Serotonergic Systems Targeted by Developmental Exposure to Chlorpyrifos: Effects during Different Critical Periods

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During brain development, serotonin (5HT) provides essential neurotrophic signals. In the present study, we evaluated whether the developmental neurotoxicity of chlorpyrifos (CPF) involves effects on 5HT signaling, as a potential mechanism underlying noncholinergic neuroteratogenic events. We evaluated four different treatment windows ranging from the neural tube stage [gestational days (GD) 9–12] and the late gestational period (GD17–20) through postnatal phases of terminal neuronal differentiation and synaptogenesis [postnatal days (PN) 1–4, PN11–14]. Exposure to CPF on GD9–12 elicited initial suppression, immediately followed by rebound elevation, of $5HT_{1A}$ and $5HT_2$ receptors as well as the 5HT transporter, all at doses below the threshold for cholinergic hyperstimulation and the resultant systemic toxicity. In contrast, with GD17–20 exposure, the initial effect was augmentation of all three components by low doses of CPF. Sensitivity of these effects declined substantially when exposure was shifted to the postnatal period. We also identified major alterations in 5HT-mediated responses, assessed for the adenylyl cyclase signaling cascade. Although GD9–12 exposure had only minor effects, treatment on GD17–20 elicited supersensitivity to both stimulatory and inhibitory responses mediated by 5HT. Our results indicate that CPF affects 5HT receptors, the presynaptic 5HT transporter, and 5HT-mediated signal transduction during a discrete critical gestational window. These effects are likely to contribute to the noncholinergic component of CPF's developmental neurotoxicity. *Key words:* adenylyl cyclase, brain development, chlorpyrifos, organophosphate insecticides, serotonin receptors, serotonin transporter. *Environ Health Perspect* 111:1736–1743 (2003). doi:10.1289/ehp.6489 available via *http://dx.doi.org/* [Online 22 August 2003]

Despite recent curtailment of production for domestic use [U.S. Environmental Protection Agency (EPA) 2000], exposures of pregnant women and children to chlorpyrifos (CPF) remain virtually ubiquitous in both agricultural and urban communities (Berkowitz et al. 2003; Curl et al. 2002; Enrique et al. 2002; Fenske et al. 2002; Whyatt et al. 2003). CPF, and likely other organophosphates, is a developmental neurotoxicant, targeting the immature brain at doses below the threshold for systemic toxicity, so exposures relevant to adverse neurobehavioral end points may go initially undetected [Jamal et al. 2002; Landrigan 2001; Landrigan et al. 1999; May 2000; National Research Council (NRC) 1993; Physicians for Social Responsibility 1995; Pope 1999; Ray and Richards 2001; Rice and Barone 2000; Slotkin 1999]. Indeed, there is increasing evidence that the mechanism for systemic toxicity, cholinergic hyperstimulation consequent to inhibition of cholinesterase, is not the primary mechanism for adverse effects of CPF on brain development (Barone et al. 2000; Lassiter et al. 1998, 2002; Moser and Padilla 1998; Pope 1999; Qiao et al. 2002; Rice and Barone 2000; Slotkin 1999). CPF itself, rather than its active metabolite that inhibits cholinesterase, affects neural cell replication and differentiation, axonogenesis and synaptogenesis, and the programming of synaptic function (Barone et al. 2000; Garcia et al. 2001, 2002, 2003; Lassiter et al. 1998, 2002; Monnet-Tschudi et al. 2000; Moser and Padilla 1998; Pope 1999; Qiao et al. 2001, 2002; Rice and Barone 2000; Slotkin 1999).

One potential noncholinergic mechanism for the developmental neurotoxicity of CPF is cell signaling mediated through adenylyl cyclase (AC), the enzyme that controls the synthesis of the second messenger cyclic AMP. CPF interacts directly with the expression and function of neurotransmitter receptors that control AC, and in addition, developmental exposure to CPF causes delayed-onset deterioration of AC-mediated cell signaling (Auman et al. 2000; Huff and Abou-Donia 1995; Meyer et al. In press; Olivier et al. 2001; Schuh et al. 2002; Song et al. 1997; Ward and Mundy 1996; Zhang et al. 2002). One of the most potent noncholinergic effects noted for CPF is the ability to affect the phosphorylation and function of nuclear transcription factors that control cell differentiation and that are themselves dependent on cyclic AMP (Crumpton et al. 2000; Garcia et al. 2001; Schuh et al. 2002). Nevertheless, even for the AC pathway, cholinergic systems have remained the main focus of many reports (Huff and Abou-Donia 1995; Huff et al. 2001; Ward and Mundy 1996; Zhang et al. 2002). In the present study, we focused instead on serotonergic (5HT) systems for two distinct reasons. First, 5HT subserves important trophic functions that control the differentiation and architectural organization of the developing brain (Azmitia 2001; Dreyfus 1998; Lauder 1985; Levitt et al. 1997; Turlejski 1996; Weiss et al. 1998; Whitaker-Azmitia 1991, 2001), so the targeting of 5HT systems represents a likely site for noncholinergic effects of CPF on brain development. Second, it is increasingly thought that environmental toxicants evoke long-term changes in the programming of 5HT function, leading to appetitive and affective disorders, and consequent increases in the incidence of obesity and depression (Slikker and Schwetz 2003; Toschke et al. 2002; von Kries et al. 2002). To our knowledge, there is only one previous report on the effects of CPF on 5HT systems in the developing brain, indicating effects on the high-affinity presynaptic 5HT transporter (5HTT) (Raines et al. 2001). Various 5HT receptor subtypes converge on the control of AC. $5HT_{1A}$ receptors inhibit AC through the inhibitory G-protein, G_i (Barnes and Sharp 1999) but also can stimulate AC through release of G-protein βγ subunits (Raymond et al. 1999). $5HT_2$ receptor stimulation leads to heterologous enhancement of AC responses mediated by other, non-5HT receptors (Morin et al. 1992; Rovescalli et al. 1993), and $5HT_7$ receptors, which cross-react with ligands for $5HT_{1A}$ receptors, are stimulatory for AC (Duncan et al. 1999; Lovenberg et al. 1993). Accordingly, in the present study we evaluated the short-term effects of developmental CPF exposure on 5HT receptors, the 5HTT site, and the ability of 5HT to evoke stimulation or inhibition of AC activity. Determinations were made in the forebrain, a brain region containing major 5HT terminal fields, as well as in the brainstem, a region containing 5HT cell bodies that project to the forebrain. Treatment regimens were chosen to span the threshold for systemic toxicity and/or inhibition of cholinesterase, and to incorporate different critical periods of exposure in both prenatal and postnatal periods (Garcia et al. 2003; Meyer et al. In press; Qiao et al. 2002, 2003; Raines et al. 2001; Roy et al. 1998; Slotkin 1999; Slotkin et al. 2001a).

