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UNRELATED DEVELOPMENTAL NEUROTOXICANTS ELICIT SIMILAR TRANSCRIPTIONAL PROFILES FOR EFFECTS ON NEUROTROPHIC FACTORS AND THEIR RECEPTORS IN AN IN VITRO MODEL

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Abstract

Diverse developmental neurotoxicants can often produce similar functional and behavioral outcomes. We examined an organophosphate pesticide (diazinon), an organochlorine pesticide (dieldrin) and a metal (Ni^{2+}) for effects on the expression of neurotrophic factors and their receptors and modulators in differentiating PC12 cells, an in vitro model of neuronal development. Each agent was introduced at 30 μM for 24 or 72 hr, treatments devoid of cytotoxicity. Using microarrays, we examined the mRNAs encoding members of the fibroblast growth factor (*fgf*) family, the neurotrophins (*ntf*s), brain-derived neurotrophic factor (*bdnf*), nerve growth factor (*ngf*), the *wnt* and *fzd* gene families, and the receptors and modulators for each class. All three agents evoked highly concordant patterns of effects on genes encoding the *fgf* family, whereas the correlations were poor for the group comprising *bdnf, ngf* and their respective receptors. For *wnt, fzd* and their receptors/modulators, the relationships between diazinon and dieldrin were highly concordant, whereas the effect of Ni^{2+} was less similar, albeit still significantly correlated with the others. Our results show that otherwise disparate developmental neurotoxicants converge on common sets of neurotrophic pathways known to control neuronal differentiation, likely contributing to similarities in functional outcomes. Further, cell culture models can provide a useful initial screen to identify members of a given class of compounds that may be greater or lesser risks for developmental neurotoxicity, or to provide an indication of agents in different classes that might produce similar effects.

Keywords

Brain-derived neurotrophic factor; Diazinon; Dieldrin; Fibroblast growth factor; *fzd* gene family; Metal neurotoxicity; Microarrays; Nerve growth factor; Neuronal development; Neurotoxicity; Neurotrophic factors; Nickel; Organochlorine insecticides; Organophosphate insecticides; PC12 cells; Tyrosine kinase receptors; *wnt* gene family

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INTRODUCTION

A wide variety of environmental contaminants and neuroactive drugs are capable of perturbing brain development, leading to lasting neurobehavioral deficits [8,20]. Although considerable attention has focused on identification of mechanisms that could define the actions of specific agents or classes of developmental neurotoxicants, there are common threads among apparently unrelated agents, so that diverse chemicals often converge on the same target pathways and regions [4,16,20,30,41,55,59-61]. In that manner, compounds of different classes may share the neurobehavioral outcomes, such as learning disabilities, attentional disorders, conduct disorders, and cognitive dysfunction. The conundrum is to establish the cellular events that unite the initially disparate insults to result in similar functional deficits. In a number of recent studies, we showed that developmental exposure to agents as varied as heroin, phenobarbital, terbutaline, nicotine and an organophosphate pesticide, chlorpyrifos, all eventually compromise cell signaling cascades that mediate the actions of numerous neurotransmitters and hormones that themselves act as trophic factors that control brain development [1,28,29,40,44,60,61]. Further, we showed that administering counteragents that ameliorate the effects on cell signaling can reverse or prevent many of the adverse neurodevelopmental effects [48].

We and other research groups have recently started to establish how neurotrophic signals even further downstream from neurotransmitter inputs participate in the convergent actions of different neurobehavioral teratogens [5,6,51,52]. Our main focus has been on neurotrophins and their receptors known to play vital roles in brain assembly and for which disruption of expression or function clearly lead to developmental abnormalities [9,14,15,22,36]. In studies where neonatal rats were exposed to organophosphate pesticides, chlorpyrifos or diazinon, we found disruption of transcriptional profiles for key neurotrophins, their receptors and signaling pathways, including those for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and the fibroblast growth factor (FGF) superfamily [51,52]. Each agent discoordinated neurotrophic factor expression at exposures below the threshold for any overt signs of exposure or even for significant cholinesterase inhibition, thus indicating a particularly high sensitivity of these pathways over and above systemic effects of the organophosphates. With chlorpyrifos, we then confirmed that the targeting of neurotrophic factors was a direct effect by comparing the results of in vivo exposure with those in neuronotypic PC12 cells, a standard in vitro model for neuronal cell replication and differentiation [10,49,52,56].

