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Neurochemical and Neurobehavioral Effects of Repeated Gestational Exposure to Chlorpyrifos in Maternal and Developing Rats

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CHANDA, S. M. AND C. N. POPE. *Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyrifos in maternal and developing rats.* PHARMACOL BIOCHEM BEHAV **53**(4) 771-776, 1996. – Acute exposure to the organophosphate pesticide chlorpyrifos (CPF) on gestation day 12 (GD12, 200 mg/kg/ml, SC) causes extensive neurochemical changes in maternal brain but lesser changes in fetal brain. In the present study, we examined the relative neurotoxicity of repeated, lower-level CPF exposures during gestation in rats. Pregnant Sprague-Dawley rats were exposed to CPF (6.25, 12.5, or 25 mg/kg per day, SC) from GD12-19 and sampled at either GD16, GD20, or postnatal day 3 (PND3) for measurement of various maternal and developmental neurochemical markers. In contrast to the high acute dose exposure, no maternal toxicity was noted with repeated lower-level dosing. Extensive acetylcholinesterase (AChE) inhibition (83-90%) was noted in maternal brain at all three time points following repeated exposures (25 mg/kg). Higher AChE inhibition (58%) was noted in fetal brain at GD20 compared to 19-25% on PND3 in treated pups cross-fostered to control dams and in control pups cross-fostered to treated dams following repeated exposures (25 mg/kg per day). Whereas similar reductions in brain muscarinic receptor binding were noted at GD20 and PND3 in dams and developing brain between acute and repeated dosing regimens, greater changes in [^{3}H]CD and [^{3}H]cytisine binding were evident following repeated exposures. Righting reflex and cliff avoidance tests were markedly altered following repeated exposures. The results suggest that lower-level repeated exposures to CPF cause extensive neurochemical and neurobehavioral changes in developing rats in the absence of maternal toxicity.

Cholinesterase inhibition Cholinergic neurochemistry Organophosphate Repeated prenatal exposure

CHLORPYRIFOS (CPF) is an organophosphate (OP) pesticide which exerts toxicity by inhibiting acetylcholinesterase (AChE, EC 3.1.1.7) in the central and peripheral nervous systems. Inhibition of AChE leads to accumulation of acetylcholine in the synapse, producing typical signs of cholinergic toxicity (e.g., excessive salivation, tremors, muscle fasciculations). Prolonged inhibition of acetylcholinesterase by OP compounds results in inactivation or internalization of cholinergic receptors, thereby leading to tolerance to organophosphate toxicity (5).

We previously noted that a high, acute dose of CPF [200 mg/kg per ml, SC, at gestational day 12 (GD12)] caused extensive inhibition of brain AChE activity in dams (>80%) and fetus (40-44%) throughout gestation (3), with a subset of dams (about 14%) exhibiting signs of acute toxicity characteristic of AChE inhibition. Maternal and fetal brain muscarinic receptor binding was also reduced 11-30% following acute exposures to CPF during gestation. The results suggested that acute exposure during gestation produces more extensive neurotoxicologic effects in the dam compared to the fetus.

Several studies have reported that repeated exposures to organophosphates during gestation can cause fetotoxicity and marked neurochemical changes in the developing brain (7, 11,12,18,23). In adult rats, repeated exposure to organophosphate pesticides leads to tolerance (5), partly dependent on a decrease in the densities of muscarinic and nicotinic receptors (6,8,22,23). In contrast, young rats were relatively resistant to the persistent neurochemical and neurobehavioral alterations seen in adult rats following repeated CPF exposures (2).

The objective of this study was to examine the relative

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neurochemical and neurobehavioral changes in developing and maternal systems following lower-level repeated exposure to CPF during gestation (GD12–19). We conclude that marked neurochemical and behavioral alterations occur in the developing organism following repeated CPF exposures in the absence of overt maternal toxicity.

METHODS

Chemicals

Chlorpyrifos (*O,O'*-diethyl *O*-(3,5,6-trichloro-2-pyridyl phosphorothioate, 98% purity) was purchased from Chem Service (Westchester, PA). [^{3}H]Acetylcholine iodide (spec act 93.2 mCi/mmol), [^{3}H]cis-methyl dioxolane (CD) (spec act 64.5 mCi/mmol), [^{3}H]cytisine (CYT) (spec act 35.5 mCi/mmol), and [^{3}H]quinuclidinyl benzilate (QNB) (spec act 43.0 mCi/mmol) were all purchased from New England Nuclear (Boston, MA). Other reagents used were of analytic grade.

