Developmental Neurotoxicity Elicited by Prenatal or Postnatal Chlorpyrifos Exposure: Effects on Neurospecific Proteins Indicate Changing Vulnerabilities

Stephanie J. Garcia, Frederic J. Seidler, and Theodore A. Slotkin

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

The developmental neurotoxicity of the organophosphate pesticide chlorpyrifos (CPF) is thought to involve both neurons and glia, thus producing a prolonged window of vulnerability. To characterize the cell types and brain regions involved in these effects, we administered CPF to developing rats and examined neuroprotein markers for oligodendrocytes (myelin basic protein, MBP), for neuronal cell bodies (neurofilament 68 kDa, NF68), and for developing axons (neurofilament 200 kDa, NF200). Prenatal CPF administration on gestational days (GDs) 17–20 elicited an immediate (GD21) enhancement of MBP and NF68; by postnatal day (PN) 30, however, there were deficits in all three biomarkers, with the effect restricted to females. Exposure in the early postnatal period, PN1–4, did not evoke significant short-term or long-term changes in the neuroproteins. However, with treatment on PN11–14, we found reductions in MBP in the immediate posttreatment period (PN15, PN20) throughout the brain, and deficiencies across all three proteins emerged by PN30. With this regimen, males were targeted preferentially. The sex-selective effects seen here for the GD17–20 and PN11–14 regimens match those reported earlier for subsequent behavioral performance. These results indicate a shift in the populations of neural cells targeted by CPF, dependent upon the period of exposure. Similarly, developmental differences in the sex selectivity of the biochemical mechanisms underlying neurotoxicant actions are likely to contribute to discrete behavioral outcomes. *Key words:* **brain development, chlorpyrifos, glia, myelin basic protein, neurofilament protein, oligodendrocytes.** *Environ Health Perspect* **111:297–303 (2003). doi:10.1289/ehp.5791 available via** *http://dx.doi.org/* **[Online 30 October 2002]**

The neurobehavioral consequences of fetal or childhood pesticide exposure are a major biomedical and societal concern (Eriksson 1997; Eriksson and Talts 2000; Landrigan 2001; Landrigan et al. 1999; May 2000; Physicians for Social Responsibility 1995). Chlorpyrifos (CPF), one of the most widely used organophosphate pesticides, is a developmental neurotoxicant specifically targeting the immature brain (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1999). Recent U.S. regulatory provisions have thus curtailed the domestic use of CPF (U.S. Environmental Protection Agency 2000), although worldwide use, particularly in agriculture, will continue for the foreseeable future. The actual mechanisms by which CPF perturbs neural development remain elusive and complicated. A variety of *in vitro* and *in vivo* model systems indicate impairment of neural cell replication and differentiation, as well as disruption of axonogenesis and synaptic function, all culminating in disruption of behavioral performance (for reviews, see Barone et al. 2000; Pope 1999; Slotkin 1999). Superimposed on neurospecific effects, CPF may exert more generalized cytotoxicity from oxidative stress that affects the developing brain because of its lower reserve of antioxidants (Bagchi et al. 1995, 1996; Crumpton et al. 2000).

One unusual property of CPF is the apparently wide window of maturational vulnerability, with adverse neurodevelopmental effects noted for exposures ranging from embryonic stages through the postweaning period (Barone et al. 2000; Buznikov et al. 2001; Dam et al. 2000; Garcia et al. 2001, 2002; Levin et al. 2001; Pope 1999; Qiao et al. 2002; Rice and Barone 2000; Roy et al. 1998; Slotkin 1999; Slotkin et al. 2001, 2002). Recent findings from our laboratory (Garcia et al. 2001, 2002; Qiao et al. 2001) and others (Barone et al. 2000; Monnet-Tschudi et al. 2000) suggest that CPF has a shifting cellular target, initially impairing development of neurons and subsequently affecting glia, which develop much later (Aschner 2000; Garcia et al. 2001, 2002; Monnet-Tschudi et al. 2000; Qiao et al. 2001). All three major classes of glia, astrocytes, oligodendrocytes, and microglia, are critical to brain development (Aschner et al. 1999; Barone et al. 2000; Compston et al. 1997; Guerri and Renau-Piqueras 1997). Astrocytes provide nutrition, structural support, and protection from oxidative stress, and additionally guide migrating neurons; oligodendrocytes ensheath axons with myelin; and microglia serve as macrophages (Aschner et al. 1999; Barone et al. 2000; Compston et al. 1997; Guerri and Renau-Piqueras 1997). In most brain regions, neurons exit the cell cycle and undergo terminal differentiation relatively early (i.e., prenatally in the rat), whereas gliogenesis and glial cell differentiation continue well into postnatal development (Aschner et al. 1999; Barone et al. 2000; Cameron and Rakic 1991; Compston et al. 1997; Guerri and Renau-Piqueras 1997; Wiggins 1986). The cerebellum, the brain region that develops last, is an exception, with a peak of neurogenesis in the second postnatal week (Rodier 1988). The finding that CPF targets glia as well as (or perhaps more than) neurons (Barone et al. 2000; Garcia et al. 2001, 2002; Monnet-Tschudi et al. 2000; Qiao et al. 2001) thus provides a partial explanation for the exceptionally long maturational period in which brain development is sensitive to this agent. Interference with the numerous roles of glia in synapse formation (Ullian et al. 2001), axon migration, and myelination (Compston et al. 1997; Riederer et al. 1992) may all contribute to eventual adverse outcomes.

