Despite recent restrictions on production for home use, chlorpyrifos (CPF) remains one of the most widely used pesticides, and there is concern over the potential consequences of fetal and childhood exposure (reviewed in Landrigan et al. 1999; Pope 1999; Slotkin 1999). The systemic toxicity of CPF primarily reflects cholinergic hyperstimulation as a result of the inhibition of cholinesterase activity (Milesen et al. 1998), and immature organisms are more susceptible to CPF-induced toxicity than are adults (Moser and Padilla 1998; Pope and Chakrabarti 1992; Pope et al. 1991; Whitney et al. 1995). Nevertheless, it is increasingly clear that the developmental neurotoxicity of CPF involves mechanisms other than the inhibition of cholinesterase, with specific targeting of neural cell replication and differentiation, axonogenesis, and synaptogenesis (Barone et al. 2000; Pope 1999; Slotkin 1999). Several recent studies suggest that CPF affects relatively late events in brain development, centered around the proliferation, differentiation, and functioning of glial cells (Garcia et al. 2001, 2002; Qiao et al. 2001), the cells that provide metabolic support for neurons and that guide axons to their proper targets within the developing central nervous system. In turn, these findings raise the issue of identifying the critical window for adverse effects of CPF on neurodevelopment. If late-occurring processes are involved, then vulnerability will extend into childhood, a period in which exposures may be particularly high (Fenske et al. 1990; Gurunathan et al. 1998; Landrigan 2001; Landrigan et al. 1999).

In our earlier work with postnatal CPF exposure in rats, we found clear-cut evidence of cell damage and loss in the immature brain, as well as interference with the development of specific neural pathways, including prominent effects on cholinergic innervation (Dam et al. 1999; Slotkin 1999; Slotkin et al. 2001). Importantly, these effects were all seen at doses that did not elicit signs of systemic toxicity (e.g., mortality, body or tissue weight loss, maternal cannibalism, interference with suckling). In the present study, we used the same approach to examine the effects of prenatal CPF exposure during two treatment windows: gestational days (GD) 9–12, corresponding to organogenesis and neural tube formation, and GD17–20, a peak period of neurogenesis in the brainstem and forebrain, the regions exhibiting highest expression of the cholinergic phenotype. In both cases, we performed dose–response studies to determine effects on cell development and cholinergic synaptogenesis at doses below the threshold for maternal or fetal toxicity, and effects on non-neural tissues such as heart and liver. Using the established relationship between DNA levels and cell number (Winick and Noble 1965), we focused on indices of cell packing density (DNA per gram of tissue) and cell number (DNA content), and cell size (protein/DNA ratio), biomarkers that have been shown to be affected by postnatal CPF exposure (Campbell et al. 1997; Dam et al. 1998; Song et al. 1998; Whitney et al. 1995). For cholinergic synaptogenesis, we assessed choline acetyltransferase (ChAT), a constitutive marker of cholinergic nerve terminals (Slotkin et al. 2001); we also measured radioligand binding to the m2-muscarinic acetylcholine receptor (m2AChR), a mediator of cholinergic signaling that typically undergoes down-regulation in the presence of cholinergic hyperstimulation (Bushnell et al. 1993; Chakrabarti et al. 1993; Ward and Mundy 1996) and that may also be a direct target for CPF actions (Bomser and Casida 2001; Huff et al. 1994). Finally, we compared effects on biomarkers of brain development with the dose–response curve for inhibition of cholinesterase in the fetal brain.

Methods

Animal treatments. All experiments were carried out in accordance with the declaration of...
Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Timed-pregnant Sprague-Dawley rats were housed in breeding cages with a 12-hr light–dark cycle and free access to food and water. CPF was dissolved in dimethyl sulfoxide 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 injections on the same schedule. One group of pregnant rats received 0, 1, 2, or 5 mg/kg daily from GD9 to GD12, and determinations were conducted on GD17 and GD21. A second group received 0, 1, 2, 5, 10, 20, or 40 mg/kg daily on GD17–20, and determinations were conducted on GD21, 24 hr after the last dose of CPF. Heart, liver, and brain were dissected from each fetus, and on GD21, the brain was separated into forebrain and brainstem. Tissues were frozen immediately in liquid nitrogen and stored at −85°C.

