Fetal and childhood exposures to widely used organophosphate pesticides, especially chlorpyrifos (CPF), have raised concerns about developmental neurotoxicity. Previously, biomarkers for brain cell number, cell packing density, and cell size indicated that neonatal rats were more sensitive to CPF than were fetal rats, yet animals exposed prenatally still developed behavioral deficits in adolescence and adulthood. In the present study, we administered CPF to pregnant rats on gestational days 17–20, using regimens devoid of overt fetal toxicity. We then examined subsequent development of acetylcholine systems in forebrain regions involved in cognitive function and compared the effects with those on general biomarkers of cell development. Choline acetyltransferase, a constitutive marker for cholinergic nerve terminals, showed only minor CPF-induced changes during the period of rapid synaptogenesis. In contrast, hemicholinium-3 binding to the presynaptic choline transporter, which is responsive to nerve impulse activity, displayed marked suppression in the animals exposed to CPF, despite a return to nearly normal values by weaning; deficits were again apparent in adolescence and adulthood. There was no compensatory up-regulation of cholinergic receptors, as m2 muscarinic cholinergic receptor binding was unchanged. CPF also elicited delayed-onset alterations in biomarkers for general aspects of cell integrity, with reductions in cell packing density, increases in relative cell size, and contraction of neuritic extensions; however, neither the magnitude nor timing of these changes was predictive of the cholinergic defects. The present findings indicate a wide window of vulnerability of cholinergic systems to CPF, extending from prenatal through postnatal periods, occurring independently of adverse effects on general cellular neurotoxicity. Key words: brain development, chlorpyrifos, choline acetyltransferase, cholinesterase, development, DNA, hemicholinium-3 binding, muscarinic m2 acetylcholine receptor. Environ Health Perspect 111:536–544 (2003). [Online 30 October 2002] doi:10.1289/ehp.5828 available via http://dx.doi.org/.

Although some uses of the organophosphate insecticide chlorpyrifos (CPF) were recently curtailed in the United States (U.S. EPA 2000), CPF and other organophosphates continue to be applied worldwide on a major scale. Studies with animal and cell culture models of CPF exposure indicate that CPF is especially damaging to the developing brain, targeting diverse events in neural development, including cell proliferation and differentiation, axonogenesis and synaptogenesis, and synaptic function (see reviews in Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1999); although some developmental toxicant effects may be unique to CPF, major features of its actions are shared by related organophosphates as well as carbamates (Mileson et al. 1998; Pope 1999; Qiao et al. 2001). The mixture of mechanisms underlying CPF’s actions renders the developing brain vulnerable to adverse effects over a broad period, spanning prenatal and postnatal stages (Barone et al. 2000; Lassiter et al. 1998, 2002; Moser and Padilla 1998; Pope 1999; Qiao et al. 2002; Rice and Barone 2000; Slotkin 1999). Indeed, interference with cell proliferation and differentiation extends to glia, which continue to proliferate into adolescence (Barone et al. 2000; Garcia et al. 2001, 2002; Monnet-Tschudi et al. 2000; Qiao et al. 2001).

We recently compared biochemical indices of brain cell damage in developing rats exposed to CPF prenatally or postnatally (Garcia et al. 2002; Qiao et al. 2002; Slotkin 1999) and found that postnatal exposure had a greater, immediate effect on the number of brain cells and on indices of synaptic development. On the surface, this seemed somewhat surprising, given that CPF readily crosses the placenta to enter the fetal brain and actually achieves higher concentrations than in the maternal brain (Hunter et al. 1999; Lassiter et al. 1998). Indeed, when we examined more selective indices of neuronal development, there was some evidence for specific disruption of acetylcholine systems after prenatal exposure, even at CPF doses below the threshold for fetal growth impairment or for inhibition of fetal brain cholinesterase (Qiao et al. 2002). Preliminary morphologic studies indicate that prenatal CPF does affect brain cell development but with a more focal pattern than is likely to be the case for postnatal CPF (Lassiter et al. 2002; White et al. 2002). Indeed, when animals given prenatal CPF were evaluated for behavioral performance in adolescence and adulthood, they displayed deficits in cognitive behaviors that depend specifically upon septo-hippocampal cholinergic function, and showed selective loss of the cholinergic components of working and reference memory (Levin et al. 2002). We previously identified similar, late-arising behavioral deficits in animals exposed to CPF postnatally (Levin et al. 2001), effects that were accompanied by delayed neurotoxic changes in neurochemical indices of cholinergic synaptic activity (Slotkin et al. 2001) and in other neurotransmitter systems regulated by cholinergic input (Slotkin et al. 2002). The neurochemical changes were most notable for regions of the forebrain (cerebral cortex, hippocampus, striatum) involved in learning and memory.

The present study takes a similar approach to the mechanisms underlying the neurobehavioral abnormalities associated with prenatal CPF exposure, addressing two specific questions: First, are there immediate or delayed deficits in cholinergic innervation or cholinergic synaptic function in the same forebrain areas that are compromised by postnatal CPF administration? Second, are these effects separable from general cellular abnormalities, such as alterations in the number of cells or in the cell protein complement? For cholinergic synaptic development, we assessed choline acetyltransferase (ChAT) activity and the binding of [3H]hemicholinium-3 (HC-3) to the high-affinity presynaptic choline transporter. ChAT, the enzyme responsible for acetylcholine biosynthesis, is a constitutive marker for cholinergic nerve terminals and serves as an archetypal measure of cholinergic innervation, but its activity does not respond to changes in impulse flow. Accordingly, ChAT increases during cholinergic synaptogenesis but does not change in response to stimuli that alter cholinergic neuronal activity (Aubert et al. 1996; Happe and Murrin 1992; Navarro et al. 1989; Slotkin et al. 1990; Zahalka et al. 1992, 1993a). In contrast, high-affinity choline uptake, as assessed with the binding of HC-3 to the presynaptic high-affinity choline transporter, is responsive to neuronal activity (Klemm and Kuhar 1979; Simon et al. 1976), and the comparative changes in ChAT and HC-3 binding or transporter function...
permit distinction between effects on synaptic outgrowth as distinct from synaptic activity (Aubert et al. 1996; Happe and Murrin 1992; Navarro et al. 1989; Slotkin et al. 1990; Zahalka et al. 1992, 1993a). These markers have been used previously to characterize effects of CPF on cholinergic systems in adult rats (Liu and Pope 1996, 1998) and to evaluate the immediate and delayed effects of postnatal CPF exposure (Dam et al. 1999; 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; 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).