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Materials and Methods

Animal treatments. All experiments using live animals were carried out in accordance with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996). Timed-pregnant Sprague–Dawley rats (Zivic Laboratories, Pittsburgh, PA, USA) were housed in breeding cages, with a 12-hr light–dark cycle and with free access to food and water. CPF (Chem Service, West Chester, PA, USA) was dissolved in dimethyl sulfoxide (DMSO) to provide rapid and complete absorption (Whitney et al. 1995) and was injected subcutaneously in a volume of 1 mL/kg body weight. For exposure during neurulation, dams were injected daily with CPF at 1 or 5 mg/kg of body weight on GD9–12, and tissues were obtained on GD17 and GD21. For later gestational exposure (GD17–20), dams were given CPF daily (1, 2, 5, 10, 20, or 40 mg/kg) and tissues were sampled on GD21. Control animals received DMSO injections on the same schedules. These doses span the threshold for inhibition of fetal brain cholinesterase activity, fetal growth impairment, or reduced maternal weight gain, all of which become evident at about 5 mg/kg (Garcia et al. 2002; Qiao et al. 2002).

For postnatal CPF treatments, all dams delivered during GD22, and pups were then randomized on the day after birth and redistributed to the dams to achieve a litter size of 10 to maintain a standard nutritional status. Randomization was repeated at intervals of several days, and in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. For studies of CPF effects in the first few days after birth, animals were given 1 mg/kg daily on PN1–4. For studies in older animals, which tolerate higher doses (Campbell et al. 1997; Pope and Chakraborti 1992; Pope et al. 1991; Whitney et al. 1995), daily treatment with 5 mg/kg was given on PN11–14. These doses have been shown previously to alter brain development without eliciting overt systemic toxicity (Campbell et al. 1997; Song et al. 1997; Whitney et al. 1995). Behavioral differences remain apparent, or may first emerge, after weaning (Dam et al. 2000; Song et al. 1997). Neither regimen evokes weight loss or mortality (Campbell et al. 1997; Dam et al. 1998; Johnson et al. 1998; Song et al. 1997), and in the present study we did not observe any changes in suckling or maternal caretaking. Animals were selected from each litter as described below, 24 hr and 6 days after the last CPF injection.

For samples obtained on GD17, we analyzed the whole brain, whereas for those obtained on GD21 the forebrain was separated from the rest of the brain by making a cut rostral to the thalamus. Because the cerebellum represents an inappreciable proportion of brain weight on GD21, the remainder was designated "brainstem." This dissection, which follows the natural planes of the fetal and neonatal rat brain, includes the corpus striatum, hippocampal formation, and neocortex within the area designated "forebrain." The region designated "brainstem" includes the midbrain, colliculi, pons, and medulla oblongata (but not cervical spinal cord), as well as the thalamus. For studies on PN5, PN10, PN15, and PN20, blunt cuts were made through the cerebellar peduncles, whereupon the cerebellum (including flocculi) was lifted from the underlying tissue. Then, cuts were made to separate the forebrain from the brainstem as described for GD21. All tissues were frozen with liquid nitrogen and maintained at –45°C until assayed.

Membrane preparation. Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, USA) in ice-cold 50 mM Tris (pH 7.4), and the homogenates were sedimented at 40,000 × *g* for 15 min. The pellets were washed twice by resuspension (Polytron) in homogenization buffer followed by resedimentation, and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in the same buffer.

5HT receptor and transporter binding. We used two radioligands (Perkin-Elmer Life Sciences, Boston, MA, USA) to determine 5HT receptor binding (Xu et al. 2002): 1 nM [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin (specific activity, 135 Ci/mmol) for $5HT_{1A}$ receptors (Park et al. 1999; Stockmeier et al. 1998), and 0.4 nM [^{3}H]ketanserin (specific activity, 63 Ci/mmol) for $5HT_2$ receptors (Leysen et al. 1982; Park et al. 1999). For $5HT_{1A}$ receptors, incubations lasted for 30 min at 25°C in a buffer consisting of 50 mM Tris (pH 8), 2 mM $MgCl_2$, and 2 mM sodium ascorbate; 400 µM 5HT (Sigma Chemical Co., St. Louis, MO, USA) was used to displace specific binding. For $5HT_2$ receptors, incubations lasted 15 min at 37°C in 50 mM Tris (pH 7.4), and specific binding was displaced with 40 µM methylsergide (Sandoz Pharmaceuticals, East Hanover, NJ, USA). Incubations were stopped by the addition of a large excess of ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto glass fiber filters that were presoaked in 0.05% polyethyleneimine (Sigma). The filters were then washed repeatedly, and radiolabel was determined. For binding to the presynaptic 5HTT (Moret and Briley 1991; Slotkin et al. 1997, 1999b, 2000; Xu et al. 2001), the membrane suspension was incubated with 85 pM [^{3}H]paroxetine (specific activity, 19.4 Ci/mmol; PerkinElmer) with or without addition of 100 µM 5HT to displace specific binding. Incubations lasted 120 min at 20°C.

