Effects of Repeated Oral Postnatal Exposure to Chlorpyrifos on Cholinergic Neurochemistry in Developing Rats

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The neurochemical effects of repeated postnatal exposure to chlorpyrifos (CPS) were studied in developing rats. Rats were gavaged daily from postnatal day (PND) 1–21 with CPS in corn oil starting at 1.5 mg/kg (low dosage group) and increasing gradually to 3 mg/kg and then to 6 mg/kg (high dosage group). Brain cholinesterase (ChE) activity was significantly inhibited on PND 6, 12, 22, and 30, with maximum inhibition on PND 6 of 49% and 59% and recovering to 18 and 33% on PND 30 in the low and high dosage groups, respectively. On PND 22 and 30, 94% or greater of the inhibited ChE could not be reactivated by the oxime TMB-4 in both treatment groups, indicating aging of the phosphorylated ChE. Total muscarinic acetylcholine receptors (mAChR) were reduced in a dose-related manner on PND 12 and 22, with substantial recovery by PND 30. M1/M3 mAChR were significantly reduced on PND 6 and 12 only in the high dosage group, and on PND 22 in both groups, while M2/M4 mAChR were reduced in the high dosage group on PND 22 and 30. On PND 30 choline acetyltransferase activity and vesicular acetylcholine transporter levels were decreased by 12 and 22%, respectively, only in the high dosage group. High-affinity choline transporter levels were decreased at all time points in the high dosage group and on PND 6, 22, and 30 in the low dosage group. The results presented here demonstrate that repeated postnatal exposures to CPS result in transient reductions of mAChR and more persistent alterations of presynaptic cholinergic neurons. In addition, the long-term reduction of brain ChE activity observed following repeated postnatal exposure to CPS is attributable to permanent inactivation or “aging” of the enzyme.

Key Words: developmental neurotoxicity; chlorpyrifos; choline acetyltransferase; organophosphate “aging”; muscarinic receptors; cholinesterase.

Chlorpyrifos (CPS) has been one of the most widely used insecticides during the last decade, in numerous agricultural and residential settings. Its use has recently been restricted because of concerns over potential developmental neurotoxicity (EPA, 2001). Like other organophosphorus (OP) insecticides, CPS exerts acute toxicity primarily through persistent inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) at cholinergic junctions within the central and peripheral nervous systems, resulting in prolonged residence time of the neurotransmitter acetylcholine (ACh) within the synapse (Ecobichon, 2001). Because of this persistent ChE inhibition by OP exposure, certain compensatory mechanisms arise to combat excess ACh present in the synapse. These homeostatic mechanisms involve alterations of both presynaptic and/or post-synaptic components of the cholinergic system, including the high affinity choline transporter (HAcHT), choline acetyltransferase (ChAT), and muscarinic acetylcholine receptors (mAChR) (Costa et al., 1982; Russell and Overstreet, 1987).

Although there have been numerous studies of compensatory responses of adult animals following exposure to OP compounds (reviewed by Costa et al., 1982; Hoskins and Ho, 1992; Russell et al., 1987), only in the past few years has attention turned to these mechanisms in developing animals. The consequences of developmental exposure to CPS is of concern because developing animals are more susceptible to the acute toxicity of CPS (Atterberry et al., 1997; Chakraborti et al., 1993; Moser and Padilla, 1998; Zheng et al., 2000), and many of the aforementioned compensatory mechanisms may participate in the process of neural development (Lauder and Schambra, 1999), which may result in neurobehavioral abnormalities in the developing animal (Carr et al., 2001; Dam et al., 2000; Levin et al., 2001, 2002). Previous studies have determined that repeated postnatal exposure of rats to CPS resulted in persistent brain ChE inhibition (i.e., ChE activity never returned to control levels during the course of the study) and transient reductions of total and M2/M4 mAChR (Liu et al., 1999; Tang et al., 1999). In addition, decreases in ChAT activity have been reported in rats exposed to CPS on PND 1 through PND 4, which persisted through PND 30 and were accompanied by decreases in HAcHT levels (Dam et al., 1999; Slotkin et al., 2001). Similar to that observed with the aforementioned studies, we have recently reported that gestational exposure to CPS results in transient decreases in mAChR and persistent reductions in ChAT activity and...
HACHT levels (Richardson and Chambers, 2003, 2004). In addition, we found that vesicular acetylcholine transporter (VACHT) levels were persistently decreased as well, suggesting that presynaptic cholinergic neurons may be especially vulnerable to gestational CPS exposure.

