Critical Periods for Chlorpyrifos-Induced Developmental Neurotoxicity: Alterations in Adenylyl Cyclase Signaling in Adult Rat Brain Regions after Gestational or Neonatal Exposure

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Developmental exposure to chlorpyrifos (CPF) alters the function of a wide variety of neural systems. In the present study we evaluated the effects in adulthood of CPF exposure of rats during different developmental windows, using the adenylyl cyclase (AC) signaling cascade, which mediates the cellular responses to numerous neurotransmitters. Animals were exposed on gestational days (GD) 9–12 or 17–20 or on postnatal days (PN) 1–4 or 11–14 and assessed at PN60. In addition to basal AC activity, we evaluated the responses to direct AC stimulants (forskolin, Mn^{2+}) and to isoproterenol, which activates signaling through β-adrenoceptors coupled to stimulatory G-proteins. CPF exposure in any of the four periods elicited significant changes in AC signaling in a wide variety of brain regions in adulthood. In general, GD9–12 was the least sensitive stage, requiring doses above the threshold for impaired maternal weight gain, whereas effects were obtained at subtoxic doses for all other regimens. Most of the effects were heterologous, involving signaling elements downstream from the receptors, and thus shared by multiple stimulants; superimposed on this basic pattern, there were also selective alterations in receptor-mediated responses, in G-protein function, and in AC expression and subtypes. Exposures conducted at GD17–20 and later all produced sex-selective alterations. These results suggest that developmental exposure to CPF elicits long-lasting alterations in cell-signaling cascades that are shared by multiple neurotransmitter and hormonal inputs; the resultant abnormalities of synaptic communication are thus likely to occur in widespread neural circuits and their corresponding behaviors. *Key words:* adenylyl cyclase, β-adrenoceptor, brain development, chlorpyrifos, organophosphate insecticides. *Environ Health Perspect* 112:295–301 (2004). doi:10.1289/ehp.6755 available via *http://dx.doi.org/* [Online 24 November 2003]

The ability of organophosphate insecticides such as chlorpyrifos (CPF) to elicit developmental neurotoxicity has received a great deal of attention in the last decade (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 b), resulting in its restricted domestic use [U.S. Environmental Protection Agency (EPA) 2000a, 2000b, 2001]. It is now evident that CPF alters brain development by a panoply of mechanisms in addition to cholinesterase inhibition (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999, in press b), compromising neural cell replication and differentiation, axonogenesis and synaptogenesis, and the programming of synaptic function (Buznikov et al. 2001; Crumpton et al. 2000; Dam et al. 1998, 1999a, 1999b; Garcia et al. 2001; Howard and Pope 2002; Huff et al. 2001; Johnson et al. 1998; Liu et al. 2002; Whitney et al. 1995; Yanai et al. 2002; Zhang et al. 2002). One prominent mechanism by which CPF acts on the developing brain is through its ability to alter the expression and function of cell-signaling proteins that control the generation of cyclic AMP (cAMP) (Crumpton et al. 2000; Garcia et al. 2001; Meyer et al. 2003; Olivier et al. 2001; Schuh et al. 2002; Song et al. 1997; Zhang et al. 2002). In turn, cAMP 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) and, during brain development, plays a pivotal role in axonal outgrowth, neural plasticity, and apoptosis (Shaywitz and Greenberg 1999; Stachowiak et al. 2003). We recently demonstrated that, during critical developmental periods, CPF alters the activity of adenylyl cyclase (AC), the enzyme responsible for cAMP production, as well as the G-proteins that couple neurotransmitter receptors to AC, along with the receptor-mediated responses to neurotransmitters that serve neurotrophic roles in brain development (Aldridge et al. 2003; Garcia et al. 2001; Meyer et al. 2003; Song et al. 1997).

The question remains as to whether the ability of CPF to alter cell signaling lasts into adulthood, or instead, whether the effects are restricted to developmental stages in which these signals affect neural cell differentiation. Certainly, CPF exposure leads to behavioral anomalies that emerge in adolescence and persist in adults (Levin et al. 2001, 2002; Richardson and Chambers 2003; Slotkin et al. 2001a, 2002). In association with the behavioral deficits, developmental CPF exposure evokes profound alterations in activity of neurotransmitter systems that operate through AC, notably cholinergic and catecholaminergic pathways (Qiao et al. 2003; Slotkin, in press a; Slotkin et al. 2001a, 2002). In the present study, we investigated the long-term impact of gestational or neonatal CPF exposure on the functioning of the AC signaling cascade, focusing on four different critical windows: the formation of the neural tube [gestational days (GD) 9–12], the late gestational period (GD17–20) in which sexual differentiation of the brain is initiated (McCarthy 1994; Mong and McCarthy 1999), and postnatal phases of terminal neuronal differentiation and synaptogenesis [postnatal days (PN) 1–4, PN11–14]; these are the same treatment windows examined for short-term effects in our previous study (Meyer et al. 2003). Doses were chosen to enable us to determine whether the threshold for effects on AC signaling lies below that for systemic toxicity and/or inhibition of cholinesterase (Aldridge et al. 2003; Garcia et al. 2003; Meyer et al. 2003; Qiao et al. 2002; Slotkin 1999, in press a).