In a recent study (Garcia et al. 2002), we evaluated the effects of CPF exposure on glial fibrillary acidic protein (GFAP), an astrocyte-associated protein (Garcia et al. 2002). In keeping with the concept that CPF targets glial development, postnatal treatment initially decreased GFAP levels. However, several weeks later we found elevations of GFAP, a pattern typically associated with gliosis in response to injury to other neural cells (Norton et al. 1992; O'Callaghan 1993). This implied that astroglia were not the only target for CPF. Furthermore, with prenatal CPF treatment, unless the dose was raised above the threshold for systemic toxicity, we did not find any changes in GFAP, despite the fact that fetal CPF exposure evokes changes in brain morphology (Lassiter et al. 2002; White et al. 2002) and subsequent behavioral anomalies (Levin et al. 2002). Accordingly, in the current study we examined the potential for CPF to perturb the development of other neural cell populations, using a design similar to our GFAP study (Garcia et al. 2002). We compared the effects of prenatal versus postnatal CPF exposure on the development and regional targeting of myelin basic protein (MBP) and the small

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

We thank C.A. Tate and M.M. Cousins for technical assistance.

Research was supported by U.S. Public Health Service grants ES10387 and ES10356 and by a STAR fellowship from the U.S. Environmental Protection Agency.

Received 28 May 2002; accepted 5 August 2002.

(68 kDa) and large (200 kDa) neurofilament proteins (NF68 and NF200). MBP, an oligodendrocyte marker, is a major component of myelin and increases with oligodendrocyte differentiation and myelination, primarily during the second and third postnatal weeks in the rat (Wiggins 1986). The neurofilament proteins are found in neurons and assemble to form intermediate filaments that regulate axon growth, axoplasmic transport, and axon caliber (Capano et al. 2001; Escurat et al. 1990; Lee and Cleveland 1996; Schlaepfer and Bruce 1990). NF68 is expressed early in the postmitotic development of neuronal cell bodies, whereas NF200 expression is associated primarily with the later growth of axons (Capano et al. 2001; Carden et al. 1987; Escurat et al. 1990; Lee and Cleveland 1996; Schlaepfer and Bruce 1990; Yang et al. 1996). By examining these neuroprotein markers, we can now elucidate how the cellular target and regional specificity for CPF-induced alterations of brain development shift with development.

Materials and Methods

Animal treatments. All experiments were conducted 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 (Zivic Laboratories, Pittsburgh, PA) were housed in breeding cages, with a 12-hr light-dark cycle and free access to food and water. CPF (Chem Service Inc., West Chester, PA) was dissolved in dimethylsulfoxide to provide rapid and complete absorption (Whitney et al. 1995) and was injected subcutaneously in a volume of 1 mL/kg body weight. For prenatal CPF exposure, dams were injected daily with CPF in doses of 1–40 mg/kg of body weight, or with vehicle. Twenty-four hours after the last injection [gestational day (GD) 21], fetuses were removed and brains were dissected. Additional dams in the control, 1 mg/kg, and 5 mg/kg treatment groups were allowed to reach term, at which point their pups were randomized within treatment groups and redistributed to the nursing dams with a litter size of 10 to maintain a standard nutritional status. Randomization was repeated at intervals of several days; in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Animals were weaned on PN21, and all determinations used no more than one male and one female from each litter.

For measurements on GD21, fetal brains were separated into forebrain and the rest of the brain by making a cut rostral to the thalamus. Because the cerebellum represents an inappreciable proportion of brain weight on GD21, the remainder was designated "midbrain + brainstem." For studies on PN5, PN10, PN15, and PN21, brains were dissected into three regions: blunt cuts were made through the cerebellar peduncles, whereupon the cerebellum (including flocculi) was lifted from the underlying tissue. Then, as for the fetal brain, a cut was made rostral to the thalamus to separate the forebrain from the midbrain + brainstem. This dissection, which follows the planes of the fetal and neonatal rat brain, includes the corpus striatum, hippocampal formation, and neocortex within the area designated "forebrain." The region designated "midbrain + brainstem" includes the midbrain, colliculi, pons, and medulla oblongata (but not cervical spinal cord), as well as the thalamus. On PN30, brains were dissected into the same three major regions, and the midbrain + brainstem and forebrain were further subdivided into midbrain, brainstem, cerebral cortex, hippocampus, and striatum.

