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Developmental neurotoxicants target neurodifferentiation into the serotonin phenotype: Chlorpyrifos, diazinon, dieldrin and divalent nickel

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ABSTRACT

Developmental exposure to organophosphates (OP) produces long-term changes in serotonin (5HT) synaptic function and associated behaviors, but there are disparities among the different OPs. We contrasted effects of chlorpyrifos and diazinon, as well as non-OP neurotoxicants (dieldrin, Ni^{2+}) using undifferentiated and differentiating PC12 cells, a well-established neurodevelopmental model. Agents were introduced at 30 μM for 24 or 72 h, treatments devoid of cytotoxicity, and we evaluated the mRNAs encoding the proteins for 5HT biosynthesis, storage and degradation, as well as 5HT receptors. Chlorpyrifos and diazinon both induced tryptophan hydroxylase, the rate-limiting enzyme for 5HT biosynthesis, but chlorpyrifos had a greater effect, and both agents suppressed expression of 5HT transporter genes, effects that would tend to augment extracellular 5HT. However, whereas chlorpyrifos enhanced the expression of most 5HT receptor subtypes, diazinon evoked overall suppression. Dieldrin evoked even stronger induction of tryptophan hydroxylase, and displayed a pattern of receptor effects similar to that of diazinon, even though they come from different pesticide classes. In contrast, Ni^{2+} had completely distinct actions, suppressing tryptophan hydroxylase and enhancing the vesicular monoamine transporter, while also reducing 5HT receptor gene expression, effects that would tend to lower net 5HT function. Our findings provide some of the first evidence connecting the direct, initial mechanisms of developmental neurotoxicant action on specific transmitter pathways with their long-term effects on synaptic function and behavior, while also providing support for in vitro test systems as tools for establishing mechanisms and outcomes of related and unrelated neurotoxicants.

Introduction

Organophosphate pesticides are undergoing increased scrutiny because of their propensity to elicit developmental neurotoxicity at exposures devoid of any signs of systemic intoxication and below the threshold for cholinesterase inhibition, the standard exposure biomarker (Physicians for Social Responsibility, 1995; Landrigan et al., 1999; Pope, 1999; Slotkin, 1999, 2004, 2005; May, 2000; Landrigan, 2001; Casida and Quistad, 2004; Weiss et al., 2004; Perera et al., 2005; Costa, 2006). Indeed, it is now clear that disruption of brain development by these agents involves mechanisms unrelated to anticholinesterase actions, largely reflecting direct effects on neural cell replication and differentiation (Pope, 1999; Slotkin, 1999, 2004, 2005; Barone et al., 2000; Qiao et al., 2002, 2003a; Yanai et al., 2002; Betancourt and Carr, 2004; Casida and Quistad, 2004; Gupta, 2004; Betancourt et al., 2006; Slotkin et al., 2007c). Accordingly, native organophosphate chemicals can actually be more active as neurodevelopmental disruptors than are their oxon metabolites, which are the forms that produce irreversible cholinesterase inhibition (Buznikov et al., 2001; Qiao et al., 2001; Jameson et al., 2007). Nevertheless, most reports still focus on the impact of organophosphates on acetylcholine systems and related behaviors (Slotkin, 1999, 2004, 2005; Jett et al., 2001; Landrigan, 2001; Levin et al., 2001, 2002; Icenogle et al., 2004; Weiss et al., 2004; Rauh et al., 2006; Eskenazi et al., 2007, 2008). Recent studies show, however, that developing serotonin (5HT) systems may be even more sensitive, leading to abnormalities of emotional, appetitive and social behaviors (Aldridge et al., 2003, 2004, 2005a, 2005b, 2005c; Ricceri et al., 2003, 2006; Slotkin and Seidler, 2005, 2007b, 2007c; Slotkin et al., 2006b, 2008b, 2008e; Moreno et al., 2008; Timofeeva et al., 2008); indeed, organophosphate exposures are now being linked to depression and suicide (London et al., 2005; Beseler et al., 2006; Jaga and Dharmani, 2007; Lee et al., 2007).

