Developmental Exposure to Chlorpyrifos Elicits Sex-Selective Alterations of Serotonergic Synaptic Function in Adulthood: Critical Periods and Regional Selectivity for Effects on the Serotonin Transporter, Receptor Subtypes, and Cell Signaling

Justin E. Aldridge, Frederic J. Seidler, and Theodore A. Slotkin

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina, USA

During brain development, serotonin (5HT) provides essential neurotrophic signals, and in earlier work, we found that developmental exposure to chlorpyrifos (CPF) elicits short-term changes in 5HT systems. In the present study, we evaluated the effects in adulthood after CPF exposures from the neural tube stage [gestational days (GD) 9–12] and the late gestational period (GD17–20) through postnatal neuronal differentiation and synaptogenesis [postnatal days (PN) 1–4 and 11–14], using treatments below the threshold for systemic toxicity. With exposure on GD9–12, CPF elicited global elevations in $5HT_{1A}$ and $5HT_2$ receptors and in the 5HT presynaptic transporter. The GD17–20 treatment elicited larger effects that displayed selectivity for regions with 5HT nerve terminals and that were preferential for males. Although similar receptor up-regulation was seen after PN1–4 exposure, the effects were larger in regions with 5HT cell bodies; in addition, the presynaptic transporter was down-regulated in the nerve terminal zones of females. The PN11–14 exposure had much smaller effects on receptors but still elicited transporter suppression with the same regional and sex selectivity. Although CPF exposure on GD17–20, PN1–4, or PN11–14 altered the ability of 5HT to modulate adenylyl cyclase, this change did not correspond with the effects on 5HT receptors, suggesting an additional set of effects on proteins that transduce the 5HT signal. Our results indicate that CPF elicits long-lasting changes in 5HT receptors, the presynaptic 5HT transporter, and 5HT-mediated signal transduction after exposure in discrete developmental windows that range from the neural tube stage through synaptogenesis. These effects are likely to contribute to neurobehavioral teratology of CPF. *Key words:* **adenylyl cyclase, brain development, chlorpyrifos, organophosphate insecticides, serotonin receptors, serotonin transporter, sex-selective effects.** *Environ Health Perspect* **112:148–155 (2004). doi:10.1289/ehp.6713 available via *http://dx.doi.org/* [Online 4 November 2003]**

Exposure of pregnant women and young children to organophosphate insecticides remains a major concern in light of their developmental neurotoxicity (Jamal et al. 2002; Landrigan 2001; Landrigan et al. 1999; May 2000; National Research Council 1993; Physicians for Social Responsibility 1995; Pope 1999; Ray and Richards 2001; Rice and Barone 2000; Slotkin 1999). Although recent restrictions have been placed on its use in the United States [U.S. Environmental Protection Agency (EPA) 2000], chlorpyrifos (CPF) remains one of the most heavily used insecticides worldwide. Of the adverse effects of CPF on neurodevelopment, cholinergic systems represent a major focus, logically because its systemic toxicity results from inhibition of cholinesterase and the consequent cholinergic hyperstimulation (Barone et al. 2000; Mileson et al. 1998; Pope 1999; Ray and Richards 2001; Slotkin 1999). Nevertheless, it is increasingly clear that CPF alters brain development through a panoply of noncholinergic mechanisms, superimposed on cholinesterase inhibition (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, In press; Rice and Barone 2000; Slotkin 1999). Accordingly, recent studies have begun to explore neurotransmitter pathways other than the cholinergic system that may be adversely affected by developmental exposure to CPF (Bloomquist et al. 2002; Dam et al. 1999a, 1999b; Karen et al. 2001; Raines et al. 2001; Sachana et al. 2001; Slotkin et al. 2002).

In an earlier study (Aldridge et al. 2003), we found that, during discrete prenatal and early postnatal periods, CPF elicits immediate alterations in the ontogenesis of serotonin (5HT) projections, characterized by adverse effects on the 5HT presynaptic transporter, 5HT receptor binding sites, and cell signaling mediated by 5HT receptors. These effects are important for three reasons. First, they were elicited at CPF exposures below the threshold for any signs of systemic toxicity and, indeed, below the levels necessary to elicit significant inhibition of cholinesterase in the fetal brain (Qiao et al. 2002). Second, 5HT serves as a neurotrophin, influencing cell differentiation and regional cytoarchitecture during brain development (Azmitia 2001; Dreyfus 1998; Lauder 1985; Levitt et al. 1997; Turlejski 1996; Weiss et al. 1998; Whitaker-Azmitia 1991, 2001); accordingly, perturbations of 5HT may be one of the contributors to noncholinergic mechanisms of CPF-induced neurobehavioral anomalies. Finally, it has been suggested that environmental toxicants that evoke long-term changes in the programming of 5HT function may contribute to appetitive and affective disorders, and consequent increases in the incidence of obesity, diabetes, and depression (Slikker and Schwetz 2003; Toschke et al. 2002; von Kries et al. 2002).

The present study was undertaken to determine if developmental CPF exposure leads to altered 5HT synaptic function in adulthood. 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 and 11–14]; these are the same treatment windows examined for short-term effects in our earlier study (Aldridge et al. 2003). We chose doses that would enable us to determine whether the threshold for effects on 5HT systems lies below that for systemic toxicity and/or inhibition of cholinesterase (Aldridge et al. 2003; Garcia et al. 2003; Qiao et al. 2002; Slotkin 1999, In press). When the rats reached adulthood (PN60), we examined factors that are critical to the functioning of 5HT synapses, all of which had been found to be affected in the immediate posttreatment period after developmental CPF exposure (Aldridge et al. 2003; Raines et al. 2001). The presynaptic 5HT transporter (5HTT) is a biomarker for the concentration of 5HT nerve terminals and is responsible for regulating the concentration of 5HT in the synapse (Cooper et al. 1996). Cell signaling is controlled through the actions of 5HT receptors; we examined two receptor subtypes, $5HT_{1A}$ and $5HT_2$. Finally, we examined the ability of 5HT receptors to control signaling through

Address correspondence to T.A. Slotkin, Box 3813 DUMC, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710 USA. Telephone: (919) 681-8015. Fax: (919) 684-8197. E-mail: t.slotkin@duke.edu

We thank I. Thillai for technical assistance.

This work was supported by National Institutes of Health grants ES10387 and ES10356 and by the Conselho Nacional de Pesquisa—CNPq/Brazil.

The authors declare they have no competing financial interests.

Received 28 August 2003; accepted 4 November 2003.

adenylyl cyclase (AC), the enzyme responsible for generation of cyclic AMP. The two subtypes evaluated here converge on the control of AC through both stimulatory and inhibitory mechanisms (Barnes and Sharp 1999; Duncan et al. 1999; Morin et al. 1992; Raymond et al. 1999; Rovescalli et al. 1993), so we evaluated the net balance of the AC response to 5HT itself. Determinations were made in brain regions with major 5HT terminal fields (cerebral cortex, hippocampus, striatum) as well as those containing 5HT cell bodies (midbrain, brainstem).

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) as adopted and promulgated by the National Institutes of Health. Timed-pregnant Sprague–Dawley rats (Zivic Laboratories, Pittsburgh, PA) 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) was dissolved in dimethylsulfoxide to provide rapid and complete absorption (Whitney et al. 1995) and was injected subcutaneously in a volume of 1 mL/kg body weight; control animals received vehicle (DMSO) injections on the same schedules. For exposure on GD9–12 or GD17–20, dams were injected daily with CPF at 1 or 5 mg/kg body weight. These doses span the threshold for inhibition of fetal brain cholinesterase activity, fetal growth impairment, and reduced maternal weight gain, all of which become evident at ≥ 5 mg/kg (Garcia et al. 2003; Qiao et al. 2002). On the day of birth, all pups were randomized within their respective treatment groups and redistributed to the dams with 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. Animals were weaned on PN21. On PN60, one male and one female were selected from each litter and were decapitated. The cerebellum, which is sparse in 5HT projections, was removed and the brain was dissected into cerebral cortex, hippocampus, striatum, midbrain, and brainstem. Tissues were frozen with liquid nitrogen and stored at –45°C.