In light of these findings, this study was designed to determine the dose-related effects of daily exposure of developing rats to CPS from postnatal day (PND) 1–21, the period in which the most rapid development of cholinergic neurons in the brain occurs (Coyle and Yamamura, 1976). In addition, since a previous study from our laboratory (Tang et al., 1999) reported that ChE inhibition was still evident 19 days after exposure using a similar dosing paradigm, we were interested in determining whether this long-term depression of ChE activity represented a developmental delay or possibly permanent inactivation or “aging” of ChE (Wilson et al., 1992). Therefore, the amount of inhibited ChE able to be reactivated by TMB-4, an oxime reactivator, was also determined (Chambers and Chambers, 1989). The results presented here demonstrate that, similar to gestational exposure, repeated postnatal exposures to CPS result in transient reductions of mAChR and more persistent alterations of presynaptic cholinergic neurons. In addition, the long-term reduction of brain ChE activity observed following repeated postnatal exposure to CPS is attributable to permanent inactivation or “aging” of the enzyme.

**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-Hemicholinium-3 (HC-3; 136 Ci/mmol), $^3$H-AH5183 (34 Ci/mmol), $^3$H-quinuclidinyl benzilate (QNB; 48 Ci/mmol), $^3$H-4-diphenylacetoxy-N-(2-chloroethyl)piperidine (4-DAMP; 80.5 Ci/mmol), $^3$H-N-methyl scopolamine (NMS; 83.5 Ci/mmol), and $^3$H-AF-DX 384 (100 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled AF-DX 384 was a generous gift of Boehringer-Ingelheim Pharmaceutical (Ridgefield, CT). ScintiLene 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 (Cr:CD(SD)BR), original stock from Charles River Laboratories (Wilmington, MA), were obtained from a breeding colony maintained at Mississippi State University. 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 food (Purina standard rat chow; Brentwood, MO) and water available ad libitum. All animal procedures were previously approved by the Mississippi State University Animal Care and Use Committee in accordance to guidelines set forth in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

Rats were bred at a ratio of three females to one male, and females were housed singly after one week with the male. Parturition was considered postnatal day (PND) 0 and litters were culled to 10–12 pups per litter by PND 3 to ensure standardized nutritional availability. The experimental design constituted a split-litter design, with equal numbers of treatment groups in each litter and no selection for sex, since previous studies have determined that there were no significant differences in ChE inhibition between the sexes (Carr et al., 2001). Pups were individually marked and gavaged daily with chlorpyrifos in corn oil at a volume of 0.5 ml/kg using a micro-syringe from PND 1 through PND 21. The treatment groups were: (1) Control Group: corn oil administered daily from PND 1 through PND 21; (2) Low Dosage Group: chlorpyrifos administered daily at 1.5 mg/kg from PND 1 through PND 21; (3) High Dosage Group: chlorpyrifos administered at 1.5 mg/kg from PND 1 through PND 5, 3 mg/kg from PND 6 through PND 13, and 6 mg/kg from PND 14 through PND 21.

This graded dosing paradigm was used to prevent the occurrence of death in the treatment groups resulting from lower levels of detoxifying enzymes in the younger animals (Atterberry et al., 1997) and to maintain a moderate amount of brain AChE inhibition (40–70%) (Tang, 2000). Treatment occurred between the hours of 0800 and 1100 h in the light phase. An equal number of pups from each litter were sacrificed 6 h after exposure on PND 6 and 12 and 24 h after the last dosage (PND 22), and 9 days after the last dosage (PND 30). Following sacrifice, brains were rapidly removed, wrapped in aluminum foil, frozen in liquid nitrogen, and stored at −70°C until determination of neurochemical parameters.

**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-bis(nitrobenzoic acid) (DTNB) as the chromogen with eserine in the blanks (Chambers et al., 1988; Ellman et al., 1961). The amount of “aged” ChE was estimated by in vitro incubation of parallel inhibited samples (500 µl) in the presence of 0.025 nM TMB-4, an oxime reactivator, or vehicle (water), similar to methods described by Chambers and Chambers (1989). A period of 30 min was allowed for reactivation, following which the homogenate was diluted 40-fold, to reduce the concentration of TMB-4 prior to assay of ChE activity, as described above. Preliminary studies determined that there was no loss or gain of activity during the 30 min incubation. ChAT activity was assayed radiometrically essentially according to the method of Fonnum (1975) as described in detail by Richardson and Chambers (2003).

