DEVELOPMENTAL NEUROTOXIC EFFECTS OF CHLORPYRIFOS ON ACETYLCHOLINE AND SEROTONIN PATHWAYS IN AN AVIAN MODEL

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Abstract

The developmental neurotoxicity of organophosphates such as chlorpyrifos (CPF) involve multiple mechanisms that ultimately compromise the function of specific neurotransmitter systems, notably acetylcholine (ACh) and serotonin (5-hydroxytryptamine, 5HT). Studies in mammalian models incorporate both direct effects on brain development and indirect effects mediated through maternal physiology and maternal/neonatal interactions. We examined the effects of CPF in an avian model, which does not share these potential confounds. Chick eggs were injected with CPF (10 or 20 mg/kg) on incubation days 2 and 6 and markers of ACh and 5HT systems were examined at hatching. The higher dose caused a reduction in cholinesterase activity but there was no consistent downregulation of m\textsubscript{2}-muscarinic ACh receptors as would have been expected from ACh hyperstimulation. Both doses evoked significant reductions in the presynaptic high-affinity choline transporter, the rate-limiting factor in ACh biosynthesis, as monitored by binding of hemicholinium-3. Choline acetyltransferase, a constitutive marker for ACh terminals, was unaffected. This suggests that CPF reduces ACh presynaptic activity rather than compromising the development of ACh projections per se. CPF exposure also reduced the expression of cerebrocortical 5HT\textsubscript{1A} receptors. These effects in the chick model recapitulate many of the actions of early gestational CPF exposure in rats, and thus suggest that CPF exerts direct actions on the immature brain to compromise the development of ACh and 5HT pathways.

Index terms

Acetylcholine systems; Brain development; Chlorpyrifos; Cholinesterase; Developing chick; Organophosphates; Serotonin systems
1. INTRODUCTION

Organophosphates are the most heavily used insecticides worldwide [9] and consequently, their propensity to produce developmental neurotoxicity remains a major concern [10,12,28,61], especially in light of the nearly-ubiquitous exposure of the human population [9]. Originally, it was thought that all agents in this class acted alike through their shared ability to inhibit cholinesterase [35] but it is increasingly evident that the adverse effects on brain development reflect families of mechanisms that are not necessarily the same for all organophosphates. These mechanisms can operate at apparently nontoxic exposures that elicit no signs or symptoms, or even below the threshold for anticholinesterase actions (reviews, [48–50]). These include oxidative stress, interference with neural cell replication and differentiation, shifts in the patterns of axonal and dendritic growth and suppression of nuclear transcription and neurotrophic factors that govern the expression of specific neurotransmitter phenotypes [7,13,25,48–51,53,55]. Because most of these mechanisms have been identified through the use of rodent models, it is essential to identify which targets represent direct effects of the organophosphates as opposed to indirect actions mediated through effects on the maternal/fetal or maternal/neonatal unit. Agents may change maternal body temperature or oxygenation [45], or induce maternal stress [62], all of which are known to affect neurobehavioral outcomes and which may or may not be shared in varying degrees by humans. For rodents, there is a distinct “litter” effect [58] related to specific patterns of maternal nest-making and pup retrieval, as well as littermate competition for teats that is not paralleled by human experience; further, suckling and maternal care are both affected if agents impair vocalization by the offspring [6,19,44].

One approach to this conundrum is to utilize in vitro models, for which maternal factors clearly do not operate, and indeed, a number of studies utilizing neural cell lines or micromass cultures have clearly demonstrated direct effects of organophosphates on neurodevelopment [5,11–13,15,25,36,39,40,53,57]. Nevertheless, these approaches have a limited ability to reproduce essential steps in brain assembly that are known to be affected by organophosphate exposure in vivo. These involve cell-to-cell neuronal or glial-neuronal interactions as well as compound mechanisms that are essential for proper wiring of neural circuits and for apoptotic modeling of brain structures. But perhaps more importantly, in vitro systems do not allow testing of the relationship of a given mechanism to behavioral deficits. In this regard, lower organisms have distinct advantages. In recent work, we developed an avian model for studies of developmental neurotoxicity of organophosphates as well as other neurotoxicants [24,64,65]. With chicks, we can administer agents directly to the medium surrounding the embryo without maternal mediation, eliminating the variables related to maternal physiology. During the early incubation period, the fluid volume of the chick egg is large compared to the embryo, so that the exposure is uniform. Cognitive performance and its neurochemical underpinnings can be tested right after hatching, before the chicks commence consumption of food and water, and are thus independent of any potential changes in self-sufficiency.

