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Transcriptional Profiles Reveal Similarities and Differences in the Effects of Developmental Neurotoxicants on Differentiation into Neurotransmitter Phenotypes in Pc12 Cells

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Abstract

Unrelated developmental neurotoxicants nevertheless converge on common functional and behavioral outcomes. We used PC12 cells, a model of neuronal development, to explore similarities and differences for organophosphate pesticides (chlorpyrifos, diazinon), an organochlorine pesticide (dieldrin) and a metal (Ni^{2+}), focusing on transcriptional profiles related to their differentiation into acetylcholine, dopamine and norepinephrine phenotypes. Agents were introduced at 30 μM for 24 or 72 hr, treatments devoid of cytotoxicity. Using microarrays, we examined the mRNAs encoding the proteins involved in neurotransmitter biosynthesis, storage, and degradation, along with the complete panoply of receptors for each transmitter. All three pesticides evoked concordant patterns of effects on genes involved in neural growth and neurite extension, with a distinctly different pattern for Ni^{2+}. All four toxicants promoted differentiation into the dopamine phenotype at the expense of the acetylcholine phenotype, involving separable effects of each agent on the various gene families; however, there were major differences in the ability of each to promote or repress the norepinephrine phenotype. Chlorpyrifos and diazinon, although displaying many similarities in their transcriptional profiles, also showed major disparities in keeping with their known differences in synaptic and behavioral outcomes after neonatal exposures to these agents in vivo. Surprisingly, there were closer similarities among diazinon, dieldrin and Ni^{2+} than for each agent to chlorpyrifos. Our results illustrate how cell culture systems, combined with microarray technology, can screen for developmental neurotoxicants, serving as a model for alternative approaches to the detection and characterization of the impact of exogenous chemicals on brain development.

Keywords

Acetylcholine; Catecholamines; Chlorpyrifos; Diazinon; Dieldrin; Dopamine; Metal neurotoxicity; Microarrays; Neuronal differentiation; Neurotoxicity; Nickel; Norepinephrine; Organochlorine insecticides; Organophosphate insecticides; PC12 cells

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Introduction

Rather than following a preordained fate, developing neurons display plasticity of the choice of neurotransmitter phenotypes, so that alterations of synaptic activity and expression of neurotrophic factors can influence the "wiring" of developing neuronal circuits [5,12,13,27]. Consequently, exposure to environmental contaminants that promote or interfere with synaptic activity or expression/function of neurotrophins can result in *miswiring*, leading to neurobehavioral anomalies. In that manner, otherwise unrelated agents might converge on common endpoints by augmenting or impairing neural activity, or by eliciting similar alterations at the level of trophic factors. The organophosphate pesticide, chlorpyrifos, provides a prime example of how these events take place. Chlorpyrifos exposure in the fetus or neonate differentially activates and suppresses acetylcholine (ACh) and monoamine systems [48,49, 51], and also has profound effects on expression and function of several families of neurotrophic factors [10,11,39,62-64]; this agent evokes inappropriate choices of neurotransmitter phenotypes in the developing brain, producing mismatches between presynaptic innervation and postsynaptic target receptors [1,2,4,23,31,37,51,54,57,59-61,65].

The developmental neurotoxicity of the organophosphates depends on mechanisms unrelated to their shared property as cholinesterase inhibitors [16,20,43,48,49,51], and consequently, there are major differences in the outcomes from exposure to agents such as diazinon or parathion as compared to chlorpyrifos [32,44,47,52,53,55,57,58,60,62,63,66,69,70]. In several recent studies, we examined the similarities and differences in the effects on trophic factors among developmental neurotoxicants within the organophosphate class as well as other classes [62-64] and found major disparities in the effects of chlorpyrifos and diazinon that could account for dissimilar outcomes; but in addition, we found surprising concordance between the effects of diazinon and the organochlorine pesticide, dieldrin [57,64]. In the current study, we explore whether these underlying effects on trophic factors dictate the differences and similarities in developmental decision-making directed toward neurodifferentiation and neurotransmitter phenotype. We chose to study PC12 cells, a standard in vitro model for neuronal development [68] that has already been shown to mimic the mechanisms and outcomes underlying organophosphate-induced developmental neurotoxicity in rodent models of neonatal exposure [7,8,21,22,24,25,31,32,38,41,44,45,56,57,63,64,67,71]. With the introduction of nerve growth factor (NGF), PC12 cells differentiate to form neuritic projections and the phenotypic characteristics of ACh and catecholamine (CA) neurons, the latter primarily involving dopamine, and to a lesser extent, norepinephrine [26,67,68]. Besides the organophosphates and dieldrin, we also evaluated the effects of a metal, Ni^{2+}, for contrast with the pesticides, since we previously found that chlorpyrifos, diazinon, dieldrin and Ni^{2+} all promote the dopamine phenotype at the expense of the ACh phenotype, but likely through different underlying mechanisms [31,57]. Here, we examined the transcriptional profiles of the entire family of genes related to these phenotypes to reveal the underlying events and to identify the mechanisms that drive the similarities and differences among developmental neurotoxicants.

Aside from their importance in testing our hypothesis, all four agents studied here appear on the registry of Superfund Chemicals [74] and thus represent significant environmental concerns. For diazinon, exposures of inner-city women during pregnancy are comparable to those seen with chlorpyrifos [76]. Organochlorines such as dieldrin produce fetal neural damage [75], in part through their interaction with $GABA_A$ receptors [14] but also through other mechanisms such as oxidative stress [34,35]. Because PC12 cells do not express $GABA_A$ receptors [28,72], any similarities in effects between dieldrin and other agents will perforce reflect these additional mechanisms which are more likely to represent convergent targets for otherwise unrelated chemicals. Nickel compounds readily cross the placenta and accumulate in fetal tissues, including the brain, at concentrations (up to 2 μg/g) comparable to

that of lead [15], greatly exceeding maternal levels [30]. Although there is little information about the developmental neurotoxicity of Ni^{2+}, it shares similar properties with lead and cadmium for blockade of calcium channels [9], specifically involving events in neurodifferentiation [42]. We recently showed similarities between the effects of Ni^{2+} on neurotransmitter choice in PC12 cells and those elicited by the organophosphates in the same model [57].

Materials and Methods

Cell cultures

Because of the clonal instability of the PC12 cell line [26], the experiments were performed on cells that had undergone fewer than five passages. As described previously [46,67], 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 [31,57,68] 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 [32,44,57,63]. 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 [44,46,67]. 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 [60,62,63]. These all involve commercial kits and standardized 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 standard procedures described previously [60,62,63]. 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 [60,62].

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 on 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. Finally, concordance of patterns of effects between different agents was evaluated by linear regression analysis.

Results

Because the comparison between effects on undifferentiated and differentiating cells were conducted with only one agent (chlorpyrifos), we performed two sets of global statistical tests. For the evaluations of chlorpyrifos, ANOVA incorporating all factors (treatment, differentiation state, time, gene) identified interactions of treatment × time ($p < 0.02$), treatment × gene ($p < 0.0001$), treatment × state × gene ($p < 0.0001$), treatment × time × gene ($p < 0.05$) and treatment × state × time × gene ($p < 0.0001$). For the entire set of 57 genes, we found 37 showing significant differences, as compared to a predicted false positive rate of <3 genes ($p < 10^{-11}$). The significant relationships held up for the two major subdivisions as well. For ACh-related genes, there were 17 showing significant differences out of a total of 26 ($p < 0.00001$), and for CA-related genes, there were 18 out of 27 ($p < 10^{-6}$). 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 ($p < 0.0001$) and interactions of treatment × time ($p <$

0.05), treatment × gene ($p < 0.0001$) and treatment × time × gene ($p < 0.0001$). Out of 57 total genes, we found significant differences for 43 ($p < 10^{-14}$ vs. the predicted false positive rate); for ACh-related genes, 19 showed significant differences out of 26 ($p < 10^{-6}$), and for CA-related genes, there were differences for 20 out of 27 ($p < 10^{-7}$). 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 to growth processes or neurotransmitter phenotypes.

Growth-related genes

We evaluated four genes directly related to neuronal growth and to the development of neuritic projections that accompanies differentiation: growth-associated protein 43 (*gap43*) and the light (*nfl*), medium (*nef3*) and heavy (*nefh*) neurofilament polypeptides. Exposure of either undifferentiated or differentiating cells to chlorpyrifos evoked upregulation of both *nfl* and *nef3*, while leaving *gap43* and *nefh* unaffected (Figure 1A). There were distinct similarities to the effects of diazinon, which also increased *nef3* (Figure 1B) and dieldrin, which enhanced both *nfl* and *nef3* (Figure 1C), although the latter also evoked a small but significant reduction in *nefh* expression. In contrast, exposure to Ni^{2+} suppressed both *gap43* and *nef3* (Figure 1D), effects that were not seen with any of the other agents.

