Neurochemical Effects of Repeated Gestational Exposure to Chlorpyrifos in Developing Rats

Jason R. Richardson\textsuperscript{1} and Janice E. Chambers\textsuperscript{2}

\textit{Center for Environmental Health Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi 39762}

Received on August 1, 2003; accepted on October 3, 2003

The neurochemical effects in developing rats exposed during gestation to the anticholinesterase organophosphorus insecticide chlorpyrifos (CPS) were determined. Pregnant rats were dosed daily with CPS (0, 3, or 7 mg/kg) in corn oil from gestation days (GD) 6–20. Pups were euthanized on postnatal days (PND) 1, 3, 6, 9, 12, and 30 for the determination of brain cholinesterase (ChE) and choline acetyltransferase (ChAT) activities, along with muscarinic receptor (mAChR) densities, the levels of the high-affinity choline uptake (HACU) system, and the vesicular acetylcholine transporter (VACHT). ChE activities were inhibited about 15 and 30\% on PND 1, in the low- and high-dosage groups, respectively, and were not different from control values by PND 6. mAChR densities on PND 1 were reduced in the high-dosage group by 30\% on PND 1, in the low- and high-dosage groups, respectively, and were not different from control levels by PND 6. ChAT activity was decreased by \( \sim 12\% \) in the high-dosage group on PND 9, 12, and 30. HACU levels, using \(^{3}\)H-methylscopolamine, \(^{3}\)H-quinuclidinyl benzilate, and \(^{3}\)H-4-DAMP, respectively, as ligands, and were not different from control levels by PND 6. ChAT activity was decreased by \( \sim 25\% \) on PND 6 in the low- and high-dosage groups, and by \( \sim 14 \) and 21\% on PND 12 and 30, only in the high-dosage group. Levels of the VACHT were reduced by a range of 13–31\% on PND 3 through 30 in the high-dosage group, using \(^{3}\)H-AH5183 (vesamicol) as the ligand. These data suggest that gestational exposure to 7 mg/kg/day CPS results in long-term alterations of presynaptic cholinergic neurochemistry.

Key Words: developmental neurotoxicity; chlorpyrifos; choline acetyltransferase; cholinesterase inhibition; cholinergic neurochemistry; organophosphate insecticide.

Chlorpyrifos \([O,O\text{-diethyl }O-(3,5,6\text{,trichloro-2-pyridinyl})\text{-phosphorothioate}]\) (CPS) is a widely used organophosphorus (OP) insecticide, with a variety of agricultural and household uses. Recent concerns over possible developmental toxicity of CPS have resulted in cancellation of residential uses of this insecticide and review of its agricultural uses in order to determine if additional measures should be undertaken to provide greater protection of children (EPA, 2001). Developing animals are more susceptible to the acute toxic effects of CPS (Atterberry \textit{et al.}, 1997; Moser and Padilla, 1998; Zheng \textit{et al.}, 2000). In addition to the acute susceptibility of juvenile animals, repeated exposures of juveniles to chlorpyrifos have resulted in long-term neurochemical or behavioral aberrations (Dam \textit{et al.}, 1999; Carr \textit{et al.}, 2001; Moser and Padilla, 1998; Slotkin \textit{et al.}, 2001; Tang \textit{et al.}, 1999; Zheng \textit{et al.}, 2000). However, there is a paucity of studies that have evaluated the effects of gestational exposure to CPS on the developing cholinergic neurochemistry of the offspring.