If the organophosphates disrupt brain development through mechanisms other than their shared property as cholinesterase inhibitors, then there is no reason they should all produce exactly the same outcomes. In a series of reports, we found that exposure of neonatal rats to pharmacodynamically-equivalent doses of chlorpyrifos, diazinon and parathion, all elicit long-term alterations in 5HT synaptic function and in 5HT-related behaviors, but that the changes

Abbreviations: 5HT, 5-hydroxytryptamine, serotonin; ANOVA, analysis of variance; NGF, nerve growth factor.

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differ substantially among the three agents, even including alterations in opposite directions (Aldridge et al., 2003, 2004, 2005a; Slotkin and Seidler, 2005, 2007a; Slotkin et al., 2006b, 2008b; Timofeeva et al., 2008). In the current study, we compared the effects of chlorpyrifos and diazinon on the expression of genes involved in 5HT biosynthesis, storage and degradation, as well as genes for the 5HT receptor subtypes. We focused on the critical period in which developing neurons first express specific neurotransmitter phenotypes, so as to determine if the differences seen with in vivo exposures reflect disparities in the direct actions of these agents. We performed the studies in PC12 cells, a well-established neurodevelopmental model (Teng and Greene, 1994) that has already been validated to reproduce the mechanisms and outcomes underlying organophosphate developmental neurotoxicity (Tuler et al., 1989; Flaskos et al., 1994; Bagchi et al., 1995, 1996; Nagata et al., 1997; Li and Casida, 1998; Song et al., 1998; Das and Barone, 1999; Crumpton et al., 2000a, 2000b; Qiao et al., 2001, 2005; Jameson et al., 2006, 2007; Slotkin et al., 2007a, 2007b, 2008c). In the presence of nerve growth factor (NGF), PC12 cells differentiate to form neuritic projections and acquire neuronal excitability, as well as specific neurotransmitter characteristics. Although acetylcholine and dopamine are the primary phenotypes for this cell line (Fujita et al., 1989; Teng and Greene, 1994; Song et al., 1998), PC12 cells also manufacture, store and secrete 5HT, exhibit expression of the 5HT biosynthetic enzymes, transporters and receptors, display receptor-mediated responses to 5HT, and show enhancement of all these features during differentiation (Furukawa et al., 1992; King et al., 1992; Li and DePetrillo, 2002; Zhang et al., 2004, 2005; Reaney and Smith, 2005; Homma et al., 2006; Lee et al., 2006).

Besides the organophosphates, we also evaluated the response to two agents from different classes, an organochlorine pesticide, dieldrin, and a metal, divalent nickel. We previously found that chlorpyrifos, diazinon, dieldrin and Ni^{2+} all produce similar changes in the balance of acetylcholine vs. dopamine phenotypes (Jameson et al., 2006; Slotkin et al., 2007b). Here, our interest was to see whether diverse neurotoxicants, outside the organophosphate class, produce similar or dissimilar effects on development of 5HT neuronal characteristics. These agents also have intrinsic interest because of significant environmental concerns about human exposure and safety (U.S. National Library of Medicine, 2006). For dieldrin, this clearly includes known endpoints for developmental neurotoxicity (Uzoukwu and Sleight, 1972; Brannen et al., 1998; Liu et al., 1998; Kitazawa et al., 2001, 2003; Slotkin et al., 2007b). Nickel accumulates in the fetus at concentrations similar to those of lead (Casey and Robinson, 1978; Jacobsen et al., 1978), and the neural actions of this metal resemble those of lead and cadmium (Benters et al., 1996).

