Pharmacology Biochemistry and Behavior, Vol. 46, pp. 219–224, 1993
Printed in the U.S.A.

0091-3057/93 $6.00 + .00

Comparative Neurochemical and Neurobehavioral Effects of Repeated Chlorpyrifos Exposures in Young and Adult Rats

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CHAKRABORTI, T. K., J. D. FARRAR AND C. N. POPE. *Comparative neurochemical and neurobehavioral effects of repeated chlorpyrifos exposures in young and adult rats.* PHARMACOL BIOCHEM BEHAV 46(1) 219–224, 1993.— Neonatal (7 days old) rats are markedly more sensitive than adults (3 months old) to the acute toxic effects of the insecticide, chlorpyrifos (CPF). In the present study, we have compared the effects of subacute CPF exposures in these same age groups. Repeated doses of CPF (40 mg/kg, SC, every 4 days, total of 4 doses) caused extensive inhibition of cortical, hippocampal, and striatal cholinesterase (ChE) activity in adult rats at 4 (90–92%) and 14 (71–78%) days after the last treatment. Rats treated similarly during postnatal maturation (beginning on day 7) showed a much lower degree of ChE inhibition (21–60%) at these time points. Muscarinic ([^{3}H]quinuclidinyl benzilate, QNB) receptor binding in cortex, hippocampus, and striatum was reduced in adult brain at 4 (30–43%) and 14 (22–32%) days after the final treatment, whereas receptor densities were only marginally affected (5–11% reduction) in young rats. Basal motor activity levels were not affected in either young or adult rats as a function of CPF exposure. CPF-treated adult rats exhibited higher activity levels after challenge with scopolamine (1 mg/kg, IP) at 2, 4, 6, and 8 weeks after treatment, whereas CPF exposure did not affect the motoric response to scopolamine in rats treated during postnatal maturation. These data suggest that although neonatal rats are more sensitive to acute lethal effects from high doses of CPF, adult rats exhibit more persistent neurochemical and neurobehavioral alterations following repeated, lower-level exposures.

Organophosphate Cholinesterase Inhibition Chlorpyrifos Muscarinic receptors Motor Activity Scopolamine Postnatal

SIGNS of acute toxicity following exposure to organophosphorus (OP) insecticides are generally attributable to their inhibition of acetylcholinesterase (AChE, E.C. 3.1.1.7) in the central and peripheral nervous system, with subsequent accumulation of acetylcholine (ACh) at synaptic terminals (11,19). There is considerable evidence to suggest that neonatal/perinatal animals are more sensitive than adults to acute exposure to a number of OPs (2,4,10,13,16,20,22,25). Using the maximum tolerated dose as a measure of sensitivity, we estimated (20) that neonates (7 day old rats) were approximately 2.3, 8.6, and 6.2 times more sensitive than adults (3 months of age) to the acute toxicity of three common OP insecticides, methyl parathion, parathion, and chlorpyrifos. Little is known, however, regarding the comparative sensitivity of developing and adult rats to subacute OP exposures.

The objective of the present study was to compare the effects of equivalent, repeated lower doses of chlorpyrifos (40 mg/kg × 4, every 4 days, SC) on cholinergic neurochemical markers in immature and adult rats. As we had previously noted a long-term change in sensitivity to the locomotor stimulant effect of scopolamine in adult rats following a single dose of chlorpyrifos (21), we also examined the effects of repeated CPF exposures on basal and scopolamine-induced locomotor activity levels.

METHOD

Animals

Adult, male Sprague–Dawley rats (3 months of age, average body weight about 375 g) were used throughout the experiment. Adult rats were maintained in community cages until 1 week before treatment, at which time they were transferred to individual steel mesh cages. Pregnant females were housed individually in plastic cages and dates of birth (postnatal day 0) were recorded. Pups were routinely randomized among the dams and culled to eight pups/dam (no selection by sex) on

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postnatal day 2. Animals were maintained on a 12L : 12D illumination cycle.