**Muscarinic receptor binding.** M1/M3, M2/M4, and total mAChR densities were determined as described previously (Richardson and Chambers, 2004). Briefly, crude membrane preparations were incubated with a single concentration (50 nM) of $^3$H-4-DAMP in the presence of AF-DX 384 (20 nM), to block possible binding of $^3$H-4-DAMP to M4 sites, for determination of M1/M3 mAChR densities. M2/M4 mAChR levels were determined using a single concentration (20 nM) of $^3$H-AF-DX 384, and total mAChR levels were determined using a single concentration (3 nM) of $^3$H-QNB. The specific binding was calculated as the total binding (incubated without 10 µM atropine sulfate) minus non-specific binding (incubated with atropine), and expressed as fmol/mg protein.

**Hemicholinium-3 and AH5183 binding.** The levels of HACHT, as determined by the binding of $^3$H-HC-3, were determined as described previously (Richardson and Chambers, 2004). Briefly, crude membrane preparations were incubated with a single concentration (10 nM) of $^3$H-HC-3. Specific binding was calculated as the total binding (incubated without 10 µM unlabeled HC-3) minus non-specific binding (incubated with 10 µM unlabeled HC-3), and expressed as fmol/mg protein. The levels of the VACHT, as determined by the binding of $^3$H-AH5183, were determined as described previously (Richardson and Chambers, 2004). Briefly, crude membrane preparations were incubated with a single concentration (100 nM) of $^3$H-AH5183 in the presence of 200 nM 1,3-di(2-tolyl)guanidine (DTG) to block binding of AH5183 to sigma receptors (Custers et al., 1997). Specific binding was calculated as the total binding (incubated without 10 µM L-vesamicol) minus non-specific binding (incubated with 10 µM L-vesamicol), and expressed as fmol/mg protein.

**Protein determination.** Protein concentrations were quantified for all preparations by the method of Lowry et al. (1951) using bovine serum albumin as the standard.
Statistical analysis. Data represent means ± SEM and are derived from individual pups each representing a different litter of origin. ChE 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 subjected to pair-wise comparisons by the Student-Newman-Keuls (SNK) post-hoc test, with statistical significance reported for the $p \leq 0.05$ level.

RESULTS

The dosing paradigm used in this study resulted in no signs of cholinergic crisis (salivation, lacrimation, tremors) at any time during the study and no mortality was observed in any of the treatment groups. In addition, there were no significant differences in body weights of any of the treatment groups on any of the days of sacrifice (data not shown).

All cholinergic markers exhibited age-related increases from PND 6 to PND 30, the time at which the cholinergic system has reached maturity (Coyle and Yamamura, 1976). Brain ChE activity increased steadily with age by about 3.1-fold from PND 6 to PND 30 (Table 1). Total mAChR levels, as measured by QNB binding, increased about 7.5-fold from PND 6 to PND 30. M1/M3 mAChR levels were present in higher levels earlier in the postnatal period than the M2/M4 mAChR subtype, and increased only 4.5-fold from PND 6 to PND 30 as compared to an 8.8-fold increase for the M2/M4 subtype. ChAT activity increased by 9.7-fold from PND 6 to PND 30 and paralleled developmental increases of HAChT levels, as measured by HC-3 binding, which increased by 4.3-fold from PND 6 to PND 22. V AChT levels, as measured by AH5183 binding, increased by about 2.6-fold from PND 6 to PND 30. As described below, changes in all the neurochemical parameters were observed following treatment, with some of these changes being transient while others were persistent.

Brain ChE activity was inhibited in a dose-related manner with inhibition of 49 and 59% on PND 6 in the low and high dosage groups, respectively (Fig. 1). Of the 49 and 59% of inhibited ChE in these groups, 42 and 40%, respectively, could not be reactivated by incubation with TMB-4, and was thus considered “aged.” On PND 12, ChE was inhibited by 43 and 57%, in the low and high dosage groups, of which 65 and 98%, respectively, could not be reactivated. ChE inhibition on PND 22 was 36 and 59%, of which 98 and 94% could not be reactivated in the low and high dosage groups, respectively. On PND 30, nine days after the last dosage, inhibition was about 18 and 33% in the low and high dosage groups, of which 100 and 98% could not be reactivated.