Lassiter \textit{et al.} (1998, 1999) orally exposed pregnant dams to 0, 3, 5, 7, or 10 mg/kg/day from gestation days (GD) 14 to 18 and determined that fetal brain ChE was much less inhibited than that of the dams on GD 18 to 21, and suggested that this difference was most likely the result of a greater rate of protein synthesis in the developing animal. Additionally, Mattsson \textit{et al.} (2000) came to a similar conclusion in dams orally exposed to 0, 0.3, 1, or 5 mg/kg/day from GD 6 through postnatal day (PND) 10, reporting that ChE activity had returned to near control levels by PND 5 in the highest-dosage group. In the first study, which explored neurochemical effects other than ChE inhibition in animals gestationally exposed to CPS, Chanda \textit{et al.} (1995) reported that a single high dosage of CPS (200 mg/kg), administered subcutaneously in oil to the dam on GD 12, resulted in \( \sim 30\% \) inhibition of ChE in the offspring on PND 3, which was accompanied by an 11\% decrease in muscarinic acetylcholine receptor (mAChR) levels as measured by \(^{3}\)H-quinuclidinyl benzilate (QNB) binding. In an additional study by Chanda and Pope (1996), dams administered 25 mg/kg/day from GD 12 through GD 19 resulted in about 20–25\% brain ChE inhibition in the offspring on PND 3 and a 27\% decrease in mAChR levels. Qiao \textit{et al.} (2003) reported that subcutaneous exposure of pregnant dams to 0, 1, or 5 mg/kg/day of CPS, administered in DMSO on GD 17 to 20, resulted in long-term decreases, up to PND 30 and 60, in the levels of the high-affinity choline uptake transporter, with no corresponding effects on choline acetyltransferase activity or M2/M4 mAChR levels. Recently, our laboratory reported...
that offspring of dams orally administered 0, 3, 5, or 7 mg/kg/day from GD 6 through GD 20 resulted in brain ChE inhibition in the offspring of ~45% on PND 1 in the highest-dosage group, with inhibition persisting through PND 9 (Richardson and Chambers, 2003). In addition, we found a 13% decrease in choline acetyltransferase (ChAT) activity, the enzyme responsible for acetylcholine (ACh) biosynthesis, on PND 12, when ChE levels were comparable with those of control animals. Therefore, it appears that while brain ChE activity in animals exposed to CPS during gestation is able to recover from inhibition fairly rapidly, there may be lingering effects on other neurochemical components of the cholinergic system.

The present study was designed to expand on the observations of our previous study (Richardson and Chambers, 2003) by the determination of the occurrence and persistence of effects on the neurochemical components of the cholinergic nervous system in the offspring of dams orally exposed to CPS during gestation. In addition to monitoring ChE inhibition, cell surface and total mAChR levels were measured by the binding of \(^{3}H\)-N-methylscopolamine (NMS) and \(^{3}H\)-QNB, respectively, to determine whether mAChR might be sequestered or downregulated similarly to that observed in animals exposed to CPS during the postnatal period (Tang et al., 1999). Since NMS and QNB nonspecifically label all subtypes (M1–M5) of (VAChT) by the binding of DAMP and \(^{3}H\)-AF-DX 384, respectively. Furthermore, to determine whether other presynaptic cholinergic markers were affected in a similar fashion to our previously reported decrease in ChAT activity in rats exposed to CPS during gestation, we measured levels of the high-affinity choline uptake transporter (HACU) and the vesicular acetylcholine transporter (VACHT) by the binding of \(^{3}H\)-hemicolinium-3 and \(^{3}H\)-AH5183, respectively. The results presented here confirm our previous observations of the effects of gestational exposure to CPS on brain ChE and ChAT activity, and extend them by demonstrating that exposure to CPS during gestation results in transient reductions of total mAChR and mAChR subtypes, but more persistent reductions of HACU and VACHT levels.

**MATERIALS AND METHODS**

**Chemicals.** Analytical grade CPS was a generous gift from Dow Agrosciences (Indianapolis, IN). \(^{3}H\)-Acetyl coenzyme A (CoA) (190 mCi/mmol) was purchased from ICN Biomedical (Irvine, CA). \(^{3}H\)-Hemicolinium-3 (HC-3; 136 Ci/mmol), \(^{3}H\)-AH5183 (vesamidol; 34 Ci/mmol), \(^{3}H\)-quinacilindin benzilate (QNB; 48 Ci/mmol), \(^{3}H\)-4-diphenylacetoxy-N-(2-chloroethyl)piperidine (4-DAMP; 80.5 Ci/mmol), \(^{3}H\)-N-methylscopolamine (NMS; 83.5 Ci/mmol), and \(^{3}H\)-AF-DX 384 (100 Ci/mmol) were purchased from New England Nuclear. Unlabeled AF-DX 384 was a generous gift from Boehringer-Ingelheim Pharmaceutical (Ridgefield, CT). ScintiLene\(^{\text{TM}}\) non-aqueous scintillation fluid was obtained from Fisher Scientific, Inc. (Houston, TX). All other chemicals, if not specified, were purchased from Sigma Chemical Co. (St Louis, MO).