Animals and Treatment

Gestation day zero of pregnancy was confirmed by sperm-positive vaginal smears. Pregnant Sprague-Dawley rats were housed individually in polycarbonate cages and maintained on a 12 L : 12 D cycle. The animals were provided commercially available rat chow (Diet 7001; TEK-Lad, Madison, WI) and water ad lib.

Pregnant rats (n = 5/treatment per time point) were injected with either peanut oil (PO) or CPF (25 mg/kg per day in peanut oil, SC) from GD12–19 and sacrificed on either GD20 or postnatal day 3 (PND3). The day of birth was considered to be postnatal day zero (PND0). For the dose-response study, rats were exposed to lower doses of CPF (either 6.25 or 12.5 mg/kg per ml per day, GD12–19) and sacrificed on GD20 for analysis of various neurochemical markers. Body weight and signs of acute toxicity in dams and pups were recorded daily.

On PND1, pups were weighed, sexed, and then randomized between dams and culled to seven to eight pups per dam. Three cross-fostered treatment groups were established: a) CDCP: control pups cross-fostered to control dams; b) CDTP: treated pups cross-fostered to control dams; and c) TDCP: control pups cross-fostered to treated dams.

Tissue Preparation and General Methods

Rats were decapitated on GD16 or GD20 and maternal whole-brain samples were collected. Fetuses were removed by ceasarian section. The fetuses were weighed and sexed before collecting fetal whole-brain samples at GD16 or GD20. Tissue samples were frozen at −55°C until assay. In postnatal studies, the pups were weighed and observed for signs of toxicity each day. For either prenatal or postnatal studies, the individual litter was the unit of analysis throughout, and tissues from pups were pooled (5–8/pool).

Acetylcholinesterase activity was assayed using a radiometric method (14) with a final concentration of 0.12 mM [^{3}H]ACh iodide. Maternal (1 : 50, w/v) and fetal or pup (1 : 30, w/v) brain homogenates were made using a Polytron PT 3000 homogenizer (28,000 rpm for 20 s; Brinkman Instruments, Westbury, NY) in 50 mM Tris (hydroxymethyl) amino methane buffer, pH 7.4, containing the following salts: NaCl, 120 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM (Tris-salts buffer). Preliminary experiments defined conditions for both incubation time and tissue concentration required for linear rates of substrate hydrolysis. Enzyme activity was calculated relative to protein concentration. Protein content was estimated by the method of Lowry and co-workers (17) using bovine serum albumin as standard.

Total muscarinic receptor (QNB) binding was measured by the method of Yamamura and Snyder (24) as described by Chanda and co-workers (3). Briefly, maternal and fetal brain samples were homogenized as before. Membrane fractions (1 : 50, w/v, for maternal brain and 1 : 30, w/v, for fetal or pup brain) were prepared. Binding of [^{3}H]QNB (0.75 nM final concentration) was determined by incubation with approximately 0.1–0.2 mg of membrane protein in the Tris-salts buffer for 60 min at 37°C in a final volume of 2 ml. The tissues were then filtered rapidly, washed using a Brandel cell harvestor (Model M-24R; Gaithersburg, MD), and collected in scintillation fluid (BCS; Amersham Corporation, Arlington Heights, IL). Radioactivity was counted the following day in a Beckman LS 3801 counter (Fullerton, CA) at 43% efficiency. Specific binding was determined by calculating the difference in binding in the presence and absence of atropine (10 μM final concentration).

[^{3}H]cis-methyldioxolane (CD, a high-affinity muscarinic agonist) binding was measured essentially by the method of Ehlert and co-workers (8) as described previously (4). Membranes were incubated with [^{3}H]CD (4 nM final concentration) in a final volume of 2 ml in Tris-salts buffer for 60 min at 37°C. Tissues were filtered and specific binding determined using atropine as described before.

