Developmental Neurotoxicity of Chlorpyrifos: What Is the Vulnerable Period?

Dan Qiao,[1] Frederic J. Seidler,[1] Stephanie Padilla,[2] and Theodore A. Slotkin[1]

[1]Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina, USA; [2]National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA

Previously, we found that exposure of neonatal rats to chlorpyrifos (CPF) produced brain cell damage and loss, with resultant abnormalities of synaptic development. We used the same biomarkers to examine prenatal CPF treatment so as to define the critical period of vulnerability. One group of pregnant rats received CPF (subcutaneous injections in dimethyl sulfoxide vehicle) on gestational days (GD) 17–20, a peak period of neurogenesis; a second group was treated on GD9–12, the period of neural tube formation. In the GD17–20 group, the threshold for a reduction in maternal weight gain was 5 mg/kg/day; at or below that dose, there was no evidence (GD21) of general fetotoxicity as assessed by the number of fetuses or fetal body and tissue weights. Above the threshold, there was brain sparing (reduced body weight with an increase in brain/body weight ratio) and a targeting of the liver (reduced liver/body weight). Indices of cell packing density (DNA per gram of tissue) and cell number (DNA content) similarly showed effects only on the liver; however, there were significant changes in the protein/DNA ratio, an index of cell size, in fetal brain regions at doses as low as 1 mg/kg, below the threshold for inhibition of fetal brain cholinesterase (2 mg/kg). Indices of cholinergic synaptic development showed significant CPF-induced defects but only at doses above the threshold for cholinesterase inhibition. With earlier CPF treatment (GD9–12), there was no evidence of general fetotoxicity or alterations of brain cell development at doses up to the threshold for maternal toxicity (5 mg/kg), assessed on GD17 and GD21; however, augmentation of cholinergic synaptic markers was detected at doses as low as 1 mg/kg. Compared with previous work on postnatal CPF exposure, the effects seen here required doses closer to the threshold for fetal weight loss; this implies a lower vulnerability in the fetal compared with the neonatal brain. Although delayed neurotoxic effects of prenatal CPF may emerge subsequently in development, our results are consistent with the preferential targeting of late developmental events such as gliogenesis, axonogenesis, and synaptogenesis. *Key words:* brain, chlorpyrifos, choline acetyltransferase, cholinesterase, development, heart, liver, muscarinic m_2-acetylcholine receptor. *Environ Health Perspect* 110:1097–1103 (2002). [Online 16 September 2002]

http://ehpnet1.niehs.nih.gov/docs/2002/110p1097-1103qiao/abstract.html

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 (Mileson et al. 1998), and immature organisms are more susceptible to CPF-induced toxicity than are adults (Moser and Padilla 1998; Pope and Chakraborti 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 synaptic markers 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), 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 synaptic development, we assessed choline acetyltransferase (ChAT), a constitutive marker of cholinergic nerve terminals (Slotkin et al. 2001); we also measured radioligand binding to the m_2-muscarinic acetylcholine receptor (m_2AChR), a mediator of cholinergic signaling that typically undergoes down-regulation in the presence of cholinergic hyperstimulation (Bushnell et al. 1993; Chakraborti 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

Address correspondence to T.A. Slotkin, Box 3813 DUMC, Dept. of Pharmacology & 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 C.A. Tate and M.M. Cousins for technical assistance.

This research was supported by USPHS ES10387 and ES10356, and by the Leon Golberg Toxicology Fellowship. This paper has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, and mention of trade names of commercial products does not constitute endorsement or recommendation for use.

Received 11 January 2002; accepted 20 March 2002.

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 –45°C.