**Assays.** All assay procedures used in this study have been detailed in previous publications; accordingly, only brief descriptions are provided here. For quantification of macromolecules, DNA was determined using a modified (Trauth et al. 2000) fluorescent dye-binding method (Labarca and Pijan 1980) and protein with Folin reagent (Lowry et al. 1951). Cholinesterase activity was assayed using an automated spectrophotometric technique with acetylthiocholine as the substrate (Padilla et al. 1998). ChAT activity was measured by the formation of radiolabeled acetylcholine from [3H]CoA, as modified (Slotkin et al. 1951). Cholinesterase activity was determined with [3H]AFDX384, displacing 1098

**Study design and data analysis.** Experiments were conducted on five different cohorts of animals. In the first group, the effects of CPF were examined in nonpregnant female rats (average starting weight, 349 ± 3 g) using 11 animals per treatment group. Two cohorts were used for CPF exposure on GD9–12, one each for the experiments carried out on GD17 and GD21. For this regimen, each treatment group in each cohort contained 7–10 animals (average starting weight, 300 ± 3 g). The final two cohorts were used for CPF exposure on GD17–20; the first cohort was used for exposures of 0, 1, 2, and 5 mg/kg and the second for 0, 10, 20, and 40 mg/kg. Each treatment group in each cohort contained 7–9 animals (average starting weight, 361 ± 5 g). For presentation purposes, control values were combined across cohorts, because they did not differ significantly from each other; however, treatment differences were established using only the control values for each matched cohort. For each determination, the individual fetus represented a single sample; to avoid bias NS, not significant. ANOVA values across all treatments appear in the bottom row.

**Table 1.** CPF treatment on GD17–20: fetal characteristics on GD21.

| Treatment (mg/kg/day) | Body weight (g) | Forebrain weight (mg) | Brainstem weight (mg) | Heart weight (mg) | Liver weight (mg) | Fetuses per dam |
|-----------------------|----------------|-----------------------|-----------------------|----------------|----------------|----------------|
| 0                     | 5.6 ± 0.1      | 116 ± 2               | 92 ± 2                | 25 ± 1          | 372 ± 9         | 12.8 ± 0.6     |
| 1                     | 5.5 ± 0.1      | 117 ± 2               | 94 ± 1                | 24 ± 1          | 377 ± 8         | 13.6 ± 0.8     |
| 2                     | 5.4 ± 0.1      | 115 ± 3               | 92 ± 1                | 25 ± 1          | 376 ± 13        | 12.3 ± 1.1     |
| 5                     | 5.5 ± 0.1      | 116 ± 2               | 92 ± 1                | 24 ± 1          | 364 ± 13        | 13.8 ± 0.7     |
| 10                    | 4.7 ± 0.4*     | 114 ± 3               | 88 ± 3                | 24 ± 1          | 284 ± 33*       | 9.5 ± 0.6*     |
| 20                    | 4.0 ± 0.2*     | 109 ± 2               | 86 ± 1*               | 22 ± 1          | 233 ± 17*       | 12.4 ± 0.9     |
| 40                    | 4.3 ± 0.3*     | 111 ± 2               | 80 ± 3                | 21 ± 1          | 241 ± 25*       | 12.8 ± 0.8     |
| ANOVA                  | p = 0.0001     | NS                    | p < 0.05              | NS             | p < 0.0001      | p < 0.001      |

ANOVA indicates no significant differences among treatments; there were also no significant differences for ratios of tissue/body weights (data not shown).

**Table 2.** CPF treatment on GD9–12: fetal characteristics on GD17.

| Treatment (mg/kg/day) | Body weight (g) | Brain weight (g) | Heart weight (mg) | Liver weight (mg) | Fetuses per dam |
|-----------------------|----------------|-----------------|-----------------|-----------------|----------------|
| 0                     | 0.84 ± 0.04    | 78 ± 2          | 5.3 ± 0.3       | 70 ± 4          | 13.6 ± 0.6     |
| 1                     | 0.89 ± 0.03    | 80 ± 3          | 5.4 ± 0.3       | 76 ± 3          | 12.4 ± 0.5     |
| 2                     | 0.91 ± 0.02    | 80 ± 3          | 5.6 ± 0.2       | 75 ± 2          | 11.7 ± 0.9     |
| 5                     | 0.92 ± 0.03    | 81 ± 4          | 5.8 ± 0.2       | 77 ± 3          | 12.9 ± 1.3     |

ANOVA indicates no significant differences among treatments; there were also no significant differences for ratios of tissue/body weights (data not shown).