For studies of CPF effects in the first few days after birth, animals were given a subcutaneous injection of 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. The same randomization procedure was followed. 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. Samples were obtained on PN60 as already described.

None of the prenatal or postnatal treatment regimens evoked a significant change in weight of any of the brain regions on PN60 (data not shown).

5HT receptor and transporter binding. Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) 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.

Two radioligands (Perkin-Elmer Life Sciences, Boston, MA) were used 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 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) 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). Incubations were stopped by the addition of 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 radiolabeling was determined. For binding to the presynaptic 5HT transporter (5HTT) (McGrath et al. 1997; Moret and Briley 1991; Slotkin et al. 1999b; Xu et al. 2001), the membrane suspension was incubated with 85 pM [^{3}H]paroxetine (specific activity, 19.4 Ci/mmol; Perkin-Elmer) 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 receptor binding assays, and the methods have been described in detail previously (Slotkin et al. 1990, 1992, 2001b; Xu et al. 2002). Membrane aliquots were incubated 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; the supernatant solution was assayed for cyclic AMP by radioimmunoassay (Amersham Biosciences, Piscataway, NJ). 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. This approach enables detection of potential inhibitory or excitatory actions (Chow et al. 2000; Slotkin et al. 1999a; Xu et al. 2002). The concentrations of all the agents used here have 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. Data are presented as means and SEs obtained from eight animals of each sex for each prenatal treatment group and six animals per sex for each postnatal treatment group; the only exceptions were striatum for GD17–20 exposure and brainstem for PN11–14 exposure, both of which had 12 animals per sex per treatment group. 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, a global

Table 1. Binding parameters and AC activities in controls.

Measure	Cerebral cortex	Hippocampus	Striatum	Midbrain	Brainstem
$5HT_{1A}$ binding[a]	88 ± 5[b]	358 ± 20	18 ± 1	39 ± 2	39 ± 1
$5HT_2$ binding[a]	152 ± 7	39 ± 1	133 ± 6	24 ± 1	27 ± 1
5HTT binding[a]	347 ± 12	393 ± 18	640 ± 40	491 ± 12	376 ± 7
Basal AC[c]	183 ± 11	146 ± 7	139 ± 5	253 ± 10	139 ± 7
Forskolin-stimulated AC[c]	1,258 ± 60	663 ± 27	4,307 ± 122	962 ± 56	395 ± 13

Values were combined across multiple cohorts (controls used for CPF administration on GD9–12, GD17–20, PN1–4, and PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort. Values shown are for males and females combined.
[a]fmol/mg protein. [b]Only one determination in one region showed a sex difference: $5HT_{1A}$ binding in the cerebral cortex, male 77 ± 5 fmol/mg protein, female 98 ± 6 ($p < 0.02$). [c]pmol/min/mg protein.

analysis of variance (ANOVA; data log transformed whenever variance was heterogeneous) was first conducted, incorporating all contributing variables: dose, exposure period, brain region, sex, and the three types of measurements made on each membrane preparation (repeated measures, because each membrane preparation was used for the multiple binding measurements). 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 test 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. Effects of CPF on the AC response to 5HT were evaluated for effects on basal activity with or without 5HT and on forskolin-stimulated activity with or without 5HT. 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 (Table 1), 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

CPF treatment on GD9–12. For this treatment regimen, across all three ligand binding measurements and all regions, multivariate ANOVA indicated a significant main effect of treatment ($p < 0.0001$) without any interactions of treatment × other variables. Exposure to the low dose of CPF elicited a significant overall elevation of $5HT_{1A}$, $5HT_2$, and 5HTT ligand binding without statistical distinction by region, measure, or sex (Figure 1A). Nevertheless, we verified that the treatment effect was significant in both males ($p < 0.006$) and females ($p < 0.004$), in each region ($p < 0.02$ in cerebral cortex, $p < 0.003$ in midbrain, $p < 0.005$ in brainstem), and for each of the individual measures ($p < 0.04$ for $5HT_{1A}$ receptors, $p < 0.0003$ for $5HT_2$ receptors, $p < 0.0001$ for the 5HTT site).

To evaluate the potential role of systemic toxicity in the effects on 5HT receptors and 5HTT, we also examined the effects of exposure to a higher dose (5 mg/kg) that evokes significant but transient maternal weight deficits but that still lies below the threshold for fetal weight impairment (Qiao et al. 2002); presumably, if the effects on 5HT systems in adulthood are secondary to systemic toxicity during the fetal exposure period, then the higher dose should give a far more robust effect. However, the effects were generally the same at 5 mg/kg, showing overall statistical significance from the control group but not from the 1 mg/kg group and, again, without interactions of treatment with other variables (Figure 1B).

Accordingly, CPF exposure during this early developmental period elicits lasting changes in 5HT receptors and 5HTT in a region containing major 5HT projections (cerebral cortex) as well as in regions containing 5HT cell bodies (midbrain, brainstem). In light of the positive findings with this early developmental treatment regimen, we expanded the scope of the next studies to include two more regions containing 5HT terminals fields, the hippocampus and striatum.

CPF treatment on GD17–20. CPF administered during late gestation elicited statistically robust effects assessed by global ANOVA across all ligand binding measures and brain regions, but in this case, the effect was interactive with the other variables: $p < 0.0001$ for the main treatment effect, $p < 0.004$ for treatment × sex, $p < 0.06$ for treatment × measure, $p < 0.007$ for treatment × sex × region, and $p < 0.0001$ for treatment × region × measure. Accordingly, the regions were examined separately for main treatment effects and interactions with the other variables.

The lower dose of CPF (1 mg/kg) does not evoke signs of maternal or fetal systemic toxicity and does not cause significant inhibition of fetal brain cholinesterase activity (Qiao et al. 2002). Nevertheless, we found robust effects on 5HT receptors and on 5HTT binding (Figure 2A). In fact, the effects were far larger than those seen with the GD9–12 treatment regimen (note the different ordinate scales in Figures 1 and 2), with increases as large as

