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DEVELOPMENTAL NEUROTOXICANTS TARGET NEURODIFFERENTIATION INTO THE SEROTONIN PHENOTYPE: CHLORPYRIFOS, DIAZINON, DIELDRIN AND DIVALENT NICKEL

Theodore A. Slotkin and Frederic J. Seidler

Department of Pharmacology & Cancer Biology Duke University Medical Center Durham, North Carolina 27710 USA

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, Ni²⁺) using undifferentiated and differentiating PC12 cells, a well-established neurodevelopmental model. Agents were introduced at 30 μ M for 24 or 72 hr, 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, Ni²⁺ 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.

Keywords

Chlorpyrifos; Diazinon; Dieldrin; Metal neurotoxicity; Microarrays; Neuronal differentiation; Neurotoxicity; Nickel; Organochlorine insecticides; Organophosphate insecticides; PC12 cells; Serotonin; Serotonin receptors

Correspondence: Dr. T.A. Slotkin, Box 3813 DUMC, Duke Univ. Med. Ctr., Durham, NC 27710, Tel 919 681 8015, Fax 919 684 8197, t.slotkin@duke.edu.

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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, 2008c, 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 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, 2008c; 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, 2008d). 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, the 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 $\mu\text{g}/\text{ml}$ 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, 2008d). 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 hr 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, 2008d). 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, 2008d). 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 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 undifferentiated vs. differentiating states. It was therefore important to consider the false positive rate and to protect against the increased probability of type 1 errors engendered by repeated testing of the same data base. Accordingly, before looking at effects on individual genes, we performed a global ANOVA incorporating all the variables in a single comparison: treatment, time, and all genes. Lower-order ANOVAs on subdivisions of the data set were then carried out as permitted by the interactions of treatment with the other variables. Finally, differences for individual treatments for a specified gene at a single time point were evaluated with Fisher's Protected Least Significant Difference. However, for a given gene where there was no interaction of treatment with other variables (time, differentiation state), only the main treatment effect was reported without subtesting of effects at a single time point. Treatment effects were considered significant at $p < 0.05$ (two-tailed, since we were interested in both increases and decreases in gene expression). In addition to these parametric tests of the direction and magnitude of changes in gene expression, we evaluated the incidence of significant differences as compared to the predicted false positive rate, using Fisher's Exact Test, applying a one-tailed criterion of $p < 0.05$, since only an increase above the false positive rate would be predicted; at the criterion of $p < 0.05$, one gene out of every 20 tested can be expected to show a difference at random. Finding a significant decrease in the incidence of detected differences relative to the false positive rate would be biologically implausible and statistically meaningless.

RESULTS

Global statistical analysis

We evaluated effects in both undifferentiated and differentiating cells for chlorpyrifos, whereas for the other three agents, studies were confined to differentiating cells only. Accordingly, we conducted two initial global statistical tests. For the evaluations of chlorpyrifos, ANOVA incorporating all factors (treatment, differentiation state, time, gene) identified a main effect of treatment ($p < 0.003$, control < chlorpyrifos) and interactions of treatment $\times$ differentiation state ($p < 0.04$), treatment $\times$ gene ($p < 0.0001$), treatment $\times$ gene $\times$ time ($p < 0.0002$) and treatment $\times$ gene $\times$ state ($p < 0.0003$). For the entire set of 19 genes, we found 13 showing significant differences, as compared to a predicted false positive rate of 1 gene ($p < 0.0005$). For the study of diazinon, dieldrin and Ni^{2+} conducted in differentiating cells, global ANOVA (factors of treatment, gene, time) identified a main effect of treatment ($p < 0.0001$; control > dieldrin $\approx$ diazinon > Ni^{2+}) and interactions of treatment $\times$ time ($p < 0.03$), treatment $\times$ gene ($p < 0.0001$) and treatment $\times$ gene $\times$ time ($p < 0.0001$). Out of 19 total genes, we found significant differences for 15 ($p < 10^{-5}$ vs. the predicted false positive rate). In light of the interactions of treatment with the other variables, we divided the data into the separate treatments for presentation, with genes grouped by functional relationships. The first group contained the genes involved in 5HT synthesis, storage and degradation: tryptophan hydroxylase (*tph*), the high-affinity presynaptic 5HT transporter (*slc6a4*), the vesicular monoamine transporter genes (*slc18a1*, *slc18a2*), and both of the monoamine oxidases (*maoa*, *maob*). The second grouping contained the genes encoding all of the 5HT receptor subtypes that passed the quality control filters: *htr1a*, *htr1b*, *htr1d*, *htr1f*, *htr2a*, *htr2b*, *htr2c*, *htr3a*, *htr3b*, *htr5a*, *htr5b*, *htr5* and *htr7*.