For postnatal CPF treatments, all pups were randomized the day after birth and redistributed to the dams as already described. For studies of CPF effects in the first few days after birth, animals were given 1 mg/kg daily on PN1–4. For studies in older animals, which tolerate higher doses (Campbell et al. 1997; Pope and Chakraborti 1992; Pope et al. 1991; Whitney et al. 1995), daily treatment with 5 mg/kg was given on PN11–14. These doses have been shown previously to alter neural function without eliciting overt systemic toxicity (Campbell et al. 1997; Song et al. 1997; Whitney et al. 1995). Behavioral differences remain apparent, or may first emerge, after weaning, despite the rapid recovery of cholinesterase activity (Dam et al. 2000; Song et al. 1997). Neither regimen evokes weight loss or mortality (Campbell et al. 1997; Dam et al. 1998; Johnson et al. 1998; Song et al. 1997), and in the current study we did not observe any changes in suckling or maternal caretaking. Animals were weaned and selected from each litter as detailed above.

Tissues were frozen in liquid nitrogen and stored at –45°C.

Assays. Neurospecific proteins were assayed by a modified (Garcia et al. 2002) dot-immunobinding technique (O'Callaghan 1991; O'Callaghan et al. 1999). Briefly, tissues were homogenized with a sonic probe (Heat Systems-Ultrasonics, Inc., Plainview, NY) in nine volumes of hot 1% sodium dodecyl sulfate (Bio-Rad, Hercules, CA) and were diluted in 120 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $NaHCO_3$, 0.7% Triton X-100, 0.2% NaN_3, and 5 mM HEPES (pH 7.4). Ten-microliter aliquots containing 2–15 µg protein were blotted onto prewashed nitrocellulose membranes (0.2 µm; Bio-Rad). Blots were dried and fixed in 25% isopropanol, 10% acetic acid, and 65% water, incubated for 5 min in Tris-buffered saline (200 mM NaCl, 50 mM Tris, 0.002% NaN_3, pH 7.4) and treated for 1 hr with a blocking solution of 0.5% gelatin (EIA grade; Bio-Rad) in Tris-buffered saline. Blots were then incubated in blocking solution containing 0.1% Triton X-100 with addition of the appropriate antibodies (Chemicon International Inc, Temecula, CA): rabbit polyclonal anti-NF68 (diluted 1:2,000), rabbit polyclonal anti-NF200 (diluted 1:2,000), or mouse monoclonal anti-MBP (diluted 1:300). Because the MBP antibody was monoclonal, blots also were incubated with rabbit anti-mouse IgG (1:500; Dako Corporation, Carpentaria, CA) in blocking solution with Triton. To assess antibody binding, blots were incubated with 20 µCi [^{125}I]Protein-A (specific activity, 382 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) in blocking solution with Triton X-100, washed repeatedly, dried overnight, and counted for radioactivity.

Each blot included serial dilutions of a single preparation of adult midbrain, which was then used to construct a standard curve to normalize the values across blots. Thus, although values are reported in relative units, quantitative comparisons across treatments, regions, and ages could be carried out. Because the development of NF200 is delayed relative to MBP and NF68 (Capano et al. 2001; Escurat et al. 1990; Lee and Cleveland 1996; Schlaepfer and Bruce 1990), determinations for this neurospecific protein were limited to PN30.

Study design and data analysis. Experiments were conducted on four different cohorts of animals. Two cohorts were used for CPF exposure on GD17–20, with each cohort comprising at least 12 dams per treatment group. One cohort received 0, 1, 2, or 5 mg/kg daily, whereas the second received 0, 10, 20, or 40 mg/kg per day. The other two cohorts, again with at least 12 litters per treatment group, were used for CPF exposures on PN1–4 and PN11–14. For presentation purposes, control values were combined across cohorts, as they did not differ significantly from each other. However, treatment differences were established using only the control values for each matched cohort.

Data were compiled as means and standard errors. Differences between treatment groups were assessed first by a multivariate ANOVA (data log-transformed because of heterogeneous variance) incorporating all relevant variables: treatment (control, CPF), treatment period (regimen), age, region, sex, and neuroprotein (MBP, NF68, NF200). Whenever the initial ANOVA indicated an interaction of CPF treatment with other variables, data were separated according to the

interactive variable(s) and lower-order ANOVAs were conducted. Individual differences between control and CPF groups were then evaluated *post hoc* by Fisher's protected least significant difference. However, in the absence of interaction terms, only main treatment effects were compiled, without subdivision into individual determinations. For convenience, some data are presented as the percentage change from the corresponding controls, but statistical significance was always assessed on the original data. Control data were combined across the different treatment regimens (GD17–20, PN1–4, PN11–14) for presentation purposes, but in all cases, CPF effects were established using only the matched control groups. For experiments involving dose–effect determinations, data were analyzed by multiple regression, incorporating all variables (dose, brain region, neuroprotein). Significance was assumed at the level of $p < 0.05$ for main effects; for interactions at $p < 0.1$, we examined lower-order main effects after subdivision of the interactive variables (Snedecor and Cochran 1967).

