Developmental Neurotoxicity Elicited by Gestational Exposure to Chlorpyrifos: When Is Adenylyl Cyclase a Target?

Armando Meyer,[1] Frederic J. Seidler,[2] Mandy M. Cousins,[2] and Theodore A. Slotkin[2]

[1]Centro de Estudos da Saúde do Trabalhador e Ecologia Humana, Escola Nacional de Saúde Pública, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil; [2]Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina USA

The developmental neurotoxicity of chlorpyrifos (CPF) involves mechanisms over and above cholinesterase inhibition. In the present study, we evaluated the effects of gestational CPF exposure on the adenylyl cyclase (AC) signaling cascade, which regulates the production of cyclic AMP, a major controller of cell replication and differentiation. In addition to basal AC activity, we assessed the AC response to direct enzymatic stimulants [forskolin, manganese (Mn^{2+})]; the response to isoproterenol, which activates signaling through β-adrenoceptors (βARs); and the concentration of βAR binding sites. CPF administered to pregnant rats on gestational days (GD) 9–12 elicited little or no change in any components of AC activity or βARs. However, shifting the treatment window to GD17–20 produced regionally selective augmentation of AC activity. In the brainstem, the response to forskolin or Mn^{2+} was markedly stimulated by doses at or below the threshold for observable toxicity of CPF or for inhibition of fetal brain cholinesterase, whereas comparable effects were seen in the forebrain only at higher doses. In addition, low doses of CPF reduced βAR binding without impairing receptor-mediated stimulation of AC. These results indicate that signal transduction through the AC cascade is a target for CPF during a discrete developmental period in late gestation, an effect that is likely to contribute to the noncholinergic component of CPF's developmental neurotoxicity. *Key words:* adenylyl cyclase, β-adrenoceptor, brain development, chlorpyrifos, organophosphate insecticides. *Environ Health Perspect* 111:1871–1876 (2003). doi:10.1289/ehp.6468 available via *http://dx.doi.org/* [Online 29 August 2003]

Along with other widely used organophosphate insecticides, chlorpyrifos (CPF) is undergoing increasing scrutiny because of its developmental neurotoxicity (Barone et al. 2000; Landrigan 2001; Landrigan et al. 1999; May 2000; Physicians for Social Responsibility 1995; Pope 1999; Rice and Barone 2000; Slotkin 1999. In press). Although originally all organophosphates were thought to elicit neurodevelopmental damage through inhibition of cholinesterase (Mileson et al. 1998; Pope 1999), it is now apparent that other mechanisms play an important, perhaps predominating role, involving concentrations below the threshold for the systemic toxicity associated with cholinergic hyperstimulation (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999, In press). CPF itself, as distinct from CPF oxon, the active metabolite that inhibits cholinesterase, disrupts the fundamental processes of brain development, such as DNA synthesis (Dam et al. 1998; Whitney et al. 1995), expression and function of macromolecular constituents and transcription factors that control cell differentiation (Crumpton et al. 2000; Garcia et al. 2001; Johnson et al. 1998; Schuh et al. 2002), and expression and function of neurotransmitters and their receptors that act as neurotrophins in the developing brain (Buznikov et al. 2001; Dam et al. 1999a, 1999b; Howard and Pope 2002; Huff et al. 2001; Liu et al. 2002; Yanai et al. 2002; Zhang et al. 2002).

Although these studies provide a reasonable doubt as to the importance of cholinesterase inhibition for developmental neurotoxicity of CPF, they leave open the issue of which cellular targets are the most critical, most sensitive, or primary in eliciting long-term changes in nervous system development. One pathway that has received much attention is that mediated by the intracellular second messenger cyclic AMP (cAMP), which ubiquitously coordinates the critical transition from cell replication to cell differentiation (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Van Wijk et al. 1973). In brain development, cAMP ultimately influences cell division, differentiation, axonal outgrowth, neural plasticity, and programmed cell death (Shaywitz and Greenberg 1999; Stachowiak et al. 2003), events known to be targeted by CPF (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999. In press). Furthermore, neurotransmitter receptors that control adenylyl cyclase (AC), the enzyme responsible for cAMP production, and AC itself have been found to be targets for CPF (Auman et al. 2000; Huff and Abou-Donia 1995; Huff et al. 1994, 2001; Olivier et al. 2001; Schuh et al. 2002; Song et al. 1997; Ward and Mundy 1996; Yanai et al. 2002; Zhang et al. 2002). One of the most sensitive effects involves changes in the transcription factors that are downstream targets for cAMP and that are known to participate in the activation of the genes necessary for cell differentiation (Schuh et al. 2002).

These findings thus raise the possibility that actions on the AC pathway are among the critical targets of CPF in the developing brain. In the present study we explore this prospect with an *in vivo* exposure model, using CPF regimens that bracket the threshold for cholinesterase inhibition and resultant maternal/fetal toxicity (Qiao et al. 2002, 2003). We concentrated on two phases of development, an early stage involving formation of the neural tube, gestational days (GD) 9–12, and a later stage involving the transition from replication to differentiation of major neuronal cell populations (GD17–20). In both periods, CPF elicits mitotic abnormalities, apoptosis, and architectural anomalies in the developing brain at exposures that are not otherwise embryotoxic (Lassiter et al. 2002; Roy et al. 1998; White et al. 2002). At lower exposure levels, CPF-induced damage is not immediately apparent, but synaptic and functional abnormalities appear later, in adolescence and adulthood (Levin et al. 2002; Qiao et al. 2002, 2003). Thus, if the production of cAMP is involved in the adverse effects of CPF on brain development, effects on the AC signaling pathway should be evident immediately upon exposure to these lower exposures, preceding the delayed-onset anomalies.

