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Research report

Developmental neurotoxicity of low dose diazinon exposure of neonatal rats: Effects on serotonin systems in adolescence and adulthood

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Abstract

The developmental neurotoxicity of organophosphate pesticides targets serotonin (5HT) systems, which are involved in emotional and appetitive behaviors. We exposed neonatal rats to daily doses of diazinon on postnatal days 1–4, using doses (0.5 or 2 mg/kg) spanning the threshold for barely-detectable cholinesterase inhibition. We then evaluated the effects on $5HT_{1A}$ and $5HT_2$ receptors, and on the 5HT transporter in cerebral cortical regions and the brainstem in adolescence through adulthood. Diazinon evoked a lasting deficit in $5HT_{1A}$ receptors in males only, whereas it caused a small but significant increase in 5HT transporters in females; neither effect showed a significant regional selectivity. This pattern differed substantially from that seen in earlier work with another organophosphate, chlorpyrifos, which at pharmacodynamically similar doses spanning the threshold for cholinesterase inhibition, evoked a much more substantial, global upregulation of 5HT receptor expression; with chlorpyrifos, effects on receptors were seen in females, albeit to a lesser extent than in males, and were also regionally distinct. The effects of diazinon were nonmonotonic, showing larger alterations at the lower dose, likely reflecting positive trophic effects of cholinergic stimulation once the threshold for cholinesterase inhibition is exceeded. Our results reinforce the idea that different organophosphates have fundamentally distinct effects on the developmental trajectories of specific neurotransmitter systems, unrelated to their shared action as cholinesterase inhibitors. The effects on 5HT circuits expand the scope of behavioral endpoints that need to be considered in evaluating the developmental neurotoxicity of organophosphates.

Keywords: Brain development; Diazinon; Organophosphate insecticides; Serotonin receptors; Serotonin transporter

1. Introduction

Organophosphate pesticides are undergoing increasing scrutiny because of their propensity to elicit developmental neurotoxicity at lower exposures than those which cause overt symptoms of intoxication, or even below the threshold for cholinesterase inhibition, the biomarker most commonly used for exposure and risk assessment [12,14,29,30,38,53–55,65–67,86]. Indeed, a wealth of information now shows that these agents disrupt neural cell replication and differentiation, interfere with axonogenesis and synaptogenesis, and impair the functional development of neurotransmitter and neurotrophin systems, culminating in aberrant behavioral performance [9–12,19,55–57,65–67,77,92]. Consequently, the organophosphates produce developmental damage extending far beyond acetylcholine systems, notably including serotonin (5HT), which appears to be particularly sensitive to disruption by fetal or neonatal organophosphate exposure [1–5,58,64,72–75]. In keeping with the known role of 5HT abnormalities in affective disorders [49,50], rats exposed to low doses of chlorpyrifos as neonates show depression-like behavioral patterns [1]; further, a clear connection appears to be emerging between human organophosphate exposure and depression and suicide [32,36].

If the developmental neurotoxicity of organophosphates resides in mechanisms other than their shared ability to inhibit cholinesterase, then it is likely that the various members of this class might evoke dissimilar effects reflecting other mechanisms. We recently compared the ability of three different organophosphates, chlorpyrifos, diazinon and parathion, to elicit immediate changes in 5HT systems after exposure of neonatal

Abbreviations: 5HT, 5-hydroxytrypamine, serotonin; ANOVA, analysis of variance; PN, postnatal day.

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rats to doses spanning the threshold for barely-detectable, nonsymptomatic inhibition of cholinesterase [4,73,79]. Although both chlorpyrifos and diazinon evoked an immediate increase in the concentration of $5HT_{1A}$ and $5HT_2$ receptors at these low doses, parathion evoked a decrease in the $5HT_{1A}$ subtype, confirming disparate actions of the three agents. Furthermore, when we examined expression patterns for the gene families encoding the 5HT biosynthetic enzymes, transporters and receptors, we also found major divergence between chlorpyrifos and diazinon, especially for the $5HT_{1A}$ and $5HT_2$ receptor subtypes [73], suggesting that functional differences might emerge later. Accordingly, in the present study, we examined the long-term effects of neonatal diazinon exposure on the developmental profile of these receptors and the 5HT transporter (5HTT) in adolescence through adulthood, the period when lasting changes emerged in our earlier studies with chlorpyrifos [1–3,5,72,74,75]. We administered diazinon during the immediate postnatal period (postnatal days PN1-4), a stage where we previously found high sensitivity of 5HT systems to disruption by chlorpyrifos [2,4,5,75]. We evaluated two nonsymptomatic diazinon regimens [69,73,79], 0.5 mg/kg/day, which produces no discernible cholinesterase inhibition, and 2 mg/kg/day, which elicits approximately 20% inhibition, equivalent to that obtained with 1 mg/kg/day of chlorpyrifos as used in our earlier work [81]. Because the effects of chlorpyrifos on 5HT systems are strongly sex-selective [1,3,5,72], we evaluated both males and females for comparable effects of diazinon. Measurements were conducted for $5HT_{1A}$ and $5HT_2$ receptors, which converge on common endpoints in 5HT cell signaling [8,47,63] and are key players in 5HT-related mental disorders, particularly depression [7,17,93,94]. In addition, we assessed binding to the 5HTT site, which regulates the synaptic concentration of 5HT and is the major target for antidepressant drugs [37,49,50]. Evaluations were conducted in the forebrain, which contains a high concentration of 5HT projections, and in the brainstem, which contains the corresponding 5HT cell bodies.