**Experimental design.** Experimentally naive adult male and female Sprague-Dawley rats (Crl:CD(SD)/BR) were obtained from Charles River Laboratories (Wilmington, MA). Rats were maintained in a temperature-controlled room at 22 ± 3°C with a 12:12-h light:dark cycle in an AAALAC-accredited facility, with both Purina standard rat chow (Brentwood, MO) and tap water available ad libitum. All procedures were approved by the Mississippi State University Animal Care and Use Committee.

Rats were bred at a female: male ratio of 3:1. Gestational day 0 (GD 0) was determined by the presence of sperm in the vaginal lavage, and females were housed individually thereafter. Dams were randomly assigned to treatment groups, weighed daily, and orally dosed from GD 6 to 20 with 0, 3 (low), or 7 mg/kg/day (high) CPS in corn oil, based on low and high dosages used previously in our laboratory (Richardson and Chambers, 2003). Treatments were administered on a vanilla wafer (Nabisco\(^{\text{TM}}\)) at 0.5 ml/kg to reduce handling stress involved with oral intubation. Treated cookies were totally consumed within 10 min of administration. Dams were allowed to give birth and litters were culled to 10–12 pups/litter on PND 1, to ensure standardized nutritional availability. Pups were sampled on PND 1, 3, 6, 9, and 12, with an equal number of pups sampled from each litter to maintain equal litter sizes; remaining pups were weaned on PND 22 and euthanized on PND 30, the time at which the cholinergic system has reached maturity (Coyle and Yamamura, 1976). There was no selection made for sex in the sampling, since previous studies have shown no differences in ChE inhibition of rats exposed to CPS during gestation (Mattson et al., 2000), and each litter was considered as an individual unit of analysis.

**Enzyme assays.** ChE was assayed spectrophotometrically in brain homogenates (whole brain without cerebellum or medulla-pons) using acetylthiocholine as the substrate and 5,5'-dithio-bisnitrobenzoic acid (DTNB) as the chromogen with eserine in the blanks (Chambers et al., 1988; Ellman et al., 1961). ChAT activity was assayed radiometrically, essentially according to the method of Fomnn (1975) as described previously (Chambers and Chambers, 1989; Richardson and Chambers, 2003). Data were expressed as nmol product formed/min/mg protein and pmol acetylcholine formed/min/mg protein for ChE and ChAT, respectively. ChE assays were performed on all treatment groups until activities had returned to control levels. ChAT assays were performed on samples from PND 6, 9, 12, and 30, only since a previous study from our laboratory determined that ChAT activity was low and unaffected by CPS treatment on PND 1 and 3 (Richardson and Chambers, 2003).

**Muscarinic receptor binding.** Crude synaptosomal fractions of brain (excluding cerebellum and medulla-pons) were prepared using the method of Gray and Whittaker (1962), as described in detail by Tang et al. (1999). Briefly, the brain was homogenized in 10 volumes of 0.32 M sucrose. The homogenate was centrifuged at 1000 × g for 10 min, the pellet discarded, and the supernatant centrifuged at 17,000 × g × 10 min. The pellet was resuspended at a concentration of ~1 mg/ml with Krebs-Ringer buffer (comprised of [in mM]: NaCl, 150; KCl, 5; CaCl\(_2\), 1.5; MgCl\(_2\), 1.3; HEPES, 20, pH 7.4; glucose, 10) to obtain a crude synaptosomal fraction, which was kept on ice and used within 1 h.