In the current study, we used developing chick embryos to evaluate two specific features of the developmental neurotoxicity of organophosphates identified earlier in rodent models: adverse effects on development of acetylcholine (ACh) and serotonin (5-hydroxytryptamine, 5HT) systems [48–50]. These pathways are highly targeted by organophosphates at otherwise nontoxic exposures, involving mechanisms unrelated to cholinesterase inhibition. For our purposes, we chose chlorpyrifos (CPF), the most widely-studied organophosphate and the one with historically the highest utilization and human exposure. We administered CPF beginning early in development, paralleling rodent models with treatment in early gestation, which are known to produce deficits in both ACh and 5HT systems and related behaviors [3,23,38,41]. In our earlier work, we showed that CPF exposure in avian embryos similarly evokes cognitive impairment, evidenced by an inability to perform an imprinting task immediately after hatching.
Here, we show that the behavioral deficits are associated with adverse effects on the development of ACh and 5HT systems, thus proving that the actions seen in mammalian models are indeed direct effects on brain development, unrelated to maternal factors. Further, imprinting behavior depends on a specific brain region, the left half of the intermediate part of the medial hyperstriatum ventrale (IMHV), so we looked for regional differences in effects on neurotransmitter systems that might be related to the corresponding behavioral outcomes.

For effects on ACh synaptic function, we assessed four markers: cholinesterase activity, m2-muscarinic acetylcholine receptors (m2AChRs), choline acetyltransferase (ChAT) activity, and binding of hemicholinium-3 (HC3) to the presynaptic high-affinity choline transporter. Inhibition of cholinesterase and the consequent ACh hyperstimulation typically lead to muscarinic receptor downregulation [22,67]. ChAT, the enzyme that synthesizes acetylcholine, is a constitutive component of cholinergic nerve terminals and thus provides a measure of the development of cholinergic projections [14,20,36,41,42,51]. In contrast, HC3 binding to the choline transporter is responsive to neuronal activity [26,47], so that measurement of both parameters enables the distinction between effects on the development of innervation from those on synaptic activity. These markers have been used previously to characterize effects of CPF on ACh systems in adult rats [32,33] and to evaluate the immediate and delayed effects of developmental CPF exposure [38,41,43,51,52]. For 5HT systems, we measured 5HT1A and 5HT2 receptors, both of which are key players in 5HT synaptic function that are known to be affected by CPF exposure in developing rodents [3,4,54].

2. METHODS

2.1 Animal treatments

Fertile Ross 708 female × Ross 344 male chicken eggs (Gallus gallus domesticus) were purchased from the Department of Poultry Science, North Carolina State University (Raleigh, NC), and an injection window was prepared by drilling a hole in the pointed end and sealing it with medical silicon (Dow Corning Type A). Eggs were weighed and, after correcting for shell weight (11%), the concentration of CPF (Chem Service, West Chester, PA) was adjusted to deliver 10 or 20 mg/kg in a volume of 3 μl on incubation days 2 and 6. CPF was dissolved in dimethylsulfoxide vehicle and control eggs received an equivalent volume of vehicle. The eggs were placed in a commercial incubator and embryonic survival was monitored via candling. At the end of the incubation period, the hatch rate and physical attributes at hatching were noted. Chicks showing deformities were excluded from the neurochemical determinations and were euthanized immediately after hatching. Experiments were run the day after hatching.

In order to perform the neurochemical determinations in a manner reproducing the conditions used in our earlier behavioral studies [24,64], we imprinted the chicks prior to harvesting the brain regions; chicks were imprinted on each other in a batch by placing groups of nine chicks in 30 cm × 30 cm × 16 cm (height) boxes maintained at 32°C. Each box was illuminated for 30 min with a 60 W fluorescent lamp suspended 25 cm above the box, followed by a 15 min period of darkness to prime the chicks for imprinting. The lamp was then turned back on for 60 min while playing hen calls at normal human speech level, followed by a 60 min rest period with the lamp off. The lamp was then switched back on for 20 min, simulating the light used during the behavioral testing period [24,64].