The potential effects of CPF on AC were assessed in several ways. First, we evaluated basal enzymatic activity. Second, we determined the response to two AC stimulants, forskolin and manganese (Mn^{2+}). Because the two stimulants act at different epitopes on the AC molecule, the preference for one over the other reflects shifts in molecular conformation, primarily influenced by the AC isoform (Zeiders et al. 1999b). Third, we probed the AC response to specific receptor-mediated activation with isoproterenol, a β-adrenoceptor (βAR) agonist that links to AC by activating the stimulatory G-protein, G_s. This receptor has defined neurotrophic roles in brain cell development and is a postulated target for CPF (Auman et al. 2000; Dreyfus 1998; Garcia et al. 2001; Kasamatsu 1985;

Address correspondence to T.A. Slotkin, Box 3813 DUMC, 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

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Kulkarni et al. 2002; Kwon et al. 1996; Morris et al. 1983; Popovik and Haynes 2000; Schwartz and Nishiyama 1994; Slotkin et al. 1989; Song et al. 1997; Yanai et al. 2002).

Materials and Methods

Materials. Animals were purchased from Zivic Laboratories (Pittsburgh, PA, USA). CPF was purchased from Chem Service (West Chester, PA, USA). [^{125}I]Iodopindolol (specific activity, 2,200 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA, USA), and cAMP radioimmunoassay kits were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). All other chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO, USA).

Animal treatments. All experiments using live animals were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996). 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. For exposure during neurulation, dams were injected daily with CPF at 1 or 5 mg/kg body weight on GD9–12. Dams were decapitated, and fetal tissues were harvested without distinction by sex on GD17 and GD21. For later gestational exposure (GD17–20), dams were given CPF daily at 1, 2, 5, 10, 20, or 40 mg/kg, and tissues were collected on GD21. Control animals received DMSO injections on the same schedules. For samples collected on GD17, we analyzed the whole brain, whereas in GD21 samples the forebrain was separated from 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 rest of the brain was considered to represent primarily the brainstem. This dissection, which follows the natural planes of the fetal and neonatal rat brain, includes the corpus striatum, hippocampal formation, and neocortex within the area designated as "forebrain." The region designated as "brainstem" includes the midbrain, colliculi, pons, and medulla oblongata (but not cervical spinal cord), as well as the thalamus. All tissues were frozen with liquid nitrogen and maintained at –45°C until assayed.

Membrane preparation. Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, USA) in 39 volumes of ice-cold buffer containing 145 mM NaCl, 2 mM $MgCl_2$, and 20 mM Tris (pH 7.5), and the homogenates were sedimented at 40,000 × *g* for 15 min. The pellets were washed twice by resuspension (Polytron) in homogenization buffer, followed by resedimentation, and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in a buffer consisting of 250 mM sucrose, 2 mM $MgCl_2$, and 50 mM Tris (pH 7.5).

Assays. To evaluate βAR binding, aliquots of membrane preparation were incubated with [^{125}I]iodopindolol (final concentration, 67 pM), in 145 mM NaCl, 2 mM $MgCl_2$, 1 mM sodium ascorbate, and 20 mM Tris (pH 7.5), for 20 min at room temperature in a total volume of 250 µL. Incubations were stopped by dilution with 3 mL of ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto Whatman GF/C filters, which were then washed with additional buffer and counted by liquid scintillation spectrometry. Nonspecific binding was assessed by displacement with 100 µM isoproterenol. Iodopindolol binds to both β_1ARs and β_2ARs equally, which is important in light of the presence of both subtypes in the developing brain and their effective linkage to AC (Erdtsieck-Ernste et al. 1991; Pittman et al. 1980; Slotkin et al. 1994, 2001).

For assessment of AC activity, aliquots of the same membrane preparation were incubated for 10 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM $MgCl_2$, 1 mg/mL bovine serum albumin, and a creatine phosphokinase-ATP–regenerating system consisting of 10 mM sodium phosphoreatine and 8 IU/mL phosphocreatine kinase, with 10 µM guanosine triphosphate (GTP) in a total volume of 250 µL. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3,000 × *g* for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay. Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay period and was linear with membrane protein concentration; concentrations of cofactors were optimal, and in particular, higher concentrations of GTP produced no further augmentation of activity. In addition to measuring basal AC activity, we assessed the response to βAR stimulation (100 µM isoproterenol), as well as the response to the direct AC stimulants forskolin (100 µM) and Mn^{2+} (10 mM). These concentrations of each stimulant produce maximal responses, as assessed in previous studies (Auman et al. 2000, 2001; Zeiders et al. 1997, 1999a).

Data analysis. Because the treatments were given to the dams, only one fetus was used from each dam, so the number of determinations represents the number of dams. The fetuses were derived from the same litters as those used in two previous studies on cell damage and cholinergic biomarkers; therefore effects on cholinesterase activity, maternal and fetal body weights, and other litter characteristics have been published elsewhere (Garcia et al. 2002; Qiao et al. 2002).

Data are presented as mean ± SE. For convenience, some results are given as the percent change from control values, but statistical evaluations were always conducted on the original data. To establish treatment differences in receptor binding or AC activity, a global analysis of variance (ANOVA; data log transformed whenever variance was heterogeneous) was first conducted across *in vivo* treatment groups, age, brain region, and the five types of measurements made on the membranes (βAR binding, AC activity under four different conditions); the AC measurements were considered to be repeated measures because each membrane preparation was used for the multiple types of determinations. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and AC measures that differed from control values; these were conducted by lower-order ANOVAs followed, where appropriate, by Fisher's protected least significant difference to identify individual values for which the CPF groups differed from the corresponding control. For all tests, we assumed significance for main treatment effects at $p < 0.05$; however, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran 1967).

For presentation, control values from GD21 samples were combined across both cohorts (controls used for CPF administration on GD9–12 and GD17–20). However,

Table 1. Development of βAR binding and AC activities in controls.