Muscarinic receptor (mAChR) levels in crude synaptosomal preparations were determined with the specific ligands \(^{3}H\)-NMS and \(^{3}H\)-QNB, using a method similar to that of Yamamura and Snyder (1974), as described by Tang et al. (1999), with a single saturating concentration (3 nM) of \(^{3}H\)-NMS or \(^{3}H\)-QNB. The specific binding was calculated as the total binding (incubated without 10 μM atropine sulfate) minus nonspecific binding (incubated with atropine), and expressed as fmol/mg protein.

For M1/M3 and M2/M4 mAChR binding, brain samples (whole brain without cerebellum and medulla-pons) were homogenized in 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), and 1 mM MgCl\(_2\) (Tris-salts buffer) in a glass mortar, using a Wheaton motorized tissue grinder at a concentration of 7 mg/kg/day (high) CPS in corn oil, based upon low and high dosages used previously in our laboratory (Richardson and Chambers, 2003). This procedure was repeated before the final pellet (crude membrane preparation) was resuspended, using the Wheaton grinder at a concentration of ~2 mg of protein/ml.

**Aberrant behaviors.** Aberrant behaviors were measured using a modified platform version of the open field test (OF) in a 19 × 19 × 10 cm arena. Rats were placed in the center of the arena and allowed to explore for 5 min per test session. The number of entries into the four quadrants of the field was counted for each test session. Data were expressed as the mean number of entries into each quadrant ± SEM for each treatment group. Three test sessions were conducted for each rat, with a 24-h interval between test sessions. Data were analyzed using one-way ANOVA followed by post hoc Dunnett’s test for the comparison of treatment groups with controls. A p value of <0.05 was considered statistically significant.
Preliminary kinetic studies indicated that the binding of 3H-4-DAMP (0.05–250 nM) was best fit to a two-site model with a value of ~0.84 ± 0.21 and 33 ± 8.74 nM, consistent with that of Araujo et al. (1991). The high-affinity site is thought to consist of primarily M3 sites, with the low-affinity site consisting of both M1 and M3 receptor subtypes, and in the presence of the specific M2/M4 antagonist, AF-DX 384 (20 nM), to block possible binding of 3H-DAMP to M4 sites. Assays were conducted at 37°C for 60 min. Specific binding was calculated as described above and expressed as fmol/mg protein. Assays were performed on all treatment groups until they returned to control levels, since data from this study indicated that there were no long-term changes in QNB or NMS levels after a return to control levels (data not shown).

M2/M4 mAChR levels were determined in the same crude membrane preparation, essentially by the method of Castoldi et al. (1991) using the selective M2/M4 antagonist 3H-AF-DX 384. Preliminary kinetic studies indicated that the binding of 3H-AF-DX 384 (0.01–30 nM) was best fit to a one-site model with a Kd of approximately 9.8 ± 0.63 nM. Therefore, binding studies were conducted with a single concentration (20 nM) of 3H-AF-DX 384 at 37°C for 60 min. Specific binding was calculated as described above, and expressed as fmol/mg protein. Assays were performed on all treatment groups until they returned to control levels.

Hemicholinium-3 and AH5183 binding. For hemicholinium-3 (HC-3) binding to the high-affinity choline uptake transporter, brain samples (whole brain without cerebellum and medulla-pons) were prepared as described above for M1/M3 mACHR binding, except that 50 mM glycyl-glycine buffer containing 200 mM NaCl and 5 mM KCl (pH 7.4) was used.

