line into 7 × 7–cm squares. The behavior was then videotaped for 20 min under red light and scored by means of a software package for collection and analysis of data (The Observer, Noldus, Wageningen 6700, The Netherlands). Frequency and duration of “crossing,” “wall rearing,” “self-grooming,” and “immobility” as described in Alleva et al. (1985) were scored and analyzed.

**Socioagonistic behavior.** On PND 60, male mice were weighed and housed individually with some of their own sawdust in a cage identical to the home cage for 2 weeks. Between days 75 and 80 one male for each combination of prenatal and postnatal treatment group (n = 10 for each combination) underwent a 20-min period of agonistic encounter with an isolated standard opponent (unfamiliar partner) of same sex, age, and strain in a cage identical to the home cage with new sawdust as bedding. Mice used as standard opponents were untreated, and a different individual was used for every test mouse. The behavior of each pair was video recorded by means of a video camera connected to a professional Sony videocassette recorder V0-5800PS. The Observer system (see above) was used for collection and analysis of observational data scoring duration and frequency of each response. Non-socia and social behaviors scored are thoroughly described (Alleva, 1993) and include items describing general activity, such as exploring, rearing,
and digging, as well as social responses, including Attack—rushing approach
carried on over the back of the partner, often accompanied by biting attempts;
Aggressive grooming; Tail rattling—rattlesnake-like movement of the tail;
Offensive Upright Posture—the mouse stands on its hind legs facing
the opponent aggressively; Defensive Upright Posture—the animal stands pushing
the aggressive opponent with its forepaws; and Submissive Upright Posture—
the animal stands with the head pulled far back and the body rigid.

**Pup-induced maternal behavior.** On PND 90, virgin females (10 for each
treatment group) underwent a maternal induction procedure. Females
were exposed to three recently fed foster pups, 1–3 days of age. The pups were
introduced in the female’s home cage, approximately 10 cm from the sleeping
nest, and female behavior was recorded for 15 min following the first sniffing
of the pups. The occurrence and duration of the following activities were
recorded: Licking the pups, Nest building, Retrieving the pup in the mouth and
carrying it to the nest, Crouching—assumption of the nursing posture over
all the three pups. The behavior of each female was video recorded and
The Observer system (see above) was used for collection and analysis of
observational data.

**Elevated plus-maze.** Four-month-old male and female mice (n = 10 for
each treatment group) were assessed in an elevated plus-maze, consisting of two
open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm) that extend from
a common central platform (5 × 5 cm). The apparatus was made of Plexiglas
(black floor, clear walls) and elevated to a height of 60 cm above the floor level.

Mice were individually placed on the central platform facing an open arm
and allowed to explore the maze for 5 min. Behavioral items recorded were
frequencies of total, open, central, and closed arm entries and duration of the
time spent in the different areas of the maze. The following additional
behaviors were recorded in each arm: Head Dipping—exploratory movement
of head/shoulders over the side of maze, and Stretched-attend posture (SAP)—
risk-assessment posture in which the body is stretched forward and then
retracted to the original position (for a detailed analysis of these items see
Pellow et al. [1985]).

**Statistical analysis.** Analyses of variance (ANOVAs) were performed on
dams’ and pups’ body weight, open-field, socioaggressive behavior, maternal
behavior, and plus-maze data. The model of such analyses (Chiarotti et al.,
1987) included prenatal treatment (three levels) as block with respect to
postnatal treatment (three levels), sex and repeated measures as within-litter
treatment factors, postnatal treatment and sex as fixed-effect factor within litter,
and repeated measures as fixed factor within subjects. Tukey tests with
Bonferroni’s correction were applied to perform *post hoc* comparisons on
ANOVA behavioral results. Biochemical data were analyzed using all pairwise
multiple comparison procedures (Student-Newman-Keuls Method).

**RESULTS**

**Maternal and Neonatal Toxicity**

No CPF doses caused any overt signs of cholinergic intoxication at either phase of treatment. CPF did not affect weight gain during pregnancy and reproductive performance, namely, number of pups delivered and sex ratio. Mean pups’
weights at birth were comparable in CPF- and vehicle-treated females. Pups receiving CPF either prenatally or postnatally did not differ from vehicle-treated controls in their body weight gain recorded from PNDs 11 to 14 (data not shown).