At the end of the imprinting protocol, chicks were decapitated and, after removing the cerebellum and optic tectum, the rest of the brain was dissected into cerebral cortex, hippocampus, striatum, brainstem, left IMHV and right IMHV. Cholinesterase measurements were made on the rest of the brain after removal of the IMHV, which represents a minuscule proportion of the total tissue weight (4 mg out of 630 mg), and for our purposes, is designated...
as “whole brain” to distinguish it from the specific regions used in the other assays. Tissues were flash-frozen in liquid nitrogen and stored at \(-45^\circ\text{C}\) until assayed.

Eight independent samples were evaluated for each variable in each treatment group, with each sample derived from a single chick, with the exception of the IMHV, which required combining tissues from 12–14 chicks for each sample.

### 2.2 Cholinesterase activity

Each tissue was thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in ice-cold 50 mM Tris (pH 7.4), and aliquots of the homogenate were withdrawn for measurement of total protein [56] and cholinesterase activity [16]. For the latter, the homogenate was diluted in 0.5% Triton X100, 0.1 M Na$_2$HPO$_4$/KH$_2$PO$_4$ (pH 8) and left on ice for 15 min to allow the Triton X100 to solubilize membrane-associated cholinesterase.

Homogenates were sedimented at 40,000 \( \times \) g for 15 min and aliquots of the supernatant solution were added to final concentrations of 0.5 mM acetylthiocholine iodide and 0.33 mM 5,5′-dithiobis(2-nitrobenzoic acid) in the same buffer without Triton (all reagents from Sigma Chemical Co., St. Louis, MO). Assays were incubated at room temperature for 4, 8, 12, 16 and 20 min, and the enzyme activity was assessed from the linear portion of the time course, reading the absorbance at 415 nm. The assay was standardized using mercaptoethanol and calculated relative to total protein.

### 2.3 ACh synaptic markers

Tissues were thawed in 79 volumes of ice-cold 10 mM sodium-potassium phosphate buffer (pH 7.4) and homogenized (Polytron). For ChAT activity [29], assays contained 60 mM sodium phosphate (pH 7.9), 200 mM NaCl, 20 mM choline chloride, 17 mM MgCl$_2$, 1 mM EDTA, 0.2% Triton X-100, 0.12 mM physostigmine, 0.6 mg/ml bovine serum albumin and 50 \( \mu \)M \[^{14}\text{C}\]acetyl-coenzyme A (PerkinElmer Life Sciences, Boston, MA; specific activity 60 mCi/mmol, diluted with unlabeled compound to 6.7 mCi/mmol). Samples were preincubated for 15 min on ice, transferred to a 37°C water bath for 30 min, and the reaction terminated by placing the samples on ice. Labeled acetylcholine was then extracted, counted and the activity determined relative to total protein [56].

For measurements of \[^3\text{H}\]HC3 binding [60], the cell membrane fraction was prepared by sedimenting an aliquot of the same tissue homogenate at 40,000 \( \times \) g for 15 min. The membrane pellet was resuspended (Polytron) in the original volume of buffer, resedimented, and the resultant pellet was resuspended using a smooth glass homogenizer fitted with a Teflon pestle, in 10 mM sodium-potassium phosphate buffer (pH 7.4) and 150 mM NaCl. Radioligand binding was evaluated with 2 nM \[^3\text{H}\]HC3 (PerkinElmer; specific activity 125 Ci/mmol), with incubation for 20 min at room temperature, followed by rapid vacuum filtration onto glass fiber filters (presoaked for 30 min with 0.1% polyethyleneimine in buffer). The nonspecific component was defined as radioligand binding in the presence of an excess concentration (10 \( \mu \)M) of unlabeled HC3 (Sigma). Binding values were expressed relative to membrane protein.

Similarly, for \(m_2\)AChR binding, aliquots of the cell membrane fraction were incubated in 10 mM sodium-potassium phosphate buffer (pH 7.4) for 60 min at room temperature, using 1 nM \[^3\text{H}\]AFDX384 (PerkinElmer; specific activity, 115 Ci/mmol) with or without 1 \( \mu \)M atropine (Sigma) to displace specific binding [41].