Measure	GD17 whole brain (n = 6)	GD21 forebrain (n = 17)	GD21 brainstem (n = 18)
βAR binding (fmol/mg protein)	4.7 ± 0.3	8.0 ± 0.3	10.9 ± 0.5[a]
Basal AC (pmol/min/mg protein)	83 ± 5	92 ± 3	589 ± 28[a]
Isoproterenol-stimulated AC (pmol/min/mg protein)	91 ± 6	94 ± 4	636 ± 27[a]
Forskolin-stimulated AC (pmol/min/mg protein)	177 ± 15	226 ± 11	1,175 ± 59[a]
Mn^{2+}-stimulated AC (pmol/min/mg protein)	484 ± 41	526 ± 11	1,814 ± 78[a]

Values were combined across both cohorts (controls used for CPF administration on GD9–12 and on GD17–20); however, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.
[a]Significant difference between GD21 forebrain and brainstem.

statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.

Results

Development of* βAR *binding and AC in controls. βARs in both the forebrain and brainstem were higher than the values in whole brain in samples collected on GD17, and regional differences were apparent, with higher binding in the brainstem (Table 1). Similarly, AC activities in GD21 samples were much higher in the brainstem than in the forebrain. To assess whether the differences in βARs corresponded to enhanced AC sensitivity to receptor stimulation, we assessed the response to the isoproterenol relative to basal activity and to the maximum G_s-sensitive AC response as assessed with forskolin (Figure 1A). Across all regions, isoproterenol caused a small but significant stimulation over basal activity (ratio > 1, $p < 0.03$). However, the response was significant only for GD17 whole brain ($p < 0.008$) and GD21 brainstem ($p < 0.002$) and not for GD21 forebrain. Similarly, although GD17 whole brain and GD21 brainstem showed an equivalent proportion of isoproterenol response relative to forskolin, the value was significantly lower for GD21 forebrain. Thus, the absolute concentration of βARs did not provide the primary determinant of the response to isoproterenol. The higher AC activity seen in the brainstem was also accompanied by differential effects on the response to the two direct AC stimulants, forskolin and Mn^{2+} (Figure 1B). Although forskolin stimulation was relatively consistent as a proportion to basal activity, the Mn^{2+}-mediated response in the brainstem was 50% lower. Calculated as the forskolin/Mn^{2+} ratio, values were 0.37 ± 0.01 in whole brain collected on GD17 and 0.43 ± 0.02 in the GD21 forebrain, whereas it was significantly higher in the GD21 brainstem (0.65 ± 0.02, $p < 0.0001$ vs. brainstem).

Systemic toxicity of CPF. As reported previously (Garcia et al. 2002; Qiao et al. 2002), the threshold for CPF-induced impairment of maternal growth was 5 mg/kg with treatment on either GD9–12 or GD17–20, but fetal brain growth was unaffected even at the highest doses (data not shown). Neither the early nor the late treatment paradigm affected the number of fetuses or fetal viability. Fetal brain cholinesterase showed significant inhibition at ≥ 5 mg/kg (Qiao et al. 2002).

CPF exposure on GD17–20. Before examining the effects of CPF on each variable and each brain region, a global ANOVA was performed across both regions and all measurements so as to avoid type 1 statistical errors that would otherwise result from multiple tests on the same data set. The overall test indicated a significant main effect of CPF ($p < 0.003$) and interactions of treatment with region and type of measurement: $p < 0.007$ for treatment × region, $p < 0.0001$ for treatment × measure, and $p < 0.03$ for treatment × region × measure. Accordingly, the results were separated into the two regions for further analysis of treatment effects on each measure.

In the forebrain, animals treated with CPF from GD17 through GD20 displayed robust (> 40%) βAR decreases at 2 mg/kg, a dose below the threshold for systemic toxicity and at which cholinesterase inhibition is barely detectable (Qiao et al. 2002) (Figure 2A). However, the response displayed distinct hormesis (i.e., was nonmonotonic), disappearing as the dose was raised above the toxicity threshold. Across all AC measures, CPF elicited a net increase in activity (main effect), but the magnitude of enhancement differed among the various stimulants (treatment × measure interaction). Basal and isoproterenol-stimulated AC activity showed no significant changes overall, whereas the responses to forskolin and Mn^{2+} showed major increases only at doses of > 10 mg/kg. When activities were determined relative to basal AC, there were some specific differences from the pattern seen for absolute AC activity, but the overall pattern was similar (Figure 2B). Isoproterenol-mediated responses were significantly elevated by small amounts, and the enhanced responses to the two direct AC stimulants were fully evident. Nevertheless, all these effects involved CPF doses of ≥ 5 mg/kg. There were no changes in the forskolin/Mn^{2+} response ratio that would have accompanied a shift in the AC isoform (Zeiders et al. 1999b).

In the brainstem, CPF elicited alterations in βAR binding and AC activities that were in the same direction as those seen in the forebrain, but the dose–effect relationships were distinctly different (Figure 3A). The decrement of βAR binding was evident even at the lowest dose of CPF, which lies below the threshold for detectable cholinesterase inhibition (Qiao et al. 2002); again, the response was hormetic and disappeared once the dose was raised above the toxicity threshold. Overall stimulation of AC displayed differential effects depending on the test stimulant (treatment × measure interaction). In this case, unlike in the forebrain, every single measure of AC showed significant augmentation after CPF treatment. Responses displayed hormesis for basal and isoproterenol-stimulated AC. For forskolin and Mn^{2+}, the enhancement was evident at 2 mg/kg, a lower dose than that required for effects in the forebrain. Because of the differential effects on disparate measures of AC activity, we reexamined the responses as relative ratios (Figure 3B). Although the absolute response to isoproterenol was augmented, the effect was actually no greater than the change

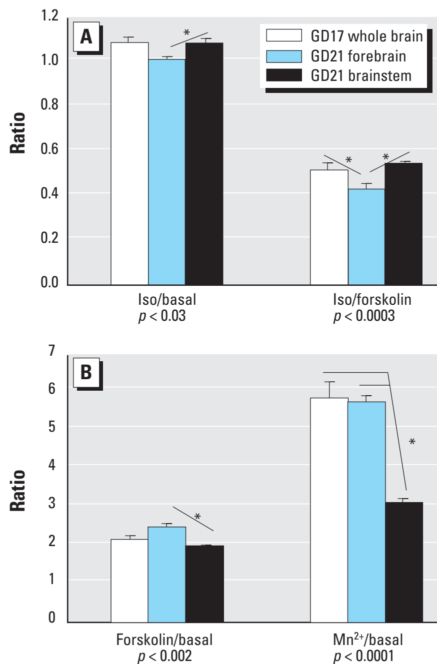

Figure 1. Development of AC responses to isoproterenol (*A*) and to forskolin and Mn^{2+} (*B*) relative to basal AC activity in controls. Iso, isoproterenol. Data are presented as mean ± SE of six determinations on samples collected on GD17 and 17–18 determinations for those collected on GD21. ANOVA results for each measure appear within the figure, and lines and asterisks denote individual groups that differ significantly from each other.