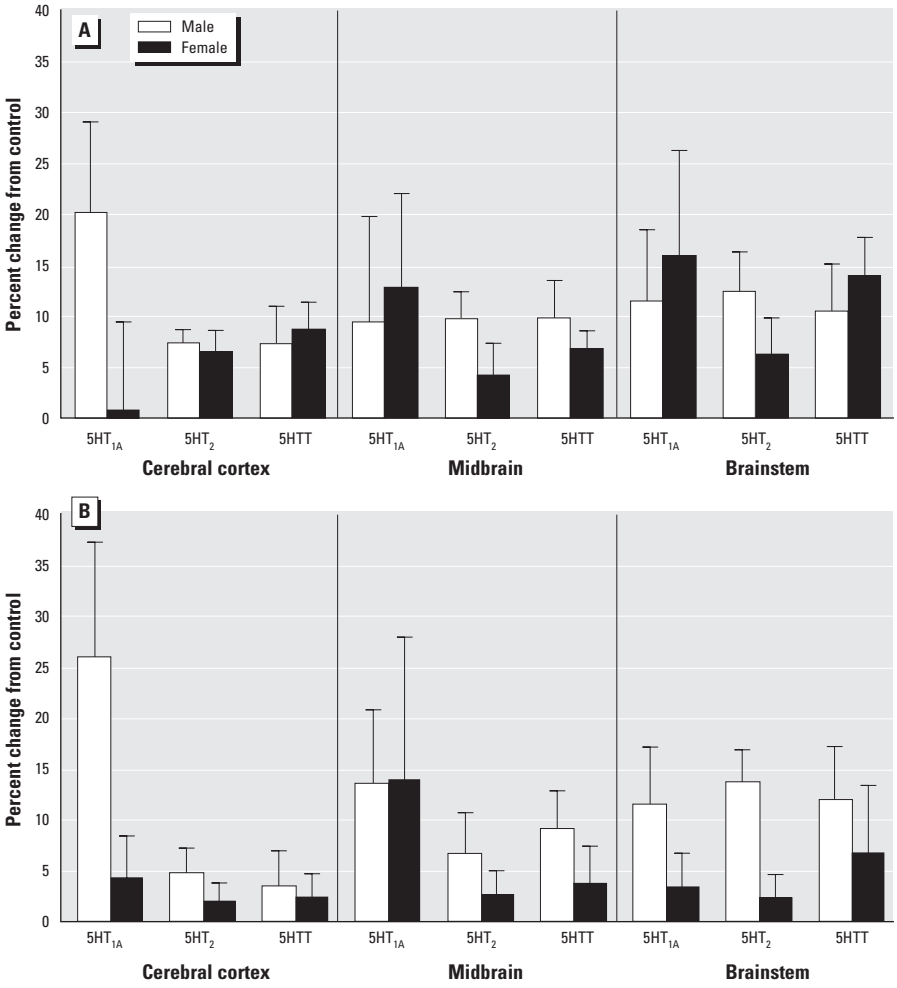

Figure 1. Effects of GD9–12 CPF exposure on 5HT receptors and 5HTT in brain regions in adulthood (PN60), presented as the percent change from control values (Table 1). (*A*) 1 mg/kg CPF (ANOVA: Rx, $p < 0.0001$). (*B*) 5 mg/kg CPF (ANOVA: Rx, $p < 0.002$). Rx, treatment. Lower-order ANOVAs for each region, measure, or sex or for individual values were not evaluated because of the absence of interactions of treatment with the other variables.

30–80% above control values in some regions. In addition, the GD17–20 regimen displayed differential effects according to the specific brain regions, individual measures, and sex. By far, the largest elevations were seen in the striatum, which also showed a strong preferential action in males. The other regions containing 5HT terminal fields, cerebral cortex and hippocampus, also displayed significant elevations but on a far more modest scale, yet still comparable with the magnitude of effect seen with the GD9–12 regimen. In the cerebral cortex, males were also affected significantly more than were females. The two regions containing 5HT cell bodies (midbrain, brainstem) showed significant increases of the same magnitude as those in the cerebral cortex and hippocampus. Although there were significant differences in the magnitude of CPF's effect for each of the ligand markers, all three showed significant elevations in multiple regions.

As before, when the CPF dose was raised above the threshold for maternal toxicity and fetal cholinesterase inhibition (5 mg/kg), the effects were still present overall but were not greater than those seen at the lower dose. In fact, most of the effects were smaller for 5 mg/kg than for 1 mg/kg, indicating that systemic toxicity, if anything, tends to reduce receptor and transporter expression rather than contributing to the increases.

CPF treatment on PN1–4. For the three binding parameters, multivariate ANOVA indicated a main effect of CPF treatment ($p < 0.0001$) that was interactive with all the other variables: $p < 0.0002$ for treatment × sex, $p < 0.0001$ for treatment × region, $p < 0.0003$ for treatment × measure, and $p < 0.0007$ for treatment × region × measure. Accordingly, the data were subdivided for lower-order analyses.

As found with the prenatal exposure regimens, postnatal CPF treatment produced an overall elevation of 5HT ligand parameters in adulthood, and as found for the GD17–20 treatment, the effects tended to be greater in males than in females (Figure 3). Modest effects were seen in the cerebral cortex and hippocampus, whereas substantially larger alterations were seen in the striatum. Notably in this case, actions in the regions containing 5HT cell bodies, the midbrain and brainstem, were among the most robust. There was one additional difference from the effects of the prenatal treatment regimens: 5HTT binding was reduced in females in all regions except the brainstem. Although only the cerebral cortex showed an individually significant difference, the magnitude of this effect was comparable in hippocampus, striatum, and midbrain, and statistical evaluations across these four regions showed a significant main effect of treatment ($p < 0.002$) without a treatment × region interaction. The brainstem was unique in showing global elevations of 20–30% for all three measures in both sexes.

CPF treatment on PN11–14. ANOVA across the three ligand binding measures indicated a significant interaction of treatment × sex ($p < 0.04$) and treatment × region × sex × measure ($p < 0.007$), necessitating examination of lower-order effects (Figure 4).

In general, the effects of this treatment regimen were smaller than the others, with statistical significance found only in two of the regions containing 5HT terminals (cerebral cortex, striatum) and in neither of the regions containing the cell bodies (midbrain, brainstem). Although only two individual measurements displayed statistical significance, there was a clear distinction of the effects on 5HTT between the regions with 5HT terminals and those with cell bodies. In females, 5HTT binding was significantly reduced across the cerebral cortex, hippocampus, and striatum ($p < 0.007$), whereas it was unaffected in the midbrain and brainstem. As was seen with the PN1–4 regimen, this effect was not shared by males.

Effects on AC signaling. In comparison with the robust effects of the different CPF regimens on 5HT receptors and 5HTT, alterations in AC signaling tended to be much less remarkable. CPF exposure on GD9–12 or on GD17–20 had no significant effect on basal or forskolin-stimulated AC activities in the absence of added 5HT (data not shown). With the PN1–4 regimen, across all brain