Total mAChR density, as measured by the binding of $^3$H-QNB, was significantly reduced ($p \leq 0.05$) on PND 12 by 10 and 17% and on PND 22 by 13 and 21% in the low and high dosage groups, respectively (Fig. 2). By PND 30, while the low dosage group had returned to control levels, the high dosage group was still reduced by about 9%. M1/M3 mAChR densities, as measured by the binding of $^3$H-4-DAMP, were significantly reduced ($p \leq 0.05$) by about 33 and 19% on PND 6 and 12, respectively, only in the high dosage group (Fig. 3). On PND 22, M1/M3 densities were reduced in a dose-related...
manner, with reductions of 12 and 20% in the low and high dosage groups, respectively, and neither treatment group was significantly different from control values on PND 30. M2/M4 mAChR densities, as measured by the binding of $^{3}$H-AF-DX 384, were significantly reduced ($p < 0.05$) by about 19 and 9% on PND 22 and 30, respectively, only in the high dosage group (Fig. 4).

ChAT activity was significantly reduced ($p < 0.05$) by 12% on PND 30, nine days after the last treatment, only in the high dosage group and was not altered at earlier time points (Fig. 5). HACHT levels, as measured by the binding of $^{3}$H-HC-3, were reduced in a dose-related manner on PND 6, with reductions of 20 and 40% in the low and high dosage groups, respectively (Fig. 6). On PND 12, levels were reduced by 18%, only in the high dosage group. HACHT levels were significantly decreased to a similar extent, 20 and 24%, on PND 22 in the low and high dosage groups. On PND 30, HACHT levels were decreased by 10 and 26% in the low and high dosage groups.

FIG. 2. Total brain muscarinic acetylcholine receptor (mAChR) levels as measured by QNB binding in rat pups exposed to 0 (Control) or 1.5 mg/kg/day CPS (Low) daily from postnatal day 1 through 21, or 1.5 mg/kg/day from postnatal day 1–5, 3 mg/kg/day from postnatal day 6–13, and 6 mg/kg/day from postnatal day 14–21 (High). Data represent mean ± SEM (n = 4–6). Within each age, bars labeled with a different letter are significantly different ($p < 0.05$) by SNK.

FIG. 3. M1/M3 brain muscarinic acetylcholine receptor (mAChR) levels as measured by 4-DAMP binding in rat pups exposed to 0 (Control) or 1.5 mg/kg/day CPS (Low) daily from postnatal day 1 through 21, or 1.5 mg/kg/day from postnatal day 1–5, 3 mg/kg/day from postnatal day 6–13, and 6 mg/kg/day from postnatal day 14–21 (High). Data represent mean ± SEM (n = 4–6). Within each age, bars labeled with a different letter are significantly different ($p < 0.05$) by SNK.

FIG. 4. M2/M4 brain muscarinic acetylcholine receptor (mAChR) levels as measured by AF-DX 384 binding in rat pups exposed to 0 (Control) or 1.5 mg/kg/day CPS (Low) daily from postnatal day 1 through 21, or 1.5 mg/kg/day from postnatal day 1–5, 3 mg/kg/day from postnatal day 6–13, and 6 mg/kg/day from postnatal day 14–21 (High). Data represent mean ± SEM (n = 4–6). Within each age, bars labeled with a different letter are significantly different ($p < 0.05$) by SNK.

FIG. 5. Specific activity of brain choline acetyltransferase (ChAT) of rat pups exposed to 0 (Control) or 1.5 mg/kg/day CPS (Low) daily from postnatal day 1 through 21, or 1.5 mg/kg/day from postnatal day 1–5, 3 mg/kg/day from postnatal day 6–13, and 6 mg/kg/day from postnatal day 14–21 (High). Data represent mean ± SEM (n = 4–10). Within each age, bars labeled with a different letter are significantly different ($p < 0.05$) by SNK.
VAChT levels, as measured by the binding of $^3$H-AH5183, were significantly reduced by 22% on PND 30, only in the high dosage group (Fig. 7).

**DISCUSSION**

These experiments were designed to determine whether inhibition of ChE by repeated oral exposure to CPS during the first three weeks of postnatal development in the rat results in transient or persistent alterations in cholinergic neurochemistry. This period, termed the brain “growth spurt” (Dobbing and Sands, 1979), represents a period of intense cholinergic synaptogenesis (Coyle and Yamamura, 1976), and is equivalent to the third trimester of gestation and early postnatal period of humans.