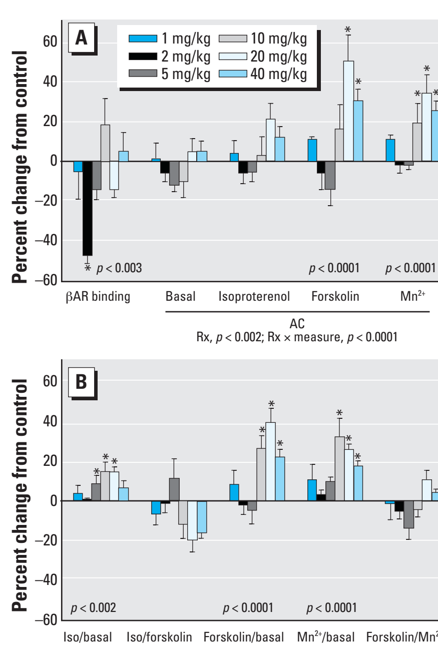

Figure 2. Effects of CPF exposure on GD17–20 on forebrain βAR binding and AC activity measured on GD21. Abbreviations: Iso, isoproterenol; Rx, treatment. (*A*) Effects on absolute activities. (*B*) Activity ratios. Data represent mean ± SE obtained from five to seven animals for each group. ANOVA results for each measure appear within the figure, and asterisks denote individual groups that differ significantly from the corresponding control.

in basal AC; accordingly, the isoproterenol/basal activity ratio was unaffected. In contrast, the isoproterenol/forskolin response ratio showed significant decrements, indicating that βAR-mediated responses were suboptimal after CPF treatment. Forskolin- and Mn^{2+}-stimulated AC activities remained significantly elevated after correction for basal AC, but the effects were not as robust until the dose was raised above 5 mg/kg. As was true in the forebrain, the brainstem also showed no change in the forskolin/Mn^{2+} activity ratio.

CPF exposure on GD9–12. For examination of the effects of CPF during neurulation, the dose range was more restricted, encompassing exposures below and up to the threshold

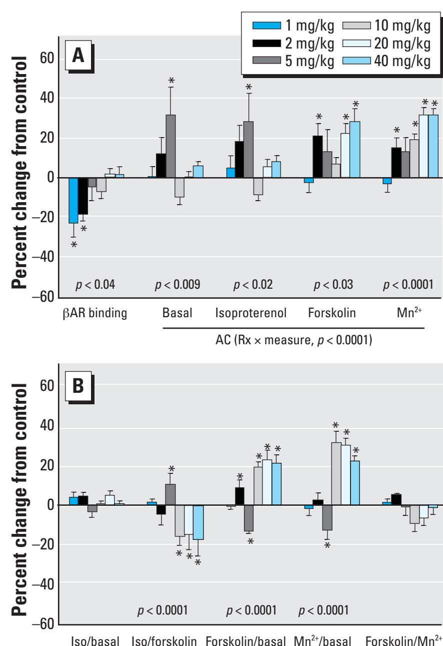

Figure 3. Effects of CPF exposure on GD17–20 on brainstem βAR binding and AC activity measured on GD21. Abbreviations: Iso, isoproterenol; Rx, treatment. (*A*) Effects on absolute activities. (*B*) Activity ratios. Data represent mean ± SE obtained from five to seven animals for each group. ANOVA results for each measure appear within the figure, and asterisks denote individual groups that differ significantly from the corresponding control.

for systemic toxicity (Qiao et al. 2002). Across all measures and the three different tissues (GD17 whole brain, GD21 forebrain, GD21 brainstem), global ANOVA indicated a significant interaction of treatment × measure ($p < 0.03$), and accordingly, we then assessed each measurement separately. This lower-order test indicated significant effects on βAR binding ($p < 0.04$ for main treatment effect, $p < 0.09$ for treatment × tissue) but not for AC activities. The absence of significant overall effects on AC should be interpreted with caution, however, because it mixes together the effects in whole brain on GD17 with those of the two separate regions on GD21. Restricting the analysis to the latter measurements, we detected a significant overall decrement in AC at the highest CPF dose ($p < 0.0006$ for main effect). In any case, the direction of change with this regimen was opposite that obtained with treatment on GD17–20 and was statistically distinguishable from those effects ($p < 0.06$ for treatment × region × regimen).

Examining each age and tissue independently, the effects of CPF on GD17 were relatively minor and did not achieve statistical significance for any of the measurements (Figure 4A). By GD21, there was significant augmentation of βAR binding in the forebrain Figure 4B), with effects fully evident at 2 mg/kg, a dose below the threshold for systemic toxicity (Qiao et al. 2002). No such effect was seen in the brainstem (Figure 4C), and the regional difference was statistically robust ($p < 0.02$ for treatment × region). As noted above, AC activities were significantly decreased overall across the two regions on GD21 at the highest dose, although the absence of a treatment × measure interaction did not permit us to examine the significance of each measurement separately.

Discussion

Results of the present study indicate that gestational exposure to CPF evokes immediate alterations in AC-mediated cell signaling in the developing brain, with a distinct regional hierarchy and critical window of vulnerability. It is highly unlikely that CPF interacts directly with the signaling proteins of this intracellular transduction cascade or that it simply causes global alterations in the expression or function of the proteins, because in those situations, effects would have been temporally and spatially uniform. Because cAMP is a pivotal control point for the trophic control of cell replication and differentiation by neurotransmitters and hormones (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Van Wijk et al. 1973), the complex series of changes in AC signaling elicited by developmental exposure to CPF provides a mechanism for deleterious outcomes.