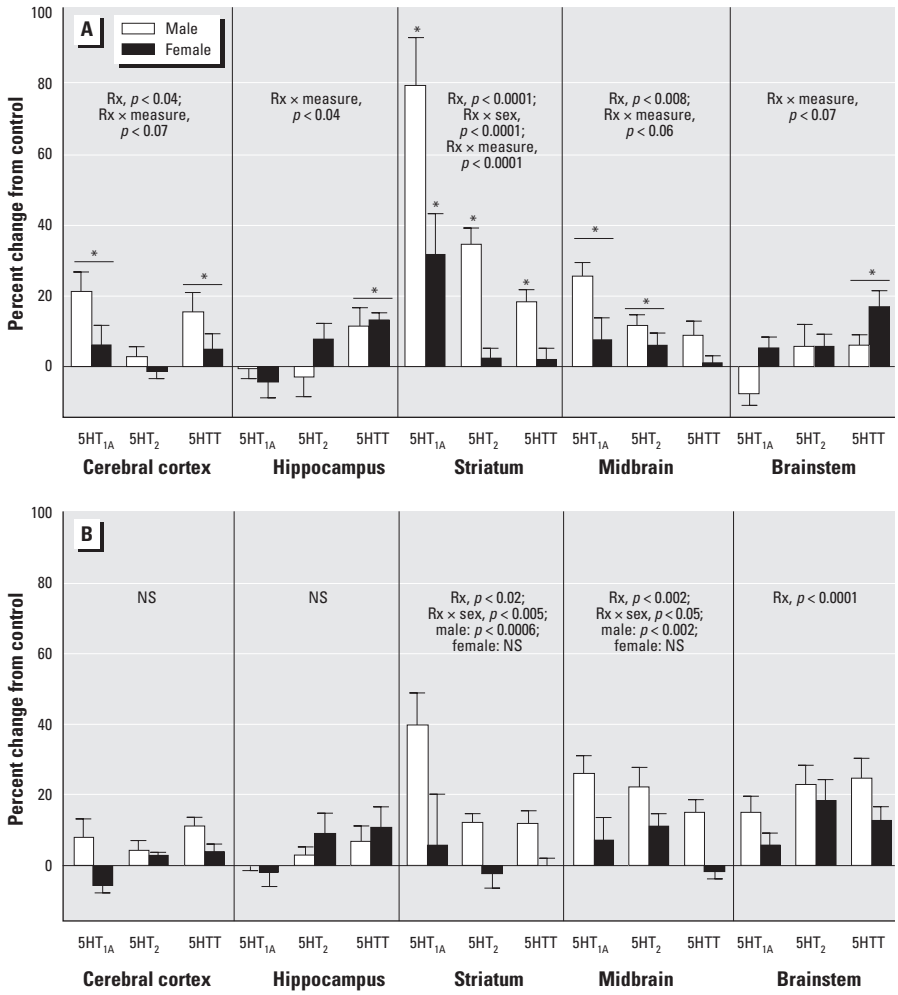

Figure 2. Effects of GD17–20 CPF exposure on 5HT receptors and 5HTT in brain regions in adulthood (PN60), presented as the percent change from control values (Table 1). (*A*) 1 mg/kg CPF. (*B*) 5 mg/kg CPF. Abbreviations: NS, not significant; Rx, treatment. Note the different ordinate scale from that in Figure 1. ANOVAs across all regions and measures and across both sexes are as follows: (*A*), Rx, $p < 0.0001$; Rx × sex, $p < 0.02$; Rx × region, $p < 0.0001$; Rx × sex × region, $p < 0.002$; Rx × measure, $p < 0.06$; Rx × region × measure, $p < 0.0001$; (*B*), Rx, $p < 0.0001$; Rx × sex, $p < 0.002$; Rx × sex × region, $p < 0.1$. ANOVAs for each region appear within the figure. Where the test for a given region did not indicate a treatment × measure interaction, only the main effect across the three measures was compiled. In the presence of a treatment × measure interaction, separate statistical evaluations were conducted for each ligand.

*Individual measures for which the CPF groups differ significantly from the corresponding control, grouped either across both sexes (in the absence of a treatment × sex interaction, indicated by an asterisk over a line connecting male and female values) or individually for males and females (where a treatment × sex interaction was detected).

regions, CPF exposure elicited a sex-related alteration in basal AC activity in the absence of added 5HT (treatment × sex, $p < 0.03$), but no significant differences values were separated by sex; there were no significant differences in forskolin-stimulated AC activities in the absence of added 5HT (data not shown). CPF exposure on PN11–14 had a significant main treatment effect on basal AC in the absence of added 5HT ($p < 0.004$), but the difference represented an average reduction of only 7% (data not shown). There was a similar effect on forskolin-stimulated AC (7% reduction, $p < 0.009$).

We evaluated the effects of 5HT on AC activity under two standard conditions: effects on basal AC and effects on forskolin-stimulated AC, so as to detect either stimulation or inhibition of activity (Chow et al. 2000; Slotkin et al. 1999a; Xu et al. 2002). For 5HT effects on basal activity, the only treatment effect was obtained in the brainstem after PN1–4 CPF exposure; there was a significant treatment × sex interaction ($p < 0.05$), but neither sex displayed a significant main effect of CPF treatment when examined separately (data not shown). For the GD9–12 regimen, we also did not see any effect of CPF treatment on the 5HT response of forskolin-stimulated AC (data not shown), but there were significant effects for all the other treatment regimens.

CPF exposure on GD17–20 elicited changes in the AC response to 5HT in the cerebral cortex and midbrain. In the cerebral cortex of control rats, 5HT exerted a net inhibitory effect on forskolin-stimulated AC activity, evidenced by a reduction in the ratio of activity with or without 5HT (Figure 5A). Low-dose (1 mg/kg) CPF exposure elicited a reduction in 5HT in males but intensified the inhibitory effect in females. Raising the dose above the threshold for systemic toxicity (5 mg/kg) did not intensify the effect and actually reduced it (data not shown). In the midbrain, the inhibitory effect of 5HT was reversed in females, and no significant differences were seen in males (Figure 5B).

CPF exposure on PN1–4 had a small but statistically significant effect on AC signaling in the brainstem (Figure 5C), and again, females were affected but males were not. In this region, the CPF group displayed a shift from a net stimulatory response to 5HT to an inhibitory response. The PN11–14 treatment regimen affected the AC response to 5HT in the striatum (Figure 5D), with a significant overall intensification of the inhibitory response.

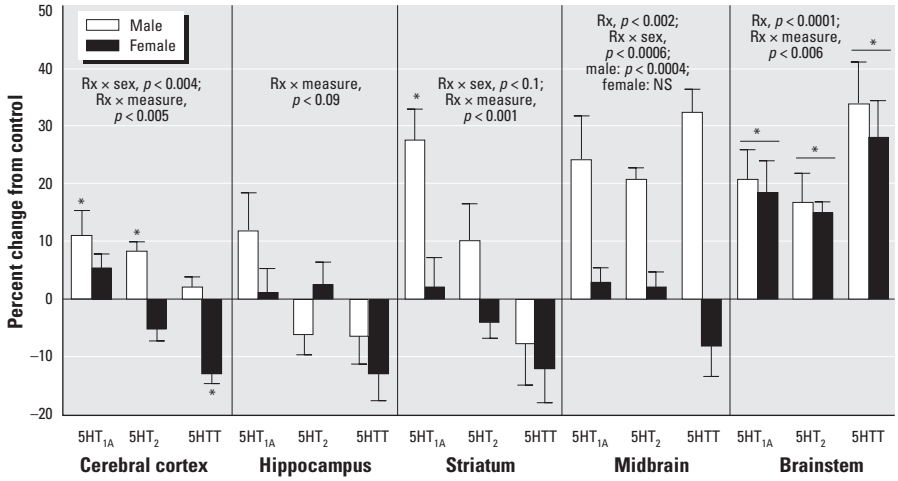

Figure 3. Effects of PN1–4 CPF exposure (1 mg/kg) on 5HT receptors and 5HTT in brain regions in adulthood (PN60), presented as the percent change from control values (Table 1). Abbreviations: NS, not significant; Rx, treatment. ANOVAs across all regions and measures and across both sexes are as follows: Rx, $p < 0.0001$; Rx × sex, $p < 0.0002$; Rx × region, $p < 0.0001$; Rx × measure, $p < 0.0003$; Rx × region × measure, $p < 0.0007$; ANOVAs for each region appear within the figure. Where the test for a given region did not indicate a treatment × measure interaction, only the main effect across the three measures was compiled. In the presence of a treatment × measure interaction, separate statistical evaluations were conducted for each ligand.
*Individual measures for which the CPF groups differ significantly from the corresponding control, grouped either across both sexes (in the absence of a treatment × sex interaction, indicated by an asterisk over a line connecting male and female values) or individually for males and females (where a treatment × sex interaction was detected).

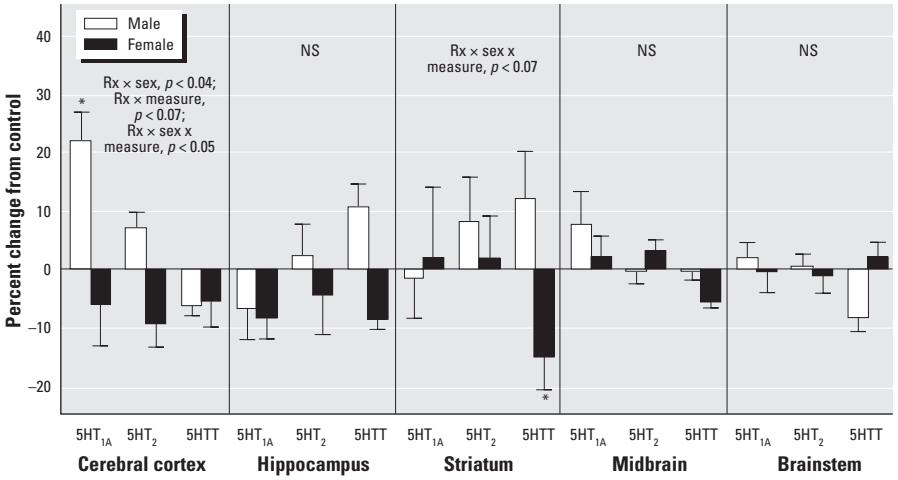

Figure 4. Effects of PN11–14 CPF (5 mg/kg) exposure on 5HT receptors and 5HTT in brain regions in adulthood (PN60), presented as the percent change from control values (Table 1). Abbreviations: NS, not significant; Rx, treatment. ANOVAs across all regions and measures and across both sexes are as follows: Rx × sex, $p < 0.04$; Rx × region × sex × measure, $p < 0.007$; ANOVAs for each region appear within the figure.
*Individual values for which the CPF group differs significantly from the control.