Development of brain ChE activity (Carr et al., 2001; Richardson and Chambers, 2003, 2004; Tang et al., 1999), total mAChR levels (Aubert et al., 1996; Coyle and Yamamura, 1976; Tang et al., 1999), M1/M3 and M2/M4 mAChR (Aubert et al., 1996), and ChAT activity (Coyle and Yamamura, 1976; Richardson and Chambers, 2003, 2004) has been reported previously and was similar to that observed here. High affinity choline uptake transporter (HACHT) levels, measured by the binding of $^3$H-HC-3, paralleled the development of ChAT activity, as has previously been suggested by $^3$H-choline uptake assays (Coyle and Yamamura, 1976) and autoradiography of $^3$H-HC-3 binding sites (Happe and Murrin, 1992). The development of vesicular acetylcholine transporter (VACHT) sites, as measured by $^3$H-AH5183 binding was similar to that determined by autoradiographic methods (Aubert et al., 1996). Consistent with previous results from our laboratory (Carr et al., 2001; Tang et al., 1999), repeated postnatal exposure to CPS resulted in dose-related inhibition of ChE, with maximum inhibition of about 59% in the high dosage group. Significant inhibition was still evident on PND 30, nine days after the last dosage, in agreement with the two previous studies above. Previously, a study from our lab had suggested that the persistent inhibition of ChE was the result of continued inhibition and did not represent a developmental delay in ChE expression (Tang et al., 1999). In this study, we found that the persistent inhibition of ChE was the result of permanent inactivation of ChE, or “aging,” a process resulting from dealkylation of phosphorylated ChE which prevents spontaneous recovery of activity, thus requiring de novo synthesis of new enzyme for activity to return to control levels (Wilson et al., 1992). Our results show a gradual increase in the proportion of “aged” ChE with time, such that by PND 22, essentially all of the inhibited ChE could not be reactivated by incubation with TMB-4, and thus was considered “aged.” Interestingly, there were no differences in the proportion of aged ChE between the low and high dosage groups, although there were significant differences in the amount of ChE inhibition in these groups, suggesting that the rate of aging is a constant and independent of the degree of ChE inhibition. These results suggest that the proportion of “aged” ChE is the primary determinant of the persistence of inhibited ChE in this study. While much of the research into aging of ChE has involved high level acute exposure situations in adult animals (Chambers and Chambers, 1989; Wilson et al., 1992), this is the first report, of which we are aware, involving lower level repeated exposures in developing animals. Therefore, the
amount of “aged” ChE may exacerbate the toxicity elicited by additional OP exposure, since there is a reduced number of ChE molecules, and thus, less physiological reserve. The persistence of the ChE inhibition is likely the result of the retention of the relatively lipophilic CPS for slow bioactivation, leading to reinhibition of reactivated ChE.

Since persistent inhibition of ChE in adult rats results in down-regulation of total mAChR levels (Russell and Overstreet, 1987), we studied the response of mAChR in developing rats repeatedly exposed to CPS. In this study, total mAChR levels, as measured by QNB binding, were significantly reduced on PND 12 and 22, similar to that reported by others (Liu et al., 1999; Tang et al., 1999; Zheng et al., 2000). By PND 30, the low dosage group had returned to control levels and the high dosage group exhibited only a slight reduction in total mAChR, suggesting that the decrease in mAChR is transient and dependent on the length and/or magnitude of ChE inhibition, as has been observed previously (Tang et al., 1999).

While the binding of \(^3\)H-QNB provides information on the total levels of mAChR, there are five subtypes (M1–M5) of mAChR which can be divided into two general subdivisions, the M1/M3 subtype, whose activation leads to the hydrolysis of inositol trisphosphate, and the M2/M4 subtype, whose activation leads to inhibition of adenyl cyclase activity and decreases in cAMP levels (Peralta, 1988). M1/M3 mAChR appeared to more readily respond to CPS exposure in this study than the M2/M4 subtypes, as there were significant decreases in M1/M3 levels at all of the sampling times, except for PND 30. The M2/M4 subtypes were significantly decreased only on PND 22 and 30 in the high dosage group; these receptors may be less responsive to the effects of CPS than are the M1/M3 subtypes because of their later development (Aubert et al., 1996; Richardson and Chambers, 2004). Using the same dosing paradigm as this study, Tang (2000) found significant reductions in the levels of M2/M4 mAChR, labeled with \(^3\)H-AF-DX 384, in cerebral cortex, hippocampus, and corpus striatum as early as PND 6. Therefore, it is possible that the brain preparation used in the current study (whole brain without cerebellum and medulla-pons) may mask some of these effects that are restricted to these particular brain regions. Liu et al. (1999) also determined that daily sc exposure of PND 7 rats to CPS for 14 days resulted in greater decreases in M2/M4 levels in the cerebral cortex and striatum than in adult rats exposed in the same manner. Taken in concert with the findings of others, results in the current study suggest that subtypes of mAChR and different brain regions may have differential susceptibility to the impact of repeated developmental exposure to CPS.