Chlorpyrifos. In both undifferentiated and differentiating cells, chlorpyrifos exposure evoked upregulation of *tph*, the gene encoding tryptophan hydroxylase, the rate-limiting enzyme in 5HT biosynthesis, and downregulation of *slc6a4*, encoding the presynaptic high-affinity 5HT transporter (Figure 1A). The effects on the vesicular monoamine transporter genes were highly dependent on differentiation state: there was a small reduction in *slc18a1* expression restricted to differentiating cells, but more robust changes in *slc18a2*, with suppression in the undifferentiated state and stimulation during differentiation. Neither isoform of monoamine oxidase showed any significant effect of chlorpyrifos exposure.

Chlorpyrifos had major effects on the expression of 5HT receptors (Figure 1B), predominantly representing a significant net increase: $p < 0.009$ for both differentiation states, $p < 0.02$ for differentiating cells. In both undifferentiated and differentiating cells, chlorpyrifos increased expression for *htr1a*, *htr1d*, *htr2b* and *htr5a*, and decreased expression for *htr3b*. More selective effects were seen for *htr1b* and *htr6* (stimulated in differentiating cells only), *htr5b* (stimulated in undifferentiated cells only) and *htr7* (small but significant decrease restricted to differentiating cells).

Diazinon

Although diazinon, like chlorpyrifos, increased *tph* expression, the effect was much smaller (Figure 2A). Diazinon also evoked significant decrements in expression of all three transporter genes (*slc6a4*, *slc18a1*, *slc18a2*), as well as *maob*. There were marked differences in the effects on 5HT receptors between the two organophosphates. Whereas chlorpyrifos evoked an overall increase in receptor expression, diazinon exposure evoked a significant ($p < 0.03$) net decrease (Figure 2B) Individually, significant reductions were seen for *htr1d*, *htr2b* and *htr6*, with *htr2c* showing a transient increase followed by a decrease, and *htr3a* showing a small but significant increase.

Dieldrin

Exposure to dieldrin during differentiation produced marked upregulation of *tph*, more than that seen with the other agents (Figure 3A). As with chlorpyrifos or diazinon, dieldrin reduced the expression of *slc6a4* and *slc18a1* but did not affect *slc18a2*, *maoa* or *maob*. For 5HT receptor expression, dieldrin evoked a significant ($p < 0.002$) overall decrease with much more widespread effects than those seen for diazinon. We found reduced expression for eight subtypes (*htr1a*, *htr1d*, *htr2b*, *htr2c*, *htr3a*, *htr3b*, *htr4b*, *htr6*), with only one subtype showing an increase (*htr5a*).

Divalent nickel

The effects of Ni^{2+} were entirely distinct from those of the other three neurotoxicants. Uniquely, Ni^{2+} reduced expression of *tph* and enhanced that of *slc18a1* (Figure 4A); however, like chlorpyrifos, diazinon and dieldrin, Ni^{2+} also evoked a suppression of *slc6a4*. Overall, Ni^{2+} exposure reduced the net expression of 5HT receptor genes (Figure 4B) even more strongly than did diazinon or dieldrin ($p < 0.0001$ vs. control, $p < 0.01$ vs. diazinon, $p < 0.05$ vs. dieldrin). Individually, significant decrements were seen for *htr1d*, *htr2a* and *htr3b*. The *htr3a* subtype showed a biphasic effect (initial suppression and a small rebound increase), whereas *htr5b* showed a transient elevation.

DISCUSSION

In our earlier, in vivo work with chlorpyrifos administered to neonatal rats, we demonstrated permanent, profound and global upregulation of 5HT receptors, the 5HT transporter, and 5HT synaptic activity, associated with major alterations in 5HT-dependent behaviors (Aldridge *et al.*, 2004, 2005a, 2005b; Slotkin and Seidler, 2005, 2007c). In contrast, equivalent neonatal exposure to diazinon produced a much more restricted spectrum of effects and a correspondingly lesser impact on 5HT-related behaviors (Roegge *et al.*, 2008; Slotkin *et al.*, 2008c; Timofeeva *et al.*, 2008); in particular, whereas the largest promotional effect for chlorpyrifos was for 5HT_{1A} receptors, diazinon produced downregulation of this subtype, and the two agents had opposite effects in anxiety tests. Here, we found that these disparities in long-term outcomes are likely to originate in differences in the direct actions of the two agents on neurodifferentiation into the 5HT phenotype. The main effect of chlorpyrifos represented an increase in the overall expression of 5HT-related genes, whereas the main effect of diazinon was to repress the same repertoire. These relationships hold up even for receptor subtypes, as chlorpyrifos produced its strongest induction for the 5HT₁ receptor genes whereas diazinon produced downregulation of 5HT_{1d}.