Discussion

Previous work demonstrated that exposure of fetal or neonatal rats to CPF elicits immediate alterations in 5HT receptors and their ability to modulate cell signaling, as well as in the expression of the high-affinity presynaptic 5HTT (Aldridge et al. 2003; Raines et al. 2001). In the present study, we found perturbations of 5HT synaptic components and 5HT-mediated responses in adult rats after CPF exposure in developmental windows ranging from the neural tube stage through the second postnatal week, with effects noted even at exposures below the threshold for any signs of maternal, fetal, or neonatal toxicity. These long-term perturbations of 5HT synaptic function display distinct regional and sex selectivities that change according to the specific period of exposure. It is therefore highly unlikely that CPF acts globally to increase or decrease the expression of the corresponding receptor, transporter, or signal transduction proteins, because in that case the same effects would be seen in every region, with every exposure window, and in both sexes. Rather, our results suggest that CPF alters the "program" for development of 5HT innervation with consequent effects on specific synaptic populations. In support of this view, although some of the features of the long-term effects of CPF on 5HT systems seen here mirror those found in the immediate posttreatment period (Aldridge et al. 2003; Raines et al. 2001),

many of them do not, and these are detailed and discussed below.

With CPF exposure during neurulation (GD9–12), we found a small (~10%) but significant promotional effect on 5HT receptors and the 5HTT site, without any selectivity for brain region or sex, and absent any corresponding alteration in 5HT-mediated AC signaling. In contrast, the initial posttreatment effects of this regimen are inhibitory for expression of the receptors and transporter in only one brain region (brainstem), followed shortly thereafter by elevations similar to those found here, accompanied by enhanced inhibition of AC by 5HT (Aldridge et al. 2003). Thus, for this exposure period, the effects on 5HT synaptic parameters in adulthood do not correspond to the immediate fetal effects.

For exposure on GD17–20, we found markedly larger, global increases in receptor and transporter expression, with emergence of sex and regional selectivities; the largest effects were seen in the regions with 5HT terminal projections, especially the striatum, and males were affected far more than were females. In this case, the effects in adulthood do match up well with those seen in the immediate posttreatment period, in terms of both the ubiquitous regional effects and the much larger magnitude of up-regulation (Aldridge et al. 2003). However, the effects on 5HT modulation of AC activity were totally distinct. First, unlike the receptor alterations, the shifts in adult signaling profiles were more prominent in females. Second, there were disjunct effects in a region with 5HT terminals compared with one containing primarily 5HT cell bodies (midbrain): 5HT-induced inhibition was enhanced in females in the cerebral cortex, whereas the response was reduced in the midbrain. Evidently, factors other than the concentration of 5HT receptors play a critical role in transduction of the receptor signal, an interpretation that is consonant with conclusions reached for other AC-linked receptors (Gao et al. 1998, 1999; Navarro et al. 1991). We are currently investigating the effects of early CPF exposure on development and function of the individual transduction proteins of the AC signaling cascade in order to clarify this issue.

With CPF exposure in the early postnatal period (PN1–4), we still found robust changes in 5HT receptors, and as with the GD17–20 treatment paradigm, males were affected much more than were females. There were some notable differences between the two regimens, however. With PN1–4 exposure, the regions with 5HT cell bodies (midbrain, brainstem) were affected far more than with the earlier treatment. In addition, deficits in the 5HTT site now emerged in females. Effects on 5HT modulation of AC were quite minor but still showed preferential effects in females. When the exposure was shifted to an even later period (PN11–14), the effects on receptor expression were far less notable, but there was still a sex-selective (female) reduction in the 5HTT site in brain regions containing the nerve terminals; AC signaling showed an enhanced inhibitory effect of 5HT in both sexes. Again, the effects of postnatal CPF exposures assessed in adulthood match some but not all aspects of their immediate effects on 5HT systems (Aldridge et al. 2003; Raines et al. 2001).

In general, then, our results indicate three distinct response families of long-term alterations in 5HT systems elicited by developmental CPF exposure. First, there is an enhancement of 5HT receptor expression, with peak effects elicited in the late gestational to early postnatal period, and preferential effects in males. Second, there are biphasic alterations in the 5HTT site: promotional effects elicited by gestational exposure but inhibition in regions containing 5HT terminal zones when exposure is shifted to the postnatal period. This pattern makes sense in light of the adverse effects of CPF on axonogenesis and synaptogenesis (Barone et al. 2000; Das and Barone 1999; Li and Casida 1998; Song et al. 1998), events that are most active postnatally in the rat (Rodier 1988). Third, there are smaller but significant effects on 5HT modulation of cell signaling that are entirely distinct from those on the 5HT receptors, in terms of both sex dependence and regional selectivity, but sharing a similar peak of sensitivity in the late gestational phase. Accordingly, the developing brain is sensitive to CPF-induced disruption of 5HT synaptic function at virtually all stages, with the pattern of effects shifting from global actions to more focal, sex-selective effects as maturation proceeds. Overall, however, the greatest long-term alterations appear to be concentrated in the late gestational to early postnatal period, a developmental stage in the rat that parallels the second trimester of human fetal brain development (Rodier 1988).

One question that is still unanswered is whether CPF specifically targets 5HT systems or whether the effects represent inclusion of 5HT in the spectrum of neurochemical alterations secondary to its adverse effects on neural cell replication, differentiation, and synaptic outgrowth (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1999, In press). CPF interacts directly with 5HT transport (Sachana et al. 2001) and elicits profound, immediate effects on fetal and neonatal 5HT systems during exposure (Aldridge et al. 2003). At the same time, CPF affects development of