Measurements of DNA and cell protein fractions were used to evaluate CPF’s general effects on cell development. Because each neural cell contains only a single nucleus (Winick and Noble 1965), the DNA content (amount of DNA in each brain region) reflects the total number of cells, and the DNA concentration (DNA per unit tissue weight) reflects the cell packing density (Bell et al. 1987; Slotkin et al. 1984; Winick and Noble 1965); these indices are affected by postnatal CPF exposure (Campbell et al. 1997; Dam et al. 1998; Song et al. 1998; Whitney et al. 1995). We also assessed the complement of cell proteins related to differentiation as opposed to cell numbers. As neurons specialize, they enlarge and develop axonal and dendritic projections. The ratio of total protein/DNA thus rises with the expansion of the cell (Bell et al. 1987; Slotkin et al. 1984). In cells that do not develop projections, the membrane surface-to-volume ratio falls as the cell enlarges, such that the membrane protein concentration falls with hypertrophy (Thai et al. 1996); however, for neural cells, the development of neuritic projections necessitates a rise in the contribution of membrane proteins relative to other cell proteins. Accordingly, we also assessed the membrane protein concentration and the ratio of membrane proteins to total cell proteins.

**Methods**

**Animal treatments.** Studies were carried out with the approval of the Duke University Institutional Animal Care and Use Committee, 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 with 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. Animals received 0, 1, or 5 mg/kg daily on gestational days 17–20 (GD17–20); these doses span the threshold for inhibition of fetal brain cholinesterase activity (Qiao et al. 2002) but lie below the threshold for fetal growth impairment or effects on fetal viability (Garcia et al. 2002; Qiao et al. 2002). On the day after birth, pups were randomized within treatment groups and redistributed to the nursing dams with a litter size of 10, so as to maintain standardized nutrition. Randomization was repeated at intervals of several days, and in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Animals were weaned on postnatal day 21 (PN21).

Animals were decapitated and brain regions were dissected using the natural landmarks of the neonatal rat brain: blunt cuts were made through the cerebellar peduncles, whereupon the cerebellum (including floculi) was lifted from the underlying tissue. A cut was then made rostral to the thalamus to isolate the forebrain, thus including the corpus striatum, hippocampal formation, and neocortex within the area designated “forebrain.” For studies on PN30 and PN60, the forebrain was divided into the cerebral cortex, hippocampus, and striatum. Tissues were frozen with liquid nitrogen and maintained at −45°C. At each age, each treatment group included 8–16 animals, evenly divided between males and females; the number of animals in each of the CPF groups was always matched to an equal number of controls, and determinations used no more than one male and one female from each litter. All assays were run such that all the animals for the control and both CPF groups were evaluated simultaneously to ensure that day-to-day variations in assays did not generate spurious treatment effects. Each assay included standards that were run with each batch to ensure day-to-day replication of values.

**Cholinergic biomarkers.** Tissues were thawed in 79 volumes of ice-cold 10 mM sodium-potassium phosphate buffer (pH 7.4) and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY). For ChAT activity, assays (Lau et al. 1988) contained 30 µL of diluted homogenate in a total volume of 60 µL with final concentrations of 60 mM sodium phosphate (pH 7.9), 200 mM NaCl, 20 mM choline chloride, 17 mM MgCl2, 1 mM EDTA, 0.2% Triton X-100, 0.12 mM phystostigmine, 0.6 mg/mL bovine serum albumin, and 50 µM [3H]acetyl-coenzyme A. Blanks contained homogenization buffer instead of the tissue homogenate. Samples were preincubated for 15 min on ice and transferred to a 37°C water bath for 30 min, and the reaction was terminated by placing the samples on ice. Labeled acetylcholine was then extracted and counted and the activity determined relative to tissue protein (Smith et al. 1985). Preliminary determinations established that enzyme activity was linear with time and tissue concentration under these conditions.

For measurements of [3H]HC-3 binding, an aliquot of the same tissue homogenate was sedimented at 40,000 × g for 15 min and the supernatant solution was discarded. The membrane pellet was resuspended (Polytron) in the original volume of buffer and resedimented, and the resultant pellet was resuspended using a smooth glass homogenizer fitted with a Teflon pestle, in 10 mM sodium-potassium phosphate buffer (pH 7.4) and 150 mM NaCl. Radioligand binding was evaluated with 2 nM [3H]HC-3 (Vickroy et al. 1984), with incubation for 20 min at room temperature, followed by rapid vacuum filtration onto Whatman GF/C filters (presoaked for 30 min with 0.1% polyethylenimine in buffer). The nonspecific component was defined as radioligand binding in the presence of an excess concentration of unlabeled HC-3 (10 µM). Binding values were expressed relative to membrane protein. The selection of a single, subsaturating concentration of radioligand for the binding analysis enables the detection of changes in either Kd or Bmax but does not permit distinction between effects on the two parameters. This strategy was necessitated by two factors. First, the amount of tissue in each neonatal brain region was insufficient for the multiple determinations required for Scatchard analysis. Second, we needed to measure binding in hundreds of membrane preparations, involving three treatment groups and four tissues at multiple ages, each involving as many as 16 individual animals in each group at each age point, while making sure to evaluate age-matched control and treated groups simultaneously. Previous work has shown that developmental changes in HC-3 binding reflect almost exclusively a change in Bmax (Zahalka et al. 1993a); however, the interpretation of results of the present study, which relate to HC-3 binding as an index of neural activity (Cheney et al. 1989; Jope 1979; Murrin 1980; Navarro et al. 1989; Shelton et al. 1979; Simon et al. 1976; Zahalka et al. 1992, 1993a), does not depend on a change in a specified parameter.