AC activity. We used the same membrane preparation as for the receptor binding assays; the methods have been previously described in detail (Slotkin et al. 1990, 1992, 2001b; Xu et al. 2002). Membrane aliquots were incubated for 10 min at 30°C with final concentrations of 40 mM Tris HCl (pH 7.4), 10 mM theophylline, 1 mM adenosine 5′-triphosphate, 10 µM guanosine 5′-triphosphate, 2 mM $MgCl_2$, 1 mg/mL bovine serum albumin, and a creatine phosphokinase-ATP–regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/mL phosphocreatine kinase, in a total volume of 250 µL. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3,000 × *g* for 15 min, and the supernatant solution was assayed for cyclic AMP by radioimmunoassay (Amersham Pharmacia Biotech, Piscataway, NJ, USA). AC activity was evaluated under four different conditions: basal activity; the response to 100 µM forskolin (Sigma), which acts directly on AC, bypassing the need for activation of neurotransmitter receptors (Seamon and Daly 1986); and both basal and forskolin-stimulated activity in the presence of 100 µM 5HT. The superimposition of effects of 5HT on

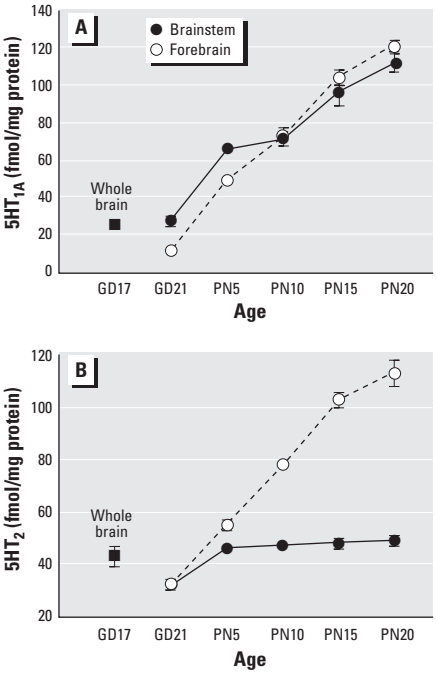

Figure 1. Development of 5HT receptor binding in control rat brain regions (mean ± SE). (*A*) $5HT_{1A}$ receptors. (*B*) $5HT_2$ receptors. Values were combined across the different cohorts used for the various CPF treatments. However, effects of CPF shown in Figures 2–7 were determined using only the appropriately matched controls. Across both receptor subtypes and both brain regions, the postnatal age points indicated significant sex differences ($p < 0.01$ for age × sex; $p < 0.02$ for age × sex × subtype), but values are shown for both sexes combined. Sex differences are discussed in the text.

those of forskolin enables detection of potential inhibitory actions, whereas the effects of 5HT on basal activity are more sensitive to excitatory effects (Chow et al. 2000; Slotkin et al. 1999a; Xu et al. 2002). The concentrations of all the agents used here have previously been found to be optimal for effects on AC and/or were confirmed in preliminary experiments (Auman et al. 2000, 2001; Xu et al. 2002; Zeiders et al. 1997, 1999).

Data analysis. For the studies with prenatal CPF treatment, because the treatments were given to the dams, we used only one fetus from each dam; therefore, the number of determinations represents the number of dams. The fetuses were derived from the same litters as those used in two previous studies on cell damage and cholinergic biomarkers (Garcia et al. 2002; Qiao et al. 2002) in which effects on cholinesterase activity, maternal and fetal body weights, and other litter characteristics were reported. For studies with postnatal treatment, the randomization procedure distributed maternal differences equally over all litters; for each experiment, only one male and one female were taken from each litter for any given determination.

Data are presented as mean ± SE obtained from five to seven determinations for each treatment group at each age and, for postnatal points, for each sex. For convenience, some of the results are given as the percent change from control values, but statistical evaluations were always conducted on the original data. To establish treatment differences in radioligand binding or AC activity, a global analysis of variance (ANOVA; data log transformed whenever variance was heterogeneous) was first conducted, incorporating all contributing variables: treatment, age, brain region, sex, and the multiple types of measurements made on each membrane preparation (three binding assays, AC activity under four different conditions). We considered the variables to be repeated measures because each membrane preparation was used for the multiple types of determinations. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and measures that differed from control values. These were conducted by lower-order ANOVAs, followed, where appropriate, by Fisher's protected least-significant difference to identify individual values for which the CPF groups differed from the corresponding control. However, in situations where there was no interaction of treatment × other variables, only main treatment effects are reported without conducting separate subtests. For all tests, significance for main treatment effects was assumed at $p < 0.05$; however, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran 1967).

For presentation, control values were combined across the different treatment cohorts (controls used for CPF administration on GD9–12, GD17–20, PN1–4, PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.

Results

Ontogenesis of 5HT receptors. In control rats, the development of 5HT receptor binding showed distinct subtype and regional hierarchies. For $5HT_{1A}$ receptors (Figure 1A), values in the brainstem or forebrain rose in parallel over the period from GD17 through PN20; the only significant sex difference was found in PN10 rats, when females (79 ± 2 fmol/mg protein) displayed slightly higher values than did males (64 ± 6 fmol/mg protein). In contrast, for $5HT_2$ receptors (Figure 1B), there was a much greater ontogenetic increase in the forebrain than in the brainstem, achieving nearly a 3-fold regional difference by PN20. Again, there were small, transient sex differences with females showing slightly higher values on PN4 (female, 49 ± 1 fmol/mg protein; male, 43 ± 1 fmol/mg protein) and PN10 (female, 49 ± 1 fmol/mg protein; male, 45 ± 1 fmol/mg protein). Binding of [^{3}H]paroxetine to the 5HTT site showed a different regional specificity for changes during fetal development. On GD17, the value in whole brain was 147 ± 7 fmol/mg protein, rising to 240 ± 21 in the brainstem on GD21 but remaining low in the forebrain (118 ± 14 fmol/mg protein). As identified in previous work (Raines et al. 2001), the largest rise in 5HTT binding occurs postnatally, with a doubling in both regions by PN20.