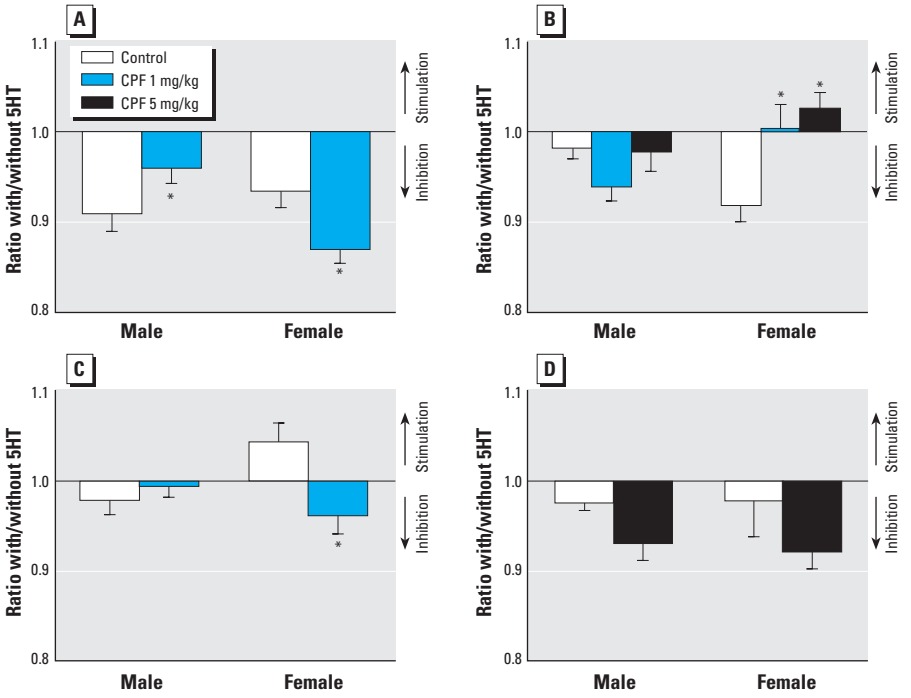

Figure 5. Alterations in the AC response to 5HT in adulthood (PN60) after CPF exposure during different developmental periods; normative AC activities in the absence of 5HT are presented in Table 1. Rx, treatment. Only the treatments and regions showing a significant difference are presented: GD17–20 exposure, cerebral cortex (*A*) and midbrain (*B*); PN1–4 exposure, brainstem (*C*); PN11–14, striatum (*D*). The response to 5HT was determined as the ratio of activity with 5HT to activity without 5HT; thus, a ratio > 1 denotes stimulation, whereas a ratio < 1 denotes inhibition. ANOVAs across treatment and sex are as follows: (*A*), Rx × sex, $p < 0.0004$; (*B*), Rx × sex, $p < 0.003$; (*C*), Rx × sex, $p < 0.03$; (*D*), Rx, $p < 0.04$. Where a significant treatment × sex interaction was detected, separate tests were carried out for each sex; in the absence of an interaction, only the main effect of treatment was compiled.

*Individual values for which the CPF group differs significantly from the control.

other neurotransmitter systems that converge on the same signaling pathways as 5HT (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 eliciting heterologous effects on the G-proteins that couple receptors to AC, or on AC itself (Auman et al. 2000; Garcia et al. 2001; Olivier et al. 2001; Song et al. 1997). It may thus be difficult to isolate a specific mechanism when it is most likely that there are multiple mechanisms superimposed on each other that mediate the net effect of CPF on any given neurotransmitter pathway. Nevertheless, the effects on 5HT systems by CPF represent an important end point, in light of the role of this neurotransmitter in appetitive and affective disorders. Accordingly, it will be vital to expand the spectrum of outcomes evaluated for CPF to include 5HT-related behaviors.

In our earlier studies (Aldridge et al. 2003; Dam et al. 2000; Garcia et al. 2002; Icenogle et al. In press; Levin et al. 2001, 2002; Meyer et al. 2003; Qiao et al. In press; Slotkin et al. 2001a, 2002), we found that sex differences in the effects of CPF emerged only when exposure occurred in late gestation or in the neonatal period, and not with exposure during neurulation (GD9–12). The present findings for 5HT systems showed the same critical period for sex selectivity. CPF lacks sufficient estrogenic activity to account directly for these effects (Andersen et al. 2002; Vinggaard et al. 2000), but it does interfere with testosterone catabolism (Usmani et al. 2003); furthermore, with exposures above the threshold for systemic toxicity, CPF evokes secondary endocrine alterations, so a marginal effect at the lower exposures here might contribute to sex differences in outcome (Guven et al. 1999). Nevertheless, because the critical period found here corresponds to the commencement of sexual differentiation of the brain (McCarthy 1994; Mong and McCarthy 1999) and specifically involves the cyclic AMP pathway (Auger 2003), CPF effects on brain development are themselves likely to influence sexual differentiation and resultant sex-related outcomes.

The present findings indicate that fetal or neonatal exposure to CPF alters the program for development of 5HT synaptic function, thus affecting 5HT systems in adulthood and extending the effects of CPF beyond cholinergic neurotransmission. Serotonergic dysfunction is involved in appetitive and affective disorders, so our results are consonant with suggestions that at least some of the incidence of these disturbances may have contributions from environmental neurotoxicant exposures (Slikker and Schwetz 2003; Toschke et al. 2002; von Kries et al. 2002). On the surface, it seems unlikely that these effects, by themselves, would trigger such multifactorial diseases, but certainly they might confer additional risk that acts in concert with other factors. Studies of CPF effects on 5HT-related behaviors, especially those operating in animal models of depression or obesity, will be needed to clarify the outcomes of the long-term alterations in 5HT synaptic function identified here.

References

Aldridge JE, Seidler FJ, Meyer A, Thillai I, Slotkin TA. 2003. Serotonergic systems targeted by developmental exposure to chlorpyrifos: effects during different critical periods. Environ Health Perspect 111:1736–1743.

Andersen HR, Vinggaard AM, Rasmussen TH, Gjermandsen IM, Bonefeld-Jorgensen EC. 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*. Toxicol Appl Pharmacol 179:1–12.

Auger AP. 2003. Sex differences in the developing brain: crossroads in the phosphorylation of cAMP response element binding protein. J Neuroendocrinol 15:622–627.

Auman JT, Seidler FJ, Slotkin TA. 2000. Neonatal chlorpyrifos exposure targets multiple proteins governing the hepatic adenylyl cyclase signaling cascade: implications for neurotoxicity. Dev Brain Res 121:19–27.

———. 2001. Regulation of fetal cardiac and hepatic β-adrenoceptors and adenylyl cyclase signaling: terbutaline effects. Am J Physiol 281:R1079–R1089.

Azmitia EC. 2001. Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation, and apoptosis. Brain Res Bull 56:413–424.

Barnes NM, Sharp T. 1999. A review of central 5-HT receptors and their function. Neuropharmacology 38:1083–1152.

Barone S, Das KP, Lassiter TL, White LD. 2000. Vulnerable processes of nervous system development: a review of markers and methods. Neurotoxicology 21:15–36.

Bloomquist JR, Barlow RL, Gillette JS, Li W, Kirby ML. 2002. Selective effects of insecticides on nigrostriatal dopaminergic nerve pathways. Neurotoxicology 23:537–544.

Campbell CG, Seidler FJ, Slotkin TA. 1997. Chlorpyrifos interferes with cell development in rat brain regions. Brain Res Bull 43:179–189.

Chow FA, Seidler FJ, McCook EC, Slotkin TA. 2000. Adolescent nicotine exposure alters cardiac autonomic responsiveness: β-adrenergic and m2-muscarinic receptors and their linkage to adenylyl cyclase. Brain Res 878:119–126.

Cooper JR, Bloom FE, Roth RH. 1996. The Biochemical Basis of Neuropharmacology. 7th ed. New York:Oxford University Press.

Dam K, Garcia SJ, Seidler FJ, Slotkin TA. 1999a. Neonatal chlorpyrifos exposure alters synaptic development and neuronal activity in cholinergic and catecholaminergic pathways. Dev Brain Res 116:9–20.

Dam K, Seidler FJ, Slotkin TA. 1998. Developmental neurotoxicity of chlorpyrifos: delayed targeting of DNA synthesis after repeated administration. Dev Brain Res 108:39–45.

———. 1999b. Chlorpyrifos releases norepinephrine from adult and neonatal rat brain synaptosomes. Dev Brain Res 118:120–133.

———. 2000. Chlorpyrifos exposure during a critical neonatal period elicits gender-selective deficits in the development of coordination skills and locomotor activity. Dev Brain Res 121:179–187.

Das KP, Barone S. 1999. Neuronal differentiation in PC12 cells is inhibited by chlorpyrifos and its metabolites: is acetylcholinesterase inhibition the site of action? Toxicol Appl Pharmacol 160:217–230.

Dreyfus CF. 1998. Neurotransmitters and neurotrophins collaborate to influence brain development. Perspect Dev Neurobiol 5:389–399.

Duncan MJ, Short J, Wheeler DL. 1999. Comparison of the effects of aging on 5-HT_7 and 5-HT_{1A} receptors in discrete regions of the circadian timing system in hamsters. Brain Res 829:39–45.

Gao MH, Lai NC, Roth DM, Zhou JY, Zhu J, Anzai T, et al. 1999. Adenylyl cyclase increases responsiveness to catecholamine stimulation in transgenic mice. Circulation 99:1618–1622.

Gao MH, Ping PP, Post S, Insel PA, Tang RY, Hammond HK. 1998. Increased expression of adenylylcyclase type VI proportionately increases β-adrenergic receptor-stimulated production of cAMP in neonatal rat cardiac myocytes. Proc Natl Acad Sci USA 95:1038–1043.