The binding of the high-affinity choline uptake inhibitor 3H-HC-3 to the high-affinity choline uptake transporter was measured in crude membrane preparations of the brain essentially as described by Sandberg and Coyle (1985), with modifications to reduce the total assay volume to 200 μl. Preliminary kinetic studies indicated that the binding of 3H-HC-3 (0.05–40 nM) was best fit to a two-site model with Kd values of approximately 1.38 ± 0.87 and 32.86 ± 3.03 nM. Therefore, studies were conducted with a single concentration (10 nM) of 3H-HC-3, to label the high-affinity site, at 37°C for 30 min. Specific binding was calculated as the total binding (incubated without 10 μM unlabeled HC-3) minus nonspecific binding (incubated with 10 μM unlabeled HC-3) and expressed as fmol/mg protein. Assays were performed on PND 6, 9, 12, and 30, since previous studies have shown that specific 3H-HC-3 binding was first detectable around PND 5–7 (Aubert et al., 1996; Happe and Murrin, 1992).

The binding of the acetylcholine transport inhibitor 3H-AH5183 (vesamicol) to the vesicular acetylcholine transporter was measured in the same synapticosomal preparations as described above for NMS and QNB binding, essentially, by the method of Meyer et al. (1993). Preliminary kinetic studies indicated that the binding of 3H-AH5183 was best fit to a single site model with a Kd of ~30.59 ± 2.97 nM, when in the presence of 200 nM 1,3-di(2-tolyl)guanidine (DTG), to block binding of AH5183 to sigma receptors (Casters et al., 1997). Therefore, assays were conducted with a single concentration (100 nM) of 3H-AH5183 in the presence of 200 nM DTG for 30 min at 37°C. Specific binding was calculated as the total binding (incubated without 10 μM L-vesamicol) minus nonspecific binding (incubated with 10 μM L-vesamicol) and expressed as fmol/mg protein.

Protein determinations. Protein concentrations for synapticosomal preparations were determined by the bicinchoninic acid method (Smith et al., 1985), with bovine serum albumin as the standard. Protein concentration of all other preparations was quantified by the method of Lowry with bovine serum albumin as the standard. Protein determinations.

Statistical analysis. Litter was considered the smallest unit of analysis, with each litter representing an independent replication (n = 4–6 litters per treatment group). AChE and ChAT activities, along with mACHR, HC-3, and AH5183 binding data for each time point were log-transformed for comparisons involving heterogeneous variance and analyzed by the general linear model (GLM) using SAS on a personal computer. If a significant F was determined, means were separated by the Student-Newman-Keuls (SNK) post hoc test, with statistical significance reported for the p ≤ 0.05 level.

RESULTS

Dams treated with CPS showed no signs of overt toxicity and there were no adverse effects on pup growth rate noted (data not shown). Likewise, there were no differences in the sex ratios of the litters or numbers of pups per litter observed between treatment groups (data not shown).

Brain ChE was inhibited in a dose-related manner in the offspring, with inhibition of ~15 and 30% in the low- and high-dosage groups on PND 1, respectively (Fig. 1). Inhibition in the low-dosage group was still ~15% on PND 3 and had returned to control levels by PND 6. Likewise, inhibition in the high-dosage group was ~27% on PND 3 and was not significantly different than control levels by PND 6.

Levels of mACHR receptors present on the cell surface, as measured by NMS binding, were decreased by ~18 and 27% on PND 1 and 3, respectively, but only in the high-dosage group (Fig. 2A). Total mACHR levels, as measured by QNB binding, were decreased by ~21 and 17% on PND 1 and 3, respectively, only in the high-dosage group (Fig. 2B). Levels of both cell surface and total mACHR had returned to control levels by PND 6.

M1/M3 mACHR levels, as indicated by 4-DAMP binding, were decreased by ~17 and 25% on PND 1 in the low- and high-dosage groups, respectively (Fig. 3A). On PND 3, these levels had returned to control values in the low-dosage group. However, levels in the high-dosage group were decreased by...
~15%, returning to control values by PND 6. In contrast, M2/M4 mAChR levels, as indicated by AF-DX 384 binding, were not significantly different from control values at any of the time points studied (Fig. 3B).

ChAT activity was unaffected by treatment until PND 9, where activity was decreased in the high-dosage group by about 11% (Fig. 4). The decrease in ChAT activity persisted in the high-dosage group through PND 30, the last sampling day, with decreases of 13 and 10% on PND 12 and 30, respectively.