Assays. All assay procedures used in this study have been detailed in previous publications; accordingly, only brief descriptions are provided here. For quantitation of macromolecules, DNA was determined using a modified (Trauth et al. 2000) fluorescent dye-binding method (Labarca and Piagen 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 [^{14}C]acetyl-coenzyme A (CoA), as modified (Slotkin et al. 2001) from earlier procedures (Lau et al. 1988). Radioligand binding to m_2AChRs was determined with [^{3}H]AFDX384, displacing specific binding with atropine (Song et al. 1997); we used a single, subsaturating ligand concentration (1 nM) to enable detection of changes in receptors reflecting either altered affinity (K_d) or altered capacity (B_{max}).

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

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	88 ± 2	23 ± 1	241 ± 25*	12.8 ± 0.8
ANOVA	$p < 0.0001$	NS	$p < 0.04$	NS	$p < 0.0001$	$p < 0.01$

NS, not significant. ANOVA values across all treatments appear in the bottom row.
*Individual values that differ significantly from the corresponding control, evaluated only where the ANOVA indicated a significant overall treatment effect.

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

Treatment (mg/kg/day)	Body weight (g)	Brain weight (mg)	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	12.6 ± 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).

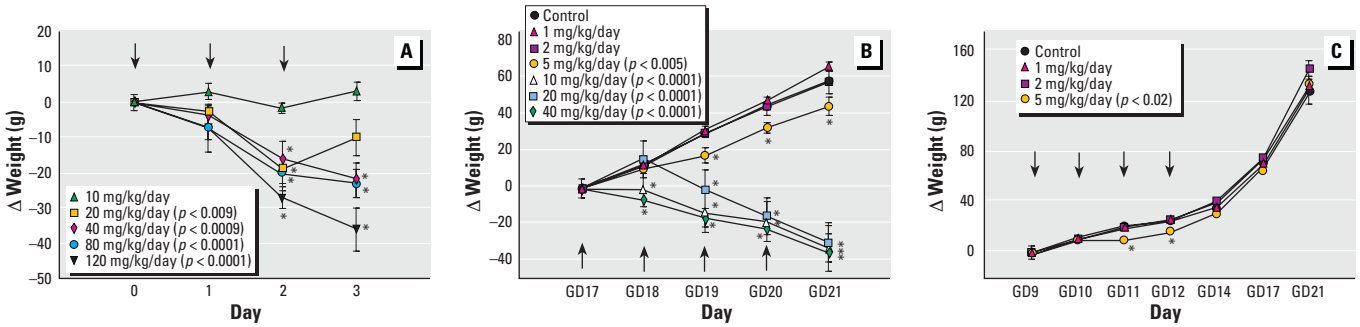

Figure 1. Effects of CPF exposure on maternal body weight gain; presented as the weight change (Δ weight) from the first day of injection. (*A*) CPF effects on nonpregnant females (ANOVA: treatment, $p < 0.0001$; treatment × time, $p < 0.003$). (*B*) Maternal body weight gain, CPF treatment GD17–20 (ANOVA: treatment, $p < 0.0001$; treatment × time, $p < 0.0001$). (*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 measures. We then subdivided the measures into three groupings: body and tissue weights, cell development markers (DNA, protein), and cholinergic markers (ChAT, m_2AChR binding, cholinesterase) and performed the nested ANOVA on each grouping. Again, there were highly significant treatment × measurement interactions. Accordingly, data were separated into the individual measures, which were then evaluated by ANOVA; where appropriate, this was followed by *post hoc* evaluations of each treatment group compared with the controls with Dunnett's *t*-test, using untransformed data. Significance was assumed at the level of $p < 0.05$ for main effects; 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).

Data are presented as means and standard errors of the mean. To facilitate comparisons across multiple tissues, ages, and variables, some results are given as the percentage change from the corresponding control group, but 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). [^{14}C]Acetyl-CoA (specific activity, 44 mCi/mmol; diluted with unlabeled compound to 6.7 mCi/mmol) and [^{3}H]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