Binding to the nicotinic agonist [^{3}H]cytisine (CYT) was performed according to the method of Pabreza and co-workers (19). Membranes were incubated with [^{3}H]CYT (4 nM final concentration) in a final volume of 0.25 ml in Tris-salts buffer for 75 min at 2°C. Specificity was determined by inclusion of nicotine (10-μM final concentration) in paired samples. Tissues were filtered and radioactivity counted as described before.

Behavioral Testing

The surface righting reflex was measured by placing the pup in a supine position and determining its ability to turn over. The time required to place all four feet on the surface was recorded (with a limit of 60 s) for each trial. Cliff avoidance behavior was measured by placing the pups on the edge of a table and noting its ability to avoid the "cliff." Turning or backing away from the edge of the table was noted to be a positive response. Again, a 60-s limit was established (13). Behavioral tests were conducted in a blinded manner.

Statistical Analyses

Brain ChE, QNB, CD, and CYT receptor densities, and fetal body weights were compared among groups for each tissue at each time point using analysis of variance (ANOVA) (21). Significant interactions were interpreted by the least-squares means procedure. Duncan's multiple-range test was used to compare receptor densities among the age groups at different time points. Repeated-measures ANOVA was used to compare maternal body weight data among the treatment groups (21). An α level of 0.05 was chosen for significance in all tests. The litter was used as the unit of analysis for all measures taken on fetuses or pups.

RESULTS

Maternal and Fetal Toxicity

Repeated exposure to CPF did not produce any overt signs of toxicity in the dams characteristic of AChE inhibition.

There was an initial decrease in maternal body weight (2%, 3 days after treatment) after which the treated rats gained weight at approximately the same rate as did the controls (Fig. 1A). As expected, fetal and neonatal body weight increased from GD16 to PND1, but there was a significant reduction in body weight of treated pups at PND1 relative to controls ($p < 0.001$, $df = 20$) (Fig. 1B).

Biochemical Studies

Cholinesterase inhibition. A significant dose-related inhibition of AChE was observed following the three dosing regimens at GD20 ($p < 0.001$, $df = 31$, $F = 45.65$) (Fig. 2A). In each case, maternal brain AChE inhibition was greater than the fetal brain AChE inhibition with all three doses (Fig. 2A). Extensive AChE inhibition was noted in both dams and fetuses or pups at all three time points following repeated exposure (25 mg/kg per day) (Fig. 3A). There was 83–90% and 42–58% inhibition of AChE in dams and fetuses at GD16 and GD20, respectively ($p < 0.001$, $df = 27$, $F = 6.968$). At PND3 there was still extensive inhibition of AChE in maternal brain (85%, $p < 0.001$) and significant AChE inhibition (19–25%, $p < 0.005$) in neonatal brain in both treated pups cross-fostered to control dams (CDTP) and control pups cross-fostered to treated dams (TDCP, $p < 0.006$, $df = 8$, $F = 13.79$) (Fig. 3A).

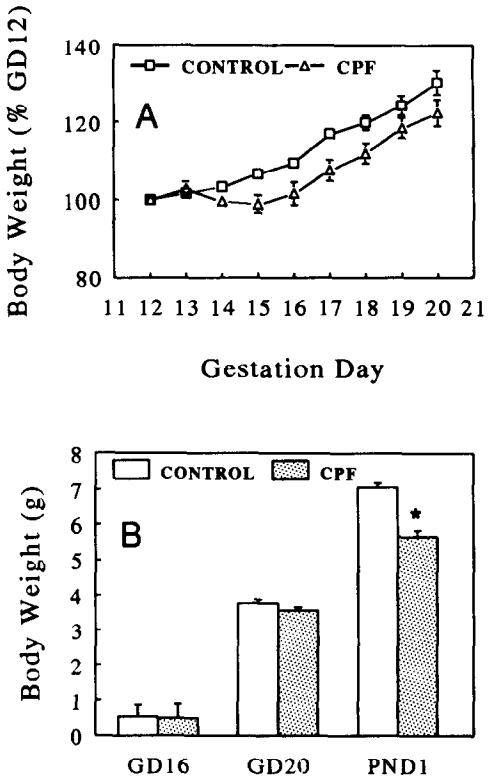

FIG. 1. (A) Changes in maternal body weights after repeated exposure to CPF (25 mg/kg per day, SC). CPF was administered from GD12–19 and body weights were recorded daily. Data represent the percent change in body weight following CPF exposure (mean ± SEM, $n = 5$/treatment group). (B) Effect of repeated exposure to CPF (25 mg/kg per day, SC, GD12–15/GD19) on fetal/pup body weight. Data represent body weights in grams (mean ± SEM, $n = 5$/treatment group). *Significant differences between control and treated ($p < 0.05$).