By far the greatest period of sensitivity was late gestation after CPF exposure on GD17–20. We observed significant βAR deficits at doses below the threshold for maternal or fetal systemic toxicity and, indeed, below the level at which significant cholinesterase inhibition can be detected in the fetal brain (Qiao et al. 2002). Nevertheless, the AC response mediated by βARs, isoproterenol-stimulated AC activity, was unaffected or even increased, indicating that receptor binding is not the primary determinant of the receptor-mediated signaling response. These results reinforce the idea that the expression and function of signaling proteins downstream from the receptor provide the primary determinants of the net cellular response to receptor activation (Gao et al. 1998, 1999; Navarro et al. 1991a, 1991b; Slotkin et al. 2001, 2003). Accordingly, we evaluated AC responses mediated by direct stimulants, which test the inherent responsiveness of AC itself. CPF exposure on GD17–20 elicited marked increases in AC responses to forskolin or Mn^{2+} but with a distinct regional hierarchy: the brainstem was far more sensitive than the forebrain. Indeed, in the brainstem, AC induction was evident with doses as low as 2 mg/kg using either AC stimulant. Because forskolin and Mn^{2+} operate through different epitopes of the AC molecule (Limbird et al. 1979; Seamon and Daly 1986; Zeiders et al. 1999b), the parallel effect of CPF on the responses to the two agents, unaccompanied

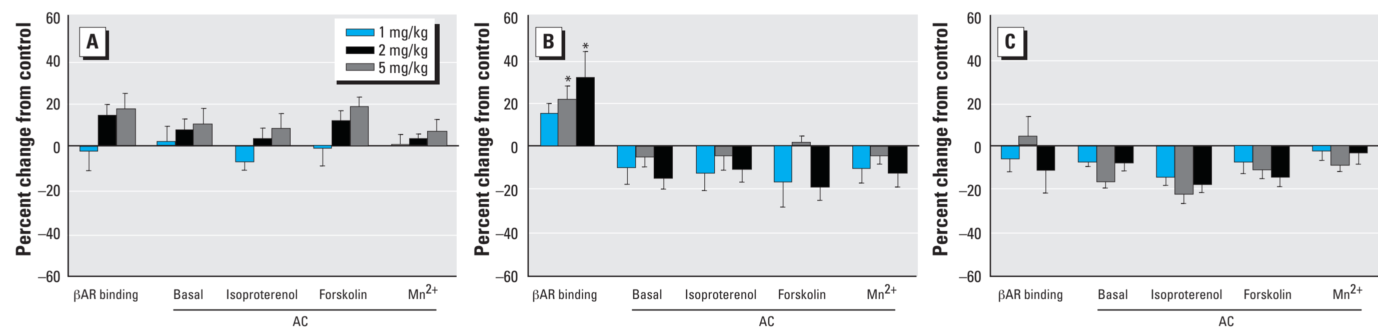

Figure 4. Effects of CPF exposure on GD9–12 assessed in whole brain on GD17 (*A*) and in forebrain (*B*) and brainstem (*C*) on GD21. Data represent mean ± SE of five or six determinations for each group at each age.
*Individual groups differ significantly from the corresponding control ($p < 0.04$; ANOVA).

by a shift in their relative activity (i.e., no change in the forskolin/Mn^{2+} response ratio), implies that CPF treatment increases (induces) the concentration of AC molecules.

A closer examination of the effects on AC in the brainstem indicates that the enzyme induction caused by CPF exposure actually masks deficits in βAR-mediated responses. If there were no changes in the receptor-mediated component, then the isoproterenol response would simply mimic the effect seen on total AC activity. Instead, the proportion of AC capable of responding to isoproterenol declined in the CPF group, evidenced by a drop in the isoproterenol/forskolin response ratio. Unlike the effects on AC itself, however, this deficit in the relative βAR response was detectable only at doses above the threshold for cholinesterase inhibition.

In contrast to the prominent effects of the GD17–20 CPF regimen on AC signaling, similar treatment on GD9–12 elicited little or no effect; if anything, AC activities tended to be reduced slightly, rather than increased. Accordingly, the window of vulnerability for CPF's effects on AC signaling appears to be concentrated in late gestation. Although our studies do not address the specific reasons for the higher liability of late gestational exposure, there are certainly major developmental differences in the two stages. Within the brain itself, the basic processes of neurogenesis, gliogenesis, axonogenesis, cell migration, and architectural organization are completely distinct in mid- versus late gestation (Rodier 1988). Further, the later period corresponds to the onset of sexual differentiation of the brain. Although CPF is only weakly estrogenic (Andersen et al. 2002; Vinggaard et al. 2000), effects on neural development are likely to influence the ontogeny of sexual dimorphism, endocrine responses, or even hormonal levels, and CPF intoxication in adults is known to have secondary endocrine effects (Guven et al. 1999). In the present study, we did not examine male and female fetuses separately. However, in previous work we found that CPF treatment on GD17–20 produces sex-dependent neurobehavioral differences that emerge in adolescence and adulthood (Levin et al. 2002). If sexual differentiation is a component of CPF's targeted effects on brain development, then we would predict that the effects of earlier exposure on GD9–12 might not show sex dependence; these studies are currently under way.