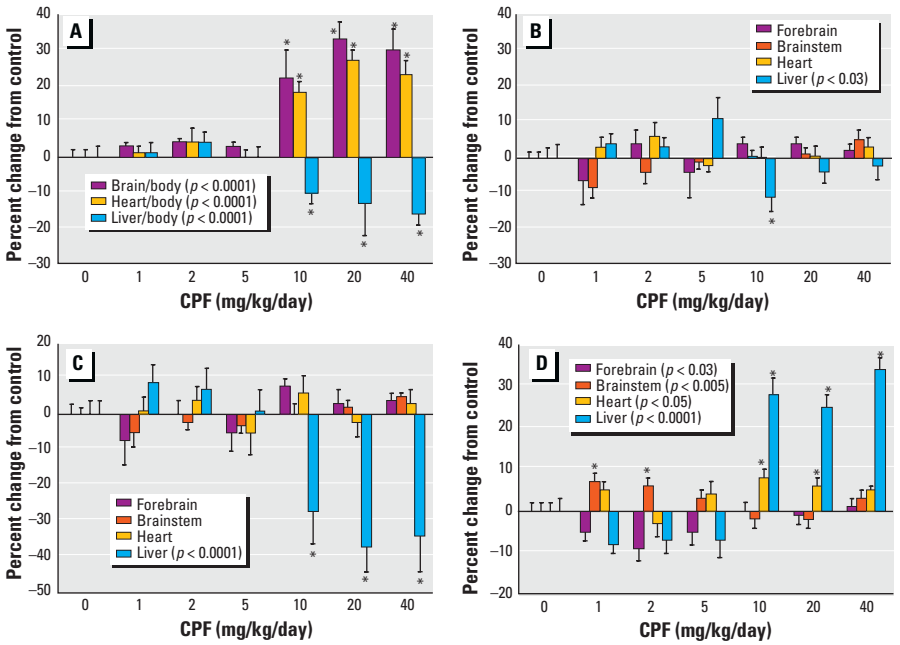

Figure 2. Effects of CPF (GD17–20) on biomarkers of cell development, assessed on GD21, presented as the percentage change from corresponding control values. (*A*) Relative tissue weights (ANOVA: treatment, $p < 0.0001$; treatment × tissue, $p < 0.0001$); (*B*) DNA concentration (ANOVA: treatment × tissue, $p < 0.009$); (*C*) DNA content (ANOVA: treatment, $p < 0.003$; treatment × tissue, $p < 0.0001$); (*D*) protein/DNA ratio (ANOVA: treatment, $p < 0.0001$; treatment × tissue, $p < 0.0001$).
*Individual points showing significant effects, tested only for those tissues showing a significant overall effect by ANOVA.

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 m_2AChRs, with up to a 20% deficit across both brain regions and a threshold of 5 mg/kg/day. Cardiac m_2AChRs 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 m_2AChR 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 m_2AChRs on GD17, as confirmed by comparisons across the two cholinergic markers: main effect of CPF ($p < 0.03$) without an interaction of treatment × 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; m_2AChR 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).