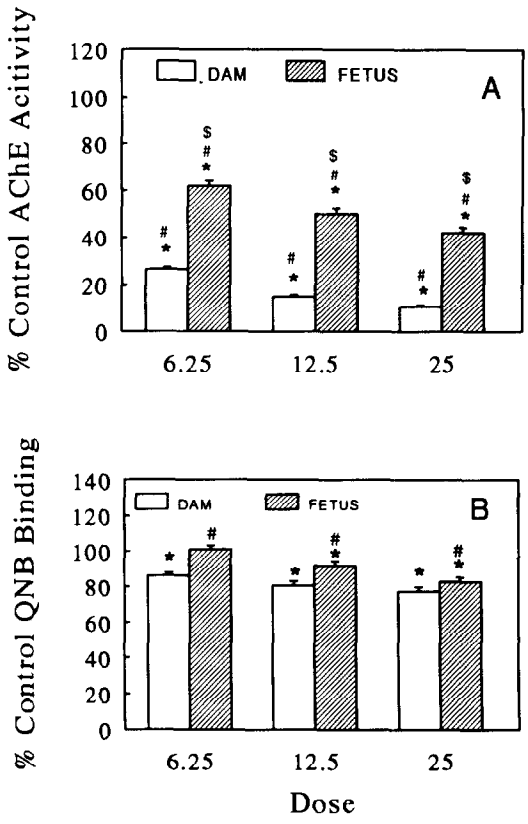

FIG. 2. (A) Dose-related inhibition of brain cholinesterase activity following repeated exposure to CPF in dams and fetus. The rats were exposed to either 6.25, 12.5, or 25 mg/kg per day CPF, SC, from GD12–19 and sacrificed on GD20. Data represent the percentage of control brain cholinesterase activity at GD20 (mean ± SEM). Control values (nanomoles of [^{3}H]acetylcholine hydrolyzed per minute per milligram of protein) 6.25 and 12.5 mg/kg per day: dam = 39.24 ± 1.04, fetus = 10.08 ± 0.28; 25 mg/kg per day: dam = 63.56 ± 2.56, fetus = 16.8 ± 0.54. (B) Dose-related effect of CPF (either 6.25, 12.5, or 25 mg/kg per day, SC, of CPF GD12–19) on QNB binding in maternal and fetal brain at GD20. Data represent the percentage of vehicle control values (mean ± SEM). Control values (pmol [^{3}H]QNB bound/mg protein) for 6.25 and 12.5 mg/kg per day: dam = 2.35 ± 0.03, fetus = 0.25 ± 0.01; 25 mg/kg per day: dam = 2.58 ± 0.16, fetus = 0.53 ± 0.005. *Significant differences between treated and control values; #Significant difference between doses; $Significant difference between dam and fetus ($p < 0.05$).

Receptor binding. A dose-related downregulation of muscarinic receptors was noted following the three different dosing regimens in maternal and fetal brain ($p < 0.001$, $df = 31$, $F = 14.04$) (Fig. 2B). [^{3}H]QNB binding was reduced in maternal (23%, $p < 0.005$) and fetal brain (17%, $p < 0.001$) at GD20 (CPF 25 mg/kg per day) (Fig. 3B). At PND3, total muscarinic receptor binding was still reduced in maternal brain (31%, $p < 0.001$) and in the CDTP group (27%, $p < 0.003$) (Fig. 3B).