Regardless of the mechanisms underlying the critical period for effects of CPF on AC, it is important to note that CPF exerts other types of developmental neurotoxicant effects in the earlier phases of development. These include abnormal patterns of cell replication and cell death during CPF exposure at the neural tube stage (Roy et al. 1998), as well as lasting neurobehavioral effects of such exposure (Icenogle et al. In Press). Our results indicate that those effects are not mediated through initial alterations in the AC cascade, but rather through other mechanisms. Similarly, the window for targeted effects on AC components shows postnatal closure. CPF treatment of neonatal rats does not augment brainstem or forebrain AC activity as was seen here for the late gestational treatment regimen (Song et al. 1997). Instead, the postnatal exposures cause delayed-onset deterioration of AC signaling that likely represents a consequence of other mechanisms contributing to altered cell development (Campbell et al. 1997; Dam et al. 1998, 1999a). Findings in the cerebellum, a region that develops much later than the brainstem or forebrain (Rodier 1988), reinforce the concept of a critical period of cell maturation in which AC is vulnerable to CPF; postnatal CPF exposure elicits the same type of immediate increase in cerebellar AC activity as seen here for the earlier-developing regions with gestational CPF treatment (Song et al. 1997).

Finally, it is interesting to note that several effects of CPF displayed distinct hormesis (i.e., the effects were nonmonotonic), with alterations apparent at low doses but disappearing once the dose was raised above the threshold for cholinesterase inhibition and systemic toxicity. A similar phenomenon has been noted for effects on biomarkers of synaptic development (Qiao et al. 2002, 2003) and for behavioral consequences of gestational or neonatal CPF treatment (Levin et al. 2001, 2002; Icenogle et al. In Press). Cholinergic input provides a positive trophic effect on brain development at the levels of cell maturation and regional architecture (Hohmann and Berger-Sweeney 1998; Lauder and Schambra 1999), and it is thus possible that raising the dose of CPF above the threshold for cholinesterase inhibition can partially offset deleterious effects mediated by noncholinergic mechanisms. Consequently, the dose–effect curve for the developmental neurotoxicity of CPF can be expected to display multiple phases, not a monotonic relationship. This also points out an inherent difficulty in ascribing any effects of CPF in an *in vivo* treatment model to a definitive "cholinergic" or "noncholinergic" mechanism. Effects on signaling pathways, such as the AC pathway, no doubt have an influence on responses mediated by cholinergic signals, which operate in part through cAMP. In turn, cholinergic effects influence AC and cAMP formation. Resolution of these issues thus ultimately requires simplified systems such as cell cultures or lower organisms (Buznikov et al. 2001; Schuh et al. 2002; Song et al. 1998).

The present study thus reinforces the idea that CPF elicits developmental neurotoxicity through mechanisms independent of, and at doses below the threshold for, cholinesterase inhibition. The AC signaling cascade represents a major control point for brain cell replication and differentiation, and CPF targets this intracellular pathway with discrete temporal and regional selectivity. In addition to immediate changes in AC signaling, CPF also has the potential to evoke delayed-onset alterations (Song et al. 1997) that may influence later maturational events such as axonogenesis, synaptogenesis, and synaptic function (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999. In press). Accordingly, future studies will need to address the issue of the long-term effects of gestational CPF exposure on the AC pathway.

References

Andersen HR, Vinggaard AM, Hoj Rasmussen T, Gjermandsen IM, Cecilie Bonefeld-Jorgensen E. 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*. Toxicol Appl Pharmacol 179:1–12.

Auman JT, Seidler FJ, Slotkin TA. 2000. Neonatal chlorpyrifos exposure targets multiple proteins governing the hepatic adenylyl cyclase signaling cascade: implications for neurotoxicity. Dev Brain Res 121:19–27.

———. 2001. Regulation of fetal cardiac and hepatic β-adrenoceptors and adenylyl cyclase signaling: terbutaline effects. Am J Physiol 281:R1079–R1089.

Barone S, Das KP, Lassiter TL, White LD. 2000. Vulnerable processes of nervous system development: a review of markers and methods. Neurotoxicology 21:15–36.

Bhat NR, Shanker G, Pieringer RA. 1983. Cell proliferation in growing cultures of dissociated embryonic mouse brain: macromolecule and ornithine decarboxylase synthesis and regulation by hormones and drugs. J Neurosci Res 10:221–230.

Buznikov GA, Nikitina LA, Bezuglov VV, Lauder JM, Padilla S, Slotkin TA. 2001. An invertebrate model of the developmental neurotoxicity of insecticides: effects of chlorpyrifos and dieldrin in sea urchin embryos and larvae. Environ Health Perspect 109:651–661.

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

Claycomb WC. 1976. Biochemical aspects of cardiac muscle differentiation. J Biol Chem 251:6082–6089.

Crumpton TL, Seidler FJ, Slotkin TA. 2000. Developmental neurotoxicity of chlorpyrifos in vivo and in vitro: effects on nuclear transcription factor involved in cell replication and differentiation. Brain Res 857:87–98.

Dam K, Garcia SJ, Seidler FJ, Slotkin TA. 1999a. Neonatal chlorpyrifos exposure alters synaptic development and neuronal activity in cholinergic and catecholaminergic pathways. Dev Brain Res 116:9–20.

Dam K, Seidler FJ, Slotkin TA. 1998. Developmental neurotoxicity of chlorpyrifos: delayed targeting of DNA synthesis after repeated administration. Dev Brain Res 108:39–45.

———. 1999b. Chlorpyrifos releases norepinephrine from adult and neonatal rat brain synaptosomes. Dev Brain Res 118:120–133.

Das KP, Barone S. 1999. Neuronal differentiation in PC12 cells is inhibited by chlorpyrifos and its metabolites: is acetylcholinesterase inhibition the site of action? Toxicol Appl Pharmacol 160:217–230.

Dreyfus CF. 1998. Neurotransmitters and neurotrophins collaborate to influence brain development. Perspect Dev Neurobiol 5:389–399.

Erdtsieck-Ernste BHW, Feenstra MGP, Boer GJ. 1991. Pre- and postnatal developmental changes of adrenoceptor subtypes in rat brain. J Neurochem 57:897–903.

Gao MH, Lai NC, Roth DM, Zhou JY, Zhu J, Anzai T, et al. 1999. Adenylyl cyclase increases responsiveness to catecholamine stimulation in transgenic mice. Circulation 99:1618–1622.