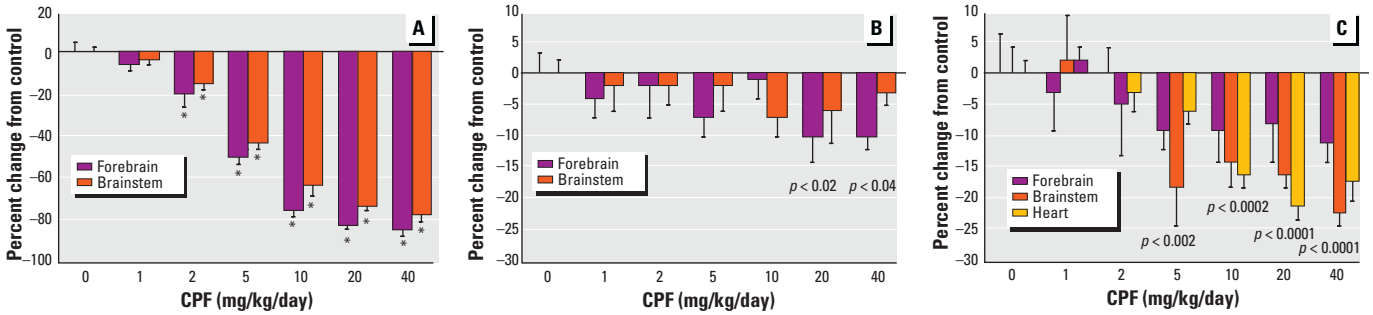

Figure 3. Effects of CPF (GD17–20) on cholinergic markers, assessed on GD21, presented as the percentage change from corresponding control values. (*A*) Cholinesterase (ANOVA: treatment, $p < 0.0001$; treatment × tissue, $p < 0.0001$); (*B*) ChAT (ANOVA: treatment × tissue, $p < 0.1$); (*C*) m_2-AChRs (ANOVA: treatment, $p < 0.0001$).

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

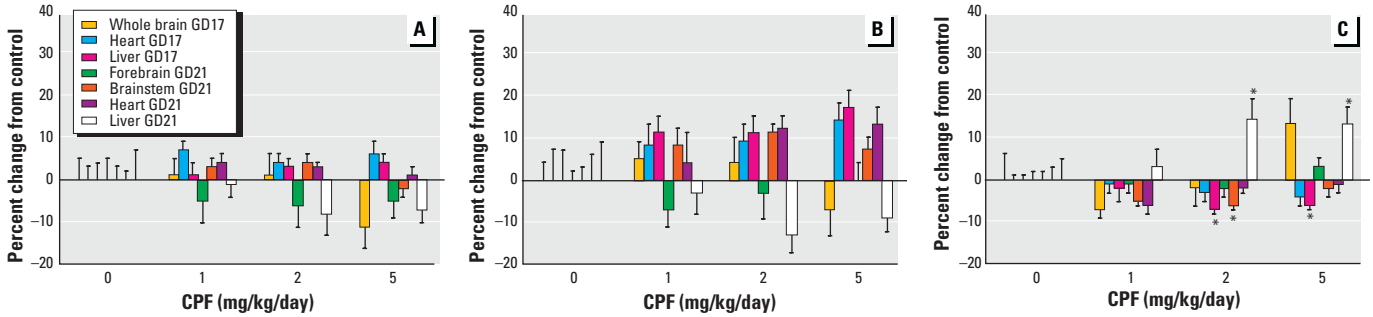

Figure 4. Effects of CPF (GD9–12) on biomarkers of cell development, assessed on GD17 and GD21, presented as the percentage change from corresponding control values. (*A*) DNA concentration (ANOVA: NS); (*B*) DNA content (ANOVA: treatment × age × tissue, $p < 0.02$; heart, $p < 0.03$; liver, $p < 0.05$); (*C*) protein/DNA ratio (ANOVA: treatment, $p < 0.03$; treatment × age × 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, 0.0121 ± 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 ± 4 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 m_2AChR 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 expeditious 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 (Guerri and Renau-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

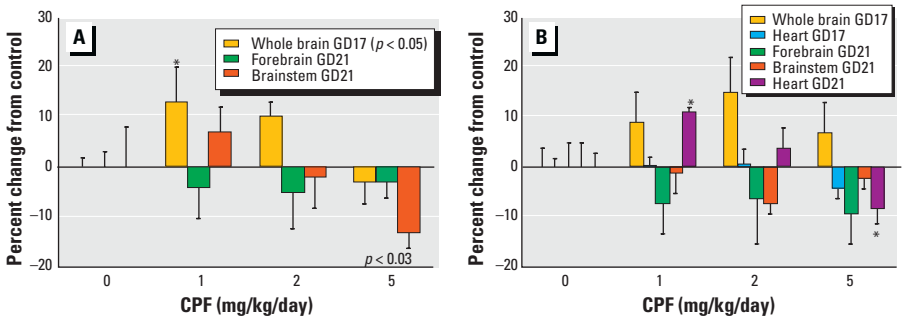

Figure 5. Effects of CPF (GD9–12) on cholinergic markers, assessed on GD17 and GD21, presented as the percentage change from corresponding control values. (*A*) ChAT (ANOVA: treatment, $p < 0.05$); (*B*) m2-AChRs (ANOVA: heart, $p < 0.005$).
*Individual values showing significant effects and main treatment effects across multiple tissues are shown the bar clusters.