ACh-related genes

For genes involved in ACh synthesis, storage and degradation, we evaluated choline acetyltransferase (*chat*), the high-affinity presynaptic choline transporter (*slc5a7*), the low-affinity choline transporter that also transports creatine (*slc6a8*), the vesicular ACh transporter (*slc18a3*), and three cholinesterases, acetylcholinesterase (*ache*), the glycolipid-anchored form of acetylcholinesterase (*hache*) and butyrylcholinesterase (*bche*). Chlorpyrifos exposure strongly suppressed expression of *chat* and *sl5a7*, regardless of whether cells were in the undifferentiated or differentiating state (Figure 2A). There were small, but significant increments in *slc6a8*, *slc18a3* and *ache*, whereas *bche* showed robust downregulation limited to undifferentiated cells. Like chlorpyrifos, diazinon evoked significant suppression of *chat* and smaller increases in *slc6a8* and *slc18a3* (Figure 2B); however, this organophosphate did not cause significant changes in any of the cholinesterase genes. Unlike the organophosphates, dieldrin failed to alter *chat* but did evoke upregulation of *slc6a8* and *slc18a3* (Figure 2C). On the other hand, the response to Ni^{2+} was completely unique, with strong downregulation of *slc18a3* and a small, significant decrease in *ache* (Figure 2D).

For muscarinic ACh receptor genes, we obtained measurements for subtypes 1 through 5: *chrm1*, *chrm2*, *chrm3*, *chrm4* and *chrm5*. Chlorpyrifos exposure affected expression of four out of the five subtypes (Figure 3A). In undifferentiated cells, there was strong suppression of *chrm1*, but the same subtype was upregulated when chlorpyrifos exposure occurred during differentiation. Regardless of differentiation state, chlorpyrifos evoked upregulation of *chrm2* expression and downregulation of *chrm3*. For *chrm5*, chlorpyrifos likewise evoked major reductions but only when exposure occurred during differentiation. The effects of diazinon on muscarinic ACh receptor gene expression in differentiating cells showed both similarities to, and differences from those of chlorpyrifos (Figure 3B). Diazinon evoked downregulation of both *chrm1* and *chrm2* but failed to affect *chrm5*. Further, diazinon evoked a small, but significant increase in *chrm4* that was not obtained with chlorpyrifos. Even though it belongs to an unrelated chemical class, dieldrin exposure actually elicited some changes that were similar to those of diazinon, notably decreases in *chrm1* and *chrm2* (Figure 3C); however, dieldrin downregulated *chrm4* and strongly upregulated *chrm5*, effects in the opposite direction from those obtained with diazinon (*chrm4*) or chlorpyrifos (*chrm5*). For this set of genes, Ni^{2+} exposure elicited some of the very same changes as seen with diazinon or dieldrin (Figure 3D): marked suppression of both *chrm1* and *chrm2*, as well as downregulation of *chrm4*.

Results were obtained for eight of the nicotinic ACh receptor α-subunits: *chrna2*, *chrna3*, *chrna4*, *chrna5*, *chrna6*, *chrna7*, *chrna9* and *chrna10*. Chlorpyrifos evoked major changes in gene expression that were highly dependent on differentiation state and restricted to specific subtypes (Figure 4A); some of these effects were substantially larger than those seen with other agents, so the reader should note that the scale in the graph for chlorpyrifos encompasses twice the range as for the others. In general, changes were much larger in undifferentiated cells, where chlorpyrifos downregulated *chrna2* and upregulated *chrna4* and *chrna10* (Figure 4A); *chrna9* showed a highly time-dependent change, with a large increase after 24h of exposure and an equally large decrease after 72h. In differentiating cells, chlorpyrifos evoked a small downregulation of *chrna5*, a large increase in *chrna10*, and a time-dependent change (decrease, then increase) in *chrna9*. The pattern obtained with exposure of differentiating cells to diazinon was completely different (Figure 4B). Three of the subtypes unaffected by chlorpyrifos showed significant decrements with diazinon (*chrna2*, *chrna3*, *chrna7*) and one other (*chrna4*) showed a time-dependent change (increased at 24h, decreased at 72h). Unlike chlorpyrifos, diazinon failed to evoke any change in expression of either *chrna9* or *chrna10*, but did share a similar suppression of *chrna5*. The response to dieldrin exposure was quite similar to that of diazinon (Figure 4C): decreased expression of *chrna2*, *chrna3* and *chrna5* and a time-dependent change in *chrna4*, (initial increase disappearing by 72h). However, dieldrin evoked a small, significant upregulation of *chrna7*, the opposite effect to that seen with diazinon. Exposure to Ni^{2+} also produced decrements in *chrna2* and *chrna3* expression akin to those obtained with diazinon or dieldrin, and elicited decreases in two other subtypes (*chrna4*, *chrna7*) that were similarly affected by diazinon but not dieldrin, (Figure 4D). Uniquely, Ni^{2+} also produced a decrease in *chrna10*.

Of the remaining nicotinic ACh receptor components, we obtained measurements for three β-subunits (*chrnb2*, *chrnb3*, *chrnb4*) as well as the δ, ε and γ subunits (*chrnd*, *chrne*, *chrng*, respectively). Chlorpyrifos exposure (Figure 5A) produced robust upregulation of *chrnb3* and had effects on *chrne* that depended highly on whether cells were undifferentiated (decreased expression) or undergoing differentiation (increased expression at 24h). The effects of diazinon were dissimilar, with significant decrements in *chrnb4* and *chrng*, and a time-dependent increase (24h) in *chrnd* (Figure 5B); like chlorpyrifos, diazinon evoked an initial rise in *chrne* expression, but the effect was much smaller and then showed a significant decrease after 72h of exposure. Exposure of differentiating cells to dieldrin evoked two changes that were similar to those of diazinon, decreases in *chrnb4* and *chrng*, but it failed to affect *chrne* (Figure 5C). The effect of dieldrin on *chrnd* bore some resemblance to that of diazinon, namely a drop between 24h and 72h; however, for diazinon, the value at 24h was significantly elevated, so the subsequent decrease reduced the 72h value nonsignificantly, whereas there was no initial increase with dieldrin and a significant decrement at 72h. Exposure to Ni_{2+} also evoked a deficit in *chrnb4* and *chrng* expression similar to those seen with diazinon or dieldrin (Figure 5D); Ni^{2+} significantly reduced *chrnb3*, an effect that was not significant for the other two agents, but it should be noted that the nonsignificant effects were in the same direction and were not themselves statistically distinguishable from the significant decrement obtained with Ni^{2+}. However, the reduction in *chrnb3* was clearly different from the effect of chlorpyrifos, which instead elicited a significant *increase*.

Catecholamine-related genes

We evaluated 11 genes related to CA synthesis and storage: tyrosine hydroxylase (*th*), dopamine β-hydroxylase (*dbh*), the presynaptic high-affinity norepinephrine transporter (*slc6a2*), the presynaptic high-affinity dopamine transporter (*slc6a3*), the vesicular monoamine transporter (*slc18a1*, *slc18a2*), chromogranins A and B (*chga*, *chgb*), monoamine oxidase A and B (*maoa*, *maob*) and catechol-O-methyltransferase (*comt*). Chlorpyrifos exposure evoked significant upregulation of both of the genes directly involved in CA biosynthesis (*th*, *dbh*) as

well as the presynaptic norepinephrine transporter (*slc6a2*), whereas for the dopamine transporter (*slc6a3*), the effects were highly dependent on differentiation state and time (Figure 6A); chlorpyrifos evoked initial upregulation of *slc6a3* in differentiating cells, followed by suppression at 72h. For the genes encoding the vesicular monoamine transporter, chlorpyrifos evoked a small reduction in *slc18a1* in differentiating cells, a strong suppression of *slc18a2* in undifferentiated cells, and a robust initial upregulation of the latter during differentiation. There were small increases in the chromogranins (significant for *chga*, nonsignificant for *chgb* because of slightly higher variability) and *comt*. With diazinon, there was a similar upregulation of *th*, but *dbh* expression was reduced instead of being increased (Figure 6B). The same dichotomy was evident in the effects of diazinon on the presynaptic transporters: diazinon failed to upregulate *slc6a2* but markedly increased *slc6a3*. Also unlike chlorpyrifos, diazinon reduced expression of both of the vesicular transporter genes (*slc18a1*, *slc18a2)*, as well as *chgb, maob* and *comt*. The pattern obtained with dieldrin was similar to that of diazinon in many respects: upregulation of *th* and *slc6a3*, and decreases in *dbh*, *slc18a1* and *chgb* (Figure 6C). However, dieldrin evoked a reduction in *slc6a2* and an increase in *comt* instead of a decrease, and failed to alter *maob*. Like the other three agents, Ni^{2+} evoked a significant increase in *th* expression but *dbh* was neither up- nor downregulated (Figure 6D). This agent also elicited significant elevations in *slc18a1* and both of the chromogranins (*chga, chgb*), as well as reductions in *slc6a2* and *comt*.