Gao MH, Ping PP, Post S, Insel PA, Tang RY, Hammond HK. 1998. Increased expression of adenylylcyclase type VI

proportionately increases β-adrenergic receptor-stimulated production of cAMP in neonatal rat cardiac myocytes. Proc Natl Acad Sci USA 95:1038–1043.

Garcia SJ, Seidler FJ, Crumpton TL, Slotkin TA. 2001. Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecule synthesis, adenylyl cyclase signaling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells. Brain Res 891:54–68.

Garcia SJ, Seidler FJ, Qiao D, Slotkin TA. 2002. Chlorpyrifos targets developing glia: effects on glial fibrillary acidic protein. Dev Brain Res 133:151–161.

Guidotti A. 1972. Adenosine 3′,5′-monophosphate concentrations and isoproterenol-induced synthesis of deoxyribonucleic acid in mouse parotid gland. Mol Pharmacol 8:521–530.

Guven M, Bayram F, Unluhizarci K, Kelestimur F. 1999. Endocrine changes in patients with acute organophosphate poisoning. Hum Exp Toxicol 18:598–601.

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

Howard MD, Pope CN. 2002. *In vitro* effects of chlorpyrifos, parathion, methyl parathion and their oxons on cardiac muscarinic receptor binding in neonatal and adult rats. Toxicology 170:1–10.

Huff RA, Abou-Donia MB. 1995. In vitro effect of chlorpyrifos oxon on muscarinic receptors and adenylate cyclase. Neurotoxicology 16:281–290.

Huff RA, Abu-Qare AW, Abou-Donia MB. 2001. Effects of subchronic in vivo chlorpyrifos exposure on muscarinic receptors and adenylate cyclase of rat striatum. Arch Toxicol 75:480–486.

Huff RA, Corcoran JJ, Anderson JK, Abou-Donia MB. 1994. Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. J Pharmacol Exp Ther 269:329–335.

Hultgårdh-Nilsson A, Querol-Ferrer V, Jonzon B, Krondahl U, Nilsson J. 1994. Cyclic AMP, early response gene expression, and DNA synthesis in rat smooth muscle cells. Exp Cell Res 214:297–302.

Icenogle LM, Christopher C, Blackwelder WP, Caldwell DP, Qiao D, Seidler FJ, et al. In press. Behavioral alterations in adolescent and adult rats caused by a brief subtoxic exposure to chlorpyrifos during neurulation. Neurotoxicol Teratol.

Institute of Laboratory Animal Resources. 1996. Guide for the Care and Use of Laboratory Animals. 7th ed. Washington, DC:National Academy Press.

Johnson DE, Seidler FJ, Slotkin TA. 1998. Early biochemical detection of delayed neurotoxicity resulting from developmental exposure to chlorpyrifos. Brain Res Bull 45:143–147.

Kasamatsu T. 1985. The role of the central noradrenaline system in regulating neuronal plasticity in the developing neocortex. In: Prevention of Physical and Mental Congenital Defects. Basic and Medical Science, Education, and Future Strategies (Marois M, ed). Progress in Clinical and Biological Research Series 163C. New York:Alan R. Liss, 369–373.

Kulkarni VA, Jha S, Vaidya VA. 2002. Depletion of norepinephrine decreases the proliferation, but does not influence the survival and differentiation, of granule cell progenitors in the adult rat hippocampus. Eur J Neurosci 16:2008–2012.

Kwon JH, Eves EM, Farrell S, Segovia J, Tobin AJ, Wainer BH, et al. 1996. β-Adrenergic receptor activation promotes process outrgrowth in an embryonic rat basal forebrain cell line and in primary neurons. Eur J Neurosci 8:2042–2055.

Landrigan PJ. 2001. Pesticides and polychlorinated biphenyls (PCBs): an analysis of the evidence that they impair children's neurobehavioral development. Mol Genet Metab 73:11–17.

Landrigan PJ, Claudio L, Markowitz SB, Berkowitz GS, Brenner BL, Romero H, et al. 1999. Pesticides and inner-city children: exposures, risks, and prevention. Environ Health Perspect 107(suppl 3):431–437.

Lassiter T, White L, Padilla S, Barone S. 2002. Gestational exposure to chlorpyrifos: qualitative and quantitative neuropathological changes in the fetal neocortex. Toxicologist 66:632.

Lauder JM, Schambra UB. 1999. Morphogenetic roles of acetylcholine. Environ Health Perspect 107(suppl 1):65–69.

Levin ED, Addy N, Baruah A, Elias A, Christopher NC, Seidler FJ, et al. 2002. Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations. Neurotoxicol Teratol 24:733–741.

Levin ED, Addy N, Christopher NC, Seidler FJ, Slotkin TA. 2001. Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. Dev Brain Res 130:83–89.

Limbird LE, Hickey AR, Lefkowitz RL. 1979. Unique uncoupling of the frog erythrocyte adenylate cyclase system by manganese. J Biol Chem 254:2677–2683.

Liu J, Chakraborti T, Pope C. 2002. In vitro effects of organophosphorus anticholinesterases on muscarinic receptor-mediated inhibition of acetylcholine release in rat striatum. Toxicol Appl Pharmacol 178:102–108.

May M. 2000. Disturbing behavior: neurotoxic effects in children. Environ Health Perspect 108:A262–A267.

Mileson BE, Chambers JE, Chen WL, Dettbarn W, Ehrich M, Eldefrawi AT, et al. 1998. Common mechanism of toxicity: a case study of organophosphorus pesticides. Toxicol Sci 41:8–20.

Morris G, Seidler FJ, Slotkin TA. 1983. Stimulation of ornithine decarboxylase by histamine or norepinephrine in brain regions of the developing rat: evidence for biogenic amines as trophic agents in neonatal brain development. Life Sci 32:1565–1571.

Navarro HA, Kudlacz EM, Kavlock RJ, Slotkin TA. 1991a. Prenatal terbutaline treatment: tissue-selective dissociation of perinatal changes in β-adrenergic receptor binding from regulation of adenylate cyclase activity. Life Sci 48:269–274.