In adulthood (PN60), we examined the effects of CPF on AC in several ways. First, we evaluated basal enzymatic activity. Second, we determined the response to two AC stimulants, forskolin and 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. 1999). Third, we probed the AC response to specific receptor-mediated activation with isoproterenol, a β-adrenoceptor (βAR) agonist linked to AC by 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; Kulkarni et al. 2002; Kwon et al. 1996; Morris et al. 1983; Popovik and

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Haynes 2000; Schwartz and Nishiyama 1994; Slotkin et al. 1989; Song et al. 1997). Our studies also characterized the regional specificity and sex selectivity of the long-term effects of CPF on cell signaling.

Materials and Methods

Animal treatments. All experiments using live animals were carried out in accordance with the Declaration of Helsinki (World Medical Association 2002) and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996) 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 DMSO 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 (DMSO) injections on the same schedules. For exposure on GD9–12 or GD17–20, dams were injected daily with CPF at 1 or 5 mg/kg body weight. These doses span the threshold for inhibition of fetal brain cholinesterase activity, fetal growth impairment, and reduced maternal weight gain, all of which become evident at ≥ 5 mg/kg (Garcia et al. 2003; Qiao et al. 2002). On the day of birth, all pups were randomized within their respective treatment groups and redistributed to the 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. On PN60, animals were decapitated and the brain was dissected into cerebral cortex, hippocampus, striatum, midbrain, brainstem, and cerebellum, which were frozen with liquid nitrogen and stored at –45°C.

For studies of CPF effects in the first few days after birth, animals were given 1 mg/kg by subcutaneous injection 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. The same randomization procedure was followed. 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 present study we did not observe any changes in suckling or maternal caretaking. Samples were obtained on PN60 as described above.

None of the prenatal or postnatal treatment regimens evoked a significant change in weight of any of the brain regions on PN60, nor were there any deficits in body weight (data not shown).

Membrane preparation and assays. All of the assay methodologies used in this study have been described previously (Aldridge et al. 2003; Auman et al. 2000, 2001; Meyer et al. 2003; Slotkin et al. 2001b; Zeiders et al. 1997, 1999), so only brief descriptions are provided here. Tissues were thawed and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY), and cell membranes were prepared and washed by sequential sedimentation at 40,000 × *g*. The membrane pellets were dispersed with a smooth-glass homogenizer and used for ligand binding and AC assays. $[^{125}I]$Iodopindolol (67 pM) was used to determine βAR binding, and nonspecific binding was assessed by displacement with 100 μM isoproterenol. AC activity was determined by enzymatic generation of cAMP, which was then measured by radioimmunoassay. 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; Slotkin et al. 2001b; Zeiders et al. 1997, 1999).

Data analysis. Data are presented as means and SEs obtained from eight animals of each sex for each prenatal treatment group and six animals per sex for each postnatal treatment group. For convenience, 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 the *in vivo* treatment groups, sexes, regions, and measurements made on the membranes (βAR binding, AC activity under four different conditions); the latter were considered to be repeated measures because each membrane preparation was used for the multiple conditions under which AC was determined. 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, significance for main treatment effects was assumed 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 (Table 1) were combined across the multiple cohorts (controls used for administration on GD9–12, GD17–20, PN1–4, PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.

Table 1. Binding parameters and AC levels in controls.