Systemic toxicity of CPF. As reported previously for prenatal CPF exposure (Garcia et al. 2002; Qiao et al. 2002), the threshold for CPF-induced impairment of maternal growth was 5 mg/kg with treatment on either GD9–12 or GD17–20, but fetal brain growth was unaffected even at the highest doses (data not shown). Neither regimen affected the number of fetuses or their viability. Fetal brain showed significant cholinesterase inhibition at ≥ 5 mg/kg (Qiao et al. 2002). Similarly, in keeping with previous results (Campbell et al. 1997; Slotkin et al. 2001a; Song et al. 1997; Whitney et al. 1995), neither of the postnatal CPF regimens evoked any signs of systemic toxicity, nor did they affect body or brain region weights (data not shown).

CPF treatment on GD9–12. Across all three ligand-binding measurements and the three different tissues (whole brain on GD17, brainstem or forebrain on GD21), multivariate ANOVA indicated interactions of treatment × region ($p < 0.0001$), treatment × measure ($p < 0.06$), and treatment × region × measure ($p < 0.002$). Accordingly, separate analyses were conducted for each region (Figure 2). In samples collected on GD17, CPF treatment elicited robust, dose-dependent reductions in $5HT_{1A}$ and $5HT_2$ receptor binding. Binding to the 5HTT site also showed large reductions that were already maximal at the lowest of

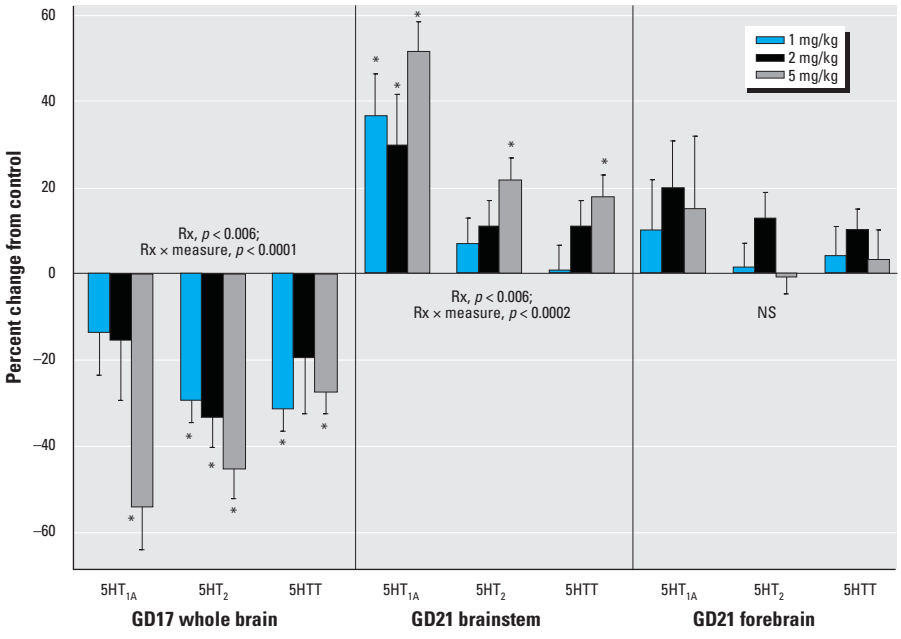

Figure 2. Effects of GD9–12 CPF exposure on development of 5HT receptors and 5HTT, presented as the percent change from control values (mean ± SE). Abbreviations: Rx, treatment; NS, not significant. ANOVA results across all regions are as follows: Rx × region, $p < 0.0001$; Rx × measure, $p < 0.06$; Rx × region × measure, $p < 0.002$. Lower-order ANOVAs for each region appear within the figure.
*Individual values for which the CPF groups differ significantly from the corresponding control.

CPF. By GD21, however, values for all three parameters showed rebound elevations that were statistically significant in the brainstem but not the forebrain.

Across all four AC measures, CPF elicited treatment effects that were regionally selective (treatment × region, $p < 0.06$), necessitating separate evaluations across the three regions. There were no effects on either basal or forskolin-stimulated AC activity (data not shown). Under basal conditions, addition of 5HT to the incubation medium elicited a net stimulation of AC activity, such that the ratio of activity with/without 5HT was significantly greater than 1 ($p < 0.007$ for the control group, $p < 0.0001$ across all treatment groups; Figure 3A). CPF administration interfered with this response only marginally ($p < 0.07$), with the strongest relationship evident in the brainstem (Figure 3A). When AC was evaluated in the presence of forskolin (Figure 3B), 5HT was not stimulatory but rather elicited no net change (controls) or an overall decrease ($p < 0.002$ across all treatment groups). CPF treatment tended to enhance the inhibitory effect of 5HT, at the margin of significance ($p < 0.08$).

CPF treatment on GD17–20. CPF administered during late gestation elicited statistically robust effects assessed by global ANOVA across all three ligand-binding measures and both regions: $p < 0.0001$ for the main treatment effect, and for interactions of treatment × region, treatment × measure, and treatment × region × measure. Accordingly, each binding parameter was assessed separately for the brainstem and forebrain (Figure 4). In the brainstem, CPF elicited significant elevations in $5HT_{1A}$ and $5HT_2$ receptor binding, as well as in [^{3}H]paroxetine binding to the 5HTT site. However, in each case, unlike the situation with the earlier gestational regimen, significant effects were obtained only with doses of ≥ 10 mg/kg, exceeding the threshold for systemic toxicity (Qiao et al. 2002). In contrast, the forebrain was far more sensitive: CPF administration evoked much larger increases that were statistically significant even at the lowest doses, well below the threshold for maternal or fetal toxicity or, indeed, for inhibition of fetal brain cholinesterase (Qiao et al. 2002). An additional, unusual feature was the distinct hormesis displayed by the 5HTT site in the forebrain, with significant increases evoked by low doses of CPF, and massive reductions once the threshold for systemic toxicity was exceeded.