Next, we evaluated the genes encoding five of the dopamine receptor subtypes: *drd1a*, *drd2, drd3, drd4* and *drd5*. Chlorpyrifos exposure evoked upregulation of *drd1a* expression in both undifferentiated and differentiating cells, with a stronger effect in the former (Figure 7A). Similarly, the *drd3* subtype showed small, but significant increases in response to chlorpyrifos in undifferentiated PC12 cells but not when cells were differentiating. In contrast, exposure of differentiating cells to any of the other agents produced dissimilar responses: diazinon's effect was limited to transient downregulation of *drd2* (Figure 7B), whereas dieldrin upregulated *drd3* (Figure 7C) and Ni^{2+} suppressed *drd4* and *drd5* (Figure 7D).

We assessed multiple subforms of the α- and β-adrenergic receptors, along with two of the β-receptor kinases that modulate the coupling of these receptors to G-proteins: *adra1a, adra1b, adra2a, adra2b, adra2c, adrb1, adrb2, adrb3, adrbk1* and *adrbk2*. The effects of chlorpyrifos were highly dependent on differentiation state (Figure 8A). In undifferentiated cells, chlorpyrifos evoked strong upregulation of *adra1a, adra1d* and *adrb2*, with lesser effects on *adra2a* (increase), *adrb3* (decrease) and *adrbk2* (decrease). However, in differentiating cells, the same agent decreased the expression of *adra1a* and *adra1d*, and induced *adrb2*, an effect not seen in the undifferentiated state. For three of the genes, *adra2a, adrb3* and *adrbk2*, chlorpyrifos evoked the same changes regardless of whether the cells were undifferentiated or differentiating. With dieldrin exposure, the changes were far less notable, limited to a transient decrease in *adrb3* expression (Figure 8B), one of the genes that was also decreased by chlorpyrifos. Dieldrin showed yet another pattern, with a small but significant decrease in *adra2b*, a transient increase in *adrb1*, a decrement in *adrb2*, and a minor rise in *adrbk1* (Figure 8C). With Ni^{2+} exposure, we saw much more widespread changes: a transient decrease in *adra1b*, elevations in *adra2a* and *adra2c*, strong suppression of *adrb2*, and a small rise in *adrbk1* (Figure 8D).

Discussion

Our results indicate a number of key findings about the underlying mechanisms and interrelationships among diverse developmental neurotoxicants. First, all four agents affected differentiation into neuronal phenotypes in a manner distinct from their effect on general cell differentiation and neuritic outgrowth. Second, although they each elicited different transcriptional patterns, the effects were all consistent with a switch from the ACh to one or

both of the CA phenotypes, the same conclusion reached from comparisons of their effects on enzymatic activity of tyrosine hydroxylase and choline acetyltransferase [31,57]. Third, and perhaps most interestingly, the transcriptional profiles readily explain many of the observed differences in outcomes from exposure to chlorpyrifos vs. diazinon in vivo [32,44,47,52,53, 55,57,58,60,62,63,66,69,70], and at the same time predict that neonatal exposure to dieldrin will have effects quite similar to those of diazinon, even though the two compounds are otherwise unrelated. For the latter conclusion, correlation analysis provides a convenient way of summarizing these types of relationships (Table 1). Using the values for all of the genes and time points, we identified a small, but significant correlation between the effects of chlorpyrifos and diazinon, and essentially no relationship between chlorpyrifos and the other two agents. In contrast, the best correlation was between the effects of diazinon and dieldrin; the worst correlations, not surprisingly, were for any of the agents in relation to Ni^{2+}. In discussing each of the sets of data, we will further utilize correlation analysis to emphasize similarities and differences involving separate families of genes.

For chlorpyrifos, there was relatively little relationship ($r = 0.08$, not significant) between responses in the undifferentiated state vs. cells undergoing differentiation when considering all of the genes we evaluated. This is consistent with previous observations of a peak of vulnerability to chlorpyrifos during the initial stages of differentiation [31,44,45,57,67], which corresponds to the critical window for targeting of brain development in vivo [3,4,40,48,49, 51]. On the other hand, there was a highly consistent effect for undifferentiated vs. differentiating cells on the values for the genes that specifically define the emergence of the ACh phenotype, *chat*, *sl5a7* and *slc18a3*: $r = 0.93$, $p < 0.008$. This relationship represented primarily a suppression of the ability to synthesize ACh, since there were overall reductions in the genes for *chat*, the enzyme required for ACh biosynthesis, and for *sl5a7*, the high-affinity presynaptic choline transporter that represents the rate-limiting factor in ACh biosynthesis [18,36]. The other ACh-related genes, although representative of the emergence of neuronal characteristics, are not selective for the ACh phenotype, since the low-affinity choline/creatine cotransporter, the cholinesterases and cholinergic receptors are all associated with ACh target cells as well as ACh neurons themselves. In contrast to the positive results for the ACh phenotypic genes, we did not observe a significant overall correlation for effects of chlorpyrifos in the undifferentiated state vs. differentiating cells for the corresponding set of genes related to CA synthesis and storage (*th, dbh, slc6a2, alc6a3, slc18a1, slc18a2*). That is not to say that there were not significant effects, but only that there is no overall coordination of the effects among the entire set of phenotype-specific genes. Chlorpyrifos did cause a parallel upregulation of four of the genes defining the CA phenotype in both undifferentiated and differentiating cells (*th, dbh, slc6a2, comt*), but those relationships did not hold for the remaining key genes (*slc6a3, slc18a1, slc18a2*). As for ACh, we evaluated many other CA-related genes that are not expressed solely in CA neurons: monoamine oxidase is a ubiquitous mitochondrial protein; the chromogranins, although directly involved in CA storage, are found in many other secretory granules and are actually prohormones [77]; and the dopamine and adrenergic receptors, like the ACh receptors, are also expressed on the target cells for CA neurons. Our findings for the key genes for ACh and CA phenotypes thus provide important clues as to one of the important outcomes of chlorpyrifos exposure in vivo: a net switch from the ACh to the CA phenotype that will, perforce, produce miswiring of neuronal circuits.

During neurodifferentiation, chlorpyrifos exposure disrupts the patterns of neuritic outgrowth, promoting dendrite formation at the expense of axonogenesis [29,78]. Here, we found upregulation of the genes commonly associated with the lower molecular weight neurofilament proteins that concentrate in the shorter projections (*nfl, nef3*), with essentially no change in the heavy neurofilament protein (*nefh*) or in *gap43*, which is associated with growth in general. Diazinon, too, is known to reduce axonogenesis both in vitro [6] and in vivo [55]. Here, we found a significant correlation between the effects of chlorpyrifos and diazinon on growth-

related genes (Table 1), but the effects of diazinon were distinctly less, with no upregulation of *nfl* and a smaller increase in *nef3*. We therefore predict that diazinon will ultimately prove to have less of a promotional effect on dendrite formation than chlorpyrifos. By the same measures, dieldrin should have even greater effects; the correlation between dieldrin and chlorpyrifos was not only higher than that for diazinon, but also represented greater net effects of dieldrin on *nfl* and *nef3*, combined with a significant reduction in *nefh*, the protein most associated with axonal projections. In contrast, the growth-associated effects of Ni^{2+} did not correlate with those of any other agent; Ni^{2+} reduced the expression of both *gap43* and *nef3*, indicating the likelihood of impaired general growth as well as reduced neurite formation.

In our earlier work delineating the effects of these four agents on neurodifferentiation, we found that all of them elicited a switch away from the ACh and toward the dopamine phenotype, as defined by the corresponding activities of neurotransmitter-specific enzymes [31,55]. Here, we observed significant correlations between the actions of chlorpyrifos and diazinon on expression of the genes defining these two phenotypes, and even more so between diazinon and dieldrin, but not for any other combinations (Table 1). Diazinon, like chlorpyrifos, suppressed *chat* expression while enhancing *th*, effects that clearly underlie the corresponding and parallel changes seen at the enzyme level for both agents [31,55]; both agents also evoked robust but transient stimulation of the gene encoding the presynaptic dopamine transporter, *slc6a3*. These are all consistent with promotion of the dopamine phenotype at the expense of the ACh phenotype. However, when we examined the genes selective for the norepinephrine phenotype, we found substantial differences between the two organophosphates. Chlorpyrifos increased expression of *dbh*, which is responsible for the conversion of dopamine to norepinephrine, as well as *slc6a2*, the presynaptic norepinephrine transporter, whereas diazinon reduced *dbh* and had no effect on *slc6a2*. We therefore anticipate that the effects of diazinon will diverge from those of chlorpyrifos specifically as they relate to the impact on noradrenergic neurons. Dieldrin had actions on the emergence of neurotransmitter phenotypes quite similar to those of diazinon, as indicated by the stronger correlation between these two agents (Table 1). Dieldrin, like diazinon, elevated *th* and *slc6a3* expression but failed to reduce *chat*. Thus, although dieldrin, like the organophosphates, promotes a switch to the dopamine phenotype at the expense of the ACh phenotype, it does so solely by promoting the dopamine-related genes, rather than by a combination of enhanced dopaminergic character and suppressed ACh character. Also like diazinon, dieldrin suppressed *dbh* expression, and in addition, reduced expression of the norepinephrine transporter (*slc6a2*), so we would again anticipate that the CA-promotional effect would be limited to the dopamine subtype, with opposite effects for differentiation into norepinephrine neurons. Ni^{2+} enhanced *th* expression without affecting *chat*, but also showed some unique features, with strong induction of one of the vesicular monoamine transporter genes (*slc18a1*) and suppression of the vesicular ACh transporter gene (*slc128a3*). Ni^{2+} also significantly reduced expression of the norepinephrine transporter (*slc6a2*), so that taken together, this agent again appears to enhance the dopamine phenotype but not the norepinephrine phenotype, while suppressing different aspects of the ACh phenotype from the other agents. Thus, we have four different agents that ultimately all promote a switch in neurotransmitter “choice,” but by a variety of originating mechanisms, each represented by transcriptional events that show some similarities, but also major differences.