For m2AChR binding, membranes from the same tissue homogenate were prepared by a slightly different protocol from that used for HC-3 binding (Zahalka et al. 1993b). The original tissue homogenate was diluted with an equal volume of 10 mM

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sodium-potassium phosphate buffer (pH 7.4) and sedimented at 40,000 x g for 10 min. The resultant pellet was resuspended in phosphate buffer, and the membranes were incubated for 60 min at room temperature, using 1 mM [3H]AFDX384 with or without 1 μM atropine to displace specific binding.

**Macromolecules.** DNA was determined in aliquots of the same tissue homogenates used for cholinergic biomarkers, using a modified (Trauth et al. 2000) fluorescent dye–binding method (Labarca and Piagner 1980). Aliquots were homogenized in 50 mM sodium phosphate, 2 M NaCl, 2 mM EDTA (pH 7.4) and sonicated briefly (Virtis, Gardiner, NY). Hoechst 33258 was added to a final concentration of 1 μg/mL. Samples were then read in a spectrofluorometer using an excitation wavelength of 356 nm and an emission wavelength of 458 nm, and were quantitated using standards of purified DNA. The total concentration of tissue proteins was assayed from the original homogenate spectrophotometrically with bichinonic acid (Smith et al. 1985); in addition, we assessed the concentration of membrane proteins, averaging the values obtained for the two membrane preparations used for HC-3 and m2AChR radioligand binding.

**Data analysis.** To avoid type I statistical errors in subdividing the data into the different measures, brain regions, ages, and sexes, we first performed global analyses of variance (ANOVA) on data groupings corresponding to the four classes of measurements: weights, cholinergic biomarkers (ChAT, HC-3 binding, m2AChR binding), indices dependent on the number of cells (DNA content and concentration), and indices related to cell proteins (total protein/DNA, membrane protein concentration, membrane protein total protein). Because each tissue homogenate contributed multiple assessments in each category, the various determinations were treated as repeated

| Table 1. Development of biomarkers in control brain regions (males and females combined). |
|------------------------------------------|-------------------|-------------------|-------------------|-------------------|
| **Control brain regions**                | **Cholinergic biomarkers** | **DNA biomarkers** | **Cell protein biomarkers** |
|                                          | **ChAT** | **HC-3** | **m2AChR** | **DNA concentration** | **DNA content** | **Total Protein/DNA** | **Membrane protein** | **Membrane/total protein** |
|                                          | (pmol/mg protein/min) | (fmol/mg protein) | (fmol/mg protein) | (mg/g tissue) | (μg/region) | | (mg/g tissue) | | |
| Forebrain                                |          |          |          |          |          |          |          |          |          |
| PN4                                      | 20 ± 1   | 10.0 ± 0.7 | 121 ± 6 | 1.54 ± 0.03 | 475 ± 13 | 67 ± 1 | 19.3 ± 0.4 | 0.189 ± 0.004 |
| PN10                                     | 120 ± 2  | 10.4 ± 0.6 | 307 ± 6 | 1.06 ± 0.01 | 700 ± 10 | 68 ± 1 | 25.3 ± 0.5 | 0.353 ± 0.007 |
| PN15                                     | 267 ± 9  | 10.8 ± 0.6 | 439 ± 7 | 0.99 ± 0.02 | 821 ± 18 | 91 ± 2 | 31.9 ± 0.6 | 0.357 ± 0.009 |
| PN21                                     | 606 ± 5  | 12.2 ± 0.8 | 441 ± 5 | 0.92 ± 0.01 | 863 ± 15 | 103 ± 2 | 37.2 ± 0.7 | 0.397 ± 0.009 |
| ANOVA age, p < 0.0001; sex, p < 0.0001 | NS       | age, p < 0.001 |          |          |          | age, p < 0.001 | age, p < 0.0001 | age, p < 0.0001 |
| Repeated measures                        |          |          |          | sex, p < 0.04; measure, p < 0.0001; age × measure, p < 0.0001 | age × measure, p < 0.0001 |          |          |          |
| Cerebral cortex                          |          |          |          |          |          |          |          |          |
| PN30                                     | 436 ± 7  | 17.9 ± 0.6 | 495 ± 12 | 0.91 ± 0.01 | 710 ± 9 | 108 ± 2 | 34.9 ± 0.5 | 0.355 ± 0.006 |
| PN60                                     | 405 ± 11 | 14.0 ± 0.5 | 377 ± 6 | 0.68 ± 0.02 | 615 ± 19 | 154 ± 5 | 42.3 ± 0.9 | 0.413 ± 0.009 |
| ANOVA age, p < 0.02; sex, p < 0.05       | age, p < 0.005; age, p < 0.001 | age, p < 0.001; sex, p < 0.001 |          |          |          | age, p < 0.0001 | age, p < 0.0001 | age, p < 0.0001 |
| Hippocampus                              |          |          |          |          |          |          |          |          |
| PN30                                     | 418 ± 16 | 12.3 ± 0.6 | 392 ± 8 | 0.75 ± 0.02 | 79 ± 3 | 116 ± 4 | 32.1 ± 0.6 | 0.368 ± 0.006 |
| PN60                                     | 499 ± 12 | 14.3 ± 1.3 | 310 ± 4 | 0.64 ± 0.01 | 76 ± 3 | 135 ± 2 | 38.5 ± 0.5 | 0.452 ± 0.007 |
| ANOVA age, p < 0.001; sex, p < 0.005     | NS       | age, p < 0.001 | age, p < 0.001 | age × sex, p < 0.05 |          | age, p < 0.001 | age, p < 0.0001 | age, p < 0.0001 |
| Striatum                                 |          |          |          |          |          |          |          |          |
| PN30                                     | 1,088 ± 30 | 75.3 ± 4.3 | 538 ± 12 | 0.87 ± 0.01 | 91 ± 5 | 110 ± 2 | 38.6 ± 0.4 | 0.408 ± 0.006 |
| PN60                                     | 1,229 ± 84 | 67.3 ± 5.4 | 427 ± 17 | 0.57 ± 0.03 | 73 ± 6 | 133 ± 3 | 41.0 ± 2.1 | 0.570 ± 0.042 |
| ANOVA age, p < 0.009; sex, p < 0.003     | region, p < 0.001; age × region, p < 0.001 | region, p < 0.001; age × region, p < 0.001 |          |          |          | age, p < 0.001; age × region, p < 0.007 | age, p < 0.001; age × region, p < 0.001 | age, p < 0.001; age × region, p < 0.001 |
| ANOVA (3 regions)                        |          |          |          |          |          |          |          |          |
| Repeated measures                        |          |          |          |          |          |          |          |          |
| ANOVA (three regions)                    |          |          |          |          |          |          |          |          |