Across all four AC measures, late gestational CPF treatment elicited significant, regionally selective effects that influenced the response to 5HT: $p < 0.03$ for the main treatment effect, $p < 0.01$ for treatment × region, and $p < 0.0001$ for treatment × measure. In the absence of 5HT, basal AC activity was generally unaffected except at one intermediate dose of CPF in the brainstem (data not shown). On the other hand, forskolin-stimulated AC activity showed consistent increases ($p < 0.03$ for the brainstem, $p < 0.0001$ for the forebrain) (Table 1). The effects of CPF on the AC response to forskolin-stimulated AC activity are discussed elsewhere (Meyer et al. In press). Again, 5HT elicited a net stimulation of basal AC activity (Figure 5A). CPF administration augmented the stimulatory

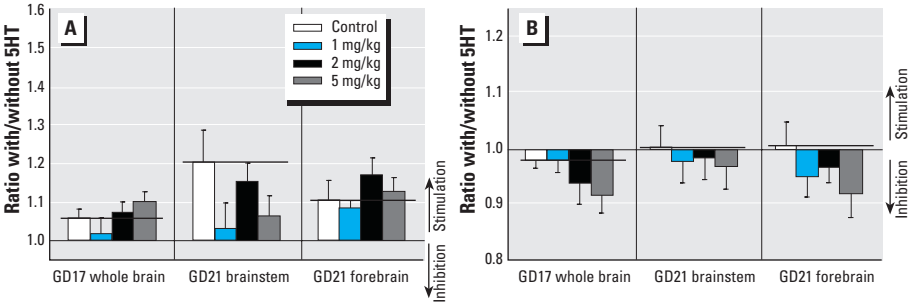

Figure 3. Effects of GD9–12 CPF exposure on the AC response to 5HT; results are presented as the response ratio (± SE; ratios > 1 indicate stimulation, and ratios < 1 indicate inhibition); the horizontal line indicates the response obtained in controls. (*A*) Basal AC response determined with or without the addition of 5HT (ANOVA results across all regions: not significant; $p < 0.07$). (*B*) Forskolin-stimulated AC with or without the addition of 5HT (ANOVA results across all regions: not significant, $p < 0.08$).

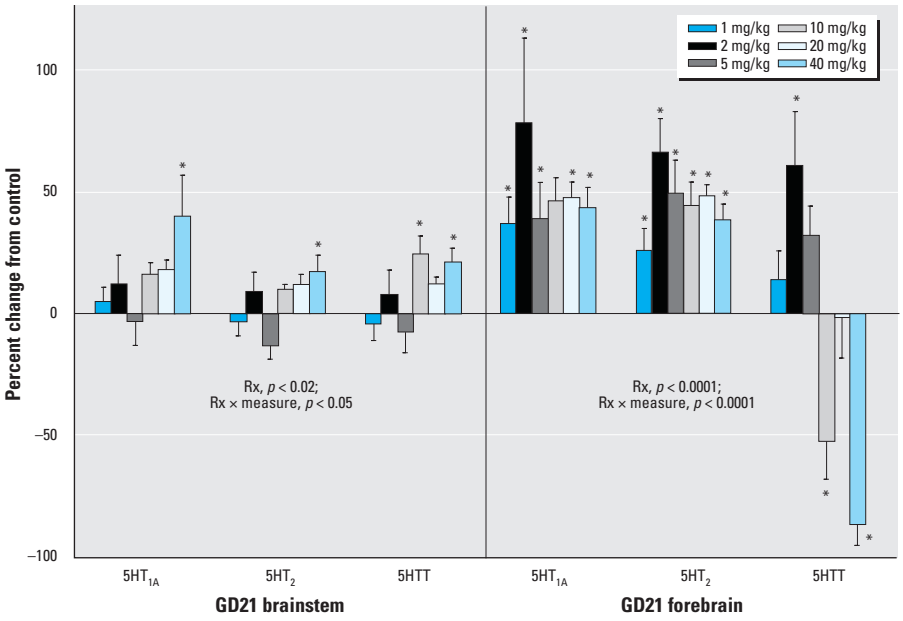

Figure 4. Effects of GD17–20 CPF exposure on development of 5HT receptors and 5HTT, presented as the percent change from control values (mean ± SE). Rx, treatment. ANOVA results across both regions are as follows: Rx, $p < 0.0001$; Rx × region, $p < 0.0001$; Rx × measure, $p < 0.0001$; Rx × region × measure, $p < 0.0001$. Lower-order ANOVAs for each region appear within the figure.
*Individual values for which the CPF groups differ significantly from the corresponding control.

Table 1. Increase in forskolin-stimulated AC activity related to CPF dose on GD17–20.

Treatment	Brainstem (pmol/min/mg protein)	Forebrain (pmol/min/mg protein)
Control	700 ± 47	178 ± 8
CPF		
1 mg/kg	684 ± 33	197 ± 19
2 mg/kg	845 ± 39[a]	168 ± 14
5 mg/kg	791 ± 79	153 ± 13
10 mg/kg	749 ± 23	206 ± 21
20 mg/kg	851 ± 36[b]	267 ± 23[c]
40 mg/kg	896 ± 32[d]	231 ± 10[d]

[a] $p < 0.04$. [b] $p < 0.003$. [c] $p < 0.0001$. [d] $p < 0.007$.

effect of 5HT in the brainstem at low doses, an effect that was offset once the dose was raised above the threshold for systemic toxicity. The forebrain was far less sensitive, with smaller increases apparent only at higher CPF doses. As before, 5HT had little or no effect on forskolin-stimulated AC activity in control brain regions obtained on GD21 (Figure 5B). Animals exposed to the late gestational CPF regimen showed a dose-dependent shift toward greater inhibition in response to 5HT that was statistically significant at every dose studied.