Although the neurotransmitter degradative enzymes and receptors can not be used to define whether cells are differentiating into specific neurotransmitter phenotypes, they all nevertheless represent important aspects of neuronal development that definitively influence cellular responses to neurotransmitter input. For degradative enzymes, although there were differences among the four neurotoxicants, there were only minor changes overall. However, genes encoding the receptors showed robust effects that were substantially dissimilar among the various agents. For muscarinic ACh receptors, chlorpyrifos stood out as unique, enhancing expression of *chrm1* and *chrm2* while suppressing *chrm3* and *chrm5*; the other three agents all

suppressed *chrm1* and *chrm2*, while differing in their effects on the other muscarinic subtypes. We saw the same dichotomy for nicotinic ACh receptor α, β, γ, δ and ε subunits, with diazinon and dieldrin showing quite similar patterns, Ni^{2+} showing lesser similarities to diazinon and dieldrin, but chlorpyrifos displaying a pattern unique from those of the other agents (Table 1). The relationships were least strong for CA receptors, where the only significant correlation was between dieldrin and Ni^{2+} (Table 1); this means that each agent basically produces a unique pattern of changes in expression of the genes encoding dopamine and norepinephrine receptors. The main conclusion of the receptor evaluations, then, is that exposure of differentiating neuronotypic cells to each of the four agents alters the expression of these key neuronal features, effects that can be expected to contribute to disruption of synaptic function over and above changes in the neurotransmitter phenotype; and again, we would predict critical differences in the outcomes of exposure to agents of the same class (chlorpyrifos, diazinon) and surprising similarities among agents of different classes (diazinon, dieldrin, Ni^{2+}).

Our results thus provide the mechanistic underpinnings that explain how exposure to two different organophosphates, chlorpyrifos and diazinon, given at the same time in brain development, at comparable doses that produce similar systemic effects, nevertheless can elicit substantially divergent outcomes in terms of neurochemistry, synaptic function and behavior [32,44,47,52,53,55,57,58,60,62,63,66,69,70]. The dissimilarities clearly reside in mechanisms other than their shared property as cholinesterase inhibitors, and as shown here, are likely to involve direct effects on developing neurons during the critical stage in which cells begin to differentiate into specific neurotransmitter phenotypes. Equally important, our results explain how diverse agents can nevertheless converge on a common set of outcomes, albeit through disparate originating mechanisms or through differential effects on members of key gene families that produce the same net outcome. We found surprising similarities in the effects of diazinon, dieldrin and Ni^{2+}, agents whose developmental neurotoxicant actions are far less studied than are those of chlorpyrifos; the present results thus provide guidelines for the types of outcomes that are the best candidates for future evaluations of each of these toxicants with in vivo animal models. Finally, our results illustrate how cell culture systems combined with microarray technology, can provide screening techniques for developmental neurotoxicants, serving as a model for further design of alternative approaches to the detection and characterization of the impact of exogenous chemicals on brain development [17,19,33,50, 73].

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References

1. Aldridge JE, Levin ED, Seidler FJ, Slotkin TA. Developmental exposure of rats to chlorpyrifos leads to behavioral alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression. Environ Health Perspect 2005;113:527–531. [PubMed: 15866758]
2. Aldridge JE, Meyer A, Seidler FJ, Slotkin TA. Alterations in central nervous system serotonergic and dopaminergic synaptic activity in adulthood after prenatal or neonatal chlorpyrifos exposure. Environ Health Perspect 2005;113:1027–1031. [PubMed: 16079074]
3. Aldridge JE, Seidler FJ, Meyer A, Thillai I, Slotkin TA. Serotonergic systems targeted by developmental exposure to chlorpyrifos: effects during different critical periods. Environ Health Perspect 2003;111:1736–1743. [PubMed: 14594624]
4. Aldridge JE, Seidler FJ, Slotkin TA. Developmental exposure to chlorpyrifos elicits sex-selective alterations of serotonergic synaptic function in adulthood: critical periods and regional selectivity for effects on the serotonin transporter, receptor subtypes, and cell signaling. Environ Health Perspect 2004;112:148–155. [PubMed: 14754568]

5. Asmus SE, Parsons S, Landis SC. Developmental changes in the transmitter properties of sympathetic neurons that innervate the periosteum. J Neurosci 2000;20:1495–1504. [PubMed: 10662839]
6. Axelrad JC, Howard CV, McLean WG. The effects of acute pesticide exposure on neuroblastoma cells chronically exposed to diazinon. Toxicology 2003;185:67–78. [PubMed: 12505446]
7. Bagchi D, Bagchi M, Hassoun EA, Stohs SJ. In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology 1995;104:129–140. [PubMed: 8560491]
8. Bagchi D, Bhattacharya G, Stohs SJ. In vitro and in vivo induction of heat shock (stress) protein (Hsp) gene expression by selected pesticides. Toxicology 1996;112:57–68. [PubMed: 8792849]
9. Benters J, Schafer T, Beyersmann D, Hechtenberg S. Agonist-stimulated calcium transients in PC12 cells are affected differentially by cadmium and nickel. Cell Calcium 1996;20:441–446. [PubMed: 8955559]
10. Betancourt AM, Burgess SC, Carr RL. Effect of developmental exposure to chlorpyrifos on the expression of neurotrophin growth factors and cell-specific markers in neonatal rat brain. Toxicol Sci 2006;92:500–506. [PubMed: 16675515]
11. Betancourt AM, Filipov NM, Carr RL. Alteration of neurotrophins in the hippocampus and cerebral cortex of young rats exposed to chlorpyrifos and methyl parathion. Toxicol Sci 2007;100:445–455. [PubMed: 17893397]
12. Black IB, Adler JE, Dreyfus CF, Jonakait GM, Katz DM, LaGamma EF, Markey KM. Neurotransmitter plasticity at the molecular level. Science 1984;225:1266–1270. [PubMed: 6147894]
13. Borodinsky LN, Spitzer NC. Activity-dependent neurotransmitter-receptor matching at the neuromuscular junction. Proc Natl Acad Sci 2007;104:335–340. [PubMed: 17190810]
14. Brannen KC, Devaud LL, Liu JP, Lauder JM. Prenatal exposure to neurotoxicants dieldrin or lindane alters *tert*-butylbicyclophosphorothionate binding to GABA(A) receptors in fetal rat brainstem. Dev Neurosci 1998;20:34–41. [PubMed: 9600388]
15. Casey CE, Robinson MF. Copper, manganese, zinc, nickel, cadmium and lead in human foetal tissues. Br J Nutrition 1978;39:639–646. [PubMed: 638131]
16. Casida JE, Quistad GB. Organophosphate toxicology: safety aspects of nonacetylcholinesterase secondary targets. Chem Res Toxicol 2004;17:983–998. [PubMed: 15310231]
17. Coecke S, Goldberg AM, Allen S, Buzanska L, Calamandrei G, Crofton K, Hareng L, Hartung T, Knaut H, Honegger P, Jacobs M, Lein P, Li A, Mundy W, Owen D, Schneider S, Silbergeld E, Reum T, Trnovec T, Monnet-Tschudi F, Bal-Price A. Workshop report: incorporating *in vitro* alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies. Environ Health Perspect 2007;115:924–931. [PubMed: 17589601]
18. Cooper, JR.; Bloom, FE.; Roth, RH. The Biochemical Basis of Neuropharmacology. Vol. 7th. Oxford University Press; New York: 1996.
19. Costa LG. Neurotoxicity testing: a discussion of in vitro alternatives. Environ Health Perspect 1998;106:505–510. [PubMed: 9599699]
20. Costa LG. Current issues in organophosphate toxicology. Clin Chim Acta 2006;366:1–13. [PubMed: 16337171]
21. Crumpton TL, Seidler FJ, Slotkin TA. Developmental neurotoxicity of chlorpyrifos in vivo and in vitro: effects on nuclear transcription factor involved in cell replication and differentiation. Brain Res 2000;857:87–98. [PubMed: 10700556]
22. Crumpton TL, Seidler FJ, Slotkin TA. Is oxidative stress involved in the developmental neurotoxicity of chlorpyrifos? Dev Brain Res 2000;121:189–195. [PubMed: 10876031]
23. Dam K, Garcia SJ, Seidler FJ, Slotkin TA. Neonatal chlorpyrifos exposure alters synaptic development and neuronal activity in cholinergic and catecholaminergic pathways. Dev Brain Res 1999;116:9–20. [PubMed: 10446342]
24. Das KP, Barone S. Neuronal differentiation in PC12 cells is inhibited by chlorpyrifos and its metabolites: is acetylcholinesterase inhibition the site of action? Toxicol Appl Pharmacol 1999;160:217–230. [PubMed: 10544056]
25. Flaskos J, McLean WG, Hargreaves AJ. The toxicity of organophosphate compounds towards cultured PC12 cells. Toxicol Lett 1994;70:71–76. [PubMed: 8310459]