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 the fetal liver because of its extremely rapid rate of cell acquisition and because this tissue achieves high levels of CPF and its metabolites (Hunter et al. 1999). Additionally, the oxidative stress caused by CPF is likely to evoke hepatic cell damage and loss (Crumpton et al. 2000; Garcia et al. 2001; Jett and Navoa 2000), so sensitivity could depend on the relative state of development of enzymes generating reactive oxygen species, compared with those required for the deactivation of free radicals and/or catabolism of CPF (Padilla et al. 2000). In any case, the effects on the fetal liver stand in direct contrast to the pattern seen for exposure in the neonatal period: postnatal CPF, which has a profound effect on brain cell development (Barone 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 neuronal/hormonal inputs using the affected signaling cascades.

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.

References

Aschner M. 2000. Interactions between pesticides and glia: an unexplored experimental field. Neurotoxicology 21:175–180.

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.

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.

Bomser JA, Casida JE. 2001. Diethylphosphorylation of rat cardiac M2 muscarinic receptor by chlorpyrifos oxon in vitro. Toxicol Lett 119:21–26.

Bushnell PJ, Pope CN, Padilla S. 1993. Behavioral and neurochemical effects of acute chlorpyrifos in rats: tolerance to prolonged inhibition of cholinesterase. J Pharmacol Exp Ther 266:1007–1017.

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

Cermak JM, Blusztajn JK, Meck WH, Williams CL, Fitzgerald CM, Rosene DL, et al. 1999. Prenatal availability of choline alters the development of acetylcholinesterase in the rat hippocampus. Dev Neurosci 21:94–104.

Chakraborti TK, Farrar JD, Pope CN. 1993. Comparative neurochemical and neurobehavioral effects of repeated chlorpyrifos exposures in young and adult rats. Pharmacol Biochem Behav 46:219–224.

Chanda SM, Pope CN. 1996. Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyrifos in maternal and developing rats. Pharmacol Biochem Behav 53:771–776.

Crumpton TL, Seidler FJ, Slotkin TA. 2000. Is oxidative stress involved in the developmental neurotoxicity of chlorpyrifos? Dev Brain Res 121:189–195.

Dam K, Garcia SJ, Seidler FJ, Slotkin TA. 1999. 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.

Fenske RA, Black KG, Elkner KP, Lee C, Methner MM, Soto R. 1990. Potential exposure and health risks of infants following indoor residential pesticide applications. Am J Public Health 80:689–693.

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.

Guerri C, Renau-Piqueras J. 1997. Alcohol, astroglia, and brain development. Mol Neurobiol 15:65–81.

Gurunathan S, Robson M, Freeman N, Buckley B, Roy A, Meyer R, et al. 1998. Accumulation of chlorpyrifos on residential surfaces and toys accessible to children. Environ Health Perspect 106:9–16.

Hohmann CF, Berger-Sweeney J. 1998. Cholinergic regulation of cortical development and plasticity: new twists to an old story. Perspect Dev Neurobiol 5:401–425.

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.

Hunter DL, Lassiter TL, Chanda SM, Barone S, Padilla S. 1998. Pharmacokinetics of chlorpyrifos and its metabolites in maternal and fetal brain and liver tissue following gestational exposure. Toxicologist 42:157–158.

Hunter DL, Lassiter TL, Padilla S. 1999. Gestational exposure to chlorpyrifos: comparative distribution of trichloropyridinol in the fetus and dam. Toxicol Appl Pharmacol 158:16–23.