Postnatal CPF treatment. CPF administration to newborn rats on PN1–4 evoked significant increases in $5HT_{1A}$ and $5HT_2$ receptor binding evaluated 24 hr after the last dose (Figure 6), but the effects were considerably smaller than those seen with the gestational CPF exposures. By PN10, increases were still evident in the forebrain of males, whereas females tended to show net decreases in the brainstem. When CPF treatment occurred later in the postnatal period (PN11–14), the increases in 5HT receptor binding assessed 24 hr after the last dose were no longer apparent in males, but females still showed vestiges of the effect (Figure 7A). By PN20, receptor binding was significantly subnormal across both brain regions and both subtypes. The deficits were confirmed in additional membrane preparations by Scatchard determinations over ligand concentrations ranging from 0.25 to 2 nM (Figure 7B). Multiple regression incorporating the independent variables (binding, treatment, sex) gave a high correlation (r^2 = 0.97), indicating that these parameters closely defined the binding values. There was a main effect of CPF treatment (p < 0.0002) that reflected the small, but highly statistically significant reductions in the concentration of binding sites (B_{max}, the intercepts on the abscissa) without a shift in receptor affinity for the ligand (determined from the slope of the lines).

For the postnatal CPF treatments, [^{3}H]paroxetine binding to the 5HTT site was assessed in a previous study (Raines et al. 2001). Exposure on PN1–4 elicited small (5–10%) decreases in the brainstem and equally small increases in the forebrain of females, but both effects were transient and were no longer evident by PN10. CPF given on PN11–14 evoked a generalized, small decrease in 5HTT binding in males, whereas females showed transient changes.

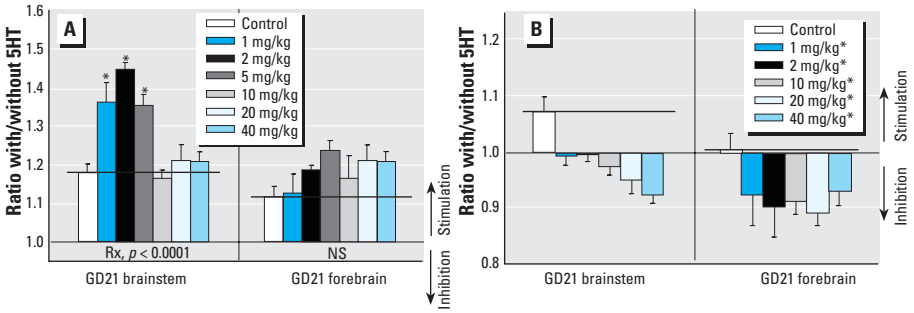

Figure 5. Effects of GD17–20 CPF exposure on the AC response to 5HT; results are presented as the response ratio (± SE; ratios > 1 indicate stimulation, and ratios < 1 indicate inhibition); the horizontal line indicates the response obtained in controls. Abbreviations: Rx, treatment; NS, not significant. (*A*) Basal AC response determined with or without addition of 5HT. ANOVA results across both regions are as follows: Rx, p < 0.0001; Rx × region, p < 0.002. Lower-order ANOVAs for each region were carried out for basal AC measurements because of the significant treatment × region interaction, and are shown within the figure. (*B*) Forskolin-stimulated AC with or without addition of 5HT. ANOVA results were calculated across both regions (Rx, p < 0.0002) but the effects were not examined separately for each region because there was only a main treatment effect.
*Individual values for which the CPF groups differ significantly from the corresponding control.

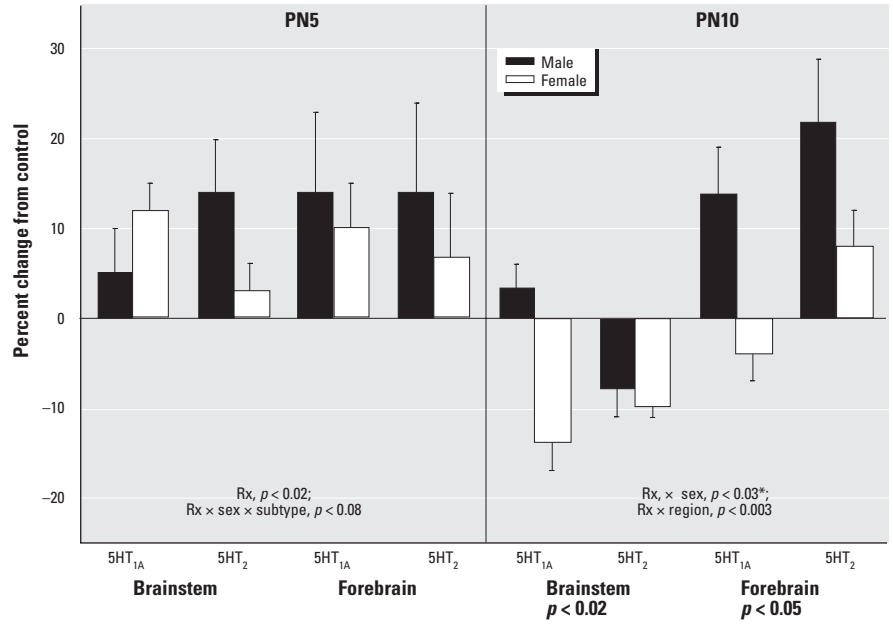

Figure 6. Effects of PN1–4 CPF exposure (1 mg/kg) on development of 5HT receptors, presented as the percent change from control values (mean ± SE). Rx, treatment. ANOVA results across both regions, both subtypes, and both age points are as follows: Rx, p < 0.02; Rx × sex, p < 0.1; Rx × region, p < 0.05; Rx × age, p < 0.06; Rx × region × age, p < 0.09; Rx × sex × age × subtype, p < 0.03. Lower-order ANOVAs appear within the figure. Separate tests for each region were not carried out for PN5 data because of the absence of a treatment × region interaction; similarly, for PN10 data, separate analyses could not be carried out absent a treatment × subtype interaction. However, the main treatment effects for each region are shown at the bottom for PN10.
*Rx × region significant for both sexes.