26. Fujita K, Lazarovici P, Guroff G. Regulation of the differentiation of PC12 pheochromocytoma cells. Environ Health Perspect 1989;80:127–142. [PubMed: 2647474]
27. Gomez-Lira G, Lamas M, Romo-Parra H, Gutierrez R. Programmed and induced phenotype of the hippocampal granule cells. J Neurosci 2005;25:6939–6946. [PubMed: 16049169]
28. Hales TG, Tyndale RF. Few cell lines with $GABA_A$ mRNAs have functional receptors. J Neurosci 1994;14:5429–5436. [PubMed: 8083746]
29. Howard AS, Bucelli R, Jett DA, Bruun D, Yang DR. Chlorpyrifos exerts opposing effects on axonal and dendritic growth in primary neuronal cultures. Toxicol Appl Pharmacol 2005;207:112–124. [PubMed: 16102564]
30. Jacobsen N, Alfheim I, Jonsen J. Nickel and strontium distribution in some mouse tissues. Passage through placenta and mammary glands. Res Comm Chem Pathol Pharmacol 1978;20:571–584.
31. Jameson RR, Seidler FJ, Qiao D, Slotkin TA. Chlorpyrifos affects phenotypic outcomes in a model of mammalian neurodevelopment: critical stages targeting differentiation in PC12 cells. Environ Health Perspect 2006;114:667–672. [PubMed: 16675418]
32. Jameson RR, Seidler FJ, Slotkin TA. Nonenzymatic functions of acetylcholinesterase splice variants in the developmental neurotoxicity of organophosphates: chlorpyrifos, chlorpyrifos oxon and diazinon. Environ Health Perspect 2007;115:65–70. [PubMed: 17366821]
33. Kimmel GL. In vitro testing in developmental toxicity risk assessment. Teratology 1998;58:25–26. [PubMed: 9787401]
34. Kitazawa M, Anantharam V, Kanthasamy AG. Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. Free Radical Biol Med 2001;31:1473–1485. [PubMed: 11728820]
35. Kitazawa M, Anantharam V, Kanthasamy AG. Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cδ in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. Neuroscience 2003;119:945–964. [PubMed: 12831855]
36. Klemm N, Kuhar MJ. Post-mortem changes in high affinity choline uptake. J Neurochem 1979;32:1487–1494. [PubMed: 438818]
37. Levin ED, Addy N, Christopher NC, Seidler FJ, Slotkin TA. Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. Dev Brain Res 2001;130:83–89. [PubMed: 11557096]
38. Li WW, Casida JE. Organophosphorus neuropathy target esterase inhibitors selectively block outgrowth of neurite-like and cell processes in cultured cells. Toxicol Lett 1998;98:139–146. [PubMed: 9788582]
39. Mauro RE, Zhang L. Unique insights into the actions of CNS agents: lessons from studies of chlorpyrifos and other common pesticides. CNS Agents Med Chem 2007;7:183–199.
40. Meyer A, Seidler FJ, Aldridge JE, Tate CA, Cousins MM, Slotkin TA. Critical periods for chlorpyrifos-induced developmental neurotoxicity: alterations in adenylyl cyclase signaling in adult rat brain regions after gestational or neonatal exposure. Environ Health Perspect 2004;112:295–301. [PubMed: 14998743]
41. Nagata K, Huang CS, Song JH, Narahashi T. Direct actions of anticholinesterases on the neuronal nicotinic acetylcholine receptor channels. Brain Res 1997;769:211–218. [PubMed: 9374188]
42. Nikodijevic B, Guroff G. Nerve growth factor-stimulated calcium uptake into PC12 cells: uniqueness of the channel and evidence for phosphorylation. J Neurosci Res 1992;31:591–599. [PubMed: 1374475]
43. Pope CN. Organophosphorus pesticides: do they all have the same mechanism of toxicity? J Toxicol Environ Health 1999;2:161–181.
44. Qiao D, Seidler FJ, Slotkin TA. Developmental neurotoxicity of chlorpyrifos modeled *in vitro*: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells. Environ Health Perspect 2001;109:909–913. [PubMed: 11673119]
45. Qiao D, Seidler FJ, Slotkin TA. Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. Toxicol Appl Pharmacol 2005;206:17–26. [PubMed: 15963341]
46. Qiao D, Seidler FJ, Violin JD, Slotkin TA. Nicotine is a developmental neurotoxicant and neuroprotectant: stage-selective inhibition of DNA synthesis coincident with shielding from effects of chlorpyrifos. Dev Brain Res 2003;147:183–190. [PubMed: 14741763]

47. Roegge CS, Timofeeva OA, Seidler FJ, Slotkin TA, Levin ED. Developmental diazinon neurotoxicity in rats: later effects on emotional response. Brain Res Bull 2008;75:166–172. [PubMed: 18158111]
48. Slotkin TA. Developmental cholinotoxicants: nicotine and chlorpyrifos. Environ Health Perspect 1999;107:71–80. [PubMed: 10229709]
49. Slotkin TA. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol 2004;198:132–151. [PubMed: 15236950]
50. Slotkin TA. Guidelines for developmental neurotoxicity and their impact on organophosphate pesticides: a personal view from an academic perspective. NeuroToxicology 2004;25:631–640. [PubMed: 15183016]
51. Slotkin, TA. Developmental neurotoxicity of organophosphates: a case study of chlorpyrifos. In: Gupta, RC., editor. Toxicity of Organophosphate and Carbamate Pesticides. Elsevier Academic Press; San Diego: 2005. p. 293-314.
52. Slotkin TA, Bodwell BE, Levin ED, Seidler FJ. Neonatal exposure to low doses of diazinon: long-term effects on neural cell development and acetylcholine systems. Environ Health Perspect 2008;116:340–348. [PubMed: 18335101]
53. Slotkin TA, Bodwell BE, Ryde IT, Levin ED, Seidler FJ. Exposure of neonatal rats to parathion elicits sex-selective impairment of acetylcholine systems in brain regions during adolescence and adulthood. Environ Health Perspect. 2008in press
54. Slotkin TA, Cousins MM, Tate CA, Seidler FJ. Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. Brain Res 2001;902:229–243. [PubMed: 11384617]
55. Slotkin TA, Levin ED, Seidler FJ. Comparative developmental neurotoxicity of organophosphate insecticides: effects on brain development are separable from systemic toxicity. Environ Health Perspect 2006;114:746–751. [PubMed: 16675431]
56. Slotkin TA, MacKillop EA, Ryde IT, Seidler FJ. Ameliorating the developmental neurotoxicity of chlorpyrifos: a mechanisms-based approach in PC12 cells. Environ Health Perspect 2007;115:1306–1313. [PubMed: 17805420]
57. Slotkin TA, MacKillop EA, Ryde IT, Tate CA, Seidler FJ. Screening for developmental neurotoxicity using PC12 cells: comparisons of organophosphates with a carbamate, an organochlorine and divalent nickel. Environ Health Perspect 2007;115:93–101. [PubMed: 17366826]
58. Slotkin TA, Ryde IT, Levin ED, Seidler FJ. Developmental neurotoxicity of low-dose diazinon exposure of neonatal rats: effects on serotonin systems in adolescence and adulthood. Brain Res Bull 2008;75:640–647. [PubMed: 18355640]
59. Slotkin TA, Seidler FJ. The alterations in CNS serotonergic mechanisms caused by neonatal chlorpyrifos exposure are permanent. Dev Brain Res 2005;158:115–119. [PubMed: 16024092]
60. Slotkin TA, Seidler FJ. Comparative developmental neurotoxicity of organophosphates in vivo: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. Brain Res Bull 2007;72:232–274. [PubMed: 17452286]
61. Slotkin TA, Seidler FJ. Prenatal chlorpyrifos exposure elicits presynaptic serotonergic and dopaminergic hyperactivity at adolescence: critical periods for regional and sex-selective effects. Reprod Toxicol 2007;23:421–427. [PubMed: 17267174]
62. Slotkin TA, Seidler FJ, Fumagalli F. Exposure to organophosphates reduces the expression of neurotrophic factors in neonatal rat brain regions: similarities and differences in the effects of chlorpyrifos and diazinon on the fibroblast growth factor superfamily. Environ Health Perspect 2007;115:909–916. [PubMed: 17589599]
63. Slotkin TA, Seidler FJ, Fumagalli F. Targeting of neurotrophic factors, their receptors, and signaling pathways in the developmental neurotoxicity of organophosphates in vivo and in vitro. Brain Res Bull 2008;76:424–438. [PubMed: 18502319]
64. Slotkin TA, Seidler FJ, Fumagalli F. Unrelated developmental neurotoxicants elicit similar transcriptional profiles for effects on neurotrophic factors and their receptors in an in vitro model. Neurotoxicol Teratol. 2008submitted
65. Slotkin TA, Tate CA, Cousins MM, Seidler FJ. Functional alterations in CNS catecholamine systems in adolescence and adulthood after neonatal chlorpyrifos exposure. Dev Brain Res 2002;133:163–173. [PubMed: 11882346]

66. Slotkin TA, Tate CA, Ryde IT, Levin ED, Seidler FJ. Organophosphate insecticides target the serotonergic system in developing rat brain regions: disparate effects of diazinon and parathion at doses spanning the threshold for cholinesterase inhibition. Environ Health Perspect 2006;114:1542–1546. [PubMed: 17035140]

67. Song X, Violin JD, Seidler FJ, Slotkin TA. Modeling the developmental neurotoxicity of chlorpyrifos *in vitro*: macromolecule synthesis in PC12 cells. Toxicol Appl Pharmacol 1998;151:182–191. [PubMed: 9705902]

68. Teng, KK.; Greene, LA. Cultured PC12 cells: a model for neuronal function and differentiation. In: Celis, JE., editor. Cell Biology: A Laboratory Handbook. Academic Press; San Diego: 1994. p. 218-224.