Measure, sex	Cerebral cortex	Hippocampus	Striatum	Midbrain	Brainstem	Cerebellum
βAR binding (fmol/mg protein)						
Male	39.9 ± 0.9	17.3 ± 0.7	44.1 ± 1.0	15.8 ± 0.3	10.0 ± 0.3	27.2 ± 0.6
Female	41.4 ± 0.9	18.1 ± 0.7	46.3 ± 1.6	16.6 ± 0.3	10.2 ± 0.3	27.1 ± 0.7
Basal AC (pmol/min/mg protein)						
Male	177 ± 11	146 ± 6	141 ± 6	240 ± 9	147 ± 9	267 ± 12
Female	186 ± 10	142 ± 4	137± 7	242 ± 10	133 ± 7	248 ± 12
Isoproterenol-stimulated AC (pmol/min/mg protein)						
Male	197 ± 12	153 ± 6	151 ± 6	254 ± 9	153 ± 8	328 ± 14
Female	208 ± 12	148 ± 5	153 ± 8	250 ± 10	141 ± 7	301 ± 12
Forskolin-stimulated AC (pmol/min/mg protein)						
Male	1,206 ± 93	576 ± 27	3,817 ± 194	1,040 ± 51	421 ± 14	997 ± 40
Female	1,340 ± 113	594 ± 21	3,960 ± 199	1,049 ± 41	428 ± 15	1,019 ± 47
Mn^{2+}-stimulated AC (pmol/min/mg protein)						
Male	1,811 ± 119	1,174 ± 60	2,145 ± 72	1,485 ± 60	828 ± 24	1,932 ± 94
Female	2,007 ± 135	1,184 ± 42	2,292 ± 98	1,481 ± 66	817 ± 28	2,015 ± 106

Values were combined across multiple cohorts (controls used for CPF administration on GD9–12, GD17–20, PN1–4, and PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort. None of the sex differences is statistically significant.

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 Biosciences (Piscataway, NJ, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Results

CPF exposure on GD9–12. CPF administration during the neural tube stage had no long-term effects on βAR binding in any of the brain regions (Figure 1A). In contrast, there were significant effects on AC activity that depended on the type of AC measurement (significant interaction of treatment × measure). Examining this effect for each dose group, it was apparent that the alterations were confined to those receiving 5 mg/kg ($p < 0.0002$; not significant for 1 mg/kg group). Because the statistics could not distinguish significant selectivity for sex or brain region (i.e., no interaction of treatment × sex or treatment × region), we characterized the differential effect on AC measures by calculating the specific changes elicited by each stimulant, namely, the ratio of activity with the added stimulant to basal AC (Figure 1B). CPF exposure elicited small but significant increases in the response to isoproterenol, forskolin, and Mn^{2+}. Additionally, the effect on the isoproterenol response was significantly smaller than that for forskolin ($p < 0.03$). There was no change in the forskolin:Mn^{2+} activity ratio (data not shown). In light of the positive findings with this first treatment regimen, the scope of the regions examined was extended to include hippocampus and striatum for the subsequent studies.

CPF exposure on GD17–20. In contrast to the effects of GD9–12 CPF exposure, shifting the exposure period to later in gestation had a profound, sex-dependent effect on βAR binding in adulthood. Overall, ANOVA identified treatment interactions with sex and brain region: $p < 0.006$ for treatment × sex, $p < 0.02$ for treatment × region, $p < 0.02$ for treatment × sex × region. Furthermore, the effects were distinguishable both at the low dose (1 mg/kg) of CPF ($p < 0.002$ for treatment × sex) as well as at the higher (5 mg/kg) dose ($p < 0.03$, $p < 0.004$, $p < 0.004$, for the three interactions, respectively). In light of the consistent sex differences, we subdivided the data into males and females for presentation and further analysis (Figure 2). Males showed a treatment × region interaction, reflecting small elevations in the striatum and midbrain, as well as a reduction in the cerebellum in the high-dose group. In contrast, females showed significant overall reductions (main treatment effect) that were robustly significant even with the lower dose of CPF ($p < 0.02$ across all regions). The largest individual changes were obtained for the striatum and brainstem.

CPF exposure on GD17–20 also influenced AC measures in a fashion that interacted with brain region (treatment × region × measure, $p < 0.1$), so values were subdivided into the individual regions and reexamined for treatment effects and interactions. CPF effects were identified in the hippocampus (main treatment effect, $p < 0.05$), striatum (treatment × measure, $p < 0.02$; treatment × sex, $p < 0.1$), midbrain (treatment × sex, $p < 0.1$), and cerebellum (treatment × sex, $p < 0.07$), and these are displayed in Figure 3; results for the other regions are not shown. For the hippocampus, CPF treatment evoked an overall elevation of AC, without preferential effects on any of the stimulants (Figure 3A); effects were significant only at 5 mg/kg. In the striatum, there was a sex disparity, with males tending to show increases in AC and females showing decreases. For individual measures, males exposed to 5 mg/kg CPF showed significant elevations in the responses to the two direct AC stimulants forskolin and Mn^{2+}, but the isoproterenol response was unaffected. In the midbrain, CPF exposure similarly tended to elevate AC in a sex-selective manner (treatment × sex interaction), but considered separately, neither sex passed the threshold for statistically significant differences (Figure 3C); the sex difference reflected the relatively greater effect of the lower dose of CPF in males compared with that in females. In the cerebellum, CPF exposure had a preferential effect on AC in females, evoking significant elevations at either 1 or 5 mg/kg (Figure 3D); the effects were exerted across all AC measures, without preference for different stimulants. Males did not show any significant differences.