Chemicals

Chlorpyrifos (*O,O'*-diethyl-*O*-3,5,6-trichloro-2-pyridyl-phosphorothioate) was purchased from Chem Service, West Chester, PA and was 99% pure. [^{3}H]Acetylcholine iodide (specific activity 100 mCi/mmol) and [^{3}H]quinuclidinyl benzilate [QNB (44.90 Ci/mmol)] were purchased from New England Nuclear, Boston, MA. All other chemicals were reagent grade.

Treatments

Chlorpyrifos was dissolved in peanut oil and rats were administered equivalent doses (40 mg/kg × 4, SC, 1 ml/kg) at an interval of 4 days (total of 4 doses). Treatments were initiated at 7 days of age (young rats) or 3 months of age (adults). Control animals were treated with peanut oil only. Six animals were treated per age group per time point and were sacrificed at either 4, 14, 28, or 56 days after the fourth treatment for biochemical analyses. Forty animals (n = 10/treatment/age group) were treated for neurobehavioral assessment.

Biochemical Assays

The animals were decapitated at 4, 14, 28, or 56 days after the last treatment and whole brain was rapidly dissected. Cortex, hippocampus, and striatum were isolated essentially as described by Glowinski and Iversen (12), and tissue samples were frozen at −55°C for less than 2 weeks before use. On the day of the assay, samples were thawed at room temperature and suspended in 50 mM Tris (hydroxymethyl) aminomethane buffer, pH 7.4 (25°C) containing NaCl, 120 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM. Tissues were homogenized on ice with a Polytron PT 3000 homogenizer (Brinkman Instruments, Westbury, NY) at 32,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × g for 10 min at 4°C with a Beckman J2-21 centrifuge. The pellets were washed twice in an equivalent amount of Tris buffer by rehomogenization and centrifugation as before.

Cholinesterase activity was measured essentially by the radiometric method of Johnson and Russell (14) using [^{3}H]acetylcholine iodide as substrate (0.12 mM final concentration). Each reaction mixture (0.1 ml final volume) contained 0.6% Triton X-100 to facilitate membrane disruption.

Muscarinic receptor densities in the membrane samples were determined essentially by the method of Yamamura and Snyder (26) using approximately 0.1 mg membrane protein/rxn (15). Membranes were incubated with [^{3}H]QNB (0.75 nM final concentration) at 37°C for 1 h in the presence or absence of atropine (10 μM final concentration), filtered rapidly, and washed (3 ml × 3) with ice-cold Tris buffer over Whatman GF/C paper using a receptor binding harvestor (Brandel model M-24, Gaithersburg, MD). The filters were collected in scintillation fluid (BCS, Amersham Corporation, Arlington Heights, IL) and radioactivity was counted the following day in a Beckman LS 3801 counter. Specific binding was determined by calculating the difference in binding in the presence and absence of atropine and was reported as pmol [^{3}H]QNB bound/mg protein.

Measurement of Locomotor Activity

The effects of chlorpyrifos exposure on locomotor activity were assessed essentially as described by Finn and coworkers (9) as reported before (21). Individual rats were placed into polycarbonate cages (20 × 22 × 44 cm) that were centered on capacitance boxes (No. SA 1566, Stoelting Co., Wood Dale, IL). The sensitivity of each box was calibrated each day with a metronome. Activity was measured for 30 min (following a 5-min acclimation period) between 1000 and 1300 h, 5 days a week during the light phase of the light/dark cycle.

Scopolamine Challenge

Beginning 14 days after the final chlorpyrifos treatment, animals were placed in the boxes and activity was measured as described above. On the following day, half of the rats received scopolamine (1.0 mg/kg, IP, in saline, 1 ml/kg) while the other half received saline only, and locomotor activity was measured for a 30-min period (beginning 30 min after scopolamine injection). On the next day, the order was reversed such that rats previously receiving scopolamine were