Discussion

Results of this study indicate that 5HT systems represent a major target for CPF in the developing brain, likely contributing to the noncholinergic components of neuroteratogenicity. The critical windows for effects on 5HT receptors, the presynaptic 5HTT site, and cell signaling mediated by 5HT extended over a wide developmental period ranging from the embryonic neural tube stage to terminal differentiation and synaptogenesis and, importantly, were detectable at exposures below the threshold for symptoms of cholinergic hyperstimulation or systemic toxicity. In this article we focus on the specific spectra of effects for each component of 5HT synaptic communication, but the central finding is that 5HT systems, major neurotrophic controllers of brain development (Azmitia 2001; Dreyfus 1998; Lauder 1985; Levitt et al. 1997; Turlejski 1996; Weiss et al. 1998; Whitaker-Azmitia 1991, 2001), are indeed a primary target for CPF.

Using radioligand binding, we assessed three different proteins that are essential for 5HT synaptic function: the presynaptic 5HTT site and two of the major receptor subtypes, $5HT_{1A}$ and $5HT_2$. For the gestational treatment, all three proteins shared similar

patterns of effects with the same critical windows. With CPF exposure on GD9–12, there was initial suppression of receptor and 5HTT binding, followed by rebound elevations by GD21. In contrast, later gestational exposure on GD17–20 elicited increases in all three proteins. Later, postnatal exposures also tended to increase receptor binding but with a much smaller magnitude of effect and less consistency. Accordingly, there are at least two distinct categories of effects, suppression during the neural tube stage and augmentation with later exposure, followed by declining sensitivity during postnatal phases of development. There are three essential factors that provide information about the mechanisms underlying these effects. First, for both phases, the alterations were apparent at CPF doses below the threshold for signs of systemic toxicity or, indeed, for significant inhibition of cholinesterase (Qiao et al. 2002). It is therefore highly unlikely that the effects on 5HT systems are secondary to cholinergic hyperstimulation. Second, the fact that all three proteins showed the same direction of change implies that CPF does not directly alter the transcription of individual genes in the 5HT system, because it is implausible that the same exact effect would be seen for the three separate genes. Accordingly, it is most probable that CPF affects 5HT cell differentiation, synaptic function, and/or synaptogenesis with a consequent, parallel impact on the diverse 5HT-associated proteins. This conclusion is reinforced by the third mechanistic observation, regional selectivity, best exemplified by the GD17–20 regimen, where the forebrain was affected much more than the brainstem. If the effects of CPF were mediated directly at the level of gene expression, the effects would instead have been uniform across the different regions and, indeed, would also not display the temporal disparities discussed above.

We also obtained clear evidence for disruption of 5HT-mediated cell signaling by developmental CPF exposure. Attribution of these effects to specific 5HT receptor changes is problematic, in view of the large number of receptor subtypes in the 5HT family and their diverse impact on signaling pathways as exemplified by AC (Barnes and Sharp 1999; Duncan et al. 1999; Lovenberg et al. 1993; Morin et al. 1992; Raymond et al. 1999; Rovescalli et al. 1993). In the present study we took a straightforward approach, examining the net effect of 5HT on AC activity (i.e., summation of excitatory and inhibitory signals) by evaluating the response to the natural agonist itself, 5HT. However, we made the assessments under conditions conducive to detecting stimulatory responses (basal AC with and without 5HT) and inhibitory responses (forskolin-stimulated AC with and without 5HT). Again, our findings indicated global effects on 5HT-mediated signals, occupying a distinct critical developmental window. CPF exposure on GD9–12 elicited only minor changes in AC responses to 5HT but tended toward enhanced inhibition. When the exposure was shifted to GD17–20, we obtained marked enhancement of both the excitatory and inhibitory responses to 5HT, connoting global supersensitivity. The effect of late gestational treatment thus is in the predicted direction for the effects of CPF on 5HT receptor binding: augmented receptor concentrations would lead to enhanced responsiveness. However, receptor concentrations alone cannot provide an adequate explanation because the effects on the stimulatory AC responses were greater in the brainstem, whereas the receptor effects were greater in the forebrain. Obviously, then, CPF elicits other changes in AC signaling that influence the response to 5HT. This conclusion is bolstered by the fact that, as noted above, CPF elicited significant increases in forskolin-stimulated AC even in the absence of added 5HT; other, non-5HT–related changes in AC signaling have also been noted after prenatal CPF treatment (Meyer et al. In press). Again, however, as was true for receptor binding, the critical window for effects on 5HT-mediated signaling appears to center around the late gestational period, although delayed-onset deterioration of signaling was apparent even with the postnatal treatment regimens.

It is noteworthy that two of the targets of GD17–20 CPF exposure displayed distinct hormesis, with effects elicited at low doses that were then offset at higher doses: 5HTT binding in the forebrain and basal AC responses to 5HT in the brainstem. On the surface, the reversal of the two effects corresponds to doses exceeding the threshold for systemic toxicity (Qiao et al. 2002). However, several other studies indicate hormetic effects of CPF that do not surpass that threshold (Levin et al. 2001, 2002; Meyer et al. In press; Qiao et al. 2002, 2003), and it would be worthwhile to pursue the actual reason for the nonmonotonic dose–effect relationship for 5HT systems. One possibility rests on the positive trophic effects of cholinergic input. Choline supplementation, which enhances cholinergic neurotransmission, can improve neural performance