NS, not significant.
measures. As described in “Results,” this initial test indicated treatment effects that differed among the different measures, so data were then examined separately for each measure, again using a multivariate ANOVA (treatment, region, age, sex). Where appropriate, this was followed by post hoc evaluations of each treatment group compared with the controls, with Fisher’s protected least significant difference; however, where treatment effects did not interact with other variables, only the main effect was recorded without testing of individual differences. 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).

Values from birth to PN21 were determined in the forebrain, whereas in older animals the forebrain was subdivided into its constituent subregions. Accordingly, the global tests incorporated two data groupings corresponding to these separable regions and ages. However, the cerebral cortex constituted approximately 80% of the forebrain weight; accordingly, we verified differences across the two age groupings by performing ANOVAs incorporating all ages and comparing treatment effects in the forebrain and cerebral cortex.

Data are presented as means and standard errors. To facilitate comparisons across multiple tissues, ages, and variables, the effects of CPF are given as the percentage change from control values (Table 1), at the postnatal ages (days) indicated on the abscissa. Until weaning, because of the absence of a treatment effect, only the main effect was recorded without testing of individual differences. 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).

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### Results

#### Development of biomarkers in control brain regions

Variables reflecting cholinergic synaptic outgrowth showed distinctly different ontogenetic profiles from those delineating general cell development. Table 1 shows comparisons among the different biomarkers for control rat brain regions from PN4 through PN60; statistical evaluations were conducted first by repeated-measures ANOVA for the three groupings (cholinergic biomarkers, DNA biomarkers, cell protein biomarkers) and then, as justified by the interactions of development (age) with other variables, by ANOVA across regions for each separate marker, and finally by ANOVA for each region. At each sequential stage, we looked for sex differences, and where there were none, we disregarded any sex differences that appeared in lower-order tests so as to avoid type I statistical errors. Where the sex differences were maintained, we examined individual values for which males and females differed. The values represent males and females combined because, although sex was significant in overall testing for some variables, there were actually very few individual differences, and these are identified below.

### 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 Mallinkrodt Baker (Paris, KY). \( [14C] \) Acetyl-coenzyme A (specific activity, 44 mCi/mmol; diluted with unlabeled compound to 6.7 mCi/mmol), \( [3H] \) HC-3 (specific activity, 161 Ci/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 for all other reagents.

Not surprisingly, body and brain region weights increased monotonically with development (data not shown), with significant differences between males and females that showed increasing divergence with age: body weight, main effect of sex \( (p < 0.0001) \), age \( \times \) sex interaction \( (p < 0.0001) \); forebrain from PN4 to PN21, main effect of sex \( (p < 0.02) \); brain regions on PN30 and PN60, main effect of sex \( (p < 0.02) \), age \( \times \) sex interaction \( (p < 0.02) \). By PN60, males weighed approximately 400 g, whereas females weighed about 260 g. Brain region weights showed smaller sex differences, ranging from only a few percentage points to 10% lower values in females by PN60 (data not shown).

ChAT activity showed marked developmental increases in the forebrain over the first 3 postnatal weeks, corresponding to the period of rapid synaptogenesis (Table 1). Values in the three subregions then stabilized by PN30, such that only minor changes were evident thereafter; the striatum showed the highest ChAT activity. HC-3 binding, which is responsive to nerve impulse activity, showed a different pattern, with only a small increase over the first 3 weeks and larger increases thereafter. The subregions again showed marked differences from each other, with striatal values much higher than those in the cerebral cortex or hippocampus. m2AChR binding, like ChAT activity, showed substantial increases in the forebrain between PN4 and PN21; values then declined slightly in all subregions between PN30 and PN60. There were significant sex differences for two of the cholinergic biomarkers: females averaged 5% higher values for ChAT in the cerebral cortex (main effect of sex), and 13% higher for the hippocampus (main effect of sex); striatal HC-3 binding showed overall sex differences

### Figure 1

Effects of prenatal CPF exposure on postnatal development of cholinergic biomarkers: (A) ChAT activity; (B) HC-3 binding; and (C) m2AChR binding. NS, not significant. Data are presented as percentage change from control values (Table 1), at the postnatal ages (days) indicated on the abscissa. Until weaning, measurements were made in the whole forebrain; for determinations in adolescence and adulthood, the forebrain was divided into its constituent subregions: cerebral cortex, hippocampus, striatum. ANOVA results across ages and regions appear at the bottom of A–C. Tests of individual points where the CPF group differs from the corresponding control were carried out only where the global test indicated an interaction of treatment \( \times \) age; this occurred only for the forebrain, which showed a main treatment effect across the first three age points in the low-dose group (arrows) and a decrease on PN21 (asterisk). Testing of individual subregions on PN30 and PN60 was not conducted because of the absence of a treatment \( \times \) region interaction. Results for males and females were combined because of the absence of a treatment \( \times \) sex effect.
(age × sex interaction), but the effects were inconsistent because males had lower values than females on PN30 but higher values on PN60.