69. Timofeeva OA, Roegge CS, Seidler FJ, Slotkin TA, Levin ED. Persistent cognitive alterations in rats after early postnatal exposure to low doses of the organophosphate pesticide, diazinon. Neurotoxicol Teratol 2008;30:38–45. [PubMed: 18096363]

70. Timofeeva OA, Sanders D, Seemann K, Yang L, Hermanson D, Regenbogen S, Agoos S, Kallepalli A, Rastogi A, Braddy D, Wells C, Perraut C, Seidler FJ, Slotkin TA, Levin ED. Persistent behavioral alterations in rats neonatally exposed to low doses of the organophosphate pesticide, parathion. Brain Res Bull. 2008submitted

71. Tuler SM, Hazen AA, Bowen JM. Release and metabolism of dopamine in a clonal line of pheochromocytoma (PC12) cells exposed to fenthion. Fund Appl Toxicol 1989;13:484–492.

72. Tyndale RF, Hales TG, Olsen RW, Tobin AJ. Distinctive patterns of $GABA_A$ receptor subunit mRNAs in 13 cell lines. J Neurosci 1994;14:5417–5428. [PubMed: 8083745]

73. U.S. Environmental Protection Agency. Opportunities to Improve Data Quality and Children's Health Through the Food Quality Protection Act. Report no 2006-P-00009. 2006 [7 July 2006]. Available: http://www.epa.gov/oig/reports/2006/20060110-2006-P-00009.pdf

74. U.S. National Library of Medicine. Superfund Chemicals in TOXMAP. 2006 [9 August 2006]. Available: http://toxmap.nlm.nih.gov/toxmap/main/sfChemicals.jsp

75. Uzoukwu M, Sleight SD. Dieldrin toxicosis: fetotoxicosis, tissue concentrations, and microscopic and ultrastructural changes in guinea pigs. Am J Vet Res 1972;33:579–583. [PubMed: 5014466]

76. Whyatt RM, Camann DE, Kinney PL, Reyes A, Ramirez J, Dietrich J, Diaz D, Holmes D, Perera FP. Residential pesticide use during pregnancy among a cohort of urban minority women. Environ Health Perspect 2002;110:507–514. [PubMed: 12003754]

77. Winkler H, Fischer-Colbrie R. The chromogranins A and B: the first 25 years and future perspectives. Neuroscience 1992;49:497–528. [PubMed: 1501763]

78. Yang D, Howard A, Bruun D, Ajua-Alemanj M, Pickart C, Lein PJ. Chlorpyrifos and chlorpyrifos-oxon inhibit axonal growth by interfering with the morphogenic activity of acetylcholinesterase. Toxicol Appl Pharmacol 2008;228:32–41. [PubMed: 18076960]

Abbreviations

ACh
: acetylcholine

ANOVA
: analysis of variance

CA
: catecholamine

NGF
: nerve growth factor

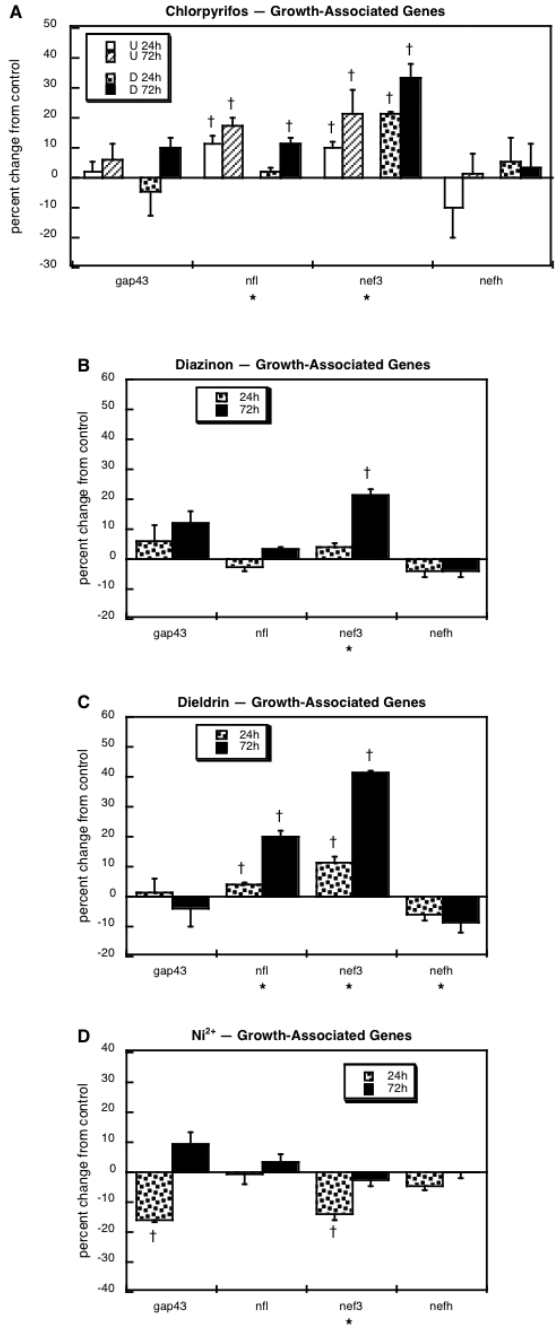

Figure 1.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on expression of growth-associated genes. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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.

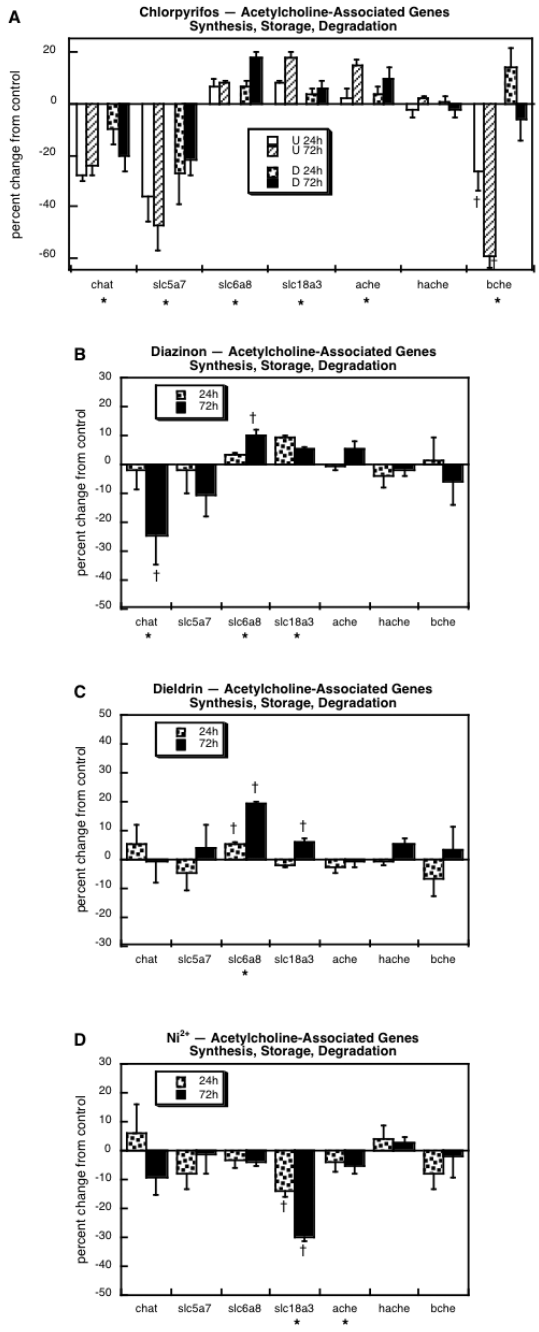

Figure 2.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on expression of genes involved in the synthesis, storage and degradation of ACh. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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.