Results

Development of neuroproteins in controls. Both MBP and NF68 were measurable at all ages from GD21 through PN30, whereas accurate determinations of NF200 were limited to PN30. In control brain, MBP (Figure 1A) was low in the fetus, without significant distinctions between the midbrain + brainstem and the forebrain. Regional differences emerged postnatally, with the greatest increases evident in postnatal weeks 2–4, in agreement with earlier findings (Wiggins 1986). By PN30, MBP in the midbrain + brainstem was 5 times that in the forebrain or cerebellum. Further division of the brain into subregions on PN30 revealed even larger distributional differences: values were highest in the brainstem, followed by the midbrain and striatum, whereas low values were seen in the cerebral cortex and hippocampus. In keeping with the fact that neurogenesis precedes myelination (Rodier 1988), NF68 showed an earlier ontogenetic increase than MBP (Figure 1B), with significant regional differences present as early as GD21. Again, by PN30, there were large disparities between the midbrain + brainstem and the forebrain or cerebellum; with regional subdivision, the rank order was brainstem > midbrain > striatum > cerebral cortex > hippocampus. On PN30, NF200 (Figure 1C) showed a similar hierarchy. These patterns for ontogeny and regional specialization of the neurofilament proteins are in agreement with earlier results (Capano et al. 2001; Schlaepfer and Bruce 1990). None of the regions showed significant overall sex differences or interactions of sex × other variables, so control values were combined across males and females. However, as shown below, sex differences did emerge in the effects of CPF on these neuroproteins.

Global statistical analyses of chlorpyrifos effects. We evaluated several data groupings for main treatment effects and interactions prior to subdividing data into separate treatment regimens, sexes, and brain regions. We first compared effects across all three regimens (GD17–20, PN1–4, and PN11–14), limiting the determinations to two brain regions (midbrain + brainstem and forebrain), one time point (24 hr posttreatment), and without regard to sex, as all three regimens shared only these variables. The result (CPF × regimen, $p < 0.1$) indicated the need to examine each regimen separately. Next, on PN30, we compared the effects of CPF on the striatum, a region for which all three regimens and all three neuroproteins (MBP, NF68, and NF200) were evaluated, this time including sex as a factor. The outcome indicated the need to subdivide the data by regimen and sex (CPF × regimen × sex, $p < 0.01$). The next grouping evaluated effects on PN30 across all six subregions (midbrain, brainstem, cerebral cortex, hippocampus, striatum, and cerebellum) for all three neuroproteins, determinations that were shared only by the two postnatal treatment regimens (PN1–4, PN11–14), again including the sex variable. This also indicated the need to look for treatment-related differences after separating the data by regimen and sex (CPF × sex, $p < 0.06$; CPF × regimen × sex, $p < 0.06$). Finally, because MBP and NF68 were evaluated across three time points (24 hr after the last injection, 5 days later, PN30) for both of the postnatal treatment regimens, we conducted analyses for those two regimens, weighting the values for subregions on PN30 to obtain estimates for forebrain (cerebral cortex + hippocampus + striatum) and midbrain + brainstem. For the PN11–14 treatment group, we obtained interaction terms indicative of treatment effects separable by sex and by specific neuroprotein (CPF × sex, $p < 0.04$; CPF × sex × neuroprotein, $p < 0.06$).

Chlorpyrifos treatment on GD17–20. CPF exposure from GD17–20 had a significant effect on MBP and NF68 on GD21, 24 hr after the last injection, characterized by a significant increase (main effect of CPF), assessed across both of the neuroproteins and both regions (Figure 2A). Because there was no interaction of treatment × other variables, we did not conduct lower-order statistical analyses for separate regions or neuroproteins, but *post hoc* analysis across those factors indicated significant increments at 5, 20 and 40 mg/kg/day of CPF. Multiple regression analysis, including the factors of dose, neuroprotein type, and brain region, confirmed the significant relationship between dose and effect ($p < 0.0001$). Earlier studies defined 10 mg/kg/day as the threshold for fetal weight deficits (Garcia et al. 2002; Qiao et al. 2002), so it was evident that the immediate effects of CPF on MBP and NF68 in the fetus involve doses spanning that threshold. Accordingly, we next assessed whether there might be