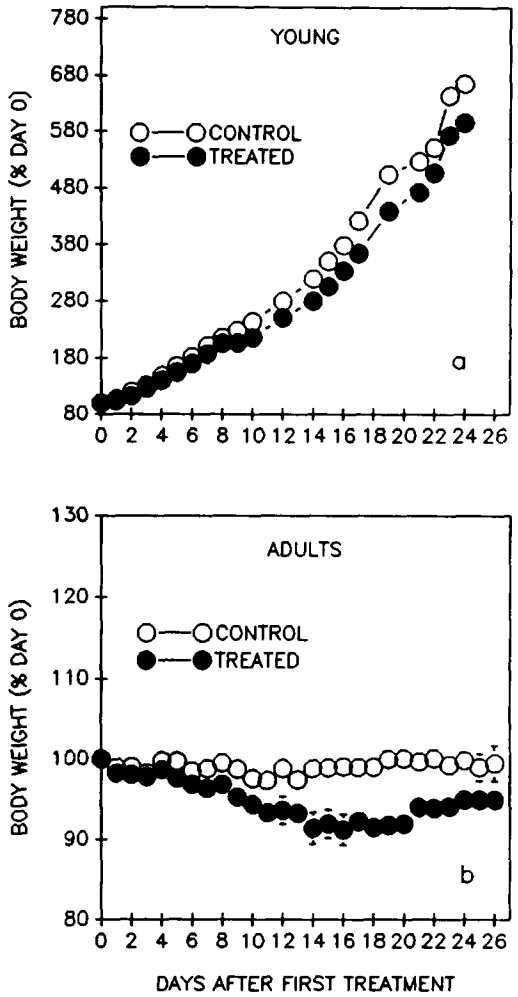

FIG. 1. Body weights in (a) young and (b) adult rats after similar, repeated chlorpyrifos exposures (40 mg/kg × 4, 4 days apart, SC). (a) Young rats (7 days old) were treated with either CPF or peanut oil; body weights were recorded daily and were plotted as percent of weight on day 0 (mean ± SE). (b) Adult rats (3 months of age age) were treated similarly with either CPF or peanut oil and body weights were plotted as above.

given saline, whereas those previously receiving saline were administered scopolamine. Scopolamine challenges were continued every other week for 8 weeks after treatment.

Data Analysis

Brain ChE and QNB binding data were compared between the age groups for each tissue at different time points by factorial analysis of variance (ANOVA) using the SAS General Linear Model (GLM) procedure (24). Significant interactions were interpreted by the LSmeans procedure. Duncan's multiple range test was used to compare receptor densities among the age groups at different time points. Treatment groups were compared at each time point by Student's *t*-test. Repeated measures ANOVA was used to compare body weight and locomotor activity data among the two treatment groups (CPF and control) in young and adult animals using the SAS GLM procedure.

RESULTS

No overt signs of toxicity were noted in young or adult rats treated with chlorpyrifos. Figure 1 shows the body weights of young and adult rats after repeated, equivalent doses of CPF. There was a slight decrease in body weight in adult rats after CPF treatments (8.5% maximal reduction, main effect of chlorpyrifos, $p < 0.01$), but no significant effect was observed in young rats ($p > 0.05$).

The degree of cholinesterase inhibition in cortex, hippocampus, and striatum from 4–56 days after treatment is shown in Table 1. ChE activity was significantly depressed in all three brain regions in both age groups at 4 and 14 days after the last treatment when compared to age-matched controls. Four days after the last CPF treatment, ChE activity was inhibited 90–92% and 55–60% in adults and young rats, respectively. Fourteen days after the last treatment, there was still 71–78% inhibition in adult brain regions but only 20–32% inhibition in brain regions of young animals. A significant age by time interaction ($p < 0.003$) indicated a difference in the rate of ChE recovery in brain between adults and young rats. In adults, brain regional ChE activity did not return to control levels even by 56 days (25–30% inhibition, $p < 0.02$) after the last treatment.

Figure 2 shows the effects of repeated CPF exposures on brain regional muscarinic receptor density in young and adult rats. The degree of receptor downregulation in young and adult rats was significantly different at both time points ($p < 0.0002$). About 30–43% and 7–12% reduction in binding was seen in adult and young rats, respectively, 4 days after the last treatment. Adult brain showed some degree of recovery in receptor density 14 days after the final treatment (22–32% reduction), while young rats still exhibited only a marginal (5–10%) reduction in receptor density. Muscarinic receptor density was still significantly reduced (cortex = 12.4 ± 1.0%; hippocampus = 8.7 ± 0.9%; striatum = 16.9 ± 0.6%, $p < 0.002$) in adult brain regions at 28 days after the last CPF treatment. Fifty-six days after the final treatment, receptor densities were not significantly different from control levels in adult brain in either hippocampus or striatum (data not shown), whereas a slight reduction (5.7 ± 1.0%, $p < 0.05$) was still evident in cortex.