Garcia SJ, Seidler FJ, Crumpton TL, Slotkin TA. 2001. Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecule synthesis, adenylyl cyclase signaling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells. Brain Res 891:54–68.

Garcia SJ, Seidler FJ, Qiao D, Slotkin TA. 2002. Chlorpyrifos targets developing glia: effects on glial fibrillary acidic protein. Dev Brain Res 133:151–161.

Garcia SJ, Seidler FJ, Slotkin TA. 2003. Developmental neurotoxicity elicited by prenatal or postnatal chlorpyrifos exposure: effects on neurospecific proteins indicate changing vulnerabilities. Environ Health Perspect 111:297–303.

Guven M, Bayram F, Unluhizarci K, Kelestimur F. 1999. Endocrine changes in patients with acute organophosphate poisoning. Human Exp Toxicol 18:598–601.

Huff RA, Abou-Donia MB. 1995. In vitro effect of chlorpyrifos oxon on muscarinic receptors and adenylate cyclase. Neurotoxicology 16:281–290.

Huff RA, Abu-Qare AW, Abou-Donia MB. 2001. Effects of subchronic in vivo chlorpyrifos exposure on muscarinic receptors and adenylate cyclase of rat striatum. Arch Toxicol 75:480–486.

Huff RA, Corcoran JJ, Anderson JK, Abou-Donia MB. 1994. Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. J Pharmacol Exp Ther 269:329–335.

Icenogle LM, Christopher C, Blackwelder WP, Caldwell DP, Qiao D, Seidler FJ, et al. In press. Behavioral alterations in adolescent and adult rats caused by a brief subtoxic exposure to chlorpyrifos during neurulation. Neurotoxicol Teratol.

Institute of Laboratory Animal Resources. 1996. Guide for the Care and Use of Laboratory Animals. 7th ed. Washington, DC:National Academy Press.

Jamal GA, Hansen S, Julu PO. 2002. Low level exposures to organophosphorus esters may cause neurotoxicity. Toxicology 181–182:23–33.

Johnson DE, Seidler FJ, Slotkin TA. 1998. Early biochemical detection of delayed neurotoxicity resulting from developmental exposure to chlorpyrifos. Brain Res Bull 45:143–147.

Karen DJ, Li W, Harp PR, Gillette JS, Bloomquist JR. 2001. Striatal dopaminergic pathways as a target for the insecticides permethrin and chlorpyrifos. Neurotoxicology 22:811–817.

Landrigan PJ. 2001. Pesticides and polychlorinated biphenyls (PCBs): an analysis of the evidence that they impair children's neurobehavioral development. Mol Genet Metab 73:11–17.

Landrigan PJ, Claudio L, Markowitz SB, Berkowitz GS, Brenner BL, Romero H, et al. 1999. Pesticides and inner-city children: exposures, risks, and prevention. Environ Health Perspect 107(suppl 3):431–437.

Lassiter T, White L, Padilla S, Barone S. 2002. Gestational exposure to chlorpyrifos: qualitative and quantitative neuropathological changes in the fetal neocortex [Abstract]. Toxicologist 66:632.

Lassiter TL, Padilla S, Mortensen SR, Chanda SM, Moser VC, Barone S. 1998. Gestational exposure to chlorpyrifos: apparent protection of the fetus? Toxicol Appl Pharmacol 152:56–65.

Lauder JM. 1985. Roles for neurotransmitters in development: possible interaction with drugs during the fetal and neonatal periods. In: Prevention of Physical and Mental Congenital Defects (Marois M, ed). New York:Alan R. Liss, 375–380.

Levin ED, Addy N, Baruah A, Elias A, Christopher NC, Seidler FJ, et al. 2002. Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations. Neurotoxicol Teratol 24:733–741.

Levin ED, Addy N, Christopher NC, Seidler FJ, Slotkin TA. 2001. Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. Dev Brain Res 130:83–89.

Levitt P, Harvey JA, Friedman E, Simansky K, Murphy EH. 1997. New evidence for neurotransmitter influences on brain development. Trends Neurosci 20:269–274.

Leysen JE, Niemegeers CJ, Van Nueten JM, Laduron PM. 1982. [^{3}H]Ketanserin (R41468), a selective ^{3}H-ligand for serotonin$_2$ receptor binding sites: binding properties, brain distribution, and functional role. Mol Pharmacol 21:301–314.

Li WW, Casida JE. 1998. Organophosphorus neuropathy target esterase inhibitors selectively block outgrowth of neurite-like and cell processes in cultured cells. Toxicol Lett 98:139–146.

May M. 2000. Disturbing behavior: neurotoxic effects in children. Environ Health Perspect 108:A262–A267.

McCarthy MM. 1994. Molecular aspects of sexual differentiation of the rodent brain. Psychoneuroendocrinology 19:415–427.

McGrath KE, Seidler FJ, Slotkin TA. 1997. Convergent control of serotonin transporter expression by glucocorticoids and cocaine in fetal and neonatal rat brain. Dev Brain Res 104:209–213.

Meyer A, Seidler FJ, Cousins MM, Slotkin TA. 2003. Developmental neurotoxicity elicited by gestational exposure to chlorpyrifos: when is adenylyl cyclase a target? Environ Health Perspect 111:1871–1876.

Mileson BE, Chambers JE, Chen WL, Dettbarn W, Ehrich M, Eldefrawi AT, et al. 1998. Common mechanism of toxicity: a case study of organophosphorus pesticides. Toxicol Sci 41:8–20.

Mong JA, McCarthy MM. 1999. Steroid-induced developmental plasticity in hypothalamic astrocytes: implications for synaptic patterning. J Neurobiol 40:602–619.

Monnet-Tschudi F, Zurich MG, Schilter B, Costa LG, Honegger P. 2000. Maturation-dependent effects of chlorpyrifos and parathion and their oxygen analogs on acetylcholinesterase and neuronal and glial markers in aggregating brain cell cultures. Toxicol Appl Pharmacol 165:175–183.

Moret C, Briley M. 1991. Platelet ^{3}H-paroxetine binding to the serotonin transporter is insensitive to changes in central serotonergic innervation in the rat. Psychiat Res 38:97–104.

Morin D, Sapena R, Zini R, Tillement JP. 1992. Serotonin enhances the β-adrenergic response in rat brain cortical slices. Eur J Pharmacol 225:273–274.

Moser VC, Padilla S. 1998. Age- and gender-related differences in the time course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicol Appl Pharmacol 149:107–119.

National Research Council. 1993. Pesticides in the Diets of Infants and Children. Washington, DC:National Research Council.

Navarro HA, Kudlacz EM, Slotkin TA. 1991. Control of adenylate cyclase activity in developing rat heart and liver: effects of prenatal exposure to terbutaline or dexamethasone. Biol Neonate 60:127–136.

Olivier K, Liu J, Pope C. 2001. Inhibition of forskolin-stimulated cAMP formation *in vitro* by paraoxon and chlorpyrifos oxon in cortical slices from neonatal, juvenile, and adult rats. J Biochem Mol Toxicol 15:263–269.

Park S, Harrold JA, Widdowson PS, Williams G. 1999. Increased binding at 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} receptors and 5-HT transporters in diet-induced obese rats. Brain Res 847:90–97.

Physicians for Social Responsibility. 1995. Pesticides and Children. Washington, DC:Physicians for Social Responsibility.

Pope CN. 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity? J Toxicol Environ Health 2:161–181.

Pope CN, Chakraborti TK. 1992. Dose-related inhibition of brain and plasma cholinesterase in neonatal and adult rats following sublethal organophosphate exposures. Toxicology 73:35–43.

Pope CN, Chakraborti TK, Chapman ML, Farrar JD, Arthun D. 1991. Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. Toxicology 68:51–61.