**AChE Assays**

At birth, brain AChE activity was unchanged by prenatal CPF treatment (both doses), while serum AChE activity was significantly decreased in a dose-dependent fashion (CPF3 vs.
CPF0: t = 5.33, p < 0.001; CPF6 vs. CPF0: t = 6.43, p < 0.001; 
CPF3 vs. CPF6: t = 2.20, p < 0.05).

On PND 15, prenatal or postnatal treatment or their combination did not affect 24 h after termination of treatment, brain AChE. However, a significant dose-dependent
inhibition of serum AChE was found for both CPF postnatal treatments (Fig. 1). Pups exposed to preCPF3-postCPF1 appeared to be more susceptible to CPF, as for AChE inhibition
(preCPF3-postCPF1 vs. preVeh-postCPF1: q = 3.745, p = 0.026). As for brain AChE, it is possible that, given the
rapid rate of enzyme synthesis in the immature rodent, recovery from inhibition had occurred by 24 h. Thus, it cannot
be excluded that the same assay performed at shorter time intervals from the last CPF treatment (1–4 h) would have
revealed some degree of brain AChE inhibition. While this possibility has been excluded for animals administered with
CPF on PNDs 11–14 (see Ricceri et al., 2003), it still remains to be verified in mice undergoing gestational or combined
gestational + postnatal exposure.

![FIG. 1. Cholinesterase (AChE) activity in brain and serum of 15-day-old mice, exposed on GDs 15–18 to Veh, CPF3, or CPF6 and on PNDs 11–14 to Veh, CPF1, or CPF3. * Indicates p < 0.05 significantly different from control values (100%). # Indicates p < 0.05 preCPF3-postCPF1 versus preVeh-postCPF1. AChE is expressed as the percent change from control (preVeh-postVeh) values. Brain AChE control values were 6.39 ± 0.20 (mean ± SE; micromoles of AcThCh hydrolyzed per minute per gram of tissue) and serum AChE control values were 2.54 ± 0.11 (mean ± SE; micromoles of AcThCh hydrolyzed per minute per milliliter of blood).](attachment:image)
ANOVA on crossing frequency yielded a main effect of prenatal treatment ($F_{2,27} = 3.54, p = 0.04$). Post hoc comparisons revealed that preCPF6-postVeh treatment induced a significant increase in locomotion ($p < 0.05$). A significant three-way interaction, prenatal treatment $\times$ postnatal treatment $\times$ 5-min blocks, was found ($F_{12,162} = 1.89, p = 0.04$). Post hoc comparisons performed on this interaction (1) revealed a hyperactivating effect of the prenatal CPF6 dose in animals receiving either vehicle or CPF1 postnatally and (2) evidenced in the first 5 min of the test a hyperactivating effect of the postnatal CPF3 dose, only in those animals receiving either vehicle or prenatal CPF3, but not prenatal CPF6 (see Fig. 2). No significant effect of either prenatal or postnatal CPF was found on rearing, wall rearing, and self-grooming.

**Open Field**

Overall, CPF treatment enhanced aggressive items of behavior (Fig. 3). The effects of prenatal and postnatal CPF exposure, though on different items, were in the same direction. A main effect of postnatal treatment ($F_{2,50} = 3.84, p = 0.02$) was found for Attack duration. Post hoc comparisons revealed that such effect was due to the longer duration of attack episodes by postnatal CPF3 mice in comparison to postnatal vehicles ($p < 0.05$). A similar effect of postnatal CPF3 was also found for Attack frequency. Post hoc comparisons performed on the postnatal treatment main effect ($F_{2,50} = 2.42, p = 0.09$) confirmed the proaggressive effect of the high postnatal CPF dose ($p < 0.05$). Neither a significant prenatal treatment effect nor an interaction between prenatal and postnatal treatment was found for this behavior.

A main effect of prenatal treatment was found only for Offensive Posture frequency ($F_{2,25} = 3.458, p = 0.047$), with a significant increase in mice exposed to CPF6 dose ($p < 0.05$ after post hoc comparisons). Neither a significant postnatal treatment effect nor an interaction between prenatal and postnatal treatment was found for this behavior. Defensive and Submissive postures’ frequency and duration were not significantly affected by either pre- or postnatal CPF but tended to decrease in CPF-treated animals, suggesting that the effects of CPF were specific and not associated to hyperactivity (Fig. 3).