At GD20, binding to [^{3}H]CD and [^{3}H]cytisine was significantly reduced in dams (7–26%, $p = 0.015$ and $p = 0.036$, respectively) (Fig. 3C and D). The fetal brain [^{3}H]CD binding was reduced by 11% at GD20 ($p < 0.001$) (Fig. 3C). At

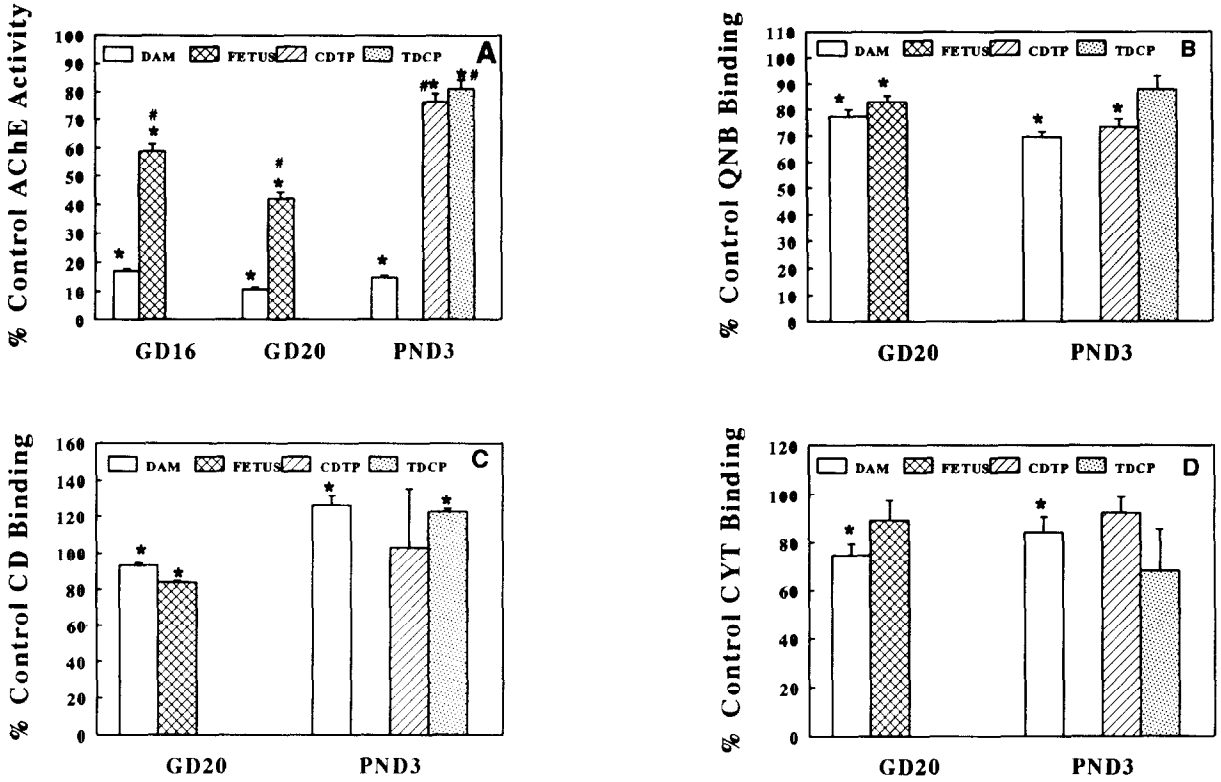

FIG. 3. (A) Effect of repeated exposure to CPF (25 mg/kg per day, SC, GD12–15/GD19) on maternal and fetal or pup brain cholinesterase activity at GD16, GD20, and PND3. Data represent the percentage of control values (mean ± SEM). Control values (nanomoles of [^{3}H]acetylcholine hydrolyzed per minute per milligram of protein) at GD16: dam = 52.2 ± 1.59, fetus = 6.46 ± 4.8; GD20: dam = 63.6 ± 2.56, fetus = 16.8 ± 0.54; PND3: dam = 47.03 ± 1.74, pup = 19.07 ± 0.54. (B) Effect of repeated exposure to CPF (25 mg/kg per day, SC, GD12–15/GD19) on maternal and fetal or pup brain QNB binding at GD20 and PND3. Data represent the percentage of control values (mean ± SEM). Control values (picomoles of [^{3}H]QNB bound per milligram of protein) at GD20: dam = 2.58 ± 0.16, fetus = 0.53 ± 0.005; PND3: dam = 2.07 ± 0.07, pup = 0.54 ± 0.005. (C) Effect of repeated exposure to CPF (25 mg/kg per day, SC, GD12–15/GD19) on maternal and fetal or pup brain CD binding at GD20 and PND3. Data represent the percentage of control values (mean ± SEM). Control values (picomoles of [^{3}H]CD bound per milligram protein) at GD20: dam = 0.39 ± 0.006, fetus = 0.048 ± 0.001; PND3: dam = 0.003 ± 0.0003, pup = 0.0003 ± 0.00004. (D) Effect of repeated exposure to CPF (25 mg/kg per day, SC, GD12–15/GD19) on maternal and fetal or pup brain CYT binding at GD20 and PND3. Data represent the percentage of control values (mean ± SEM). Control values (picomoles of [^{3}H]CYT bound per milligram protein) at GD20: dam = 0.015 ± 0.001, fetus = 0.019 ± 0.001; PND3: dam = 0.015 ± 0.0009, pup = 0.029 ± 0.013. *Significant differences between treated and control values; #Significant difference between dam and fetus or pups. (CDTP = treated pups cross-fostered to control dams, TDCP = control pups cross-fostered to treated dams, $p < 0.05$).