**Table 3.** CPF treatment on GD9–12: fetal characteristics on GD21.

| Treatment (mg/kg/day) | Body weight (g) | Forebrain weight (mg) | Brainstem weight (mg) | Heart weight (mg) | Liver weight (mg) | Fetuses per dam |
|-----------------------|----------------|-----------------------|-----------------------|----------------|----------------|----------------|
| 0                     | 5.7 ± 0.2      | 116 ± 2               | 90 ± 2                | 26 ± 1          | 401 ± 12        | 11.5 ± 1.0     |
| 1                     | 5.8 ± 0.2      | 117 ± 3               | 93 ± 2                | 26 ± 1          | 411 ± 15        | 11.4 ± 0.6     |
| 2                     | 6.0 ± 0.1      | 121 ± 4               | 93 ± 1                | 28 ± 1          | 414 ± 6         | 12.8 ± 0.8     |
| 5                     | 5.9 ± 0.1      | 121 ± 6               | 94 ± 1                | 29 ± 1          | 410 ± 9         | 11.9 ± 0.7     |

ANOVA indicates no significant differences among treatments; there were also no significant differences for ratios of tissue/body weights (data not shown).

**Figure 1.** Effects of CPF exposure on maternal body weight gain; presented as the weight change (Δ weight) from the first day of injection. (A) CPF effects on non-pregnant females (ANOVA; treatment, p < 0.001; treatment × time, p < 0.003). (B) Maternal body weight gain, CPF treatment GD9–12 (ANOVA: treatment, p < 0.001; treatment × time, p < 0.001). (C) Maternal body weight gain, CPF treatment GD9–12 (ANOVA: treatment, p < 0.003; treatment × time, p < 0.07). Arrows denote CPF injections.

*Individual time points showing significant effects, tested only for those dose regimens showing a significant overall effect by ANOVA.
from repeated sampling of the same litter, each dam contributed only one fetus to a given determination. In general, differences among treatment groups were established by multivariate analysis of variance (ANOVA; treatment, age, tissue), with data log-transformed because of heterogeneous variance. However, before separate examination of the measurement variables, we performed a nested ANOVA across all measurements for the fetuses from a given dam and found significant treatment × measurement interactions (p < 0.0001), justifying the separation of the statistical comparisons were conducted only on the original data.

Materials. Animals were purchased from Zivic Laboratories (Pittsburgh, PA), and CPF was obtained from Chem Service Inc. (West Chester, PA). Dimethyl sulfoxide was purchased from Mallinckrodt Baker (Paris, KY). [14C]Acetyl-CoA (specific activity, 44 mCi/mmol; diluted with unlabeled compound to 6.7 mCi/mmol) and [3H]AFDX384 (specific activity, 133 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). Sigma Chemical Co. (St. Louis, MO) was the source of all other chemicals.

Results
Repeated administration of CPF, given to nonpregnant female rats, had no effect on body weights at 10 mg/kg/day, but animals lost significant amounts of weight at doses of 20 mg/kg/day or higher (Figure 1). In contrast, pregnant rats were more sensitive to CPF, as assessed by this index of systemic toxicity. Treatment on GD17–20 showed impaired weight gain at 5 mg/kg/day and outright weight loss at 10, 20, and 40 mg/kg/day. Similarly, impaired weight gain was seen at 5 mg/kg/day when CPF was given on GD9–12, but the pregnant rats regained normal weights by GD14. The differences in maternal weight gain evoked by CPF treatment did not simply reflect a reduction in the weight of the fetuses (Tables 1–3). For example, on GD21, dams in the three highest dose groups for treatment on GD17–20 showed weight deficits of 80 g, whereas the reductions in fetal weight totaled only 15–25 g. This discrepancy indicated that the dose–response relationship for fetotoxicity differed from that of maternal toxicity; accordingly, we examined growth parameters and neurochemical characteristics of the fetuses exposed to CPF in the two treatment windows.