As expected by the transition of neural cells from mitosis to differentiation, the DNA concentration in the forebrain was high on PN4 and fell over the ensuing 3 weeks (Table 1). Nevertheless, because the regional weight increased 3-fold over the same period, the DNA content approximately doubled, representing the continued acquisition of new cells through at least PN21. The DNA concentration and content in the subregions continued to decline slightly between PN30 and PN60. DNA content showed significant sex differences that reflected the slightly larger brain region weights in males: males averaged 5% higher values in the forebrain during the preweaning period, rising to 15% in the cerebral cortex and hippocampus by PN60.

In keeping with postnatal growth of neural cells and the expansion of cell surface area attending axonogenesis and synaptogenesis, all three indices of cell proteins showed substantial age-related increments (Table 1). The ratio of total cell proteins to DNA (protein per cell) nearly doubled between PN4 and PN21 in the forebrain, and the subregions showed further increments between PN30 and PN60. The membrane protein concentration, which more closely represents the expansion of the cell surface area, showed larger proportional increases; this was verified by comparison of ratio of membrane protein to total protein, which showed significant augmentation with age in the forebrain and across the three subregions. Although development of the cell protein markers showed an overall sex dependence, none of the values was significant when assessed individually for the forebrain, and only one difference was noted in the subregions (higher values for females in the striatum on PN60).

All the biomarkers chosen to reflect cholinergic synaptic development and function, cell numbers, and cell protein complement showed robust developmental changes from birth to adulthood. Accordingly, these indices were evaluated to characterize the potential for delayed neurotoxicity after prenatal CPF exposure.

**General effects of CPF.** In agreement with earlier results (Qiao et al. 2002), the two CPF doses used here straddled the threshold for impairment of maternal growth but did not alter litter size, neonatal viability, or the sex ratio. The weight gain from the start of treatment (GD17) to the last day of treatment (GD20) was 49 ± 2 g in controls (n = 35) and 46 ± 2 g in the group receiving 1 mg/kg/day of CPF (n = 35, not significant vs. control) but only 31 ± 3 g in the 5 mg/kg/day group (n = 32, p < 0.0001 compared with controls or the low-dose group). Nevertheless, there was no reduction in the number of offspring (12.9 ± 0.3 in controls, 12.7 ± 0.3 in the 1 mg/kg/day group, 12.4 ± 0.3 in the 5 mg/kg/day group, not significant), nor were there any alterations in neonatal viability. Body weight of the offspring showed no significant differences throughout the period from PN4 to PN60 (data not shown). There was a statistically significant overall difference in forebrain weights (p < 0.02 for main treatment effect; p < 0.03 for treatment × age), but after subdivision into the individual ages, only one age displayed differences: on PN4, control forebrain weight was 310 ± 4 mg, compared with 309 ± 4 mg in the group exposed to the low dose of CPF (not significant) and 284 ± 6 mg in the high-dose group (p < 0.002). All other ages were not significant, nor were there any statistically significant differences across the three brain regions for measurements on PN30 and PN60 (data not shown).

Before evaluating the effects of CPF on each individual biomarker, global statistical analyses were conducted across the three classes of measurements to protect against type I statistical errors and to validate the subdivision into separate determinations. For the cholinergic assessment battery (ChAT, HC-3 binding, mAChR binding), repeated-measures ANOVA across all ages in the forebrain indicated a main treatment effect of CPF (p < 0.05) and interactions of treatment × measure (p < 0.005) and treatment × age × measure (p < 0.06). Examination of the three subregions on PN30 and PN60 showed similar global effects: main treatment effect (p < 0.007) and treatment × measure interaction (p < 0.0003). Because the cerebral cortex represents 80% of the forebrain tissue mass, we also evaluated the cholinergic markers across all six age points for the forebrain and cerebral cortex, and again obtained the same effects and interactions: main treatment effect (p < 0.0009), treatment × measure (p < 0.0001), treatment × age × measure (p < 0.1).

For the grouping of variables related to the number of cells (DNA concentration, DNA content), values from PN4 through PN21 for the forebrain displayed a main treatment effect (p < 0.03) and interactions of treatment × measure (p < 0.02) and treatment × age × measure (p < 0.03). For the subregions on PN30 and PN60, although the main effect of CPF was at the margin of significance (p < 0.06), the interaction of treatment with the other variables was statistically significant (treatment × age × region × measure, p < 0.05). Combining the measurements in the forebrain with those in the cerebral cortex so as to evaluate effects over all six age points, CPF affected the DNA-related variables in an interactive manner: treatment × age (p < 0.03), treatment × measure (p < 0.1), treatment × age × measure (p < 0.04), treatment × age × sex × measure (p < 0.03).

The measures of cell proteins (total protein/DNA, membrane protein concentration, membrane/tissue protein) similarly showed effects at all levels. For the preweaning age points in the forebrain, there were interactions of treatment × measure (p < 0.004) and treatment × age × measure (p < 0.07). The subregions on PN30 and PN60 displayed interactions of treatment × age (p < 0.1), treatment × age × sex (p < 0.08), treatment × measure (p < 0.002), and treatment × age × region × sex × measure (p < 0.02). The full time course, evaluated across the forebrain...
and cerebral cortex, exhibited a significant main CPF effect \( (p < 0.03) \), with interactions of treatment \( \times \) age \( \times \) sex \( (p < 0.0009) \) and treatment \( \times \) sex \( \times \) measure \( (p < 0.07) \).

Based on the interaction terms obtained in the global statistical tests, we subdivided the data into the separate measures for each of the three classes of determinations. Lower-order tests were then conducted, and except where noted, these did not show interactions of treatment \( \times \) sex or treatment \( \times \) sex \( \times \) other variables. Accordingly, data were combined for males and females for presentation, but the factor of sex was retained in the statistical design.