2. Methods

2.1. Animal treatments

All experiments were carried out humanely and with regard for alleviation of suffering, with protocols approved by the Institutional Animal Care and Use Committee and in accordance with all federal and state guidelines. Timed-pregnant Sprague–Dawley rats (Charles River, Raleigh, NC) were housed in breeding cages, with a 12 h light–dark cycle and free access to food and water. On the day after birth, all pups were randomized and redistributed to the dams with a litter size of 10 (five males, five females) to maintain a standard nutritional status. Because of its poor water solubility, diazinon (Chem Service, West Chester, PA) was dissolved in dimethylsulfoxide to provide consistent absorption [69,73,79,89] and was injected subcutaneously in a volume of 1 ml/kg once daily on postnatal days (PN) 1–4; control animals received equivalent injections of the dimethylsulfoxide vehicle, which does not itself produce developmental neurotoxicity [89]. Doses of 0.5 and 2 mg/kg/day were chosen because they lie below the threshold for signs of systemic toxicity in developing rats as evidenced by impaired viability or reduced weight gain [69] and they straddle the threshold for barely-detectable cholinesterase inhibition [73,79]. These treatments thus resemble the nonsymptomatic exposures reported in pregnant women [16] and are pharmacodynamically comparable to expected fetal and childhood exposures after routine home application or in agricultural communities [20,51]. Randomization of pup litter assignments within treatment groups was repeated at intervals of several days up until weaning, and in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Offspring were weaned on PN21.

On PN30, 60 and 100, one male and one female were selected from each litter of origin and were decapitated. The cerebellum (including flocculi) was removed and the midbrain/brainstem was separated from the forebrain by a cut rostral to the thalamus. The striatum and hippocampus were then dissected from these larger divisions and the midbrain and brainstem were divided from each other. The cerebral cortex was divided down the midline and then further sectioned into anterior and posterior regions (frontal/parietal cortex and temporal/occipital cortex, respectively). The current studies were performed on the frontal/parietal cortex and temporal/occipital cortex, which contain the major cerebrocortical 5HT projections, and the brainstem, which contains 5HT cell bodies; the remaining regions were reserved for future work. Tissues were frozen with liquid nitrogen and stored at −45 °C.

2.2. Assays

Assays were conducted on each individual tissue, so that each determination represented a value from the corresponding brain region of one animal. 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 [80]. The remaining homogenate was sedimented at 40,000 × *g* for 15 min and the resultant pellet was washed by resuspension (Polytron) in homogenization buffer followed by resedimentation, and was then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in 50 mM Tris buffer (pH 7.4). An aliquot was withdrawn for the determination of membrane protein [80]. Two radioligands were used to determine 5HT receptor binding [91]: 1 nM [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin (Perkin-Elmer Life Sciences, Boston, MA; specific activity, 135 Ci/mmol) for $5HT_{1A}$ receptors [52,82], and 0.4 nM [^{3}H]ketanserin (Perkin-Elmer; specific activity, 63 Ci/mmol) for $5HT_2$ receptors [35,52]. For $5HT_{1A}$ receptors, incubations lasted for 30 min at 25 °C in a buffer consisting of 50 mM Tris (pH 8), 2 mM $MgCl_2$ and 2 mM sodium ascorbate; 100 μM 5HT (Sigma) was used to displace specific binding. For $5HT_2$ receptors, incubations lasted 15 min at 37 °C in 50 mM Tris (pH 7.4) and specific binding was displaced with 10 μM methylsergide (Sandoz Pharmaceuticals, E. Hanover, NJ). Incubations were stopped by the addition of a large excess of ice-cold buffer and the labeled membranes were trapped by rapid vacuum filtration onto glass fiber filters that were pre-soaked in 0.15% polyethyleneimine (Sigma). The filters were then washed repeatedly and radiolabel was determined. For binding to the presynaptic 5HTT [46,70,71,76,90], the membrane suspension was incubated with 85 pM [^{3}H]paroxetine (Perkin-Elmer; specific activity 19.4 Ci/mmol) with or without addition of 100 μM 5HT to displace specific binding, and incubations lasted 120 min at 20 °C. Binding was calculated relative to membrane protein.

2.3. Data analysis

Data were compiled as means and standard errors. Because we evaluated multiple neurochemical variables that were all related to 5HT synapses, the initial comparison was conducted by a global ANOVA (data log-transformed because of heterogeneous variance among ages, regions and measures) incorporating all the variables and measurements so as to avoid an increased probability of type 1 errors that might otherwise result from multiple tests of the same data set: treatment, age, sex, region and the three repeated measures ($5HT_{1A}$ receptors, $5HT_2$ receptors, 5HTT). Where we identified interactions of treatment with the other variables, data were then subdivided for lower-order ANOVAs to evaluate individual treatments that differed from the corresponding control. Significance for all tests was assumed at the level of $p < 0.05$. For convenience, some of the results are presented as the percent change from control values but statistical comparisons were conducted only on the original data. For reference, the corresponding control values are shown in Table 1.