CPF treatment on GD17–20. CPF given during the later treatment window failed to cause fetal weight reductions at doses up to the threshold for maternal weight deficits (5 mg/kg/day) but did evoke fetal weight loss above that dose (Table 1). The weight deficits were present regardless of the fact that there was little or no fetal resorption. The forebrain, brainstem, and heart all showed relative growth sparing, because, with only one exception, there were no significant differences at any dose level. In contrast, liver weights showed robust reductions at doses above the threshold for maternal toxicity. The sparing of brain and heart and targeting of the liver were readily apparent when weight characteristics were compared as ratios to fetal body weight (Figure 2): At doses above 5 mg/kg/day, the brain/body and heart/body weight ratios were significantly elevated, whereas the liver/body weight ratio was reduced. Examination of biomarkers of cell development gave insight into the underlying mechanisms. DNA concentration showed only small, inconsistent changes, indicating maintenance of cell packing density. Accordingly, the hepatic DNA content was reduced, indicating that the reductions in weight reflected a deficit in the total number of cells; this was partially offset by an increase in the relative size of the remaining cells, evidenced by augmentation of the protein/DNA ratio. The cell size marker also indicated that, despite the sparing of brain weight, cell packing density, and cell number, CPF evoked alterations in cell size at doses below the threshold for general maternal or fetal toxicity. At the two lowest CPF doses, cell size was enhanced in the brainstem and reduced in the forebrain, effects that were statistically significant overall by ANOVA as well as individually in post hoc tests. Signs of cell enlargement were also seen in the heart but only at doses exceeding the threshold for general growth impairment.

The heterogeneity of cell and neuron types in the brain limits the degree of change for biomarkers of general cell development, because affected subpopulations are diluted with unaffected cells or regions. Accordingly, given the profound effect of CPF on acetylcholine systems, we next examined effects on...
markers of cholinergic synaptic development (Figure 3). Administration of 1 mg/kg/day of CPF on GD17–20 had no significant effect on cholinesterase activity in fetal brain, assessed on GD21, 24 hr after the last dose. However, significant inhibition (15–20%) was seen at 2 mg/kg/day, rising to 80% inhibition at 40 mg/kg/day. There was a consistent difference in the degree of inhibition between the two brain regions, with slightly greater effects on the forebrain compared with the brainstem. In contrast to the inhibition of cholinesterase, CPF had a smaller effect on ChAT activity, a marker for development of presynaptic cholinergic nerve terminals. Reductions in ChAT were seen at all doses but did not achieve statistical significance until a threshold of 20 mg/kg/day, and even then, the maximum deficit was 10%. Larger deficits were seen for effects of CPF on developing m2AChRs, with up to a 20% deficit across both brain regions and a threshold of 5 mg/kg/day. Cardiac m2AChRs showed similar deficits.

Control values from which the CPF-induced changes were calculated were, for tissue weight ratios, 0.0371 ± 0.0006 for brain/body, 0.00439 ± 0.00007 for heart/body, 0.067 ± 0.002 for liver/body; for DNA concentration, 2.84 ± 0.07 mg/g for forebrain, 1.55 ± 0.04 mg/g for brainstem, 2.33 ± 0.06 mg/g for heart, 1.77 ± 0.07 mg/g for liver; for DNA content, 0.33 ± 0.01 mg for forebrain, 0.139 ± 0.003 mg for brainstem, 0.055 ± 0.002 mg for heart, 0.67 ± 0.02 mg for liver; for protein/DNA ratio, 23.4 ± 0.4 for forebrain, 39.8 ± 0.6 for brainstem, 41.2 ± 0.8 for heart, 56 ± 2 for liver; for cholinesterase, 1,342 ± 64 nmol/min/g tissue for forebrain, 4,827 ± 111 for brainstem; for ChAT, 18.8 ± 0.6 pmol/min/mg protein for forebrain, 52 ± 1 for brainstem; for m2AChR binding, 88 ± 5 fmol/mg protein in forebrain, 202 ± 9 in brainstem, 303 ± 7 in heart.