High-affinity choline uptake (HACU) transporter levels, as measured by HC-3 binding, were decreased by about 27 and 25% on PND 6 in the low- and high-dosage groups, respectively (Fig. 5). HACU levels in the low- and high-dosage groups were not significantly different from control levels on PND 9. However, HACU levels in the high-dosage group were decreased compared to controls by about 14% on PND 12, and by about 21% on PND 30.
Synaptosomal levels of the vesicular acetylcholine transporter (VACht), as measured by AH5183 binding, were decreased at all sampling times except PND 1 in the high-dosage group (Fig. 6). Decreases of 13, 10, 12, 31, and 26% compared to controls were observed on PND 3, 6, 9, 12, and 30, respectively. AH5183 binding in the low-dosage group was not different from that in controls at any sampling point.

**DISCUSSION**

Previously, we had established that exposure to CPS during gestation resulted in relatively persistent inhibition of brain ChE, and a delayed decrease in ChAT after ChE activity had returned to control levels (Richardson and Chambers, 2003). Therefore, this study was undertaken to determine if other neurochemical components of the cholinergic system are affected by gestational exposure to CPS and what the persistence of these effects would be.

ChE activity was inhibited in a dose-related manner, with inhibition values similar to those of Mattsson et al. (2000) and Lassiter et al. (1998, 1999), although somewhat less in this study compared with our previous study (Richardson and Chambers, 2003). Inhibition persisted in both treatment groups through PND 3 and had returned to control values by PND 6. Previously we had suggested that the relatively persistent inhibition of brain ChE might be the result of the phosphorylated ChE being permanently inactivated or “aged” (Richardson and Chambers, 2003). Therefore, we tested the ability of the oxime reactivator, TMB-4 (Chambers and Chambers, 1989), to reactivate inhibited ChE from these animals, and we determined that all of the inhibited ChE could be reactivated on PND 1 and 3 (data not shown). Therefore, either lactational exposure to CPS or residual CPS present in the neonates, or a combination of the two, was responsible for the ChE inhibition in the offspring after cessation of treatment. Mattsson et al. (2000) reported that exposure of dams to 5 mg/kg/day CPS from GD 6 through PND 10 resulted in milk levels of 3 μg/ml on PND 1, and estimated that the pups would be exposed to approximately 0.126 mg/kg/day CPS, similar to the threshold for plasma ChE inhibition in adult rats (Breslin et al., 1996). In this study, brain ChE activity returned to control values by PND 6, while the rats were still nursing, similar to that observed by Mattsson et al. (2000), in which brain ChE activity of the offspring had returned to control levels by PND 5. Therefore, it is likely that the observed short-term ChE inhibition noted in the offspring on PND 1 and 3 was the result of a combination of residual CPS present in the neonates from in utero exposure concurrent with lactational exposure.

Accumulation of ACh resulting from ChE inhibition in adult animals can result in decreased levels (downregulation) of mAChR (Russell and Overstreet, 1987). In the present study, total mAChR levels, as measured by QNB binding, were only affected in the high-dosage group and had returned to control levels by PND 6, suggesting that the decreases were dependent on ChE inhibition. The decreases in mAChR noted in this study on PND 3 (21%) were similar to those of Chanda and Pope (1996), who observed a 27% decrease on PND 3 in the offspring of dams exposed to 25 mg/kg/day CPS subcutaneously from GD 12–19. While decreases in QNB binding in adult animals represent downregulation of mAChR following sustained hyperstimulation, decreases in cell surface mAChR, as measured by the hydrophilic ligand NMS, represent the sequestration of these receptors from the cell surface in response to short-term hyperstimulation (Cioffi and El-Fakahany, 1986). In this study, decreases in NMS binding were observed on PND 1 and 3, only in the high-dosage group (7 mg/kg/day), with a return to control levels on PND 6. Unpublished data from our laboratory with a limited number of samples suggest that this response is dependent on the magnitude of ChE inhibition, as the offspring of dams exposed to a lower dosage, 5 mg/kg/day, of CPS during gestation exhibited decreases in NMS levels on PND 1 and 3, and with no corresponding effect on QNB levels.