PND3, there was an increase in [^{3}H]CD binding in dam (26%) and TDCP (22%) but no significant change in the CDTP group ($p < 0.05$, $p < 0.012$) (Fig. 3C). A significant (15%) reduction in [^{3}H]cytisine was noted in dams only at PND3 ($p < 0.05$) (Fig. 3D).

Behavioral Analyses

CPF exposure caused extensive alterations in both surface-righting reflex and cliff avoidance at PND1 and PND3. There was a significant increase in righting reflex time and a decrease in the percent cliff avoidance ($p < 0.001$) (Fig. 4A and B).

DISCUSSION

Previous studies from our laboratory have demonstrated that a high, single dose of CPF (200 mg/kg per ml, SC, at GD12) causes significant changes in maternal and fetal/neonatal neurochemistry (3). In contrast to adult male rats, in which few sign of cholinergic toxicity are noted, a subset of dams exposed to the high acute dose exhibited signs of cholinergic crisis. Lower-level repeated exposure studies were performed to compare maternal toxicity and examine the relative effect of such a dosing regimen on developing brain neurochemistry and neurobehavior of the progeny. For repeated exposures, the total acute dose (200 mg/kg) was separated into eight smaller doses (25 mg/kg per day, SC) and injected daily over an 8-day period (GD12–19). In contrast to the acute exposure, repeated CPF treatments did not produce any typical signs of cholinergic toxicity, and there was only a marginal decrease in maternal body weight and no change in fetal body weight at GD16 or GD20. There was a slight decrease in pup body weight at PND1, however. This may indicate that the fetus is more protected before birth than the pup after parturition.

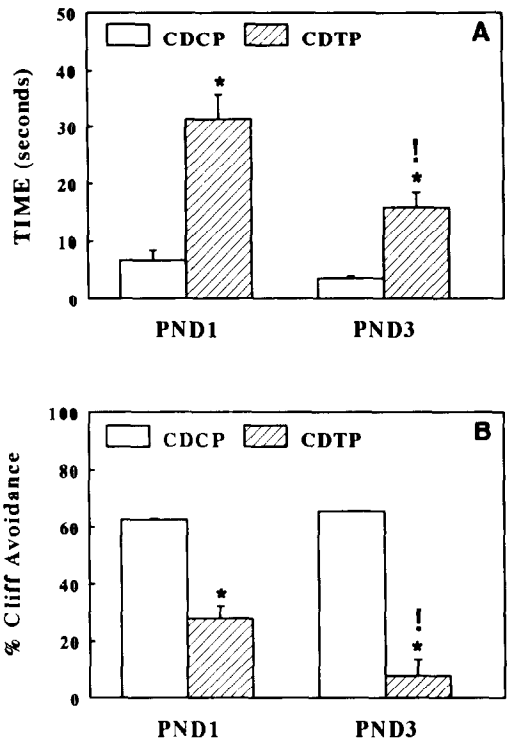

FIG. 4. (A) Effect of gestational exposure to CPF (25 mg/kg per day, SC, GD12-19, SC) on surface-righting reflex in the neonatal rats. Surface-righting reflex was measured on PND1 and PND3 in two groups, control pups cross-fostered to control dams (CDCP) and treated pups cross-fostered to control dams (CDTP). Data represent time (s) taken by the pups to return to an upright position (mean ± SEM). (B) Effect of gestational CPF (25 mg/kg per day, SC, GD12-19, SC) exposure on cliff avoidance behavior in the neonatal rats. Data represent the percentage of pups avoiding the "cliff" (mean ± SEM). *Significant differences between the treatment groups, !Significant differences between the time points ($p < 0.05$).