**CPF treatment on GD9–12.** In light of the threshold of 5 mg/kg/day for maternal toxicity, we limited our examinations of CPF effects in the earlier treatment window to doses up to that threshold. With these regimens, CPF had no significant effects on fetal body or tissue weights assessed on GD17 and GD21, nor were there effects on the number of fetuses (Tables 2 and 3); ratios of tissue to body weights were unaltered (data not shown). As noted for exposure on GD17–20, treatment on GD9–12 did not affect DNA concentration at doses up to the threshold for maternal weight impairment (Figure 4A). However, total cell number, as indexed by DNA content (Figure 4B), showed significant changes in multiple tissues. There was an increase in cardiac DNA content, whereas the liver showed a biphasic effect, with increases on GD17 and decreases on GD21. There was also an increase in hepatic cell size (protein/DNA ratio; Figure 4C) on GD21, and with this regimen, the threshold for the effect was lower than with the later CPF treatment (2 vs. 10 mg/kg/day). For the brain, there was a consistent overall pattern (p < 0.04) of reduced cell size at low doses of CPF (Figure 4C), with loss or reversal of the effect at the highest dose.

As with GD17–20 treatment, these subtle abnormalities suggested that more robust changes might be revealed for developmental markers specific for cholinergic systems (Figure 5). Low doses of CPF given on GD9–12 evoked significant elevations of ChAT in whole brain assessed on GD17, and again we saw a loss or reversal of the effect at the higher dose of 5 mg/kg/day. A similar pattern was seen for m2AChRs on GD17, as confirmed by comparisons across the two cholinergic markers: main effect of CPF (p < 0.03) without an interaction of treatment x measure; p < 0.04 for control versus CPF 1 mg/kg/day; p < 0.02 for control versus CPF 2 mg/kg/day; no significant difference for 5 mg/kg/day. By GD21, elevations of ChAT were no longer evident, and suppression was seen at the highest dose; m2AChR binding no longer showed any elevations and tended to be reduced (not significant by itself but also statistically indistinguishable from the significant reductions seen for ChAT).

**Figure 3.** Effects of CPF (GD17–20) on cholinergic markers, assessed on GD21, presented as the percentage change from corresponding control values. (A) Cholinesterase (ANOVA: treatment, p < 0.0001; treatment x tissue, p < 0.0001); (B) ChAT (ANOVA: treatment x tissue, p < 0.01); (C) m2-AChRs (ANOVA: treatment, p < 0.0001).

*Individual values showing significant effects, tested only where the ANOVA indicated a significant interaction of treatment x tissue; in the absence of an interaction, we tested only the main treatment effect at each dose, shown below each cluster of bars.

**Figure 4.** Effects of CPF (GD9–12) on biomarkers of cell development, assessed on GD17 and GD21, presented as the percentage change from corresponding control values. (A) DNA concentration (ANOVA: NS); (B) DNA content (ANOVA: treatment x age x tissue, p < 0.02; heart, p < 0.03; liver, p < 0.05); (C) protein/DNA ratio (ANOVA: treatment, p < 0.03; treatment x age x tissue, p < 0.007; brain, p < 0.04; liver, p < 0.005).

*Individual values showing significant effects, determined only where the ANOVA indicated a significant overall difference for the specified tissue.*
Control values from which the CPF-induced changes were calculated were, for DNA concentration, 2.3 ± 0.1 mg/g tissue for brain on GD17, 1.28 ± 0.07 for heart on GD17, 2.73 ± 0.06 for liver on GD17, 3.28 ± 0.02 for forebrain on GD21, 1.68 ± 0.04 for brainstem on GD21, 2.67 ± 0.07 for heart on GD21, 1.46 ± 0.11 for liver on GD21; for DNA content, 0.176 ± 0.008 mg for brain on GD17, 0.0211 ± 0.0008 for heart on GD17, 0.56 ± 0.03 for liver on GD17, 0.384 ± 0.007 for forebrain on GD21, 0.145 ± 0.004 for brainstem on GD21, 0.069 ± 0.004 for heart on GD21, 0.61 ± 0.05 for liver on GD21; for protein/DNA ratio, 25 ± 1 for brain on GD17, 28.9 ± 0.5 for heart on GD17, 48.7 ± 0.7 for liver on GD17, 18.8 ± 0.4 for forebrain on GD21, 31.6 ± 0.8 for brainstem on GD21, 40 ± 1 for heart on GD21, 71 ± 0.7 for liver on GD21; for ChAT, 13.6 ± 0.3 pmol/min/mg protein for brain on GD17, 25.7 ± 0.7 for forebrain on GD21, 69 ± 6 for brainstem on GD21; for m2AChR binding, 45 ± 2 fmol/mg protein for brain on GD17, 124 ± 2 for heart on GD17, 80 ± 4 for forebrain on GD21, 197 ± 9 for brainstem on GD21, 276 ± 9 for heart on GD21.