Figure 3 shows the effect of repeated CPF exposures on the locomotor response to scopolamine. Adult rats previously exposed to CPF demonstrated higher levels of activity in response to scopolamine at 2, 4, 6, and 8 weeks after CPF treatment compared to vehicle-treated controls. In contrast, CPF exposure had no significant effect on locomotor response to scopolamine in young animals at any time point (Fig. 4).

DISCUSSION

Previous studies in our laboratory estimated the acute maximum tolerated dose (MTD, an index of acute sensitivity) of three commonly used pesticides in adult (3 months of age) and neonatal (7 days of age) rats: in each case, the immature rats were found to be more sensitive than the adults (20). For example, the MTD (subcutaneous administration) for chlorpyrifos was 45 mg/kg and 279 mg/kg in neonatal and adult rats, respectively. In this study, we have examined the neuro-

TABLE 1

CHOLINESTERASE ACTIVITY IN CORTEX, HIPPOCAMPUS, AND STRIATUM AFTER SIMILAR, REPEATED CHLORPYRIFOS EXPOSURES (40 mg/kg × 4, EVERY 4 DAYS, SC) IN YOUNG AND ADULT RATS

		Time After Treatment			
Tissue*		4 Days	14 Days	28 Days	56 Days
COR	Adult	8.8 ± 0.5†	23.4 ± 0.5	39.7 ± 1.1	75.4 ± 5.9
	Young	45.9 ± 2.8	78.6 ± 3.7	ND	ND
HIP	Adult	10.3 ± 0.7	28.9 ± 1.7	51.1 ± 1.7	69.3 ± 1.7
	Young	45.7 ± 2.5	79.4 ± 2.8	ND	ND
STR	Adult	8.2 ± 0.8	21.5 ± 1.2	42.2 ± 1.9	76.8 ± 1.7
	Young	40.7 ± 1.9	68.1 ± 1.8	ND	ND

*Rats were treated and sacrificed at 4, 14, 28, or 56 days after the last treatment. Different brain regions were isolated as described in the Method Section.

†Values represent mean percent of control ChE activity (±SE). Adult brain control ChE activity (mean nmol of [^{3}H]acetylcholine hydrolyzed per min per mg of protein ±SE): cortex (COR) = 38.1 ± 0.9; hippocampus (HIP) = 31.1 ± 1.7; striatum (STR) = 253.8 ± 4.0. Young brain control ChE activity: COR = 19.5 ± 0.8; HIP = 32.3 ± 0.6; STR = 228.9 ± 14.2. Enzyme values for CPF-treated rats are significantly different ($p < 0.01$) from control for both age groups at all time points measured.

ND, not determined.