CPF exposure on PN1–4. In contrast to the effects of GD17–20 exposure, shifting the period of CPF treatment to the early neonatal period resulted in no significant long-term alterations of βAR binding on PN60 (data not shown). However, there was a significant overall elevation of AC (main treatment effect, $p < 0.05$) that again depended on sex, brain region, and AC measure ($p < 0.03$ for treatment × sex, $p < 0.03$ for treatment × measure, $p < 0.07$ for treatment × sex × measure, $p < 0.009$ for treatment × region × measure). Accordingly, we performed lower-order assessments on each region, looking for main treatment effects and interactions of treatment

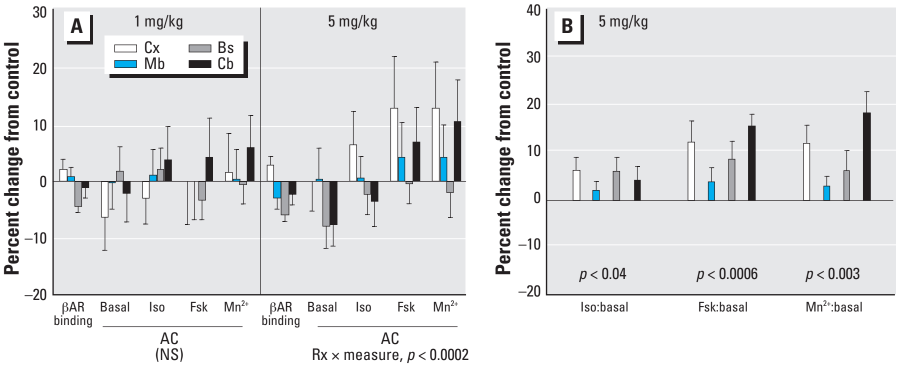

Figure 1. Effects of GD9–12 CPF exposure on PN60 (*A*) βAR binding and AC activity (ANOVA for Rx × measure, $p < 0.002$) and (*B*) AC activity ratios. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Fsk, forskolin; Iso, isoproterenol; Mb, midbrain; NS, not significant; Rx, treatment. Data are means and SEs of the percent change from corresponding control values (Table 1). Although effects on βAR binding were not significant, ANOVA indicated a significant treatment effect that depended on the specific AC measure, and the effects were restricted to the group exposed to 5 mg/kg CPF. To characterize the differential effects on AC measures, activity ratios were calculated relative to basal AC activity (*B*). ANOVAs for each measure are shown within the figure. Separate statistical evaluations for males and females or for each region were not carried out because of the absence of treatment interactions with these variables.

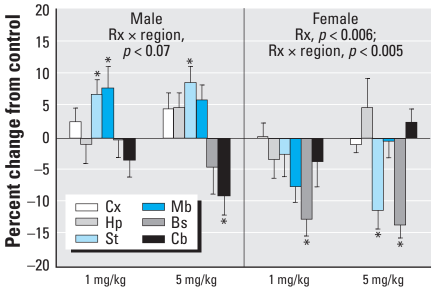

Figure 2. Effects of GD17–20 CPF exposure on PN60 βAR binding. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Hp, hippocampus; Mb, midbrain; Rx, treatment; St, striatum. Data are means and SEs of the percent change from corresponding control values (Table 1). Across all treatments, all regions, and both sexes, ANOVA indicated a significant treatment effect that differed between the two sexes (Rx × sex, $p < 0.006$; Rx × region, $p < 0.02$; Rx × sex × region, $p < 0.02$), so values were separated for males and females. Lower-order ANOVAs for each sex appear within the figure.

*Individual groups differ significantly from the control.

with other variables. Two of the regions that were targeted by GD17–20 exposure, the hippocampus and striatum, showed no significant overall effects on AC with the PN1–4 regimen (data not shown). The cerebral cortex and brainstem each showed a significant treatment × measure interaction without sex selectivity (no treatment × sex interaction), so results for males and females were combined for presentation (Figure 4A). Both regions showed significant elevations of AC but with different preference for the various stimulants. In the cerebral cortex, significant elevations were seen for direct AC stimulants (forskolin, Mn^{2+}), whereas in the brainstem, the effects were preferential for isoproterenol. Unlike the other two regions, there were sex-selective effects in the cerebellum, necessitating separate analysis of males and females (Figure 4B). Males displayed significant deficits in basal and isoproterenol-stimulated AC activity, whereas females showed a global elevation of AC.