ChAT activity provides insight into the potential rate of ACh biosynthesis and is often used as a prototypical marker for functional cholinergic neurons. Decreases in ChAT activity in this study were restricted to PND 30, nine days after the last dosage and a time at which ChAT activity has been reported to reach adult levels (Coyle and Yamamura, 1976). Similar to the effect on ChAT in this study, Slotkin et al. (2001) reported reductions in ChAT activity on PND 30 and 60 following sc exposure to CPS from PND 1 through 4. Since the decrease in ChAT activity was observed on PND 30, a time when ChE activity was in the process of returning to control values, and since no reduction in ChAT activity was seen at earlier sampling times when greater ChE inhibition was present, reduction in ChAT activity appears to be unrelated to the level of ChE inhibition, arguing against a compensatory mechanism. This reduction in ChAT activity could be related to changes in high affinity choline uptake, as discussed below.

Since high-affinity choline transporter by the high-affinity choline transporter (HACHT) is the rate limiting step in ACh biosynthesis (Simon et al., 1976), it could be hypothesized that the decrease in ChAT activity observed in this study may be the result of decreased HACHT. Indeed, Dam et al. (1999) and Slotkin et al. (2001) reported decreases in \(^3\)H-HC-3 binding following postnatal exposure to CPS, which was interpreted as a developmental effect on cholinergic tone. In the present study, HACHT levels were reduced at all time points in the high dosage group, and were only accompanied by a reduction in ChAT activity on PND 30, when ChAT activity is reported to reach adult levels (Coyle and Yamamura, 1976). Taken in concert, these data suggest that reductions in HACHT levels are not sufficient to reduce ChAT activity at the early time points. However, it is possible that the rapid synthesis of ChAT occurring during development, almost six-fold from PND 6 to PND 22, may have masked alteration of enzyme activity at these earlier sampling points, and changes could only be detected after enzyme activity was stable and at adult levels on PND 30.

The VACHT functions to transport newly synthesized ACh into synaptic vesicles for release (Parsons et al., 1993). Previously we have reported that gestational exposure to CPS resulted in decreases of VACHT levels that occurred before and during decreases of ChAT activity (Richardson and Chambers, 2004). In this study, VACHT levels were significantly reduced only in the high dosage group on PND 30, which was accompanied by a decrease in ChAT activity. However, decreases in ChAT activity were not observed at earlier time points in this study, in contrast to what we previously observed with gestational exposure. This finding suggests that although the presynaptic components of the cholinergic system appear to be more sensitive to developmental exposure to CPS regardless of exposure time, there are distinct windows of exposure which result in differential timing of alterations in ChAT activity and VACHT levels.

As stated previously, we (Richardson and Chambers, 2003, 2004) and others (Qiao et al., 2004; Slotkin et al., 2001) have found that developmental exposure to CPS results in long-term alterations of presynaptic cholinergic neurochemistry. Although the mechanism behind the reduction of ChAT activity and HACHT and VACHT levels is unclear, dysfunction of
presynaptic cholinergic neurons may play a role in the behavioral abnormalities observed after developmental exposure to CPS (Carr et al., 2001; Chanda and Pope, 1996; Dam et al., 2000; Levin et al., 2001, 2002). Indeed, dysfunction of presynaptic cholinergic neurons is associated with behavioral deficits in aged rats (Sherman et al., 1981; Sherman and Friedman, 1990) and in experimentally induced hypothyroidism in developing animals (Sawin et al., 1998). However, a causal relationship between the neurochemical alterations observed with developmental CPS exposure and the resultant behavioral abnormalities remains to be established.

In summary, repeated developmental exposure to CPS from PND 1 through 21 resulted in persistent inhibition of ChE, which is primarily the result of permanent inactivation, or aging, of ChE. Transient alterations of mAChR densities were also observed, with the M1/M3 subtype most significantly affected. In addition, reductions in presynaptic components of cholinergic neurons showed various degrees of response to this persistent inhibition, with HACHT levels appearing to be the most sensitive of these components to inhibition of ChE. However, the most striking reductions in presynaptic components occurred on PND 30, nine days after the last treatment, suggesting that presynaptic components of the cholinergic system may be particularly vulnerable to developmental exposure to CPS. Further study is required to determine the persistence of these effects and what relationship, if any, alterations of presynaptic cholinergic neurochemistry have with the behavioral deficits observed following developmental CPS exposure.

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