Methods

Cell cultures. Because of the clonal instability of the PC12 cell line (Fujita et al., 1989), the experiments were performed on cells that had undergone fewer than five passages. As described previously (Song et al., 1998; Qiao et al., 2003b), PC12 cells (American Type Culture Collection, 1721-CRL, obtained from the Duke Comprehensive Cancer Center, Durham, NC) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% inactivated horse serum (Sigma Chemical Co., St. Louis, MO), 5% inactivated fetal bovine serum (Sigma), and 50 μg/ml penicillin streptomycin (Invitrogen). Incubations were carried out with 7.5% CO_2 at 37 °C, standard conditions for PC12 cells. To initiate neurodifferentiation (Teng and Greene, 1994; Jameson et al., 2006; Slotkin et al., 2007b) twenty-four hours after seeding, the medium was changed to include 50 ng/ml of 2.5 S murine NGF (Invitrogen). Along with the NGF, we added 30 μM of each of the test agents: chlorpyrifos (Chem Service, West Chester, PA), diazinon (Chem Service), dieldrin (Chem Service) or $NiCl_2$ (Sigma). The concentration was chosen from earlier studies that demonstrated adverse effects on differentiation of PC12 cells without outright cytotoxicity (Qiao et al., 2001; Jameson et al., 2007; Slotkin et al., 2007b, 2008c). Because of the limited water solubility of the three insecticides, these agents were dissolved in dimethylsulfoxide (final concentration 0.1%), which was also added to the control cultures and to cultures containing $NiCl_2$; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation (Song et al., 1998; Qiao et al., 2001, 2003b). Cultures were examined 24 and 72 h after commencing exposure, with 5–8 independent cultures evaluated for each treatment at each time point. We used two time points so as to be able to evaluate changes in gene expression regardless of whether the mRNA for a given gene has a rapid turnover (and hence can rise rapidly) or a slower turnover that would require a longer period to show corresponding increases or decreases. For chlorpyrifos, we evaluated the effects both on undifferentiated cells and during NGF-induced differentiation, whereas for the other agents, we studied only the effects during differentiation.

Microarray determinations. Our earlier studies detailed all the techniques for mRNA isolation, preparation of cDNA, conversion to cRNA incorporating cyanine-3 (reference RNA) or cyanine-5 (sample RNA), verification of RNA purity and quality, hybridization to the microarrays, washing and scanning (Slotkin and Seidler, 2007a; Slotkin et al., 2007c, 2008c). These all involve commercial kits and procedures, and since the current studies were done identically, the techniques will not be described here. The mRNA used for the reference standard was created by pooling aliquots from each of the samples in the study so as to ensure measurable levels of all genes expressed over the background. Similarly, array normalizations and error detection were carried out by procedures described previously (Slotkin and Seidler, 2007a; Slotkin et al., 2007c, 2008c). We used Agilent Whole Rat Genome Arrays (Agilent Technologies, Palo Alto, CA), type G4131A for the studies of chlorpyrifos in undifferentiated and differentiating cells, and type G4131F for the studies of diazinon, dieldrin and nickel in differentiating cells. The two chips contain exactly the same sequences but the latter has a lower detection threshold; however, all the genes reported here passed the quality control filters with both arrays.

For many of the genes, the arrays contain multiple probes for the same gene and/or replicates of the same probe in different locations on the chip, and these were used to verify the reliability of values and the validity of the measures on the chip. In these cases, to avoid artificially inflating the number of positive findings, we limited each gene to a single set of values, selecting those obtained for the probe showing the smallest intragroup variance. The other values for that gene were used only to corroborate direction and magnitude of change. We also validated the readings on the arrays through the use of duplicate arrays for selected samples (Slotkin and Seidler, 2007a; Slotkin et al., 2007c). Values reported for four of the genes presented here (*maoa, maob, slc18a1, slc18a2*) are also being used in a study of catecholamine phenotypes because of their relation to monoamines in general.

Statistical procedures. Because of the requirement to normalize the data across arrays and within each gene, the absolute values for a given gene are meaningless, so only the relative differences between treatments can be compared. Accordingly, results are presented as means and standard errors of the percentage change from control values to allow for visual comparison of the effects across families of genes. However, statistical comparisons were based on the actual ratios (log-transformed, since the data are in the form of ratios) rather than the percent change.

Our design involved multiple planned comparisons of four agents at two time points, as well as the effects of one agent (chlorpyrifos) in