Discussion

Previous work established the selective developmental neurotoxicity of CPF in the postnatal period, characterized by cell damage and loss, impaired synaptogenesis, and deficits in synaptic function and related behaviors (Barone et al. 2000; Pope 1999; Slotkin 1999), all of which occur with threshold doses below those required for growth impairment. In contrast, the present results with fetal CPF exposure indicate relative sparing of major aspects of brain development beyond the point of outright fetotoxicity. Accordingly, with late gestational CPF treatment, fetal brain cell number, cell packing density, and cell size were all maintained even at 40 mg/kg/day, a dose that caused 80% inhibition of brain cholinesterase and significant fetal growth impairment. In fact, at least in terms of growth, the fetus was generally spared compared with the dam: fetal weight was reduced only at or above 10 mg/kg/day, a dose that produced >50% inhibition of fetal brain cholinesterase, whereas maternal weight gain was impaired at 5 mg/kg/day. Our results for biomarkers of general aspects of brain cell development are compatible with the sparing of fetal and brain growth noted in earlier studies with different routes of administration (Maurissen et al. 2000).

We are thus faced with the likelihood, at least with these particular biomarkers, that the neonate is more sensitive to CPF-induced developmental neurotoxicity than is the fetus. There are several potential reasons for this basic difference. First, the fetus recovers far more readily from cholinesterase inhibition than do older animals because of the expediency of replacement of cholinesterase molecules during rapid brain growth (Lassiter et al. 1998; Meneguz et al. 1989); accordingly, systemic toxicity related to cholinergic hyperactivity is likely to be less persistent in the fetus. Differences in the rate of resynthesis of cholinesterase may also account for the smaller effects on enzyme activity in the brainstem compared with forebrain; certainly, pharmacokinetic disparities between these two regions would seem extremely unlikely. Second, recent data suggest that CPF targets development of glial cells to a greater extent than it does neuronal cell development (Aschner 2000; Garcia et al. 2001, 2002; Monnet-Tschudi et al. 2000; Qiao et al. 2001); glial development peaks during the postnatal period (Guerrier and Renaux-Piqueras 1997; Valles et al. 1997; Zawia and Harry 1996), whereas neurogenesis occurs much earlier (Rodier 1988), so the neonatal brain may be inherently more sensitive to CPF than is the fetal brain. Third, in light of the recent recognition that CPF itself is a developmental neurotoxicant (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1999), over and above inhibition of cholinesterase by its metabolite, chlorpyrifos oxon, there are likely to be substantial differences of effects reflecting the dissimilarities in fetal versus neonatal pharmacokinetics (Hunter et al. 1998, 1999; Moser et al. 1998; Padilla et al. 2000); it is highly likely that more unchanged CPF reaches the neonatal brain after direct administration to pups than is achieved with fetal exposure paradigms that involve maternal administration (Hunter et al. 1998).

Notwithstanding these factors, it is also likely that the basic markers of cell development used here provide an inadequate basis for concluding that there are no adverse effects of prenatal CPF exposure. DNA and protein-based biomarkers require the use of fairly large brain regions, so effects on a targeted subregion or population of cells can be masked by a relatively larger, unaffected population. In that case, effects would only emerge at high doses that elicit general fetotoxicity. Indeed, when we examined markers specific to cholinergic synaptic development, we saw more robust changes at lower doses of CPF, effects similar in magnitude to those elicited by postnatal CPF exposure (Chakraborti et al. 1993; Dam et al. 1999; Slotkin et al. 2001). With these markers, we found significant deficits with treatment on GD17–20, even at 5 mg/kg/day, a dose that did not evoke fetal growth impairment. It is therefore likely that examination of smaller subregions, combined with the use of dynamic markers that provide sensitive indices of cholinergic synaptic function (Dam et al. 1999; Slotkin et al. 2001), will reveal fetal anomalies. Indeed, two preliminary reports suggest that prenatal CPF exposure can disrupt architectural organization of specific subregions, including apoptosis and changes in cell migration (Lassiter et al. 2002; White et al. 2002). It also must be noted that, even with postnatal CPF exposure, many of the neurotoxic effects appear only after a delay (Barone et al. 2000; Landrigan et al. 1999; Pope 1999; Ray and Richards 2001; Rice and Barone 2000; Slotkin 1999), whereas in the present study we have assessed only the immediate fetal effects. Accordingly, a definitive evaluation of the consequences of fetal exposure will require a longitudinal study from early development through adulthood, using doses spanning the threshold for fetotoxicity; a few reports that have appeared on this issue suggest that neurobehavioral deficits emerge after fetal CPF exposure (Chanda and Pope 1996; Muto et al. 1992), but no underlying synaptic mechanisms have as yet been identified.