CPF exposure on PN11–14. With this treatment regimen, βAR binding showed significant overall decreases (main treatment effect, $p < 0.03$) that were distinctly sex selective (treatment × sex, $p < 0.03$). Separating the values for males and females indicated a small but consistent decrement in females but not males (Figure 5). Similarly, AC activities in this treatment group did not indicate regionally selective CPF effects but did indicate the need to examine males and females separately for differential effects on the various AC measures (treatment × measure, $p < 0.07$; treatment × sex × measure, $p < 0.002$). In males, CPF treatment evoked significant, 10–20% decrements in basal and isoproterenol-stimulated AC activity with relatively smaller effects on the responses to forskolin and Mn^{2+} (Figure 6A). Females showed a more uniform pattern, with a significant overall decrease (main effect of CPF) and specific reductions in basal and forskolin-stimulated activity (Figure 6B); the cerebral cortex showed the greatest overall effect and was the only region in which the reductions achieved statistical significance individually ($p < 0.02$). Again, to characterize the differences in the effects of CPF directed toward specific AC stimulants, we calculated AC activity ratios (Figure 6C). In males, the response to isoproterenol was suppressed relative to that to forskolin, with significant deficits in striatum and cerebellum. In contrast, the same measurement in females indicated overall increases. Additionally, females showed a significant reduction in the forskolin:Mn^{2+} AC activity ratio.

Discussion

Results of the present study indicate that exposure to CPF during development elicits long-term alterations in AC-mediated cell signaling in the central nervous system, with differential effects according to sex and brain region that depend upon the exposure period. This effectively rules out the possibility that CPF simply interacts directly with the neurotransmitter receptors or proteins of the AC signaling cascade (Huff and Abou-Donia 1995; Huff et al. 1994; Ward and Mundy 1996), in which case the alterations would have been similar in every region, for both sexes, and for each regimen. Instead, our findings point to disruption of the program for development of cell signaling, with attendant targeting of specific regions for each sex that depend upon maturational phases of vulnerability of various neural cell populations (Garcia et al. 2002, 2003; Rodier 1988; Slotkin 1999, in press a). Indeed, CPF affects replication and differentiation of both neurons and glia, thus eliciting neurotoxicant actions over all the exposure periods studied here (Aldridge et al. 2003; Garcia et al. 2002, 2003; Icenogle et al., in press; Levin et al. 2001, 2002; Meyer et al. 2003; Qiao et al. 2003; Raines et al. 2001; Slotkin 1999, in press a; Slotkin et al. 2001a, 2002). Nevertheless, because AC signaling is a common final pathway for the transduction of diverse neural and hormonal signals involved in neural cell differentiation, axonal outgrowth, synaptic plasticity, and apoptosis (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Shaywitz and Greenberg 1999; Stachowiak et al. 2003; Van Wijk et al. 1973), lasting disruption of this pathway by CPF is likely to contribute directly to neurobehavioral

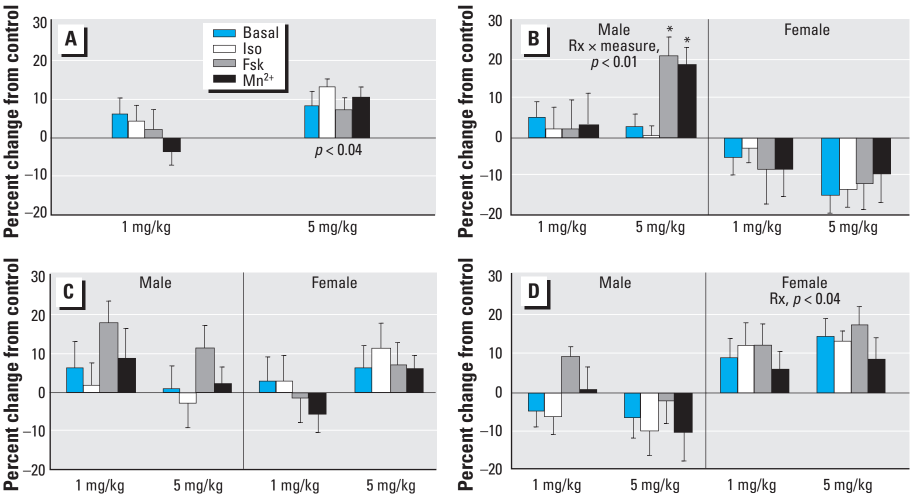

Figure 3. Effects of GD17–20 CPF exposure on PN60 AC activity in (*A*) hippocampus, (*B*) striatum, (*C*) midbrain, and (*D*) cerebellum. Abbreviations: Fsk, forskolin; Iso, isoproterenol; Rx, treatment. Data are means and SEs of the percent change from corresponding control values (Table 1). ANOVA results for each region across all variables are as follows: for (*A*) Rx, $p < 0.05$; for (*B*) Rx × measure, $p < 0.02$; Rx × sex × measure, $p < 0.1$; for (*C*) Rx × sex, $p < 0.1$; for (*D*) Rx × sex, $p < 0.07$; subdivision into the two sexes was carried out only when the ANOVA indicated an interaction of treatment × sex. Lower-order tests are shown within the figure.