Within the set of 5HT-related genes, the patterns of up- and downregulation give important clues as to the immediate functional consequences of chlorpyrifos or diazinon exposure. With chlorpyrifos, we found upregulation of *tph*, the gene encoding the rate-limiting enzyme for 5HT biosynthesis, combined with downregulation of *slc6a4*, the high-affinity 5HT transporter responsible for terminating the actions of 5HT after its release (Cooper *et al.*, 1996). An increase in 5HT synthesis, combined with a decrease in synaptic reuptake and superimposed on the induction of 5HT receptors, would clearly tend to enhance 5HT actions. The reason this is important is that 5HT plays a critical morphogenetic role in the developing brain (Lauder, 1985; Whitaker-Azmitia, 1991; Dreyfus, 1998). Thus, the fact that chlorpyrifos directly augments the actions of 5HT is likely to provide one of the key factors in more global disruption of brain development. Indeed, this may be one of the reasons that chlorpyrifos appears to elicit greater long-term disruption than diazinon or parathion (Qiao *et al.*, 2001; Slotkin *et al.*, 2006a, 2006b, 2007b, 2007c, 2008a, 2008b, 2008c, 2008d; Jameson *et al.*, 2007; Roegge *et al.*, 2008; Timofeeva *et al.*, 2008). Here, unlike the impact on genes for the 5HT receptors, diazinon evoked changes in 5HT synthesis and uptake in the same direction as chlorpyrifos

(increased *tph*, decreased *slc6a4* and *slc18a1*), but with a smaller magnitude of effect for *tph*. Consequently, diazinon might be expected to elicit similar disruption of some neurotrophic events related to 5HT function, but to a lesser extent than for chlorpyrifos.

In addition to our evaluations of chlorpyrifos and diazinon, we performed studies comparing the effects of the organophosphates to two unrelated developmental neurotoxicants, dieldrin and Ni^{2+} . Dieldrin produced some effects that were surprisingly similar to those of diazinon, with a significant overall decrease in the expression of 5HT-related genes, largely reflecting receptor downregulation. Like diazinon and chlorpyrifos, dieldrin also induced *tph* while suppressing the transporter genes, *slc6a4* and *slc18a1*. We might therefore expect that the long-term synaptic and behavioral consequences of neonatal dieldrin exposure will resemble those of diazinon, but like chlorpyrifos, might be much greater because of the higher degree of *tph* induction; these predictions need to be tested in future work. In contrast, the effects of Ni^{2+} were totally distinct from those of the other three agents, with strong downregulation of *tph* and induction of *slc18a1*, combined with overall suppression of 5HT receptor gene expression, effects that would tend to reduce net 5HT function. Consequently, we would predict a very different spectrum of neurobehavioral abnormalities from developmental exposure to this metal.

Our combined use of cell cultures and microarrays introduce a number of interpretational limitations. First, since PC12 cells are a transformed cell line, they are typically less sensitive to toxicants in general, requiring higher concentrations than those experienced with typical in vivo exposures. Further, since treatments occur over a very short time-span, rather than throughout brain development as would occur in vivo, higher concentrations are required to produce corresponding changes. To offset some of these problems, two of the test compounds, chlorpyrifos and diazinon, have been thoroughly studied and found to elicit parallel outcomes in the PC12 model and in developing rat brain (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, 2008d). Indeed, although the concentration used here, 30 μM , is approximately an order of magnitude higher than those found in newborn babies after nonsymptomatic environmental exposures in agricultural communities (Ostrea *et al.*, 2002), the cultures contain high concentrations of serum proteins; accordingly, less than 10% of the nominal concentration is actually available to diffuse into the cells (Qiao *et al.*, 2001). As with all studies of gene expression, the fact that microarrays detect mRNA also produces some limitations in that the transcriptional changes may not correspond exactly to alterations at the protein level. Again, the main conclusions reached here for chlorpyrifos and diazinon have been verified with in vivo exposures (Aldridge *et al.*, 2003, 2004, 2005a; Slotkin and Seidler, 2005, 2007a; Slotkin *et al.*, 2006b, 2008c; Timofeeva *et al.*, 2008). As just one example, chlorpyrifos exposure in neonatal rats enhances 5HT turnover, which obligates an increase in 5HT biosynthesis (Aldridge *et al.*, 2005b; Slotkin and Seidler, 2007c), paralleling the upregulation of tryptophan hydroxylase observed in the present study. Nevertheless, it is important, as done here, to render interpretations based on changes in multiple genes within a given pathway, rather than relying on a single gene. Finally, a number of genes did not show monotonic effects but rather exhibited biphasic alterations (e.g. *slc18a2* for chlorpyrifos in differentiating cells, *htr2c* for diazinon, *htr1d* for dieldrin, *htr5b* for Ni^{2+}). This is not unexpected, since genes that have fast turnover times are likely to show equally rapid increases or decreases, followed by compensatory changes in the opposite direction from the initial effect. Indeed, this is the reason we chose to evaluate the temporal course of effects, rather than relying on a single time point, as is more often done in microarray studies. Similar factors operate for many of the genes that show statistically significant alterations despite a small magnitude of effect: genes that are tightly controlled will naturally show smaller coefficients of variation and correspondingly smaller degrees of change over a short time-span; yet, these