Our findings suggest that the effects of CPF on fetal brain development are fundamentally different for exposure in early (GD9–12) compared with late (GD17–20) gestation. Exposure during the earlier period evoked an augmentation in cholinergic synaptic markers instead of the deficits seen with later treatment. The biphasic nature of the dose–response curve for effects of treatment on GD9–12 gives some clue as to the
underlying events: Promotional effects were seen only at low doses (1 or 2 mg/kg/day) but were offset by a higher dose (5 mg/kg/day) that impaired maternal weight gain. During brain development, acetylcholine serves as a trophic factor, regulating the differentiation of target cells containing cholinergic receptors (Hohmann and Berger-Sweeney 1998; Morley and Happe 2000; Navarro et al. 1989; Slotkin 1999), and its precursor and breakdown product, choline, also augments neural plasticity (Cermak et al. 1999; Montoya et al. 2000). It is thus possible that during early gestation, low CPF exposures elicit promotional effects on neural cell differentiation that are offset when doses are raised to the point of cellular or general fetotoxicity; indeed, we found previously that high CPF exposures elicit apoptosis in fetal brain during this stage of development (Roy et al. 1998). Generation of reactive oxygen species provides an additional potential mechanism for dual effects of CPF. Mild oxidative stress can induce cell differentiation (Katoh et al. 1997), whereas excessive formation of reactive oxygen species results in cell damage. Careful examination of the biomarkers for the later exposure paradigm (GD17–20) also provides some indication for a dual spectrum of CPF actions. Low doses of CPF evoked a slight but significant enhancement of cell growth in the brainstem (increased protein/DNA ratio), an effect that was lost when the dose was raised above the threshold for fetotoxicity. Our results thus point to a shifting spectrum of CPF effects on neurodevelopment, dependent both on the exposure window and on the dose.

In addition to the impact on neurodevelopment, CPF had robust effects on the fetal liver. Indeed, the deficits in liver weight were larger than those for body weight, so this tissue was selectively targeted. The underlying mechanism involved a decline in the number of liver cells (reduced DNA), with some compensatory cell enlargement (protein/DNA), so the degree of tissue weight loss was less than would have been expected from the deficit in cell number. Although we do not yet have information about the reason for hepatic cell loss, there are a number of likely possibilities. The antimitotic effects of CPF (Barone et al. 2000; Campbell et al. 1997; Dam et al. 1998; Garcia et al. 2001; Qiao et al. 2001; Song et al. 1998; Whitney et al. 1995) could target cells containing cholinergic receptors (Auman et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1999), does not evoke substantial deficits in liver weight (Auman et al. 2000); however, it does alter cell signaling cascades linked to neurotransmitter receptors (Auman et al. 2000), reinforcing the concept that, as in the developing brain, disruption of CPF-induced cell-to-cell communication is separable from generalized toxicity or growth impairment. Obviously, future work should address the potential impact of fetal or neonatal CPF exposure on liver function and, in particular, hepatic responses to neurotrophic factors. The threshold for fetotoxicity. Our results thus point to a shifting spectrum of CPF effects on neurodevelopment, dependent both on the exposure window and on the dose.

In conclusion, our results indicate that fetal CPF exposure elicits far less widespread cell damage and loss in the fetal nervous system compared with postnatal CPF exposure. Nevertheless, we found indications of more subtle alterations in specific regions and cell or synaptic populations that suggest the need for further identification of the neurotransmitter systems affected by CPF exposure. Longitudinal studies of later-emerging synaptic and behavioral alterations will be required to characterize the functional consequences of these effects.

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