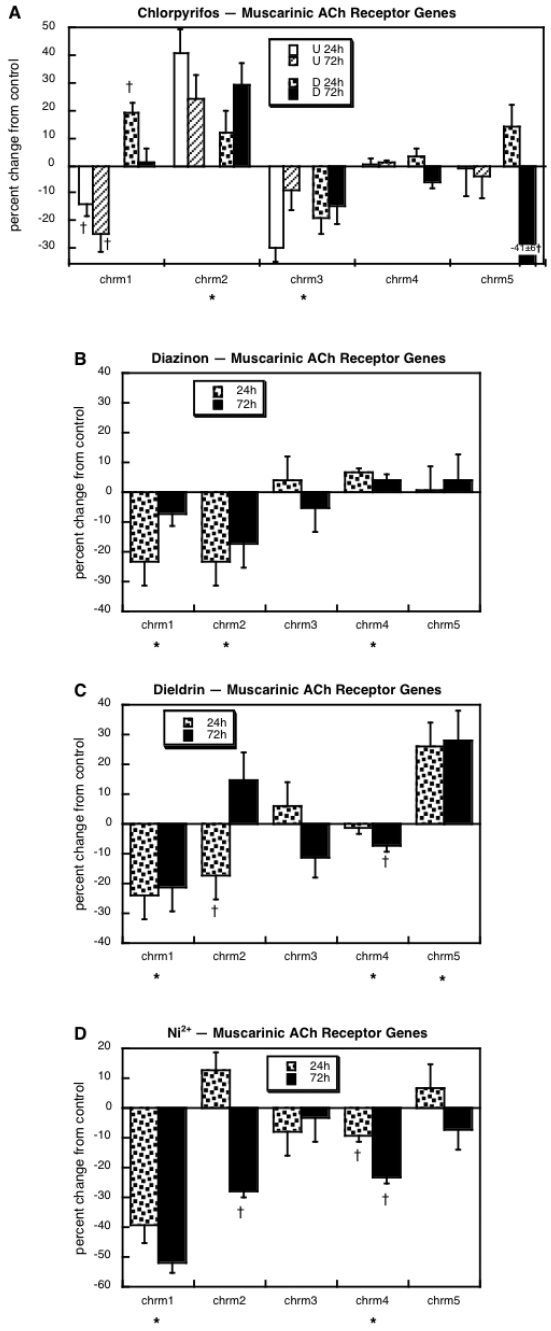

Figure 3.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on expression of genes encoding the muscarinic ACh receptors. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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.

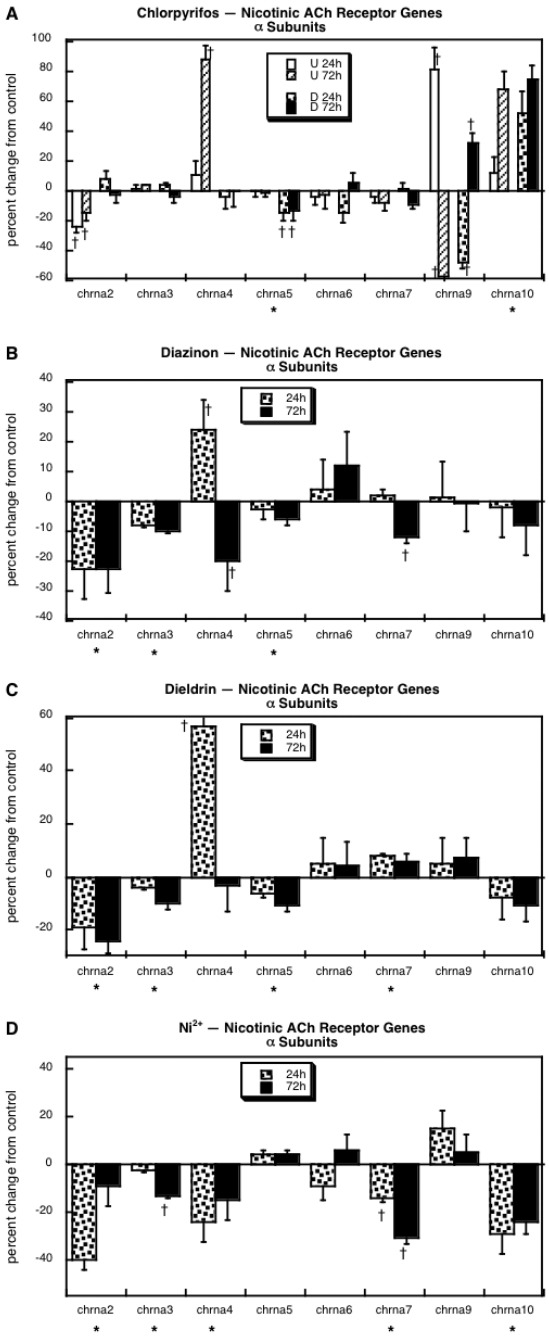

Figure 4.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on genes encoding the nicotinic ACh receptor α subunits. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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. Note the different scale for (A), which covers a two-fold larger range of changes than the other panels.

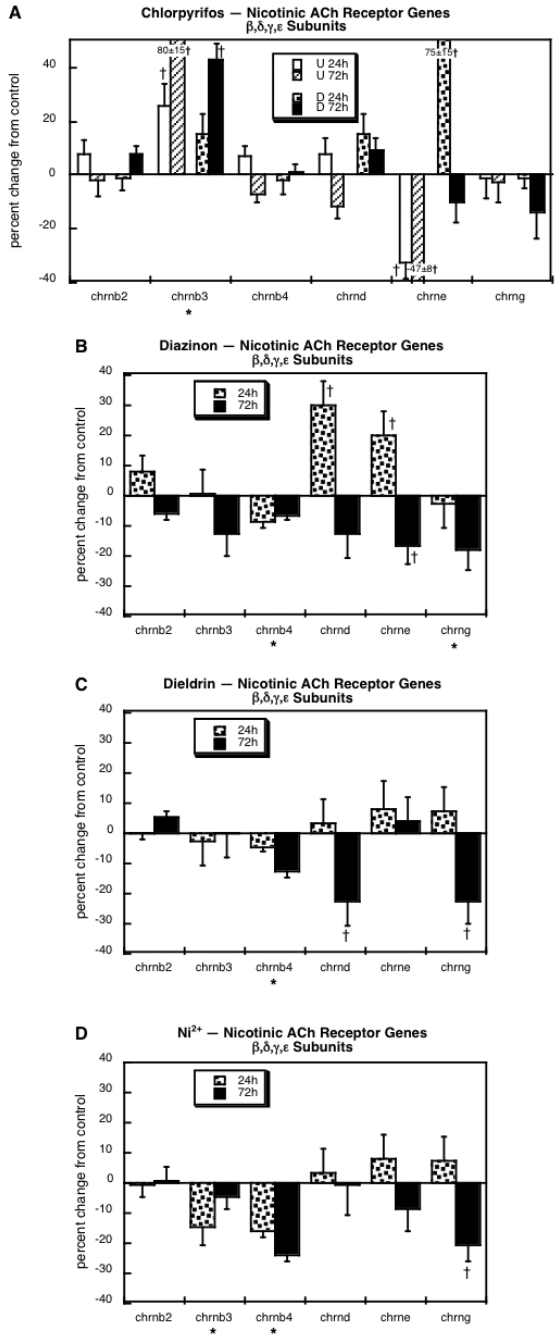

Figure 5.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on genes encoding the nicotinic ACh receptor β, δ, ε and γ subunits. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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.