Jett DA, Navoa RV. 2000. *In vitro* and *in vivo* effects of chlorpyrifos on glutathione peroxidase and catalase in developing rat brain. Neurotoxicology 21:141–145.

Katoh S, Mitsui Y, Kitani K, Suzuki T. 1997. Hyperoxia induces the differentiated neuronal phenotype of PC12 cells by producing reactive oxygen species. Biochem Biophys Res Commun 241:347–351.

Labarca C, Piagen K. 1980. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102:344–352.

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. 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.

Lau C, Seidler FJ, Cameron AM, Navarro HA, Bell JM, Bartolome J, et al. 1988. Nutritional influences on adrenal chromaffin cell development: comparison with central neurons. Pediatr Res 24:583–587.

Lowry OH, Rosebrough NJ, Farr AL, Randall R. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–270.

Maurissen JPJ, Hoberman AM, Garman RH, Hanley TR. 2000. Lack of selective developmental neurotoxicity in rat pups from dams treated by gavage with chlorpyrifos. Toxicol Sci 57:250–263.

Meneguz A, Bisso GM, Michalek H. 1989. Alterations in the distribution of cholinesterase molecular forms in maternal and fetal brain following diisopropyl fluorophosphate treatment of pregnant rats. Neurochem Res 14:285–291.

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.

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.

Montoya DAC, White AM, Williams CL, Blusztajn JK, Meck WH, Swartzwelder HS. 2000. Prenatal choline exposure alters hippocampal responsiveness to cholinergic stimulation in adulthood. Dev Brain Res 123:25–32.

Morley BJ, Happe HK. 2000. Cholinergic receptors: dual roles in transduction and plasticity. Hearing Res 147:104–112.

Moser VC, Chanda SM, Mortensen SR, Padilla S. 1998. Age- and gender-related differences in sensitivity to chlorpyrifos in the rat reflect developmental profiles of esterase activities. Toxicol Sci 46:211–222.

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.

Muto MA, Lobelle F, Bidanset JH, Wurpel JN. 1992. Embryotoxicity and neurotoxicity in rats associated with prenatal exposure to Dursban®. Vet Hum Toxicol 34:498–501.

Navarro HA, Seidler FJ, Eylers JP, Baker FE, Dobbins SS, Lappi SE, et al. 1989. Effects of prenatal nicotine exposure on development of central and peripheral cholinergic neurotransmitter systems. Evidence for cholinergic trophic influences in developing brain. J Pharmacol Exp Ther 251:894–900.

Padilla S, Buzzard J, Moser VC. 2000. Comparison of the role of esterases in the differential age-related sensitivity to chlorpyrifos and methamidophos. Neurotoxicology 21:49–56.

Padilla S, Lassiter TL, Hunter D. 1998. Biochemical measurement of cholinesterase activity. Methods Mol Med 22:237–245.

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, 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.

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

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):511–533.

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

Roy TS, Andrews JE, Seidler FJ, Slotkin TA. 1998. Chlorpyrifos elicits mitotic abnormalities and apoptosis in neuroepithelium of cultured rat embryos. Teratology 58:62–68.

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

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

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.

Trauth JA, Seidler FJ, Slotkin TA. 2000. An animal model of adolescent nicotine exposure: effects on gene expression and macromolecular constituents in rat brain regions. Brain Res 867:29–39.

Valles S, Pitarch J, Renau-Piqueras J, Guerri C. 1997. Ethanol exposure affects glial fibrillary acidic protein gene expression and transcription during rat brain development. J Neurochem 69:2484–2493.

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.

White L, Lassiter T, Das K, Barone S. 2002. Prenatal exposure to chlorpyrifos alters neurotrophin immunoreactivity and apoptosis in rat brain. Toxicologist 66:633.

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

Winick M, Noble A. 1965. Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in the rat. Dev Biol 12:451–466.

Zawia NH, Harry GJ. 1996. Developmental exposure to lead interferes with glial and neuronal differential gene expression in the rat cerebellum. Toxicol Appl Pharmacol 138:43–47.