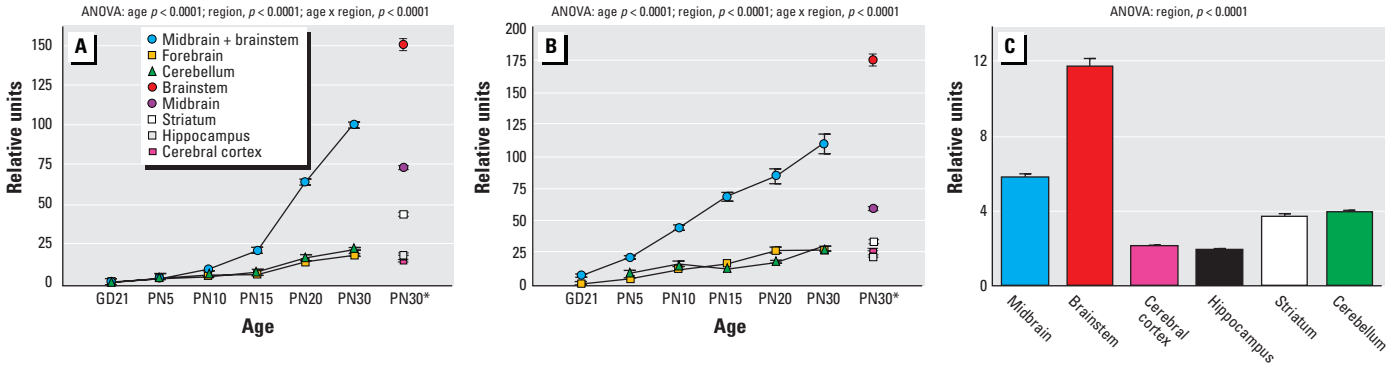

Figure 1. Development of (*A*) MBP, (*B*) NF68, and (*C*) NF200 in control rat brain regions. Data represent means and standard errors obtained from 8 animals on GD21 and from 8 to 16 animals for postnatal ages. ANOVA across regions, ages, and sex appears at the top of each panel There were no significant sex differences (no main effect of sex or interaction of sex × other variables), so the values are reported for the two sexes combined. For MBP (*A*) and NF68 (*B*), values at the right represent subdivision of the midbrain + brainstem and forebrain into their component subregions. NF200 (*C*) was evaluated only on PN30.
*Values after division of the midbrain + brainstem and forebrain into their subregions.

delayed effects of CPF after gestational administration, concentrating on the striatum, a region that we previously found to be particularly susceptible to subsequent emergence of CPF-induced damage (Garcia et al. 2002; Slotkin et al. 2002). Studies were limited to doses below the threshold for fetal growth impairment (1 or 5 mg/kg/day). On PN30, measurements across all three neuroproteins (MBP, NF68, NF200) indicated sex-dependent effects of CPF (Figure 2B). Females exposed to 5 mg/kg/day during fetal development showed a significant decrease in striatal neuroproteins on PN30. Although effects were not significant for 1 mg/kg/day when compared with control values, the lower-dose group also could not be distinguished statistically from the high-dose group, so an effect at the lower dose could not be ruled out. In contrast to females, gestational CPF administration failed to cause significant deficits in males and in fact tended to elevate the values. Again, these results were confirmed by multiple regression analysis (factors of dose and neuroprotein type), which indicated a significant dose–effect relationship in females ($p < 0.008$) but not in males.

Postnatal chlorpyrifos treatment. CPF administration on PN1–4 did not evoke statistically significant changes in MBP or NF68, assessed 24 hr after the last injection (PN5) or 5 days later on PN10 (Figure 3A). Although there was an increase in the average MBP value in the cerebellum on PN5, the results for this region were highly variable at this age, a likely consequence of its small size and especially rapid rate of growth. Similarly, there were no changes in MBP, NF68, or NF200 in any of the subregions evaluated on PN30 (Figure 3B). The absence of statistically significant effects for this treatment regimen was itself distinguishable from the significant differences seen for gestational CPF treatment: comparing the significant values for GD21 with those for PN5 (24 hr after the last injection for each regimen), treatment × regimen, $p < 0.02$. In the striatum on PN30, values for the sex-dependent effect of gestational CPF were similarly distinguishable from the lack of effect of the PN1-4 regimen in the same region, as shown by a significant interaction of treatment × regimen × sex, $p < 0.05$.

In contrast to the relative insensitivity to CPF in the early postnatal treatment group, CPF administration later in the neonatal period, PN11–14, had sex-dependent effects apparent in the immediate posttreatment period (treatment × sex × neuroprotein, $p < 0.06$) as well as on PN30 (treatment × sex, $p < 0.02$). Male rats showed significant deficits in MBP across all brain regions 24 hr after the last CPF dose (PN15) as well as 5 days later on PN20 (Figure 4A). NF68 did not show significant deficits and, if anything, was slightly increased in the midbrain + brainstem. Examination of subregions on PN30 (Figure 4B) confirmed that the deficits in MBP persisted, now accompanied by equivalent deficiencies in NF68 and NF200 (i.e., significant treatment effect across all three neuroproteins with no distinction among them). In contrast to the effects in males, female rats showed no statistically significant effects of CPF treatment on PN11–14. Rather than decreasing, values for MBP tended to increase (albeit nonsignificantly) in the forebrain on PN15 and cerebellum on PN20 (Figure 4C). Similarly, for females, there were no consistent changes across the three protein markers in brain subregions on PN30 (Figure 4D).

The preferential effect for males seen for CPF administration on PN11–14 was distinct from the lack of significant effect seen with the earlier postnatal regimen (PN1–4) or from the preferential effect on females seen with the gestational regimen: on PN30, comparing all subregions for the two postnatal regimens, treatment × sex × regimen, $p < 0.06$; on PN30, comparing values in the striatum across all three regimens (CPF treatment on GD17–20, PN1–4, PN11–14), treatment × sex × regimen, $p < 0.02$.