**Effects on cholinergic biomarkers.** During the rapid forebrain growth spurt, when ChAT activity was increasingly dramatic (Table 1), animals exposed prenatally to CPF showed small but statistically significant changes (Figure 1A). Animals receiving the low-dose regimen displayed initial enhancement of ChAT, followed by deficits on PN21. Examining the subregions on PN30 and PN60 revealed no significant difference across the cerebral cortex, hippocampus, or striatum. However, regarding the cerebrocortical values as a continuation of those in the forebrain indicated that the delayed deficits persisted through PN30: ANOVA across all ages for the forebrain and cerebral cortex indicated a treatment \( \times \) age interaction \( (p < 0.01) \), with significant deficits for PN21 and PN30 \( (p < 0.007) \). By PN60, ChAT activities were not distinguishable from control values.

In contrast to the small changes in ChAT, prenatal CPF exposure had marked effects on HC-3 binding (Figure 1B). During the preweaning phase, there were initial deficits of 20–30%, and although values tended to resolve to normal limits by weaning, binding was again subnormal in adolescence and adulthood. Again, comparing across all six age points by incorporating the forebrain in the preweaning period with the cerebral cortex for PN30 and PN60 gave the same result: a significant main treatment effect of CPF \( (p < 0.0001) \). The magnitude of the effect of CPF on HC-3 binding was statistically distinguishable from that on ChAT \( (p < 0.02) \) for treatment \( \times \) measure in the forebrain or in the three subregions.

Because HC-3 binding is responsive to cholinergic nerve impulse activity, whereas ChAT is a static marker for nerve terminals, the ratio of HC-3 binding to ChAT activity represents an index of activity per nerve terminal (Aubert et al. 1996; Happe and Murrin 1992; Navarro et al. 1989; SLOTKIN ET AL. 1990; ZAHALKA ET AL. 1992, 1993a). Accordingly, we also evaluated the effects of CPF on this ratio, using the primary data from Figure 1. For the forebrain values in preweaning animals, CPF elicited significant reductions in the HC-3:ChAT ratio \( (p < 0.02 \text{ overall}; p < 0.008 \text{ for control vs. CPF 1 mg/kg/day}; p < 0.02 \text{ for control vs. CPF 5 mg/kg/day}) \). Similarly, the subregional determinations on PN30 and PN60 indicated a reduction in the activity ratio \( (p < 0.02 \text{ overall}; p < 0.04 \text{ for control vs. CPF 1 mg/kg/day}; p < 0.004 \text{ for control vs. CPF 5 mg/kg/day}) \).

The changes in ChAT activity and HC-3 binding were selective in that they were not shared by a different cholinergic marker, mAChR binding (Figure 1C). The lack of significant differences was apparent during both the phase of rapid receptor acquisition (PN4–21) and subsequent postweaning decline (PN30–60). The same result was obtained when values for the forebrain and cerebral cortex were evaluated together. The lack of statistically significant effects on mAChR binding did not result from higher variability, because the significant deficits of HC-3 binding were readily distinguishable from the absence of effects on receptors \( (p < 0.03 \text{ for treatment \( \times \) measure in the forebrain}; p < 0.0004 \text{ for the three subregions}) \).

**Effects on DNA biomarkers.** If the effects of CPF on cholinergic systems are secondary to general impairment of cell development, then it would be expected that DNA biomarkers would show adverse effects preceding, or occurring simultaneously with, those for cholinergic markers. However, during the neonatal growth spurt, in which the forebrain was experiencing a doubling of cell number (Table 1), we did not find any significant changes in DNA concentration (Figure 2A) or content (Figure 2B) in the group exposed to 1 mg/kg/day of CPF. With the higher CPF exposure (5 mg/kg/day), there was no significant change in the marker of cell packing density (DNA concentration), but because the forebrain weight was reduced by about 10% on PN4, the DNA content was similarly subnormal; this difference resolved by PN10. In adolescence (PN30) and adulthood (PN60), there was a significant overall reduction in DNA concentration across the three subregions. Although the differences in DNA content were not significant by themselves, this negative result should be interpreted with caution, because the significant differences in DNA concentration could not be distinguished from the absence of significant differences in DNA content: none of the regions showed a treatment \( \times \) measure interaction when the two DNA variables were compared in a repeated-measures test. Indeed, comparing values for the forebrain and cerebral cortex across all six age points indicated a significant treatment \( \times \) age interaction for DNA content \( (p < 0.02) \).

![Figure 3](image-url)  
**Figure 3.** Effects of prenatal CPF exposure on postnatal development of cell protein biomarkers for (A) relative cell size (total protein/DNA) and membrane surface area (B) membrane protein concentration, (C) membrane total protein. NS, not significant. Data are presented as percentage change from control values (Table 1), at the postnatal ages (days) indicated on the abscissa. Until weaning, measurements were made in the whole forebrain; for determinations in adolescence and adulthood, the forebrain was divided into its constituent subregions. ANOVA results across ages and regions appear at the bottom of A–C. Tests of individual points where the CPF group differs from the corresponding control were not carried out because either the global test or the lower-order tests after separation of values by region failed to indicate an interaction of treatment \( \times \) age. For variables showing an interaction of treatment \( \times \) region, ANOVA results for the regions appear below the appropriate bar clusters within the panels. Results for males and females were combined because of the absence of a treatment \( \times \) sex effect after separation by region.
Nevertheless, the onset of decreased HC-3 binding in the forebrain of the low-dose group was statistically distinguishable from the absence of early changes in the DNA concentration (treatment × measure, p < 0.03) or content (p < 0.04).