### 2.4 5HT receptors

Homogenates were prepared, sedimented and washed as already described, with the final resuspension carried out in 50 mM Tris buffer (pH 7.4). An aliquot was withdrawn for the determination of membrane protein [56]. Two radioligands were used to determine 5HT receptor binding [63]: 1 nM \[^3\text{H}\]8-hydroxy-2-(di-n-propylamino)tetratin (PerkinElmer Life
For each receptor binding study, we used a single ligand concentration that lies above the $K_d$ but nevertheless is below full saturation of the binding site. With this approach, we can detect changes regardless of whether they occur in $K_d$ or $B_{max}$ but cannot distinguish between the two possibilities. The strategy required by the fact that there is simply not enough tissue in many of the regions to perform the Scatchard analyses that would be required, and in addition, the experimental design requires binding analysis for multiple ligands in hundreds of membrane preparations, thus making it technically impossible to determine $K_d$ and $B_{max}$ in each one.

2.5 Data analysis

All data are presented as means and standard errors. Treatment effects on neurochemical variables were examined by ANOVA, incorporating factors of treatment and brain region, and Fisher’s Protected Least Significant Difference Test was applied post-hoc to evaluate specific treatment groups that differed significantly from the corresponding control values. Failure to hatch and birth defects were compared using the $\chi^2$ test for observed vs. expected frequencies. Significance was assumed at $p < 0.05$.

3. RESULTS

In agreement with earlier findings [24], CPF exposure reduced the hatch rate and increased the incidence of birth defects (Fig. 1). There were no significant CPF effects on body or brain region weights of the newly-hatched chicks (data not shown).

Exposure of chick embryos to 10 mg/kg CPF on incubation days 2 and 6 did not produce any significant reduction in cholinesterase activity at hatching but raising the dose to 20 mg/kg elicited a 20% decrement that did achieve statistical significance (Fig. 2A). However, we did not observe a global downregulation of $m_2AChRs$ as would have been expected from cholinergic hyperstimulation resulting from cholinesterase inhibition (Fig. 2B). There were no significant effects in the cerebral cortex or hippocampus, and although the striatum showed a reduction at the low dose of CPF, the effect was inconsistent, since no decrease was found at the higher dose. Only in the brainstem did we see any consistent $m_2AChR$ downregulation as evidenced by a small (10%) but statistically significant reduction in the group given 20 mg/kg CPF.

In contrast to the relative lack of effect on $m_2AChRs$, CPF at either dose evoked a significant reduction in HC3 binding, with a clear-cut dose-effect relationship (Fig. 3A). At the low dose, there was an overall effect ($p < 0.05$ for the main treatment effect) even though no individual brain region achieved statistical significance. At the high dose, the main effect was far more statistically robust ($p < 0.0001$) and individually significant effects were seen in the hippocampus, striatum and brainstem. Notably, there was no significant effect for the IMHV for either hemisphere, and the only tendency was for a reduction at the high dose in the right IMHV rather than the left. In contrast to the effects on HC3 binding, we did not observe any significant effects on ChAT activity (Fig. 3B).
CPF also had an adverse effect on 5HT receptors in the cerebral cortex. For the 5HT$_{1A}$ subtype, there was an overall reduction that achieved statistical significance at the higher dose of CPF (Fig. 4A); although the effect was not statistically significant at the lower dose of 10 mg/kg, this group was also indistinguishable from the 20 mg/kg group which did show a significant effect, so that the result is clearly part of a dose-effect continuum. In contrast, there were no alterations in 5HT$_2$ receptors (Fig. 4B).

4. DISCUSSION

In previous work with rodent models of prenatal CPF exposure, we and others identified deficits in synaptic function of ACh and 5HT systems that emerge over a period from days to weeks after the end of treatment [3,14,38,41–43,51]. Here, using an avian model devoid of maternal confounds, we obtained similar results, indicating that these actions represent direct interference with brain development rather than effects mediated on maternal/fetal physiology or maternal/neonatal interactions; our results thus reinforce findings from in vitro models that similarly indicate vulnerability of these specific neurotransmitter phenotypes [15,25,36,53].