Neither prenatal nor postnatal CPF treatment altered non-social categories of behavior of male mice. Self-directed behavior, such as grooming, and items reflecting general activity, such as moving around the cage, rearing, and digging, were not affected by prenatal, postnatal, or the interaction between CPF treatments.

**Socioagonistic Behavior**

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**Pup-Induced Maternal Behavior in Virgin Females**

The effects of CPF on maternal behavior were mainly due to the postnatal exposure, and they were in the direction of enhanced maternal responding (Fig. 4). Females exposed postnatally to CPF displayed lower number of Licking episodes but of longer duration than vehicle-treated females. Both postnatal CPF1 and CPF3 significantly (1) decreased Licking frequency ($F_{2,40} = 29.10, p < 0.01$; postVeh vs. postCPF1 and postVeh vs. postCPF3 $p$ values $< 0.01$ after post hoc comparisons) and (2) increased Licking duration ($F_{2,40} = 13.52, p < 0.01$; postVeh vs. postCPF1 and postVeh vs. CPF3 $p$ values $< 0.01$ after post hoc comparisons).

Postnatal CPF treatment also increased occurrence of Crouching posture. Both postnatal CPF1 and CPF3 significantly
increased Crouching frequency ($F_{2,40} = 26.69, p < 0.01$; postVeh vs. postCPF1 and vs. CPF3 $p$ values $< 0.01$ after post hoc comparisons) and duration ($F_{2,40} = 25.80, p < 0.01$; postVeh vs. postCPF1 and vs. CPF3 $p$ values $< 0.01$ after post hoc comparisons). Postnatal CPF treatment significantly reduced sniffing, an investigative behavior that normally precedes the expression of maternal responses (frequency: $F_{2,40} = 266.140, p < 0.01$; duration: $F_{2,40} = 319.36, p < 0.01$), and both postnatal CPF doses were equally effective ($p$ values $< 0.01$ after post hoc comparisons for both frequency and duration). Neither a main effect of prenatal CPF nor an interaction between prenatal and postnatal treatment was found for pup-directed behaviors. No effects of CPF were found on latency, frequency, and duration of Nest building and pups’ Retrieving to the nest (data not shown).

**Plus-Maze**

ANOVA on the percentage of time spent in the open arms yielded a main effect of postnatal treatment ($F_{2,54} = 4.15, p = 0.02$). Post hoc comparisons performed on this effect revealed that mice exposed postnatally to CPF3 spent more time in open arms than controls ($p < 0.05$), and CPF1-treated animals showed a similar trend. In addition, a postnatal treatment $\times$ sex interaction was found ($F_{2,54} = 3.38, p = 0.04$), in that females spent a lower percentage of time in the open arms than males (females vs. males $p < 0.05$) while CPF3 increased time spent in the open by females (postVeh females vs. postCPF3 females $p < 0.01$). An effect of prenatal CPF3 was found as for Head Dipping frequency that was decreased in males (interaction prenatal treatment $\times$ sex: $F_{2,27} = 2.98, p = 0.06$, post hoc comparisons $p < 0.05$) (Fig. 5).

Neither a main effect of prenatal CPF nor an interaction between prenatal and postnatal treatment was found for time spent in the open arms. No CPF effects were found on frequency and duration of SAP and for activity levels in both sexes (data not shown).

**DISCUSSION**

The present findings confirm and extend previous results showing long-term effects on the behavior of developmental exposure to CPF at subtoxic doses in the mouse species. CPF enhances socioagonistic behavior in males and maternal responsiveness to pups in nonlactating females. In addition, CPF reduced anxiety levels in both sexes, with females more affected than males, and induced hyperactivity in males. With the exception of this latter measure, CPF effects were for the most part due to the postnatal exposure, and the prenatal exposure did not increase the magnitude of such effects.