Discussion

In an earlier study (Garcia et al. 2002) with the astrocyte marker GFAP, we found that postnatal CPF treatment evoked an initial deficit, which is consistent with the view that CPF targets the replication and differentiation of these particular glial cells (Barone et al. 2000; Garcia et al. 2001, 2002; Monnet-Tschudi et al. 2000; Qiao et al. 2001). However, later in development, GFAP was elevated (Garcia et al. 2002), likely indicating a subsequent phase of reactive gliosis (Norton et al. 1992; O'Callaghan 1993), and thus implying that other types of neural cells were also affected adversely. In the current study, we found that either prenatal or postnatal CPF exposure evoked abnormalities in the

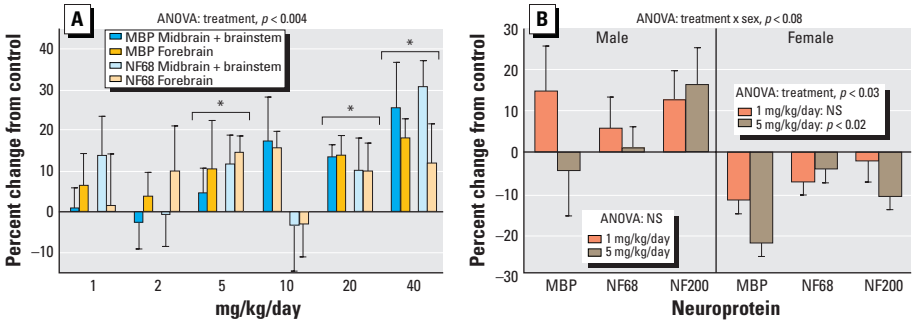

Figure 2. Effects of gestational CPF exposure (GD17–20), assessed on GD21, 24 hr after the last CPF injection (*A*), or in the striatum on PN30 (*B*). Data represent means and standard errors obtained from four animals for each determination, presented as the percent change from control values (Figure 1). For GD21 (*A*), ANOVA across both brain regions and both neuroproteins appears at the top of the panel. Separate analyses were not conducted for each region or neuroprotein because of the absence of a treatment × region or a treatment × neuroprotein interaction. For PN30 (*B*), ANOVA (shown above the panel) across all neuroproteins and both sexes indicated a separation of CPF effects by sex. Separate ANOVAs for males and females are shown within the panel.
*Doses whose values differ significantly from the controls (assessed across both regions and both neuroproteins).

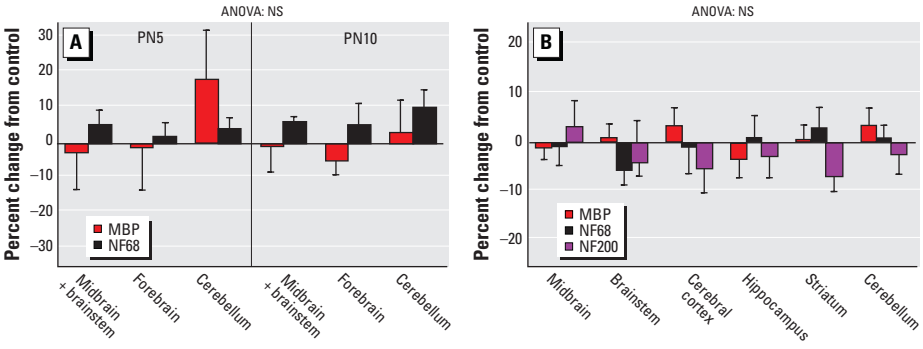

Figure 3. Effects of early neonatal CPF exposure (PN1–4) on neurospecific proteins. (*A*) Effects on MBP and NF68, assessed on PN5 (24 hr after the last CPF injection) and PN10. (*B*) MBP, NF68, and NF200 in brain subregions assessed on PN30. Data represent means and standard errors obtained from 8 to 16 animals for each determination, presented as the percent change from control values (Figure 1). ANOVA across regions, ages and neuroproteins appears at the top of each panel. Values were combined for males and females because of the absence of a treatment × sex interaction.

expression of neuroproteins associated with oligodendrocytes (MBP), with neuronal cell bodies (NF68), and with axonal projections (NF200). Furthermore, exposure during different developmental periods exerted distinct patterns; whereas prenatal or late postnatal treatment had significant effects, CPF exposure in the immediate neonatal period did not. Superimposed on these effects were differences in sex selectivity of effects for each exposure period. The finding of two separable developmental phases in which CPF affects MBP, NF68, and NF200 thus confirms the hypothesis that the developing brain represents a shifting target for CPF in which multiple processes are compromised over a wide span of vulnerability.