*Individual groups differ significantly from the control (calculated only when lower-order tests indicated a significant treatment × measure interaction).

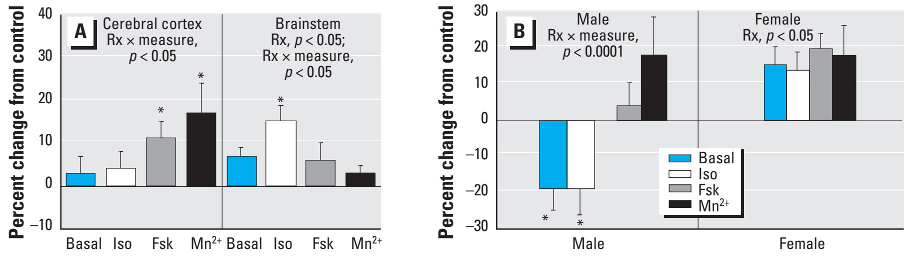

Figure 4. Effects of PN1–4 CPF exposure (1 mg/kg/day) on PN60 AC activity in (*A*) the cerebral cortex and brainstem, and (*B*) the cerebellum. Abbreviations: Fsk, forskolin; Iso, isoproterenol; Rx, treatment. Data are means and SEs of the percent change from corresponding control values (Table 1). ANOVA results across all variables are as follows for (*B*): Rx × sex, $p < 0.09$; Rx × measure, $p < 0.0004$; Rx × sex × measure, $p < 0.007$). For each region, ANOVA is shown across all variables, with subdivision into the two sexes carried out only when the ANOVA indicated an interaction of treatment × sex. Lower-order tests are shown within the figure.

*Individual groups differ significantly from the control (calculated only when lower-order tests indicated a significant treatment × measure interaction).

anomalies (Icenogle et al., in press; Levin et al. 2001, 2002). Furthermore, the fact that alterations in AC signaling are heterologous, rather than being confined to the immediate cholinergic target of CPF's actions, implies that effects will be exerted in regions, such as the cerebellum, that are sparse in cholinergic projections, and that alterations will extend to other neurotransmitter pathways. Again, this corresponds to earlier observations of disrupted cell replication and differentiation and synaptic communication in disparate brain regions (Aldridge et al. 2003; Campbell et al. 1997; Dam et al. 1999a; Garcia et al. 2003; Meyer et al. 2003; Raines et al. 2001; Slotkin 1999; Slotkin et al. 2002; Whitney et al. 1995).

There were four major features of the lasting alterations of AC signaling elicited by developmental exposure to CPF: regional selectivity, existence of a peak period of sensitivity, localization of the effects to specific signaling proteins, and preferential effects according to sex. First, the regional targeting changed dramatically with a shift in the CPF exposure period but not in a manner that would be predicted solely from the maturational timetable of each region. In general, neural maturation occurs earliest in the brainstem, later in forebrain areas, and last in the cerebellum (Rodier 1988). In contrast to that pattern, we found effects of CPF on AC signaling components in both early- and late-developing regions with either gestational or postnatal exposures, and without a distinct ontogenetic shift as would be expected from uniform targeting of a specific phase of cell development. Again, this is consistent with CPF's ability to affect different neural cell types by a variety of mechanisms ranging from mitotic inhibition to impairment of differentiation, axonogenesis, and synaptogenesis (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1999, in press a). Nevertheless, our results point to a specific phase in which CPF is most likely to disrupt long-term programming of AC function. With the earliest exposure (GD9–12), effects were seen only when the dose was raised to 5 mg/kg, above the threshold for systemic toxicity as assessed by impaired maternal weight gain (Qiao et al. 2002); even then, the magnitude of effect was only half of that seen with CPF exposure in later periods. With later gestational exposure (GD17–20), significant sex-dependent effects on AC signaling began to emerge at subtoxic exposure, best exemplified by the female cerebellum. Shifting the treatment to the postnatal period intensified the effects, with significant alterations in multiple brain regions at 1 mg/kg in animals treated on PN1–4, a dose that causes no discernible systemic toxicity (Dam et al. 1999a, 2000; Song et al. 1997); similarly, treatment with 5 mg/kg on PN11–14 elicited robust long-term alterations in AC signaling in adulthood. It thus appears that the early neonatal period in the rat, which approximates neurologic development in the third trimester and perinatal stage of human brain development (Rodier 1988), is particularly sensitive to persistent effects on AC signaling. Interestingly, this is the same conclusion that was reached from evaluations of the short-term effects on AC signaling in the fetal and neonatal brain seen immediately after CPF treatment (Meyer et al. 2003; Song et al. 1997), suggesting that the persistent effects are dependent on the earlier changes. Given the role of cAMP in neural development, it seems likely that there is a mechanistic link between early perturbations and the persistent alterations seen here in adulthood.