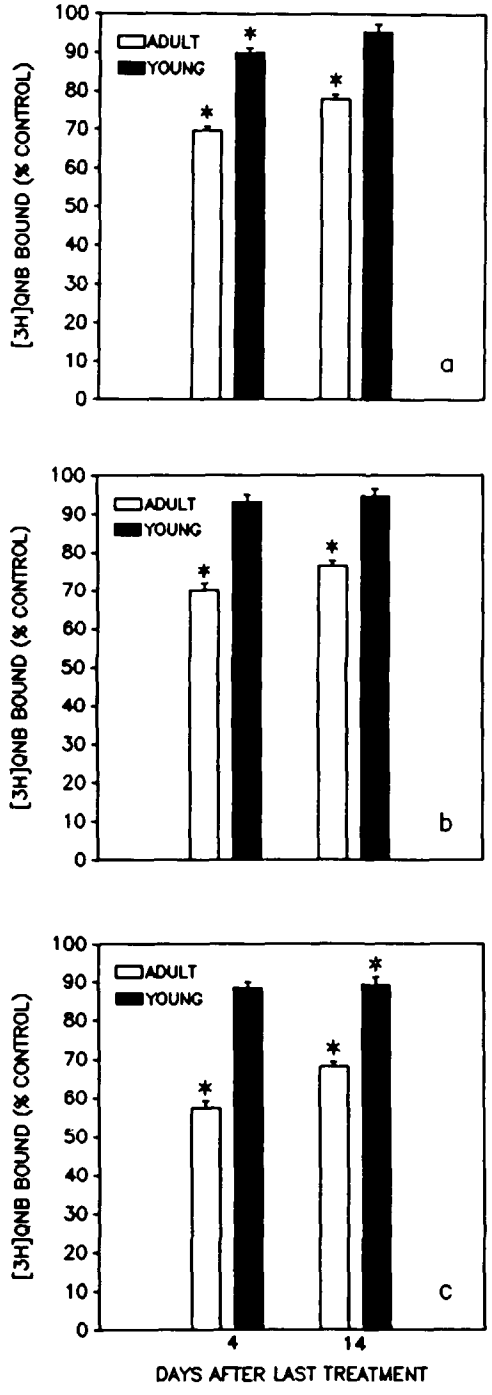

FIG. 2. Effects of equivalent, repeated doses of CPF on muscarinic receptor densities in (a) cortex, (b) hippocampus, and (c) striatum in young and adult rats. Rats were treated as in Fig. 1 and sacrificed at the specified times. Membranes were prepared from brain regions, incubated with [^{3}H]QNB, and maximal binding (mean pmol QNB bound/mg protein ± SE) was determined as described in the Method section. Values are plotted as percent of control. Muscarinic receptor densities (pmol/mg protein ± SE) in adult control brain regions: cortex = 1.55 ± 0.02; hippocampus = 1.40 ± 0.04; striatum = 2.08 ± 0.02. Control brain regional QNB binding in young rats: cortex = 1.35 ± 0.02; hippocampus = 1.39 ± 0.05; striatum = 2.29 ± 0.17. An asterisk indicates a significant difference from control ($p < 0.05$).

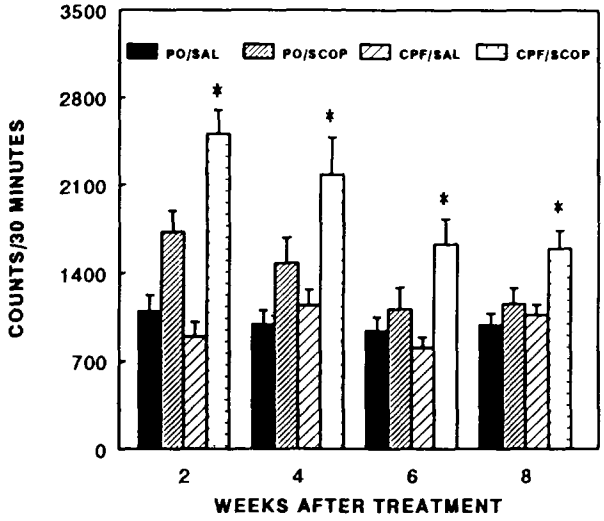

FIG. 3. Effects of chlorpyrifos treatment on the hyperactive effect of scopolamine in adult animals. At biweekly intervals beginning 2 weeks after the last treatment, rats were challenged with scopolamine (1 mg/kg, IP) in saline and locomotor activity was measured 30 min later as described in the Method section. An asterisk indicates a significant difference in response to scopolamine ($p < 0.05$) between the treatment groups. (PO = peanut oil; CPF = chlorpyrifos; SAL = saline; SCOP = scopolamine).

chemical and neurobehavioral effects of equivalent, repeated doses of chlorpyrifos (40 mg/kg × 4, every 4 days, SC) in young and adult rats. Chlorpyrifos was chosen because of the persistent neurochemical and neurobehavioral effects previously observed in adult rats following a single exposure (21), while the dose and dose interval were selected based on our earlier studies regarding acute sensitivity and the time course