**CPF Effects in Males**

The present data confirm that locomotor activity is a sensitive end point for developmental toxicity of CPF (Levin et al.,...
We have previously found a hyperactivating effect of neonatal CPF (PNDs 11–14) at the dose of 3 mg/kg in developing mice (Ricceri et al., 2003). We show here that the highest prenatal CPF dose (6 mg/kg) had a marked hyperactivating effect on adult male mice in the open-field test, an effect maintained also after reexposure to CPF 1 mg/kg on PNDs 11–14. Postnatal exposure to CPF3 also increased locomotor activity, but the combined prenatal/postnatal exposure failed to enhance the hyperactivity shown by CPF6-treated mice, possibly because of the ceiling effect produced by the highest prenatal CPF dose. CPF failed to affect habituation, as normal decrement of activity was observed throughout the open-field test. In addition, CPF-exposed mice were hyperactive neither during the socioagonistic encounters nor in the plus-maze test. The enhanced activity seemed to be specifically related to exploration of a novel environment, in line with previous experimental evidence in younger mice (Ricceri et al., 2003). Alteration of activity levels might result from the interference of CPF with early development of cholinergic synaptic function as reported for late prenatal exposure periods (Qiao et al., 2003). Furthermore, the period of postnatal exposure spans through a phase of critical importance for development of basal forebrain cholinergic pathways, modulating arousal and inhibition (Berger-Sweeney and Hohmann, 1997).

As for socioagonistic behavior, the present findings further support the hypothesis of a proaggressive role of developmental CPF exposure previously evidenced in adolescent mice (Ricceri et al., 2003). We show here that adult males exposed postnatally to CPF3 displayed increased frequency and duration of aggressive items at the expenses of defensive and submissive ones. Prenatal exposure also favored expression of offensive postures. The specificity of the proaggressive effect

FIG. 4. CPF effects on some behavioral items recorded during the 15-min maternal behavior test. Data are mean frequency and duration on 5-min time units. * Indicates $p < 0.05$ significant post hoc comparisons for the prenatal or postnatal main effect (see “Results” section): postCPF1 versus postVeh; postCPF3 versus postVeh.
of CPF is supported by the lack of significant differences in responses related to general activity during the socioagonistic encounter.

Intermale aggressive behavior is reportedly under the control of sex hormones in rodents (Archer et al., 1988). Steroid hormones facilitate expression of aggressive behavior, likely by acting as modulators on a brain circuitry involving the septum, medial preoptic area (MPOA), and anterior hypothalamus (Lu et al., 1998). However, an increasing body of evidence points to serotonin as the major neurotransmitter regulating expression of intermale aggressive behavior (Bell and Hobson, 1994). Facilitation by gonadal steroids and inhibition by serotonin receptors assure adaptive levels of aggressive behavior (Simon et al., 1998). As developmental CPF exposure produces long-term alteration of serotonergic function in the rat (Aldridge et al., 2004), it is possible that CPF exposure induces abnormal development of serotonergic pathways modulating intermale aggression in mice. In addition, the interference of CPF with hormone-dependent developmental processes involved in sex differentiation of the brain, as reported for other environmental chemicals, cannot be ruled out (Ikeda et al., 2005). Antiandrogenic activity has been reported for CPF in adult rats, but only for very high doses (Kang et al., 2004), and to the best of our knowledge, no data are available concerning the effects of developmental CPF exposure on brain steroid hormones. Recent evidence supports the view that sexual differentiation of the brain is a lifelong process in rodents (Johansen et al., 2004) and thus exposure to CPF in the late neonatal stage might still be able to affect such a process, whatever the mechanisms involved.

**CPF Effects in Females**

Data obtained from virgin females show that postnatal CPF exposure enhanced responsiveness toward pups, favoring the expression of pup-directed behaviors. Maternal behavior is a complex behavior pattern whose onset depends on both internal hormonal states and processing of a variety of external cues associated with pups (Numan, 1988). Moreover, the sensitivity to external cues may be modulated by the action of hormones and neurotransmitters in receptive brain areas, in particular at the level of the MPOA and its efferent. In the mouse species, presentation of pups evokes in virgin females activation of the same hypothalamic and limbic areas involved in maternal behavior after parturition (Calamandrei and Keverne, 1994). The response of nonlactating female mice to pups does not require hormonal priming, and it is mainly modulated by noradrenergic input to olfactory and limbic areas (Numan, 1988; Thomas and Palmiter, 1997). Recently, Olazabal et al. (2004) have suggested that serotonin has a facilitatory role in the expression of pup-care behavior in juvenile nonlactating rats, while dopaminergic mechanisms in brain areas implicated in fear response to novelty exert an opposite inhibitory role on maternal responsiveness. As maternal responses toward foster pups appear to be modulated by a complex interplay between serotonergic, dopaminergic, and oxytocinergic mechanisms in the hypothalamus, the interference of CPF on the maturation of all or one of these systems is a possibility that deserves further investigation. Analysis of maternal behavior after pregnancy and parturition in CPF-exposed females is needed to evaluate whether the enhanced responsiveness to pups is also reflected in more efficient maternal care in the proper physiological hormonal context.