OP insecticides exert toxicity through inhibition of AChE, thereby causing accumulation of acetylcholine at the synapse within the central and peripheral nervous system. CPF caused dose-related inhibition of AChE following all three gestational dosing regimens. Similar to our previous results with acute exposure to CPF, AChE was more extensively inhibited in dams compared to the fetus (3). There was some recovery of pup cholinesterase activity by PND3, whereas dam ChE activity still remained >80% inhibited. Michalek and co-workers (18) reported almost complete recovery of fetal brain AChE compared to persistent inhibition of maternal AChE activity after subchronic intoxication by diisopropylfluorophosphate (DFP). Similar to our findings following a single high dose, pups from dams which had not been exposed to CPF but were then cross-fostered to treated dams on PND1 showed significant inhibition of brain ChE activity (Fig. 3A), indicating that the pups were exposed to CPF or its active metabolite CPF-oxon during lactation.

Accumulation of acetylcholine following extensive AChE inhibition induces changes in muscarinic receptor densities (5). Downregulation of muscarinic receptors is thought to be an important mechanism for the development of tolerance to OP compounds (5). Several studies have reported a decrease in the number of QNB binding sites (B_{max}) in dam and fetus after repeated exposures to OP insecticides during gestation (12,18). In the present study, there was a dose-related decrease in QNB binding with three different dosing regimens in both maternal and fetal brain. A greater reduction in QNB binding was noted in maternal and pup brain at PND3 (31 and 27%, CDTP) compared to GD20 (23 and 17%, CDTP), respectively. Somewhat greater reductions in QNB binding were noted with repeated exposures in both dams and fetus or pups compared to our previous study using acute exposure. Repeated exposures also caused more changes in binding to other cholinergic receptor ligands [i.e., the muscarinic agonist [^{3}H]*cis*-methyldioxolane (CD) and the nicotinic agonist [^{3}H]cytisine exhibited more changes in maternal or fetal or pup brain].

The overall data suggest that repeated gestational exposure to CPF causes more profound changes in the maternal and fetal brain cholinergic system compared to a single high dose during early gestation. Similar to our previous studies, dams were more affected than the developing system by the CPF exposure. As the fetal brain cholinesterase was more sensitive to CPF than the maternal brain (3,20), therefore, the same hypothesis that the difference in maternal and fetal brain neurochemistry is due to biotransformation and/or delivery of CPF to the fetus could be extended to this study as well.

Neurochemical changes were correlated with neurobehavioral alterations in two reflex tests examined at early postnatal time points. As with the neurochemical findings, more extensive neurobehavioral changes were seen with repeated CPF exposures compared to high single dose. A similar phenomenon was reported by Bushnell and co-workers (1), in whose study repeated exposure to CPF in adult male Long-Evans rats produced marked changes in certain behavioral tests compared to a single high exposure. This suggests that even with similar changes in cholinergic neurochemistry, certain behaviors may be more sensitive to disruption by repeated exposures to CPF.

In summary, pregnant dams exposed repeatedly to CPF during gestation exhibited only a small change in body weight and no signs of cholinergic toxicity. More extensive changes in cholinergic parameters were noted in the maternal brain compared to the fetus or neonate, although such exposures were associated with persistent neurochemical and neurobehavioral changes in the developing organisms. Acetylcholine levels and other cholinergic neurochemical markers increase markedly during development (15). There is also evidence to suggest that the cholinergic processes may be involved in the organization of extracellular matrix-cell-surface interactions involved in morphogenetic movements during development (9,10). Both AChE and butyrylcholinesterase may play roles in axonal growth regulation, possibly by adhesive mechanisms: both cholinesterases appear to participate in neural proliferation and synaptogenesis in the developing nervous system (16). Exposure to anticholinesterases such as CPF during early development could therefore alter the normal development of the nervous system and lead to persistent changes in behavior in later life.

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