smaller changes may be functionally just as important as larger changes in genes whose expression is more volatile. Although it may be difficult to ascertain the impact of biphasic mRNA changes or small-magnitude changes on protein levels, these patterns nevertheless provide a “signature” for comparison of agents acting on the same gene families. Because we have many of the parallel *in vivo* findings for two of the four agents evaluated here (chlorpyrifos, diazinon), we can now make corresponding predictions about the potential effects of dieldrin and Ni²⁺ than can focus and guide future *in vivo* work.

Our findings provide some of the first evidence connecting the direct, initial mechanisms of toxicant action on specific neurotransmitter pathways with their long-term effects on synaptic function and behavior. In this instance, we were able to distinguish clearly between the actions of two different organophosphates, and to make corresponding predictions about dieldrin and Ni²⁺ that can help focus future investigations on the most appropriate synaptic and behavioral endpoints. Equally important, the results with PC12 cells provide strong support for the view that *in vitro* test systems can give key indications of lasting neurodevelopmental changes, while at the same time providing a framework that enables comparisons of mechanisms and outcomes applicable to both related and unrelated neurotoxicants.

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Abbreviations

5HT	5-hydroxytryptamine serotonin
ANOVA	analysis of variance
NGF	nerve growth factor

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Figure 1.

Effects of 30 μ M chlorpyrifos exposure on expression of genes encoding proteins for: (A) 5HT synthesis, storage and degradation, (B) 5HT receptors. Results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h). Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which treatment interacted with the other variables (differentiation state, time) and show the individual values for which treatment effects were present. Multivariate ANOVA indicates a main effect of treatment ($p < 0.003$) and interactions of treatment $\times$ differentiation state ($p < 0.04$), treatment $\times$ gene ($p < 0.0001$), treatment $\times$ gene $\times$ time ($p < 0.0002$) and treatment $\times$ gene $\times$ state ($p < 0.0003$).

Figure 2.

Effects of 30 μ M diazinon exposure on expression of genes encoding proteins for: (A) 5HT synthesis, storage and degradation, (B) 5HT receptors. Results are shown for cells undergoing NGF-induced differentiation. Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which treatment interacted with time and show the individual values for which treatment effects were present. Multivariate ANOVA indicates a main effect of treatment ($p < 0.0002$) and interactions of treatment $\times$ gene ($p < 0.03$) and treatment $\times$ gene $\times$ time ($p < 0.004$).

Figure 3.

Effects of 30 μ M dieldrin exposure on expression of genes encoding proteins for: (A) 5HT synthesis, storage and degradation, (B) 5HT receptors. Results are shown for cells undergoing NGF-induced differentiation. Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which treatment interacted with time and show the individual values for which treatment effects were present. Multivariate ANOVA indicates a main effect of treatment ($p < 0.0003$) and interactions of treatment $\times$ gene ($p < 0.0001$) and treatment $\times$ gene $\times$ time ($p < 0.0003$).

Figure 4.

Effects of 30 μM Ni^{2+} exposure on expression of genes encoding proteins for: (A) 5HT synthesis, storage and degradation, (B) 5HT receptors. Results are shown for cells undergoing NGF-induced differentiation. Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which treatment interacted with time and show the individual values for which treatment effects were present. Multivariate ANOVA indicates a main effect of treatment ($p < 0.0001$) and interactions of treatment $\times$ gene ($p < 0.0001$) and treatment $\times$ gene $\times$ time ($p < 0.0008$).