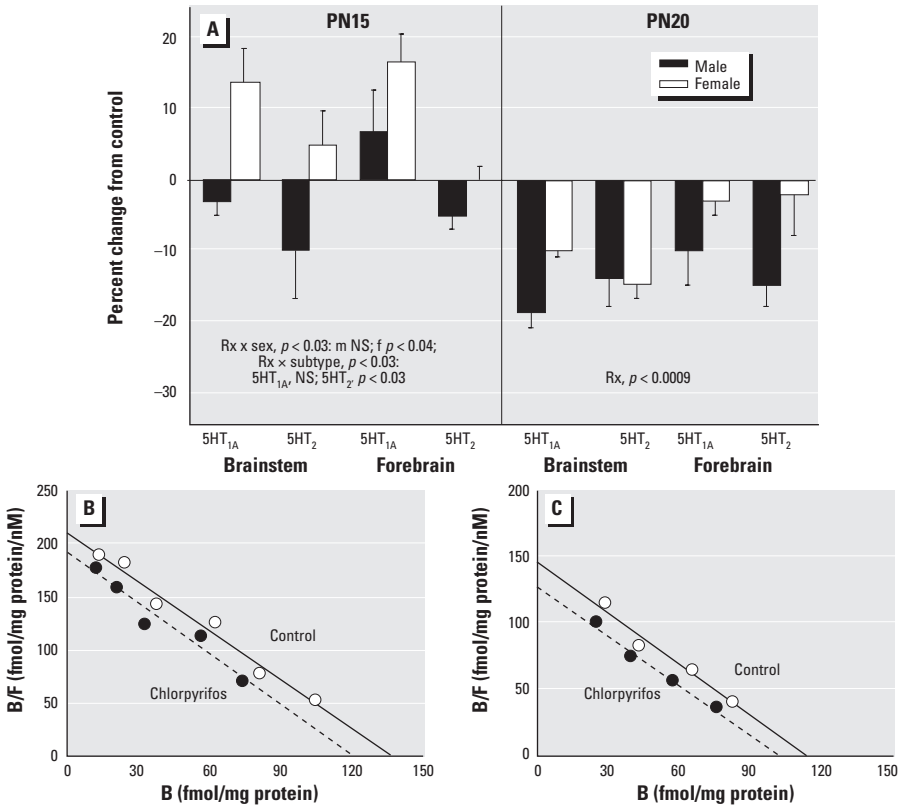

Figure 7. Effects of PN11–14 CPF exposure (5 mg/kg) on development of 5HT receptors. Abbreviations: B, bound; F, free; f, female; m, male; NS, not significant; Rx, treatment. (*A*) Effects in each region, presented as the percent change from control values (mean ± SE), shown for PN15 and PN20. ANOVA results across both regions, both subtypes, and both age points are as follows: Rx, $p < 0.05$; Rx × age, $p < 0.002$; Rx × sex, $p < 0.02$; Rx × subtype, $p < 0.06$; Rx × age × subtype, $p < 0.08$. Lower-order ANOVAs for each age point appear within the figure. Separate tests for each region were not carried out because of the absence of treatment × region interactions for the separate ages. (*B–C*) Scatchard plots for $5HT_{1A}$ receptors conducted in additional brainstem membrane preparations on PN20 for (*B*) males and (*C*) females. Across the two sets, multiple regression indicates a significant reduction in binding caused by CPF treatment ($p < 0.0002$).

and adaptability to adverse conditions (Meck and Williams 1997, 1999; Montoya et al. 2000). For behavioral effects of developmental CPF exposure, we have generally found that doses above the threshold for cholinesterase inhibition (Qiao et al. 2002) but below those for systemic toxicity have lesser effects than at either end of the dose–response curve (Levin et al. 2001, 2002, 2003). If the promotional effects of cholinergic input extend to their impact on 5HT systems, this may provide a source for nonmonotonic (hormetic) responses seen here.

At this stage, it is not clear whether the effects on 5HT systems or on AC represent a distinct targeting of these signaling elements or, instead, are included in a larger family of signaling defects elicited by developmental CPF exposure. To date, for example, only one report has appeared in which a direct interaction of CPF with 5HT uptake was evaluated in platelets (Sachana et al. 2001). Certainly, a variety of other studies have addressed signals mediated through other neurotransmitter receptors that converge on AC (Auman et al. 2000; Huff and Abou-Donia 1995; Huff et al. 1994, 2001; Ward and Mundy 1996; Zhang et al. 2002), as well as heterologous effects upon the G-proteins that couple the receptors to AC, or on AC itself, effects that are thus shared by all AC-coupled inputs (Auman et al. 2000; Garcia et al. 2001; Olivier et al. 2001; Song et al. 1997). The proteins that are phosphorylated as a result of AC activation appear to be among the most sensitive targets for developmental effects of CPF (Schuh et al. 2002). Nevertheless, effects on other signaling pathways are likely to be present (Yanai et al. 2002) but simply have not yet been examined as thoroughly as those on AC. Some studies suggest protein kinase C as an additional CPF target (Buznikov et al. 2001; Yanai et al. 2002). However, regardless of whether 5HT is a direct or indirect target, effects on behaviors mediated by 5HT systems are likely to represent an important end point for the developmental neurotoxicity of CPF.

The present study indicates that CPF targets the development of signaling proteins and their ability to elicit cellular responses for one of the major neurotrophic monoamines, 5HT, during a discrete critical period. In the future, it will be important to determine if other neurotrophins are similarly affected by CPF because these are likely to contribute to the noncholinergic components of the neuroteratogenicity of this organophosphate. However, an equally essential issue is whether the early changes in 5HT systems have an impact on the future reactivity of this neurotransmitter pathway, as suggested here by the delayed-onset deterioration of 5HT-mediated signaling after postnatal CPF exposure. Recent reports postulate that environmental toxicants, acting through discoordination of 5HT signaling, might contribute to obesity and affective disorders in childhood, adolescence, or adulthood (Slikker and Schwetz 2003; Toschke et al. 2002; von Kries et al. 2002). For CPF, this possibility is undergoing active investigation in our laboratory.

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