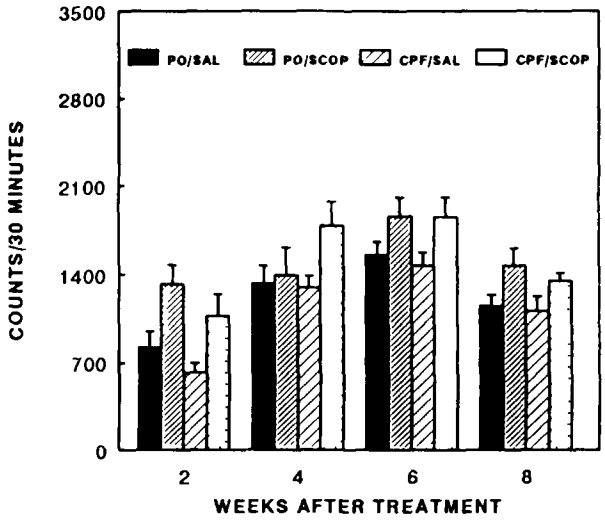

FIG. 4. Effects of chlorpyrifos treatment on the hyperactive effect of scopolamine in young rats. Rats were challenged with scopolamine (1 mg/kg, IP) as in Fig. 3. Responses to scopolamine in control and treatment groups were not significantly different at any time point. Abbreviations are the same as in Fig. 3.

of brain ChE inhibition and recovery in adult and neonatal rats (20).

Repeated, lower-level CPF exposures caused extensive (90–92%) inhibition of brain cholinesterase activity 4 days after the final dose in cortex, hippocampus, and striatum of adult rats, but markedly lower (55–60%) inhibition in similarly treated developing animals. Fourteen days after the final treatment, young rats exhibited a more extensive recovery of ChE activity compared to adults. Recovery of brain ChE activity could be due either to spontaneous reactivation of the diethylphosphorylated enzyme or to de novo protein synthesis of new enzyme. The available literature does not suggest any age-related differences in the rate of spontaneous reactivation of cholinesterase following organophosphorylation. Several reports, however, indicate that rat brain protein synthesis occurs at a much faster rate during early postnatal periods and then declines with age (7,8,18). It therefore seems likely that the age-related differences in recovery of ChE after inhibition by chlorpyrifos depend on relative rates of biosynthesis of new enzyme molecules (3,17). The more persistent inhibition of ChE in adult rats after single or repeated doses of chlorpyrifos could, however, be an indication of more extensive "depot" formation/redistribution in adults relative to young animals.

Muscarinic receptor densities in cortex, hippocampus, and striatum were also significantly reduced in adults following repeated CPF exposures. About 22–43% reduction in QNB binding was observed in adults at both time points in all three different brain regions compared to only 5–11% reduction in the brain regions of young animals. This age-related difference in the magnitude of muscarinic receptor downregulation at both time points would be expected based upon the differential extent of ChE inhibition between the age groups.

Receptor downregulation, while being a mechanism for tolerance to elevated synaptic acetylcholine levels, can increase sensitivity to cholinergic antagonists (5,21,23). Muscarinic cholinergic antagonists, such as scopolamine, are known to increase locomotor activity in laboratory rodents (1,6,21). Due to the extensive downregulation of muscarinic receptors in adult rats treated with CPF, we hypothesized that CPF-treated adult rats would be supersensitive to scopolamine's locomotor stimulant properties. Indeed, a difference in the motoric response to scopolamine was noted 2 weeks after CPF treatment. As in our previous studies using a single dose of CPF, however, differences in response to scopolamine were also observed much later (4, 6, and 8 weeks after CPF treatment). Following either an acute high dose of CPF or after repeated lower-level exposures, a change in response to scopolamine was noted even after brain muscarinic receptor levels had essentially returned to control levels. Possible mechanisms for such a persistent change in sensitivity to scopolamine are currently being investigated.

The overall data indicate more extensive neurochemical and neurobehavioral changes in adult rats compared to young rats given identical, repeated doses of chlorpyrifos. We conclude that while perinatal rats are more sensitive to lethality from high-dose CPF exposures, adult rats can exhibit more persistent neurochemical/neurobehavioral changes following lower, subacute exposures.

ACKNOWLEDGEMENTS

This research was supported by Cooperative Agreement CR-816229-01-1 with the United States Environmental Protection Agency awarded to C.N.P. This manuscript has been reviewed by the Health Effects Research Laboratory, U.S. EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendations for use.

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