Qiao D, Seidler FJ, Abreu-Villaça Y, Tate CA, Cousins MM, Slotkin TA. In press. Chlorpyrifos exposure during neurulation: cholinergic synaptic dysfunction and cellular alterations in brain regions at adolescence and adulthood. Dev Brain Res.

Qiao D, Seidler FJ, Padilla S, Slotkin TA. 2002. Developmental neurotoxicity of chlorpyrifos: what is the vulnerable period? Environ Health Perspect 110:1097–1103.

Qiao D, Seidler FJ, Slotkin TA. 2001. Developmental neurotoxicity of chlorpyrifos modeled *in vitro*: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells. Environ Health Perspect 109:909–913.

Raines KW, Seidler FJ, Slotkin TA. 2001. Alterations in serotonin transporter expression in brain regions of rats exposed neonatally to chlorpyrifos. Dev Brain Res 130:65–72.

Ray DE, Richards PG. 2001. The potential for toxic effects of chronic, low-dose exposure to organophosphates. Toxicol Lett 120:343–351.

Raymond JR, Mukhin YV, Gettys TW, Garnovskaya MN. 1999. The recombinant 5-HT_{1A} receptor: G protein coupling and signalling pathways. Br J Pharmacol 127:1751–1764.

Rice D, Barone S. 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108(suppl 3):S511–S533.

Rodier PM. 1988. Structural-functional relationships in experimentally induced brain damage. Prog Brain Res 73:335–348.

Rovescalli AC, Brunello N, Perez J, Vitali S, Steardo L, Racagni G. 1993. Heterologous sensitization of adenylate cyclase activity by serotonin in the rat cerebral cortex. Eur Neuropsychopharmacol 3:463–475.

Sachana M, Flaskos J, Nikolaidis E, Hargreaves A, Alexaki-Tzivanidou E. 2001. Inhibition of rat platelet 5-hydroxytryptamine uptake by chlorpyrifos and carbaryl. Pharmacol Toxicol 89:195–200.

Seamon KB, Daly JW. 1986. Forskolin: its biological and chemical properties. Adv Cyclic Nucleotide Protein Phosphorylation Res 20:1–150.

Slikker W, Schwetz BA. 2003. Childhood obesity: the possible role of maternal smoking and impact on public health. J Child Health 1:29–40.

Slotkin TA. 1999. Developmental cholinotoxicants: nicotine and chlorpyrifos. Environ Health Perspect 107(suppl 1):71–80.

———. In press. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol.

Slotkin TA, Cousins MM, Tate CA, Seidler FJ. 2001a. Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. Brain Res 902:229–243.

Slotkin TA, Epps TA, Stenger ML, Sawyer KJ, Seidler FJ. 1999a. Cholinergic receptors in heart and brainstem of rats exposed to nicotine during development: implications for hypoxia tolerance and perinatal mortality. Dev Brain Res 113:1–12.

Slotkin TA, McCook EC, Lappi SE, Seidler FJ. 1992. Altered development of basal and forskolin-stimulated adenylate cyclase activity in brain regions of rats exposed to nicotine prenatally. Dev Brain Res 68:233–239.

Slotkin TA, Miller DB, Fumagalli F, McCook EC, Zhang J, Bissette G, et al. 1999b. Modeling geriatric depression in animals: biochemical and behavioral effects of olfactory bulbectomy in young *versus* aged rats. J Pharmacol Exp Ther 289:334–345.

Slotkin TA, Navarro HA, McCook EC, Seidler FJ. 1990. Fetal nicotine exposure produces postnatal up-regulation of adenylate cyclase activity in peripheral tissues. Life Sci 47:1561–1567.

Slotkin TA, Tate CA, Cousins MM, Seidler FJ. 2001b. β-Adrenoceptor signaling in the developing brain: sensitization or desensitization in response to terbutaline. Dev Brain Res 131:113–125.

———. 2002. Functional alterations in CNS catecholamine systems in adolescence and adulthood after neonatal chlorpyrifos exposure. Dev Brain Res 133:163–173.

Snedecor GW, Cochran WG. 1967. Statistical Methods. Ames, IA:Iowa State University Press.

Song X, Seidler FJ, Saleh JL, Zhang J, Padilla S, Slotkin TA. 1997. Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenylyl cyclase signaling cascade. Toxicol Appl Pharmacol 145:158–174.

Song X, Violin JD, Seidler FJ, Slotkin TA. 1998. Modeling the developmental neurotoxicity of chlorpyrifos *in vitro*: macromolecule synthesis in PC12 cells. Toxicol Appl Pharmacol 151:182–191.

Stockmeier CA, Shapiro LA, Dilley GE, Kolli TN, Friedman L, Rajkowska G. 1998. Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression: postmortem evidence for decreased serotonin activity. J Neurosci 18:7394–7401.

Toschke AM, Koletzko B, Slikker W, Hermann M, von Kries R. 2002. Childhood obesity is associated with maternal smoking in pregnancy. Eur J Pediatr 161:445–448.

Turlejski K. 1996. Evolutionary ancient roles of serotonin: long-lasting regulation of activity and development. Acta Neurobiol Exp 56:619–636.

U.S. EPA. 2003. Administrator's Announcement. Washington, DC:U.S. Environmental Protection Agency. Available: http://www.epa.gov/pesticides/announcement6800.htm [accessed 5 June 2003].

Usmani KA, Rose RL, Hodgson E. 2003. Inhibition and activation of the human liver microsomal and human cytochrome P450 3A4 metabolism of testosterone by deployment-related chemicals. Drug Metab Dispos 31:384–391.

Vinggaard AM, Hnida C, Breinholt V, Larsen JC. 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol In Vitro 14:227–234.

von Kries R, Toschke AM, Koletzko B, Slikker W Jr. 2002. Maternal smoking during pregnancy and childhood obesity. Am J Epidemiol 156:954–961.

Ward TR, Mundy WR. 1996. Organophosphorus compounds preferentially affect second messenger systems coupled to M2/M4 receptors in rat frontal cortex. Brain Res Bull 39:49–55.

Weiss ER, Maness P, Lauder JM. 1998. Why do neurotransmitters act like growth factors? Perspect Dev Neurobiol 5:323–335.

Whitaker-Azmitia PM. 1991. Role of serotonin and other neurotransmitter receptors in brain development: basis for developmental pharmacology. Pharmacol Rev 43:553–561.

———. 2001. Serotonin and brain development: role in human developmental diseases. Brain Res Bull 56:479–485.

Whitney KD, Seidler FJ, Slotkin TA. 1995. Developmental neurotoxicity of chlorpyrifos: cellular mechanisms. Toxicol Appl Pharmacol 134:53–62.

Xu Z, Seidler FJ, Ali SF, Slikker W, Slotkin TA. 2001. Fetal and adolescent nicotine administration: effects on CNS serotonergic systems. Brain Res 914:166–178.

Xu Z, Seidler FJ, Cousins MM, Slikker W, Slotkin TA. 2002. Adolescent nicotine administration alters serotonin receptors and cell signaling mediated through adenylyl cyclase. Brain Res 951:280–292.

Zeiders JL, Seidler FJ, Iaccarino G, Koch WJ, Slotkin TA. 1999. Ontogeny of cardiac β-adrenoceptor desensitization mechanisms: agonist treatment enhances receptor/G-protein transduction rather than eliciting uncoupling. J Mol Cell Cardiol 31:413–423.

Zeiders JL, Seidler FJ, Slotkin TA. 1997. Ontogeny of regulatory mechanisms for β-adrenoceptor control of rat cardiac adenylyl cyclase: targeting of G-proteins and the cyclase catalytic subunit. J Mol Cell Cardiol 29:603–615.

Zhang HS, Liu J, Pope CN. 2002. Age-related effects of chlorpyrifos on muscarinic receptor-mediated signaling in rat cortex. Arch Toxicol 75:676–684.