In the brain, there are five types of mAChR (M1–M5), which are often divided into two distinct pairs, M1/M3 and M2/M4 based on their coupling to second messenger systems (Bowen and Marek, 1982). M1/M3 mAChR are coupled to the hydrolysis of inositol triphosphate and are of particular interest because of their early development (Aubert et al., 1996), and several reports indicating that M1 and M3 mAChR are more efficiently coupled to their signal transduction mechanisms in developing rats than are their adult counterparts (Balduini et al., 1987; Heacock et al., 1987). M1/M3 levels, as measured by the binding of 4-DAMP, were significantly reduced in both the low- and high-treatment groups on PND 1 and in the high-treatment group on PND 3. The return of these receptor

**FIG. 6.** Vesicular acetylcholine transporter levels as measured by 3H-AH5183 binding in rat pups exposed to 0 (control), 3 (low), or 7 (high) mg/kg/day chlorpyrifos from gestation day 6 through 20. Bars labeled with the same letter on the same sampling day are not significantly different from each other (p < 0.05) by SNK. Data are presented as the mean ± SEM (n = 4–6).
levels to control values paralleled that of ChE activity, suggesting that these reductions are the result of ChE inhibition and not a developmental delay in expression from gestational exposure to CPS. M2/M4 mAChR, which is coupled to the inhibition of adenyl cyclase (Peralta et al., 1988), typically develop later than M1/M3 receptors (Aubert et al., 1996) and are not effectively coupled to their signal transduction systems until the third postnatal week (Lee et al., 1990). In contrast to the robust effects on M1/M3 receptors, M2/M4 receptor levels were not altered at any of the time points studied, suggesting that receptors of the M1/M3 class are particularly sensitive to CPS exposure, possibly because of their earlier development, as compared to the M2/M4 subtypes.

ChAT activity, while not the rate-limiting step in ACh synthesis (Simon et al., 1976), provides insight into the potential rate of ACh synthesis and is an indirect measure of functional cholinergic neurons. We have previously reported a nonstatistically significant decrease (13%) of ChAT activity on PND 9 and a significant decrease of 13% on PND 12, a time at which ChE activity had returned to control values, in the offspring of dams exposed to 7 mg/kg/day during gestation (Richardson and Chambers, 2003). This was confirmed in the present study, with significant decreases in ChAT activity on PND 9 and 12. ChAT activity was also significantly decreased on PND 30, a time at which ChAT activity is reported to reach adult levels (Coyle and Yamamura, 1976). Thus, it appears that exposure of dams to CPS during gestation at 7 mg/kg/day but not 3 mg/kg/day causes a long-term reduction of ChAT activity in the offspring.

The high-affinity choline uptake (HACU) system develops in parallel with ChAT activity (Coyle and Yamamura, 1976) and is the rate-limiting step in the synthesis of ACh (Simon et al., 1976). In addition, HACU levels have been shown to be reduced by increased nerve impulse activity in response to ChE inhibition (Swann and Hewitt, 1988). Therefore, we sought to determine the effects of gestational exposure to CPS on the development of the HACU system. HACU levels were significantly decreased in both the low- and high-dosage groups on PND 6, and returned to near control levels by PND 9. However, starting on PND 9 there was a progressive decline in HACU levels in the high-dosage group, which reached a maximum reduction of 21% on PND 30, the last day examined. A similar long-term reduction of HACU levels has also been observed by Qiao et al. (2003) in the offspring of dams subcutaneously exposed to 1 or 5 mg/kg/day CPS in DMSO from GD 17–20. Although the study by Qiao and coworkers observed effects on HACU levels at lower dosages than that observed in this study, caution must be taken in comparing the studies because of the different vehicles used, since DMSO has been shown to inhibit ChE activity (Jacob and Herschler, 1986) and may enhance the actions of CPS.