When we administered CPF on PN11–14, a peak period for gliogenesis, myelination, and axonogenesis (Aschner et al. 1999; Barone et al. 2000; Compston et al. 1997; Riederer et al. 1992; Wiggins 1986), we found immediate deficits in MBP and eventual deficits in all three neuroproteins across all brain regions in male rats. Combined with our earlier results showing biphasic changes in GFAP with this regimen (Garcia et al. 2002), the pattern is entirely consistent with the view that, during this stage, CPF interferes with development of multiple types of cells, followed by reactive astrogliosis. Although the PN11–14 treatment window is outside the span of neurogenesis in most of the regions affected (notably excepting the cerebellum), the critical involvement of glia in neuronal integrity, axonogenesis, and synaptogenesis could account for abnormalities including neuronal markers such as NF68 and NF200. However, it is equally likely that CPF interferes directly with neurite extension and axonogenesis (Li and Casida 1998; Sachana et al. 2001). At this time, we have no definitive explanation for the sex selectivity of the effects on neurospecific proteins, but it is noteworthy that the subsequent behavioral deficits are also preferentially expressed in males (Levin et al. 2001), a completely different pattern from that seen with CPF administration in adults. Typically, CPF affects adult females more than males (Gordon and Padnos 2000; Moser 2000), most likely because of differences in CPF catabolism (Ma and Chambers 1994). One possible mechanism for sex selectivity is the differential levels of sex steroids within the brain itself. Glial cells synthesize steroids (Jung-Testas and Baulieu 1998), and the levels of GFAP and MBP (and possibly other neurospecific proteins) are hormonally regulated (Jung-Testas and Baulieu 1998; Melcangi et al. 1998). Further research may need to focus on the impact of CPF exposure on sex hormone synthesis and levels in the developing brain and their participation in neural development.

The late gestational period (GD17–20) represented an entirely separate period of vulnerability to CPF, as characterized by immediate and delayed effects on neurospecific proteins. In this case, we saw an initial promotional effect on GD21, indicating that the consequences were completely different from those seen with the PN11–14 exposure paradigm. In our earlier study with GFAP, we also saw enhancement after gestational CPF exposure but only when the dose was raised above the threshold for fetotoxicity (Garcia et al. 2002). In the current experiments, MBP and NF68 appeared to be slightly more sensitive biomarkers of the effects of CPF. Later in development (PN30), GFAP remained within normal limits, whereas we found significant deficiencies for measurements across the other markers. Additionally, with gestational CPF treatment, the sex preference was distinct from that of the later CPF regimen, as females were affected more than males. We are thus faced with a number of interpretational questions. First, why is there an initial increase in neurospecific proteins? The immediate effects of CPF are likely to comprise direct effects of CPF on neural cell development as well as indirect effects mediated by cholinesterase inhibition (Barone et al. 2000; Mileson et al. 1998; Pope 1999; Slotkin 1999). With the gestational regimens used here, there is significant cholinesterase inhibition, and thus presumptively an increase in cholinergic effects (Qiao et al. 2002). In turn, enhanced cholinergic activity in the fetus can augment synaptic plasticity and development (Meck and Williams 1997, 1999; Montoya et al. 2000), albeit at an eventual cost if cells are forced into differentiation prematurely (Navarro et al. 1989). We recently found that in the fetus low doses of CPF can enhance some biomarkers of cholinergic cell differentiation (Qiao et al. 2002), so it is likely that the effects on MBP and NF68 reflect a similar mechanism.

The second issue raised by the effects of gestational CPF exposure is why MBP, NF68, and NF200 were affected more than GFAP (Garcia et al. 2002). Here, it is important to note that delineation of the levels of any of the neurospecific proteins does not necessarily reflect just the number of each cell type, as specific protein expression is also subject to regulation (Capano et al. 2001; Compston et al. 1997). The changes seen here thus represent the net summation of damage to each type of cell, disruption of the timetable for differentiation, changes in the concentrations