For ACh systems, we did not find any significant reductions in cholinesterase activity at the lower CPF dose but raising the dose to 20 mg/kg did produce a small, but significant reduction of about 20%. This effect could reflect residual actions of CPF oxon, the active metabolite of CPF that produces irreversible cholinesterase inhibition, or alternatively, could also represent suppression of differentiation into the ACh phenotype [36]. The first possibility seems unlikely, since the determinations were done at hatching, more than two weeks after the last CPF injection; since the embryo grows by orders of magnitude over that span, nearly all the cholinesterase molecules would have been formed during the major embryonic growth phase in the latter part of incubation, rather than during the initial phase of CPF exposure. Nevertheless, we specifically tested whether the reduced cholinesterase activity reflected a functionally-important effect by determining whether m$_2$AChRs were downregulated, as would be expected from ACh hyperstimulation [22,67]. The results were negative — there were no consistent overall effects on the receptors. If the reduced cholinesterase activity instead indicates an impairment of differentiation into the ACh phenotype, then we would expect to see deficits in other biomarkers of ACh presynaptic integrity or function. ChAT, a constitutive marker for ACh terminals, was unaffected, but we found a robust, dose-dependent reduction in HC3 binding, connoting a decrease in the presynaptic, high-affinity choline transporter. Since the transporter is rate-limiting in ACh biosynthesis and is directly responsive to synaptic activity [47], our results indicate a major deficiency in ACh function, rather than a loss of ACh terminals per se, in agreement with similar findings in the rodent model [14,38,41,43,51].

It is notable that we did not see any particular targeting of ACh pathways in the left side of the IMHV; indeed the effects were not even significant in that region, whereas deficits were seen in the hippocampus, striatum and brainstem. In earlier work, we showed that the same CPF treatment impaired the ability of newly-hatched chicks to imprint [24,64,65], a behavior that depends largely on the integrity of the left IMHV [8,21], thus recapitulating the role of the mammalian hippocampus in learning and memory [8]. The left IMHV stores the required imprinting information, whereas the right IMHV acts only as a temporary or “buffer” storage site [8]. The behavioral impairment was associated with a loss of ACh function at the postsynaptic level, involving uncoupling of muscarinic ACh receptors from their ability to elicit signaling changes related to specific PKC isoforms [24,64,65]. In combination with our current findings, which do not indicate selective (or even significant) changes in presynaptic ACh function in the left IMHV, the impairment of imprinting obviously reflects primarily the postsynaptic alterations. Because we did find adverse effects on HC3 binding in other regions, it is apparent that the disruption of ACh systems by developmental CPF exposure reflects multiple targets at both the presynaptic and postsynaptic levels, with specific regional
differences. The contributions of these other regional targets to impaired behavioral function will need to be established in future studies.

For the 5HT receptors, we again found significant reductions evoked by early CPF exposure, similar to those found in the rodent model [3]; however, the effect in chicks was limited to the 5HT1A subtype, whereas it is shared by both subtypes in developing rats. Accordingly, there are some disparities, perhaps reflecting indirect mechanisms for the effects on development of 5HT2 receptor in mammals, or alternatively a basic species difference. However, it should be noted that the current study involved only a single time point, and studies in rats show progressive changes in 5HT systems after early CPF exposure [1–4], so that the apparent differences may resolve with future examination of the temporal course in the chick model. In any case, the shortfall in 5HT1A receptors caused by developmental exposure to CPF is likely to be critical to the subsequent emergence of related functional deficits: changes in the expression of the 5HT1A receptor during early development play a key role in the permanent programming of 5HT behavioral responses [27].

In agreement with earlier results [24], we found that early CPF exposure reduced the hatching rate and increased the incidence of birth defects; however, we found such effects at the high dose only, whereas both doses had significant actions previously. The two studies differ in the strain of chicken used as the source of eggs, which obviously has an impact on outcomes for viability and morphology. However, these two factors provide a prime example of endpoints that do not translate well from avian to mammalian species, since avian embryos are extremely sensitive to otherwise inconsequential environmental perturbations, such as small changes in temperature and humidity, movement and vibration, seasonal variations, or clutch-to-clutch differences. Nearly 20% of the control eggs were infertile or failed to hatch, which is also a typical percentage for commercial hatcheries. Whereas in mammalian species fetal growth retardation typically occurs at toxicant exposures below those that elicit fetal death, we found no effects on body weight in the chicks that hatched even at exposures that reduced the hatching rate, both in the previous study [24] and in the current work. Accordingly, whereas our results provide interpretable cross-species evaluations for neurodevelopmental endpoints, the specific measures of hatching rate and malformation may be of much more limited utility in comparing benchmarks for toxicant exposures.