**CPF Effects on Plus-Maze Performances**

CPF appears to reduce anxiety responses, as it increased time spent in the open arms with a significant sex-selective effect, in accordance with previous rat data (Aldridge et al., 2005). CPF effects are specific for anxiety response, as measures regarding...
activity and exploration during the test are not significantly affected. At variance from previous rat data, showing that male rats became less anxious after CPF exposure and thus more similar to females (Aldridge et al., 2004), in the mouse species CPF affects preferentially females’ behavior, raising the open-arm time in females to the same levels as found in males. There are strong species-specific differences between mice and rats, as female mice, differently from rats, are more anxious than males when confronted with a novel environment (Augustsson et al., 2005). In this light, previous rat data on reduced anxiety by CPF are confirmed by our present findings, as CPF appears to reduce anxiety levels blunting sex differences in two different rodent species.

Neural Targets for CPF Behavioral Effects

The data so far collected in rodent models indicate that CPF targets maturational CNS events that are not necessarily related to inhibition of brain cholinesterase. Behavioral changes are in fact observed following developmental exposure to doses inducing no or very limited brain AChE inhibition. It is possible that assessment of brain AChE activity shortly after treatment termination (1–4 h) would have revealed an effect of the repeated CPF treatment. However, in our previous study, using the same mouse strain and the same CPF doses as in the present study, we found only limited (20%) brain AChE inhibition 1 h after termination of PNDs 1–4 CPF treatment and no effect after treatment on PNDs 11–14 (Ricceri et al., 2003). In this perspective, even very low levels (less than 20%) of serum AChE inhibition that are considered safe from a regulatory point of view might be associated with delayed neurobehavioral changes. The search for markers of neurotoxicity further to AChE inhibition should be undertaken to take into account the complexity of effects brought about by developmental exposure to CPF and other organophosphorous pesticides. In vitro and in vivo evidence indicates that CPF impairs neural cell division and differentiation and interferes with synaptic function and intracellular signaling (Dam et al., 2000; Levin et al., 2001; Qiao et al., 2003). These effects are paralleled by different behavioral changes that include activity levels, exploration, response to novelty, socioagonistic and maternal behaviors, and cognitive functions. Such a wide spectrum of effects is not unexpected since CPF interferes with basic maturational mechanisms. However, developmental CPF exposure evokes significant upregulation of 5HT1a receptors (Aldridge et al., 2004). Recent data show that mice (Kusserow et al., 2004) overexpressing serotonin receptor during postnatal development had reduced levels of anxiety-related behaviors as measured in plus-maze and higher activity levels in the open field. Data concerning enhanced aggressive behavior in males and enhanced maternal responsiveness in females might be in line with such serotonergic hypothesis. However, the role of other systems, including the neuroendocrine mediators of social behaviors, such as oxytocin and vasopressin, and the well-known role of acetylcholine in the processing of socially relevant olfactory cues cannot be discounted on the basis of the present results.

Recent restrictions have been placed on CPF use in the United States (U.S. EPA, 2000), as it has been hypothesized that exposure to CPF might constitute a risk factor for later vulnerability to neurodevelopmental disorders in children (Berkowitz et al., 2004). The question which arises is whether rodent data collected so far on CPF neurobehavioral toxicity are relevant to children’s health. Certainly both rat and mice data point to a picture of generalized alterations in responsiveness to environmental and social cues while the impairment of cognitive performances appears to be of minor extent. While there are data showing lower spatial learning performances in CPF-exposed rats (Levin et al., 2001), we did not find any CPF-induced impairment in water maze acquisition in male mice treated with CPF as in the present study. The behavioral disturbances observed in rodents developmentally exposed to subtoxic doses of CPF might have some in common with neurodevelopmental disorder affecting the social domain and the emotional/affective responses to environmental challenges (Arnoldt et al., 2005). Furthermore, both socioagonistic behavior and maternal behavior induced by pup presentation are two sex dimorphic behavior patterns whose performance depends on the maturation of brain functional pathway subjected to sex hormone regulation. The potential endocrine-disrupting activity of CPF should be more extensively investigated, by using sensitive behavioral end points, such as those considered in this study.

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