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

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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 5HT1A and 5HT2 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 adenyl 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: adenyl 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 their 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 (Azmita 2001; Dreyfus 1998; Lauder 1985; Levitt et al. 1997; Turlejski 1996; Weiss et al. 1998; Whitaker-Azmitia et al. 1991, 2001); accordingly, perturbations of 5HT may be one of the contributors to non-cholinergic 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, 5HT1A and 5HT2. Finally, we examined the ability of 5HT receptors to control signaling through...
adenyl 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 itself. Determinations were made in brain regions with major 5HT terminal fields (cerebral cortex, hippocampus, striatum, midbrain, brainstem). Tissues were frozen with liquid nitrogen and stored at –45°C.  

**Material regimens evoked a significant change in weight of any of the brain regions 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 [3H]8-hydroxy-2-(di-n-propylamino)tetrailim (specific activity, 135 Ci/mmol) for 5HT1A receptors (Park et al. 2000; Stockmeier et al. 1998), and 0.4 nM [3H]ketanserin (specific activity, 63 Ci/mmol) for 5HT2 receptors (Leysen et al. 1982; Park et al. 1999). For 5HT1A receptors, incubations lasted 30 min at 25°C in a buffer consisting of 50 mM Tris (pH 8), 2 mM MgCl2, and 2 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.

**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 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.

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**Table 1. Binding parameters and AC activities in controls.**

| Measure                  | Cerebral cortex | Hippocampus | Striatum | Midbrain | Brainstem |
|--------------------------|-----------------|-------------|----------|----------|-----------|
| 5HT1A binding*          | 88 ± 5          | 358 ± 20    | 18 ± 1   | 39 ± 2   | 39 ± 1    |
| 5HT1 binding            | 152 ± 7         | 39 ± 1      | 133 ± 6  | 24 ± 1   | 27 ± 1    |
| 5HT2 binding            | 347 ± 12        | 393 ± 18    | 640 ± 40 | 491 ± 12 | 376 ± 7   |
| Basal AC                | 183 ± 11        | 146 ± 7     | 139 ± 5  | 253 ± 10 | 139 ± 7   |
| Forskolin-stimulated AC | 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.  

*pmol/mg protein. **Only one determination in one region showed a sex difference: 5HT1A binding in the cerebral cortex, male 77 ± 5 fmol/mg protein, female 98 ± 6 (p < 0.02). **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
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, SHTT 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...
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 were observed in any effect of CPF treatment on the 5HT system in any region. These long-term perturbations of 5HT synaptic components and 5HT-mediated responses in adult rats after CPF exposure in developmental windows 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),

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; Soklin 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.

**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 postnatal 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 < 0.0003; (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-D 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. 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; Incenoge 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 neurogenesis (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 catalysis (Usmani et al. 2003); furthermore, with exposures above the threshold for catabolism (Usmani et al. 2003); further—more, with exposures above the threshold for cell proliferation, maturation, and apoptosis. Brain Res Bull 56:413–424. Barnes NM, Sharp T. 1999. A review of central S-HT receptors and their function. 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