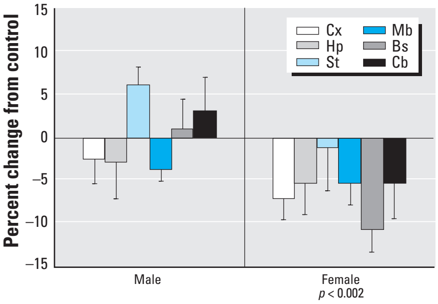

Figure 5. Effects of PN11–14 CPF exposure (5 mg/kg/day) on PN60 βAR binding. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Hp, hippocampus; Mb, midbrain; Rx, treatment; St, striatum. Data are means and SEs of the percent change from corresponding control values (Table 1). Across all treatments, all regions, and both sexes, ANOVA (Rx, $p < 0.03$; Rx × sex, $p < 0.03$) indicated a significant treatment effect that differed between the two sexes, so values were separated to conduct lower-order ANOVAs for males and females. Individual regions for which the CPF group differs from the control were not tested because of the absence of a treatment × region interaction.

CPF exposure evoked alterations in AC signaling at all loci within the pathway, displaying both temporal and regional selectivity for the targeting of specific proteins. With the earliest treatment (GD9–12), the responses to the two direct AC stimulants forskolin and Mn^{2+} were enhanced to the same extent, suggesting augmented expression and/or catalytic activity of AC itself. Because there was no change in the forskolin:Mn^{2+} response ratio, it is unlikely that there was a shift in the AC isoform, so a global increase in AC expression is probable. A similar effect was seen in the female hippocampus and male striatum after CPF exposure on GD17–20 and in the cerebral cortex after PN1–4 treatment. On the other hand, the PN11–14 treatment did produce a change in the forskolin:Mn^{2+} response ratio across multiple brain regions in females, indicating that isoform shifts can also be elicited, specifically with late postnatal exposure. Effects on the isoproterenol response, both in absolute terms and relative to the changes in the forskolin response, also indicated the targeting

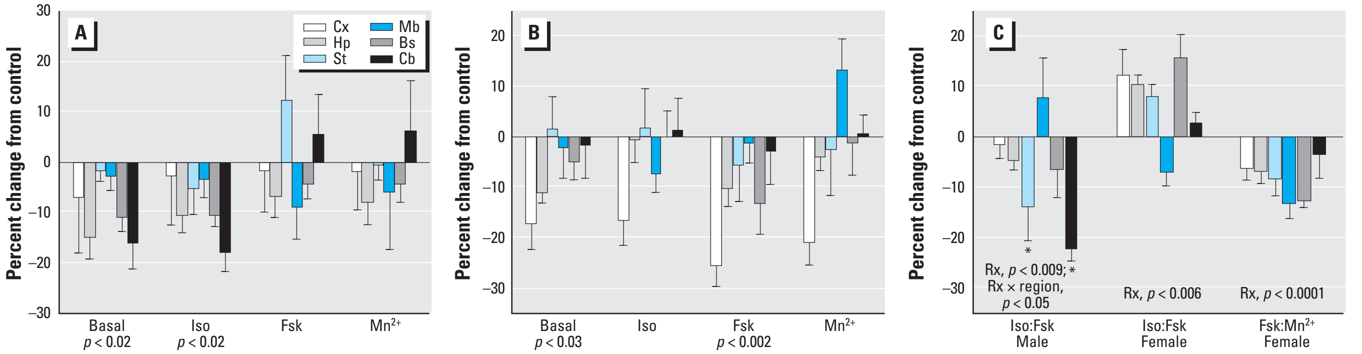

Figure 6. Effects of PN11–14 CPF exposure (5 mg/kg/day) on PN60 AC activity. (*A*) Males. (*B*) Females. (*C*) AC activity ratios. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Fsk, forskolin; Hp, hippocampus; Iso, isoproterenol; Mb, midbrain; Rx, treatment; St, striatum. Across all regions and both sexes, ANOVA indicated a significant interaction of treatment with sex, so values were separated for males (*A*) and females (*B*). Data are means and SEs of the percent change from corresponding control values (Table 1). ANOVA across all regions and measures is as follows: for (*A*) Rx × measure, $p < 0.02$; for *B*, Rx, $p < 0.04$; Rx × measure, $p < 0.02$). Lower-order tests of the individual AC measures are shown within the figure. Separate tests for each region were not carried out because of the absence of a treatment × region interaction. Activity ratios (*C*) were calculated because of the selective effects of CPF on the various AC stimulants; ANOVA across regions appears within the figure.