The VAChT is responsible for the transport of newly synthesized ACh into vesicles for release (Parsons et al., 1993) and is present at high levels early in development, suggesting a role in neural development (Aubert et al., 1996). As with ChAT activity, VAChT levels were only affected in the high-dosage group, with significant decreases on PND 3, 6, 9, 12, 22, and 30. In addition, there appeared to be a progressive decline in VAChT levels from PND 9 to PND 30 in the high-dosage group. This is the first study, of which we are aware, that reports reductions of VAChT levels in animals exposed to organophosphorus insecticides, either developmentally or as adult animals.

The reductions observed in ChAT activity, along with reductions in VAChT and HACU levels, suggest that presynaptic cholinergic neurons are especially sensitive to the effects of gestational exposure to CPS at the doses used in this study. However, the mechanism behind these selective decreases is not clear, since the time points demonstrating the most notable effects on these markers were times at which there were no corresponding decreases in brain ChE activities. However, there are two possible explanations. First, the expression of ChAT and VAChT are coordinately regulated and share a common gene locus and regulatory elements for gene expression (reviewed by Eiden, 1998). In addition, acute in vitro exposure of mouse brain slices to anticholinesterases has been shown to coordinately decrease ChAT and VAChT mRNA expression (Kauf er et al., 1999). However, there are currently no data on the effects of gestational exposure to CPS on the mRNA expression of ChAT and VAChT. Also, there are no current data on whether HACU is regulated in a similar manner to ChAT and VAChT, since the HACU has just recently been cloned (Okuda et al., 2000) and there are no data currently available on its regulatory elements. Secondly, it is possible that these reductions represent a selective loss of presynaptic cholinergic neurons. Antibodies to ChAT, VAChT, and HACU have been used to map the distribution of cholinergic neurons in mammalian brain (Armstrong et al., 1983; Gilmor et al., 1996; Kus et al., 2003) and are measures of intact cholinergic neurons; thus, the loss of these specific markers could be inferred to represent a loss of cholinergic neurons. Maurissen et al. (2000) reported that the offspring of rats exposed to CPS from GD 6 through PND 10 displayed no overt neuropathology, as determined by hematoxylin and eosin (H&E) staining. Although this observation suggests that no overt neuronal loss occurs after gestational CPS exposure, H&E staining is not specific for cholinergic neurons, and thus may not have been sensitive enough to allow for determination of cholinergic neuron loss. Likewise, our neurochemical determinations were performed on whole brain without cerebellum and medullapons, and therefore do not possess the anatomical resolution required to determine whether there is specific cholinergic loss. Therefore, further study is needed to delineate the mechanism responsible for the selective targeting of presynaptic cholinergic markers observed in the high-dosage group in this study.

In summary, repeated exposure of developing rats to CPS during gestation resulted in transient decreases in ChE activity and mAChR levels, with the M1/M3 subtypes being the most...
sensitive to exposure. In contrast to these transient effects, there were long-lasting alterations of presynaptic components of the cholinergic system, i.e., HACU levels, ChAT activity, and VACHT levels, which were still noted longer than a month after the last treatment of the dams. In addition, these effects were primarily associated with time points at which there was no corresponding ChE inhibition. Taken in concert, these data suggest that gestational exposure of rats to 7 mg/kg/day of CPS, but not 3 mg/kg/day, results in long-term alterations of presynaptic cholinergic neurochemistry.

ACKNOWLEDGMENTS

The authors wish to acknowledge the generous gifts of chlorpyrifos from Dow Agrosciences and AF-DX 384 from Boehringer-Ingelheim. The authors would also like to thank Dr. Russell Carr for assistance with preparation of this manuscript. This research was partially supported by the National Science Foundation (EPS-9874669), the National Institutes of Health (R01 ES10386), the Mississippi Agricultural and Forestry Experiment Station (MAFES) (under MAFES project MSIV-701030), and the College of Veterinary Medicine, Mississippi State University. This paper is MAFES publication J-10406 and the Center for Environmental Health Sciences publication 103.

REFERENCES