Navarro HA, Kudlacz EM, Slotkin TA. 1991b. Control of adenylate cyclase activity in developing rat heart and liver: effects of prenatal exposure to terbutaline or dexamethasone. Biol Neonate 60:127–136.

Olivier K, Liu J, Pope C. 2001. Inhibition of forskolin-stimulated cAMP formation *in vitro* by paraoxon and chlorpyrifos oxon in cortical slices from neonatal, juvenile, and adult rats. J Biochem Mol Toxicol 15:263–269.

Physicians for Social Responsibility. 1995. Pesticides and Children. Washington DC:Physicians for Social Responsibility.

Pittman RN, Minneman KP, Molinoff PB. 1980. Ontogeny of β_1- and β_2-adrenergic receptors in rat cerebellum and cerebral cortex. Brain Res 188:357–368.

Pope CN. 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity? J Toxicol Environ Health 2:161–181.

Popovik E, Haynes LW. 2000. Survival and mitogenesis of neuroepithelial cells are influenced by noradrenergic but not cholinergic innervation in cultured embryonic rat neopallium. Brain Res 853:227–235.

Qiao D, Seidler FJ, Padilla S, Slotkin TA. 2002. Developmental neurotoxicity of chlorpyrifos: what is the vulnerable period? Environ Health Perspect 110:1097–1103.

Qiao D, Seidler FJ, Tate CA, Cousins MM, Slotkin TA. 2003. Fetal chlorpyrifos exposure: adverse effects on brain cell development and cholinergic biomarkers emerge postnatally and continue into adolescence and adulthood. Environ Health Perspect 111:536–544.

Rice D, Barone S. 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108(suppl 3):511–533.

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

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

Schuh RA, Lein PJ, Beckles RA, Jett DA. 2002. Noncholinesterase mechanisms of chlorpyrifos neurotoxicity: altered phosphorylation of Ca^{2+}/cAMP response element binding protein in cultured neurons. Toxicol Appl Pharmacol 182:176–185.

Schwartz JP, Nishiyama N. 1994. Neurotrophic factor gene expression in astrocytes during development and following injury. Brain Res Bull 35:403–407.

Seamon KB, Daly JW. 1986. Forskolin: its biological and chemical properties. Adv Cyclic Nucleotide Protein Phosphorylation Res 20:1–150.

Shaywitz AJ, Greenberg ME. 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821–861.

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

———. In press. Guidelines for developmental neurotoxicity and their impact on organophosphate pesticides: a personal view from an academic perspective. Neurotoxicology.

Slotkin TA, Auman JT, Seidler FJ. 2003. Ontogenesis of β-adrenoceptor signaling: implications for perinatal physiology and for fetal effects of tocolytic drugs. J Pharmacol Exp Ther 306:1–7.

Slotkin TA, Baker FE, Dobbins SS, Eylers JP, Lappi SE, Seidler FJ. 1989. Prenatal terbutaline exposure in the rat: selective effects on development of noradrenergic projections to cerebellum. Brain Res Bull 23:263–265.

Slotkin TA, Lau C, Seidler FJ. 1994. β-Adrenergic receptor overexpression in the fetal rat: distribution, receptor subtypes and coupling to adenylate cyclase via G-proteins. Toxicol Appl Pharmacol 129:223–234.

Slotkin TA, Tate CA, Cousins MM, Seidler FJ. 2001. β-Adrenoceptor signaling in the developing brain: sensitization or desensitization in response to terbutaline. Dev Brain Res 131:113–125.

Snedecor GW, Cochran WG. 1967. Statistical Methods. Ames, IA:Iowa State University Press.

Song X, Seidler FJ, Saleh JL, Zhang J, Padilla S, Slotkin TA. 1997. Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenylyl cyclase signaling cascade. Toxicol Appl Pharmacol 145:158–174.

Song X, Violin JD, Seidler FJ, Slotkin TA. 1998. Modeling the developmental neurotoxicity of chlorpyrifos *in vitro*: macromolecule synthesis in PC12 cells. Toxicol Appl Pharmacol 151:182–191.

Stachowiak EK, Fang X, Myers J, Dunham S, Stachowiak MK. 2003. cAMP-Induced differentiation of human neuronal progenitor cells is mediated by nuclear fibroblast growth factor receptor-1 (FGFR1). J Neurochem 84:1296–1312.

Van Wijk R, Wicks WD, Bevers MM, Van Rijn J. 1973. Rapid arrest of DNA synthesis by N6,O2′-dibutyryl cyclic adenosine 3′,5′-monophosphate in cultured hepatoma cells. Cancer Res 33:1331–1338.

Vinggaard AM, Hnida C, Breinholt V, Larsen JC. 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol In Vitro 14:227–234.

Ward TR, Mundy WR. 1996. Organophosphorus compounds preferentially affect second messenger systems coupled to M2/M4 receptors in rat frontal cortex. Brain Res Bull 39:49–55.

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

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

Yanai J, Vatury O, Slotkin TA. 2002. Cell signaling as a target and underlying mechanism for neurobehavioral teratogenesis. Ann NY Acad Sci 965:473–478.

Zeiders JL, Seidler FJ, Iaccarino G, Koch WJ, Slotkin TA. 1999a. Ontogeny of cardiac β-adrenoceptor desensitization mechanisms: agonist treatment enhances receptor/G-protein transduction rather than eliciting uncoupling. J Mol Cell Cardiol 31:413–423.

Zeiders JL, Seidler FJ, Slotkin TA. 1997. Ontogeny of regulatory mechanisms for β-adrenoceptor control of rat cardiac adenylyl cyclase: targeting of G-proteins and the cyclase catalytic subunit. J Mol Cell Cardiol 29:603–615.

———. 1999b. Agonist-induced sensitization of β-adrenoceptor signaling in neonatal rat heart: expression and catalytic activity of adenylyl cyclase. J Pharmacol Exp Ther 291:503–510.

Zhang HS, Liu J, Pope CN. 2002. Age-related effects of chlorpyrifos on muscarinic receptor-mediated signaling in rat cortex. Arch Toxicol 75:676–684.