Effects on protein biomarkers. Because the adverse effects of CPF on cell development might entail changes in cell growth or neuritic differentiation, we also assessed the effects of prenatal CPF exposure on protein biomarkers of cell size and cell membrane area during the period of rapid initial growth and during the transition from adolescence to adulthood. The ratio of total cell protein to DNA was subnormal in the immediate neonatal period in animals exposed to CPF prenatally (Figure 3A). However, by adolescence and adulthood, values became elevated in the cerebral cortex and hippocampus. The membrane protein concentration showed a different pattern from that of total protein: values tended to be subnormal in both the preweaning and postweaning period (Figure 3B). To compare the specific effects on membrane proteins, we evaluated the ratio of membrane to total protein (Figure 3C): prenatal CPF exposure did not have a statistically significant effect in the forebrain during the preweaning period, but by PN30 and PN60, values became subnormal in the cerebral cortex and hippocampus. As above, combining the forebrain values with those of the cerebral cortex did not change the conclusions (total protein, p < 0.007 for treatment × age; membrane protein, p < 0.009 for the main treatment effect; membrane/total protein, p < 0.05 for treatment, p < 0.1 for treatment × age).

Discussion

Earlier work demonstrated the characteristics of CPF-induced developmental neurotoxicity consequent to postnatal exposure: brain 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. Deficits in cholinergic function appear almost immediately (Dam et al. 1999) and persist into adolescence and adulthood (Slotkin et al. 2001), accompanied by cognitive defects related to impaired cholinergic function (Levin et al. 2001). In contrast, prenatal CPF causes much less overall cell damage and loss (Qiao et al. 2002), but there may be specific, focal effects in forebrain areas populated by cholinergic neurons (Lassiter et al. 2002; Qiao et al. 2002; White et al. 2002). The present results indicate that, despite the initial sparing, prenatal CPF exposure elicits marked alterations that emerge in the postnatal period. Using treatment regimens that lie below the threshold for fetal growth impairment and that span the threshold for fetal brain cholinesterase inhibition (Qiao et al. 2002), we identified postnatal deficits in cholinergic activity that persisted into adulthood, associated with, but not necessarily caused by, delayed, generalized effects on brain cell development. Although CPF does concentrate in milk (Mattsson et al. 2000), its short biologic half-life (hours; Hunter et al. 1999; Lassiter et al. 1998) makes it extremely unlikely that the observed effects of prenatal exposure, terminated 2 days before birth, reflect an indirect postnatal exposure from residual CPF.

For ChAT activity, a constitutive marker for cholinergic nerve terminals, low-dose (1 mg/kg/day) prenatal CPF exposure elicited slight initial postnatal elevations that eventually regressed to normal or subnormal values. The same effect was noted in the fetal brain (Qiao et al. 2002), likely representing promotion of cell differentiation consequent to cholinergic trophic effects (Hohmann and Berger-Sweeney 1998; Morley and Happe 2000; Navarro et al. 1989; Slotkin 1999). Presumably, at the higher dose (5 mg/kg/day), these are offset by deleterious actions, producing an “inverted-U” dose–effect curve; the same phenomenon has been noted for behavioral outcomes of these treatments (Levin et al. 2002). Notably, however, CPF did not elicit any long-term deficits in ChAT that indicate a specific loss of cholinergic nerve terminals. In contrast, however, HC-3 binding, which is responsive to neuronal activity (Aubert et al. 1996; Happe and Murrin 1992; Klemm and Kuhar 1979; Navarro et al. 1989; Simon et al. 1976; Slotkin et al. 1990; Zahalka et al. 1992, 1993a), was markedly impaired. The reduction in presynaptic activity was not compensated by up-regulation of cholinergic receptors, as we found no significant alteration of mAChR binding. Accordingly, the major change elicited by prenatal CPF administration appears to be a reduction in cholinergic synaptic function, effects that were demonstrable even at exposure to 1 mg/kg/day, a dose that lies below the threshold for maternal and fetal growth impairment and for inhibition of fetal brain cholinesterase (Qiao et al. 2002).

The time course for the effects of prenatal CPF on HC-3 binding gave additional insight into the underlying processes. Deficits were apparent in the early neonatal period, before the formation of the majority of forebrain synapses; in this phase, ChAT was rising rapidly in the controls, whereas the activity marker, HC-3 binding, was relatively static. Therefore, the fact that CPF lowered HC-3 binding while initially promoting ChAT activity indicates two separable actions on early stages of cholinergic synaptic development. HC-3 binding was nearly normal by weaning, yet marked decrements reappeared in adolescence and adulthood. Accordingly, prenatal CPF elicits delayed-onset alterations, disrupting the “program” for the emergence of cholinergic activity. The functional significance of the later-occurring neurochemical anomalies is corroborated by behavioral deficits in cholinergic contributions to working and reference memory that emerge in adolescence and adulthood after fetal CPF exposure (Levin et al. 2002). Notably, the same exact pattern is elicited by prenatal exposure to nicotine (Zahalka et al. 1992), and therefore it is tempting to speculate that these long-term alterations reflect disruption consequent to elevated cholinergic activity during a critical period in fetal development. In addition to inhibiting cholinesterase, CPF, like nicotine, interacts directly with nicotinic cholinergic receptors (Katz et al. 1997), such that exposures that do not cause significant cholinesterase inhibition might still affect cholinergic signaling. Regardless of the underlying mechanism, the important fact is that otherwise nontoxic prenatal exposures to CPF elicit deficits in cholinergic function that influence cognitive performance in adolescence and adulthood.