In comparing the chick model to rodents, it is important to note that, although the incubation time to hatching in the chick embryo (21 days) is quite similar to the gestational period in rats (21–22 days), the chick develops far more rapidly [46]. Our treatment paradigm thus encompasses early gestational exposure but assessment at hatching is actually neurodevelopmentally equivalent to rats at about the end of the second postnatal week, which in turn approximates human development at the time of birth. Additionally, whereas the mother participates in clearing chemicals and metabolites from the mammalian fetus, the avian egg has no way to eliminate the agent totally, since it is an enclosed environment. Very early in development, encompassing the period in which we administered CPF, the chick embryo possesses both the cytochrome P450 enzymes required to form CPF oxon, and nonspecific carboxylesterases that inactivate it [17,34]. Likewise, both acetylcholinesterase and butyrylcholinesterase emerge well before ACh synaptogenesis occurs in the neural stage [30], and accordingly, phosphorothionates that, like CPF, need to be metabolically activated to the oxon form before they can inhibit cholinesterase, do evoke cholinesterase inhibition even in the early chick embryo [18]. The extent to which the timetables for these events might diverge between rodent and avian species has not been explored systematically, but clearly they could contribute to some of the differences observed here between outcomes in chicks and previous evaluations in rats [3,14,38,41–43,51]. Nevertheless, the point remains that the outcomes were basically alike, reinforcing the underlying similarities in the targeting of brain by CPF.
In conclusion, our results indicate that CPF has adverse effects on the development of ACh and 5HT systems in the chick model. These results closely resemble the deficits found for the same neurotransmitter systems in rodent models of early prenatal CPF exposure [3,14,38,41–43,51]. Since the avian model does not share the maternal confounds found in mammals, our findings reinforce the view that CPF, and likely other organophosphate pesticides, have direct, adverse actions on the developing nervous system. It is equally important to note that chick eggs are plentiful and cheap, and that automated injectors are readily available to process hundreds of eggs at a time. Our results thus point to the potential use of avian models as a means to achieve higher-throughput screening of developmental neurotoxicants.

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Abbreviations

5HT
5-hydroxytryptamine, serotonin

ACh
acetylcholine

ANOVA
analysis of variance

ChAT
choline acetyltransferase

CPF
chlorpyrifos

HC3
hemicholinium-3

IMHV
intermediate part of the medial hyperstriatum ventrale

m2AChR
muscarinic2 acetylcholine receptor

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Figure 1.
CPF effects on hatching and birth defects. Each bar shows the percentage values along with the affected/total number of chicks in each group. Asterisks denote significant differences from the corresponding control group. Birth defects for controls and CPF 10 mg/kg all were animals with externalized organs; for the CPF 20 mg/kg group, two-thirds had externalized organs and one-third had splayed legs.
Figure 2.
CPF effects on cholinesterase activity (A) and m<sub>2</sub>AChR binding. ANOVA appears at the top of each panel and asterisks denote significant differences from the corresponding control values. Abbreviations: ctx, cerebral cortex; hip, hippocampus; str, striatum; bs, brainstem.
Figure 3.
CPF effects on HC3 binding (A) and ChAT activity (B). ANOVA appears at the top of each panel and asterisks denote significant differences from the corresponding control values. Abbreviations: ctx, cerebral cortex; hip, hippocampus; str, striatum; bs, brainstem; l IMHV, left IMHV; r IMHV, right IMHV; NS, not significant.
Figure 4.
CPF effects on 5HT<sub>1A</sub> (A) and 5HT<sub>2</sub> (B) receptor binding in the cerebral cortex. ANOVA appears at the top of each panel and asterisks denote significant differences from the corresponding control values. Abbreviation: NS, not significant.