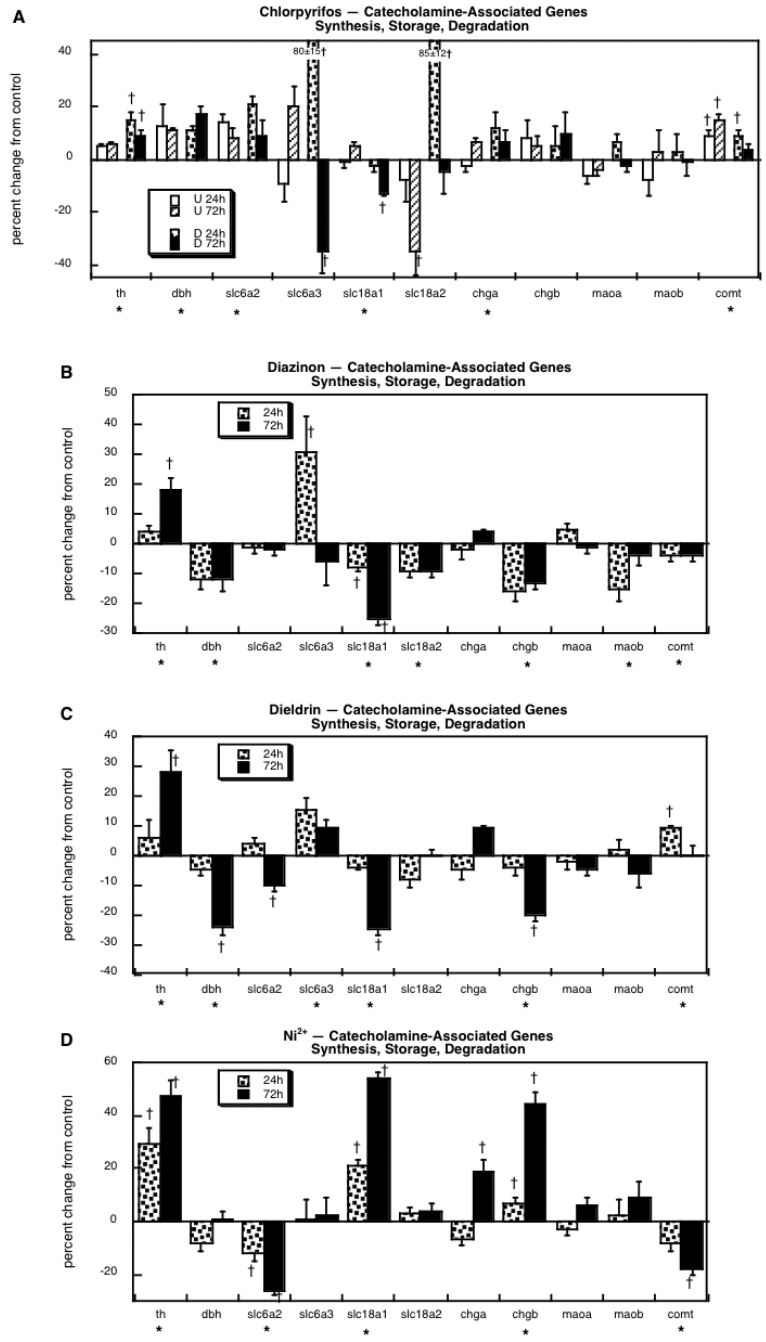

Figure 6.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on genes involved in the synthesis, storage and degradation of catecholamines. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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.

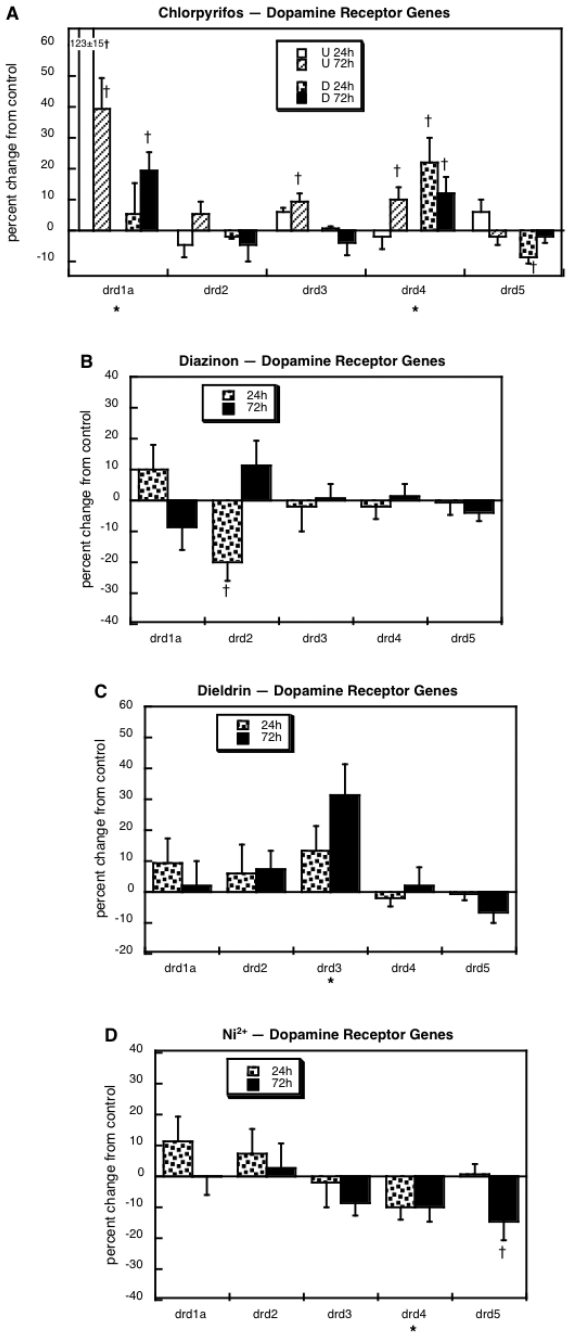

Figure 7.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on genes encoding the dopamine receptors. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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.

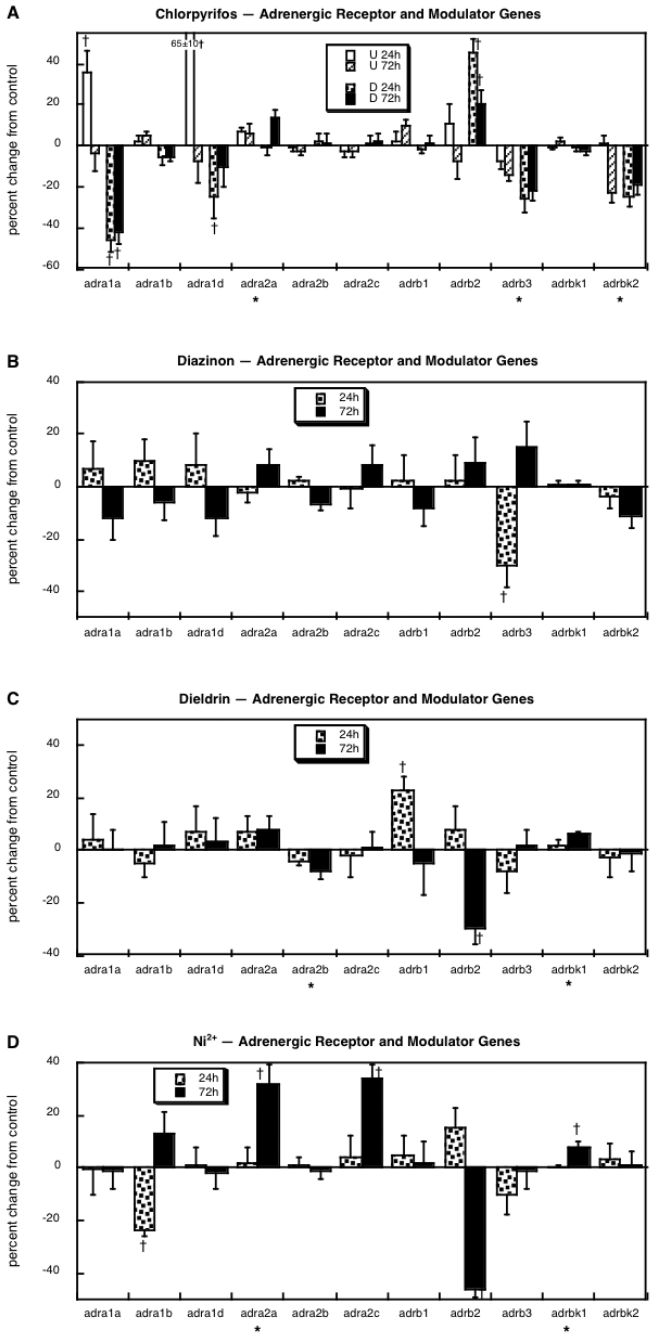

Figure 8.

Effects of 30 μM chlorpyrifos (A), diazinon (B), dieldrin (C) or Ni^{2+} (D) exposure on genes encoding the adrenergic receptors and their modulators. For chlorpyrifos, results are shown for both undifferentiated cells (U 24h, U 72h) and cells undergoing NGF-induced differentiation (D 24h, D 72h), whereas results for the other agents involve only differentiating cells. 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.

Table 1

Correlations Among Different Neurotoxicants

	Chlorpyrifos vs. Diazinon	Chlorpyrifos vs. Dieldrin	Chlorpyrifos vs. Ni^{2+}	Diazinon vs. Dieldrin	Diazinon vs. Ni^{2+}	Dieldrin vs. Ni^{2+}
All genes	r = 0.20 p < 0.04	r = 0.003 NS	r = -0.09 NS	r = 0.54 p < 0.0001	r = 0.14 NS	r = 0.16 NS
Growth genes	r = 0.70 p < 0.05	r = 0.81 p < 0.02	r = 0.23 NS	r = 0.71 p < 0.05	r = 0.15 NS	r = 0.16 NS
ACh and catecholamine phenotype*	r = 0.47 p < 0.05	r = 0.07 NS	r = -0.04 NS	r = 0.69 p < 0.002	r = -0.06 NS	r = 0.05 NS
ACh receptors	r = 0.04 NS	r = -0.09 NS	r = -0.23 NS	r = 0.60 p < 0.0001	r = 0.48 p < 0.003	r = 0.21 NS
CA receptors	r = 0.15 NS	r = -0.04 NS	r = 0.04 NS	r = 0.11 NS	r = 0.05 NS	r = 0.47 P < 0.009

*chat, slc5a7, slc18a3, th, dbh, slc6a2, slc6a3, slc18a1, slc18a2

Abbreviation: NS, not significant

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