*Individual groups differ significantly from the control (calculated only when lower-order tests indicated a significant treatment × region interaction).

of receptor-mediated AC stimulation. With GD9–12 treatment, the βAR-mediated effect was augmented to a smaller extent than that of the direct AC stimulant (decreased isoproterenol:forskolin response ratio), suggesting an impairment of receptor coupling to AC superimposed on the induction of AC itself. The same effect was seen in the striatum when CPF treatment was given on GD17–20, in the cerebral cortex and male cerebellum with the PN1–4 exposure, and in the male striatum and cerebellum for the PN11–14 exposure. We also found one instance of a specific enhancement of the isoproterenol response, in the brainstem of the PN1–4 group. Again, it is possible to make inferences about the actual locus of CPF's effects on βAR-mediated AC signaling: none of the changes correlated with the alterations in βAR binding sites, which in some cases were opposite to the effects on the isoproterenol AC response. Accordingly, these particular effects of CPF are likely to depend upon alterations in expression or function of the G-proteins that couple βARs to AC activity. Our general conclusion, then, is that the effects of CPF on AC signaling reflect actions exerted at the levels of the signaling components downstream from the receptors, the G-proteins and AC itself; therefore, the changes are heterologous, affecting all inputs that converge on this pathway. This inference is consistent with the view that development of G-protein–coupled receptor-mediated cell signaling is regulated primarily by mechanisms operating at the levels of G-proteins and AC (Gao et al. 1998, 1999; Gaudin et al. 1995; Karoor et al. 1996; Kohout and Lefkowitz 2003; Ostrom et al. 2000; Slotkin et al. 2003; Vatner et al. 1998; Watts 2002).

Finally, as in our previous studies with CPF (Aldridge et al. 2003; Dam et al. 2000; Garcia et al. 2002; Icenogle et al., in press; Levin et al. 2001, 2002; Slotkin et al. 2001a, 2002), we found distinct sex differences for exposures on GD17–20, PN1–4, or PN11–14 but not with the early gestational treatment (GD9–12). As examples, with the GD17–20 regimen, βAR binding was affected in opposite directions in males and females, as were striatal and cerebellar AC activities; for PN1–4 exposure, basal and isoproterenol-stimulated AC activities were reduced in males but enhanced in females; and for the PN11–14 treatment, βARs and forskolin-stimulated AC were reduced only in females, the isoproterenol:forskolin response ratio was affected in opposite directions in the two sexes, and only females showed an AC isoform shift (reduced forskolin:Mn^{2+} response ratio). Although it is not possible from these data alone to determine a specific mechanism for the sex differences, it is important to note that sexual differentiation of the brain commences toward the latter part of gestation in the rat (McCarthy 1994; Mong and McCarthy 1999) and specifically involves the cAMP pathway (Auger 2003). Although CPF is only weakly estrogenic (Andersen et al. 2002; Vinggaard et al. 2000), there is new evidence that it interferes with testosterone catabolism (Usmani et al. 2003). Additionally, the effects of CPF on brain development are themselves likely to influence sexual differentiation and resultant endocrine responses or hormonal levels because CPF intoxication in adults has distinct endocrine effects (Guven et al. 1999). In any case, the present findings of a critical developmental period for the lasting, sex-selective effects of CPF on AC signaling are consonant with behavioral assessments that demonstrate the same window of vulnerability (Icenogle et al., in press; Levin et al. 2001, 2002).

In summary, we found lasting alterations in AC signaling in a wide variety of rat brain regions after CPF exposure during developmental windows ranging from the earliest phases of brain development (neurulation) through postnatal stages that are comparable with human brain development in the perinatal period (Rodier 1988). Within this broad window of vulnerability, there were differences in the regional locus, sex selectivity, and the specific signaling proteins targeted by CPF that depended on the period of exposure. Given the pivotal role played by AC signaling as a final common pathway in the response to neuronal and hormonal signals, the persistent alterations seen here are likely to contribute to lasting physiologic and behavioral alterations after developmental exposure to CPF.

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