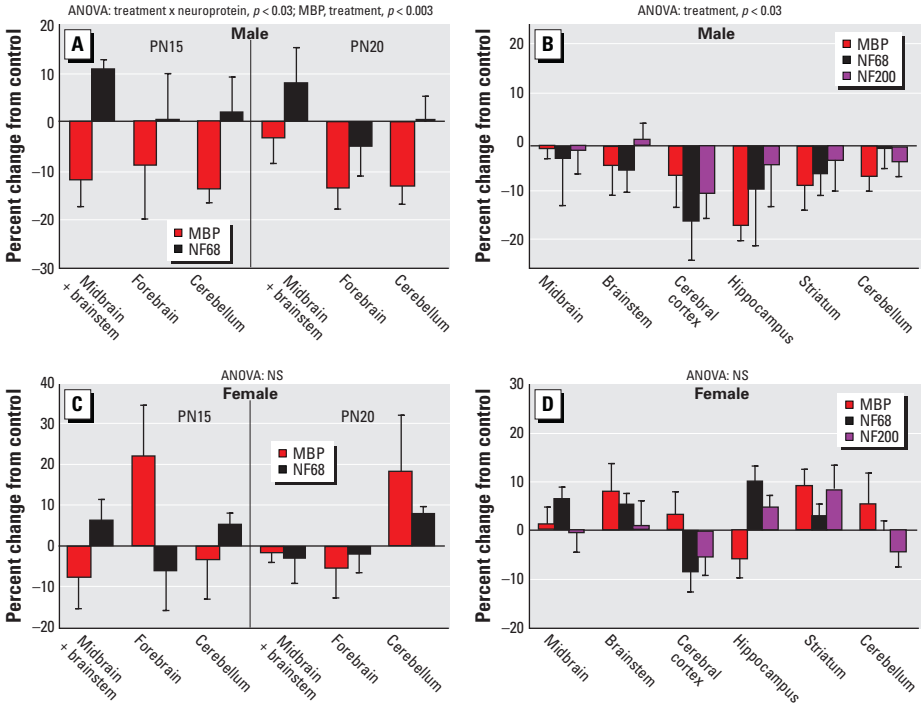

Figure 4. Effects of late neonatal CPF exposure (PN11–14) on neurospecific proteins. (*A,C*) Effects on MBP and NF68, assessed on PN15 (24 hr after the last CPF injection) and PN20. (*B,D*) MBP, NF68, and NF200 in brain subregions assessed on PN30. Data represent means and standard errors obtained from four to eight animals for each determination, presented as the percent change from control values (Figure 1). Values for males and females were separated because treatment effects were sex dependent: for PN15 and PN20 (*A,C*), treatment × sex × neuroprotein, $p < 0.06$; for PN30 (*B,D*), treatment × sex, $p < 0.02$. ANOVA across regions, ages, and neuroprotein measure appears at the top of each panel with subdivision by neuroprotein where appropriate (*A*). Individual ages or treatments for which the CPF group differs from controls were not assessed because of the absence of treatment × age or treatment × region interactions.

of each protein within its own cell type, and in the case of GFAP, the potential for reactive changes when other cells are damaged (Norton et al. 1992; O'Callaghan 1993). Axonogenesis and myelination are interrelated (Capano et al. 2001; Compston et al. 1997; Lee and Cleveland 1996), so it is likely that changes in glial markers (GFAP, MBP) interact with those for development of the neuronal neurofilament proteins. Accordingly, the greater apparent sensitivity of MBP, NF68, and NF200, compared with that of GFAP, needs to be interpreted with caution.

The sex-selective effects of gestational CPF exposure raise a third question: Are the preferential effects on females important for the eventual outcome of CPF exposure? As already discussed, a specific mechanism for sex selectivity of fetal or neonatal effects of CPF awaits elucidation. Nevertheless, it is clear that the greater effects in females seen here for neurospecific proteins have an impact on subsequent behavioral performance. We recently found that the gestational exposure paradigm, unlike postnatal treatment, selectively affects cognitive performance in females (Levin et al. 2002). Again, regardless of mechanism, our results confirm the concept that the brain represents a shifting target for CPF, with sex-selective effects that differ substantially depending upon the exposure window.

When CPF was administered in the early neonatal period, PN1–4, there were no significant alterations in the three neuroproteins, a puzzling finding in light of the clear-cut synaptic and behavioral abnormalities that result from this treatment regimen (Dam et al. 1999, 2000; Levin et al. 2001; Slotkin 1999; Slotkin et al. 2001, 2002). Indeed, in our earlier work with GFAP, we also found a limited spectrum of changes with this exposure (Garcia et al. 2002). Because the early time points involved relatively large, heterogeneous brain regions, the lack of significant effects may be misleading, as changes in discrete regions may be diluted by unaffected areas (Garcia et al. 2002). However, the same criticism could be mounted against the studies carried out in gestation or for the initial time points with the PN11–14 regimen, and we had no difficulty finding significant differences with those regimens. Certainly one possibility is that the vulnerability of the developing brain during this particular period involves other developmental processes and thus other neurospecific markers. Second, because CPF is initially promotional for MBP and NF68 with the gestational regimen and inhibitory for the PN11–14 regimen, the PN1–4 group may be in a range in which a mixture of effects is occurring, with some cell populations showing enhancement and others showing deficits. A third possibility is a technical issue: the immunoblotting technique may underestimate changes in treated tissues because of the influence of competing proteins that also change with development (O'Callaghan et al. 1999). Obviously, examination of the underlying morphologic distribution of essential neuroproteins in the affected brain regions, akin to preliminary results that have appeared for gestational exposure (Lassiter et al. 2002; White et al. 2002), will be needed to resolve these possibilities.

A similar set of circumstances probably limits the magnitude of CPF-induced changes in neuroproteins even for the regimens producing statistically significant differences. In general, we found alterations of between 10 and 30%. However, none of the probes distinguishes whether these represent changes in the numbers of oligodendrocytes (for MBP) or axons (for the neurofilament proteins), or whether protein expression changes per cell or axon. Accordingly, larger inherent alterations in cell or axon number may be partially offset by compensatory upregulation of the corresponding neuroprotein in unaffected cells. Furthermore, as shown by the control data for PN30, the individual subregions contained within the forebrain or midbrain + brainstem have widely disparate levels of neuroproteins. Thus, even a robust change in a small region, such as the hippocampus or striatum, would not be detected as a change in forebrain values, as the bulk of the tissue is cerebral cortex. Obviously, the answer to these questions lies in morphologic examinations using immunocytochemical techniques that localize the neuroproteins to anatomically discrete brain nuclei.

The results of this study reinforce the view that the critical window of vulnerability of the developing brain to CPF extends from gestational exposure through later periods of development in which glial-neuronal interactions influence brain architecture, circuitry, and function. Thus, exposures occurring during childhood are likely to be as important as those occurring prenatally. The correlations between our neurochemical findings for a shift in sex selectivity between fetal and neonatal exposures, and the resultant behavioral outcomes, point out the need to consider sex as an important variable delineating the developmental neurotoxicity of CPF.

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