Our results for biomarkers of cell development address the issue of whether the alterations in cholinergic systems represent selective actions of CPF or whether they are secondary to general disruption of brain cell proliferation and differentiation. Here, it is useful to divide development into two primary stages as defined by the normal ontogenetic patterns of cellular biomarkers. The rise in DNA content, denoting cell acquisition, was essentially complete by PN15, after which further brain region growth involved cell enlargement only (decreases in the DNA concentration and increases in cell protein markers). We did find an adverse effect of prenatal CPF exposure on DNA content on PN4, but the deficit was limited to the high-dose group and disappeared almost immediately, whereas effects on HC-3 binding occurred with both dose regimens, were present past PN4, and persisted in adolescence and adulthood. During the transition from adolescence to adulthood, there were statistically significant reductions in DNA concentration, an index of cell packing density, but these were small in magnitude and were inconsistent from region to region or between the two doses. Nevertheless, because this period is marked by synaptic remodeling and apoptosis in late-developing regions such as the hippocampus (Altman and Bayer 1990; Bayer 1983; Bayer et al. 1982; Huttenlocher 1990; McWilliams and Lynch 1983; Scheetz and Constantine-Paton 1994), it is conceivable that the late-onset phase of the deficits in
HC-3 binding is related to a more widespread, delayed neurotoxic effect. On the other hand, it is equally likely that the causal relationship is in the opposite direction, namely, that the emergence of cellular deficits reflects a primary impairment of cholinergic activity. Improper synapse formation and decreased synaptic function interfere with transsynaptic signals that are critical to the release of trophic factors that sustain neuronal integrity (Frade and Barde 1998; Schwartz 1991). Cholinergic control of nerve growth factor synthesis and release, for example, is particularly prominent in the cerebral cortex and hippocampus (Frade and Barde 1998), and disruption of cholinergic communication, precisely in the early postnatal period in which we found that CPF evoked a decrease in the HC-3 marker, evokes widespread, subsequent cellular and synaptic disruption (Berger-Sweeney and Hohmann 1997; Hohmann et al. 1988, 1991). Accordingly, one likely scenario is that focal interference with the differentiation of a small, targeted population of fetal neurons (Lassiter et al. 2002; Qiao et al. 2002; White et al. 2002) leads to deficits in cholinergic activity that emerge postnatally, with a consequent impediment to neurotrophic factors that sustain neuronal integrity, culminating in late-onset neural damage and behavioral deficits (Levin et al. 2002).

Similar interpretations can be applied to the effects of prenatal CPF exposure on protein biomarkers of cell growth (total protein/DNA) and neuritic extension (membrane/protein). Changes for these factors were more widespread and robust than those for DNA content or concentration, although again, the magnitude and timing of the effects appeared to be incompatible with their playing a causative role in the deterioration of cholinergic function. In particular, we found late-onset cell enlargement in the cerebral cortex and hippocampus (increased total protein/DNA). Because this biochemical change could represent either a larger perikaryon or more neuritic extensions, we also assessed membrane protein markers in both absolute (membrane protein concentration) and relative (membrane/protein) terms; an increase in extensions would augment membrane protein proportionally more than total protein. However, these markers indicated a decrease in absolute and relative membrane protein, consistent with a loss of membrane surface area, implicitly representative of reductions in neuritic projections. Although that interpretation needs to be confirmed with morphologic examinations, this type of defect could clearly contribute to a generalized decrease in neural activity, intensifying the loss of cholinergic function. In vitro models of neural development have demonstrated direct impairment of axonogenesis by CPF (Das and Barone 1999), but the late emergence of the deficits in protein biomarkers argues against this as an underlying mechanism and is more compatible with delayed-onset interference with neurotrophic regulation.

The biochemical approach used in the present study has two distinct limitations in interpreting the results related to measurements of DNA and cell proteins. First, homogenization of brain regions containing diverse neuronal groupings means that even drastic effects on a specific set of neurons may go unnoticed because of dilution with unaffected areas. Accordingly, the fact that we found significant alterations in biomarkers of cell number, cell packing density, and cell protein complement indicates that much larger changes may be identified when neuroanatomical approaches are taken. Second, unlike the situation with biomarkers that are specific to cholinergic innervation, measurements of DNA and protein are common to neurons and glia. Given the fact that forebrain neurogenesis is nearly completed by birth, our finding of late-onset changes in the postnatal period suggests that many of these changes involve either postmitotic damage or effects on glial cells. We recently reported that the CPF regimen used here also produces late-onset deficits in the expression of myelin basic protein, a marker that is specific for oligodendrocytes (Garcia et al. 2003), suggesting that at least some of the changes may involve glial cells. However, these changes do not rule out the possibility of late-onset neuronal apoptosis or indirect neuronal damage secondary to adverse effects on glia. Indeed, two preliminary reports suggest that prenatal CPF exposure can disrupt architectural organization of specific forebrain subregions, including apoptosis and changes in cell migration (Lassiter et al. 2002; White et al. 2002). As discussed above, such alterations are likely to trigger later neuronal loss (Berger-Sweeney and Hohmann 1997; Hohmann and Berger-Sweeney 1998; Hohmann et al. 1988, 1991). Again, neuroanatomical studies demonstrating targeted effects on different types of cells and on different brain nuclei, and specifically assessing apoptosis, are needed to resolve this issue.

In conclusion, prenatal CPF exposure compromises the subsequent development of cholinergic synaptic function, characterized by deficits in an index of neural activity (decreased HC-3 binding) without substantial loss of nerve terminals (little or no change in ChAT). These changes persist into adolescence and adulthood and correspond to the long-term defects in cholinergic components of working and reference memory (Levin et al. 2002). CPF also causes delayed abnormalities of cellular characteristics in forebrain areas involved in cognitive function, with changes indicative of reduced cell density, increased cell size, and loss of neuritic extensions. The alterations in cholinergic function do not appear to depend directly upon the timing and magnitude of the general cellular deficits; rather, the cellular effects may actually result from defective synaptic transmission. CPF has adverse effects on cholinergic synaptic function and behavior that are elicited even at exposures below the threshold for overt fetal or maternal toxicity, with a window of vulnerability extending from prenatal through neonatal stages (Dam et al. 1999; Levin et al. 2001, 2002; Slotkin et al. 2001). Accordingly, developmental neurotoxicity consequent to fetal or childhood CPF exposure may occur in settings in which immediate symptoms of intoxication are absent.

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