In the current study, we used the PC12 cell model to compare the effects of otherwise unrelated developmental neurotoxicants on the expression of neurotrophic factors, their receptors and signaling modulators. We evaluated an organophosphate pesticide, diazinon, an organochlorine pesticide, dieldrin, and a metal, Ni^{2+}, three agents that nevertheless produce similar outcomes for neurodifferentiation [24,33,49]. Because the neurotrophic factors can have overlapping functions, we used a microarray approach to evaluate multiple members of a series of neurotrophin classes, established in our earlier work as likely candidates for common downstream targets [51,52]. These include the *fgf* family and its four receptors (*fgfr1-4*), *ngf*, *bdnf*, members of the neurotrophic factor (*ntf*) family, along with the corresponding receptors and receptor modulators, the neurotrophic tyrosine receptor kinases (*ntrk* family), the low-affinity NGF receptor (*ngfr*) and its adaptor protein (*ngfrap1*). In addition, we assessed the expression of the Wingless (*wnt*) gene family, which interacts with receptors encoded by the Frizzled (*fzd*) genes [26,54], whose signals are transduced by the Disheveled (*dvl*) factors and negatively modulated by the Dickkopf (*dkk*) group [19]; all these genes are mammalian homologs of genes originally identified and named in *Drosophila melanogaster*. The *fgf* and *wnt* families are coexpressed both temporally and spatially in the developing brain and converge on common events in forebrain assembly [7,21,37-39,62]. In our earlier work with chlorpyrifos and diazinon in vivo, and with chlorpyrifos in PC12 cells in vitro, we demonstrated

highly-correlated disruption of the *fgf* and *wnt/fzd/dkk/dvl* families [52]. Here, we found that, like their effects on neuronal cell differentiation [49], diazinon, dieldrin and Ni^{2+} all share many of the same targets for expression of neurotrophic factors and their receptors in PC12 cells undergoing differentiation into neuronal phenotypes [23,49,56].

MATERIALS AND METHODS

Cell cultures

Because of the clonal instability of the PC12 cell line [18], the experiments were performed on cells that had undergone fewer than five passages. As described previously [35,53], 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 [23,49,56] 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: diazinon (Chem Service, West Chester, PA), 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 [24,33,49,52]. Because of the limited water solubility of diazinon and dieldrin, 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 differentiation [33,35,53]. Cultures were examined 24 and 72 hr after commencing exposure, with eight 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.

Microarray determinations

Our earlier studies detailed 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 [50-52]. 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. Similarly, array normalizations and error detection were carried out by procedures described previously [50-52]. We then used Agilent G4131F Whole Rat Genome Arrays (Agilent Technologies, Palo Alto, CA) to assess mRNA levels. For many of the genes, the arrays contain multiple probes for the same gene and/or replicates of the same probe in different locations on the chip, and these were used to verify the reliability of values and the validity of the measures on the chip. 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 [50,51]. We ran 8 independent samples, each on a separate chip, for each of the test conditions: 4 treatments (control, diazinon, dieldrin, Ni^{2+}) × 2 time points × n=8, for a total of 68 chips.

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; therefore, control values are not shown but were quite similar to those reported previously [52]. 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 treatments at two time points, so it was 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 treatment × time interaction, 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

For all of the gene families studied here, 58 genes passed the quality control filters, encoding 20 of the factors and receptors in the *fgf* family, 11 genes among the neurotrophins and their receptors, 14 members of the *wnt* family, and 13 of the receptors and modulators of *wnt* function (*fzd*, *dkk*, *dvl*). Of all these genes, 47 showed statistically significant treatment effects, as opposed to a false positive fate of only 3 genes ($p < 10^{-17}$). Global ANOVA, incorporating all treatments and time points in a single test, similarly confirmed the presence of highly-significant treatment differences that showed selectivity toward specific genes and time points: $p < 0.0001$ for the main treatment effect, $p < 0.0001$ for treatment × time, $p < 0.0001$ for treatment × gene, $p < 0.0001$ for treatment × gene × time. These relationships were maintained for each of the agents individually: diazinon, $p < 0.0002$ for treatment, $p < 0.0001$ for treatment × time, $p < 0.0001$ for treatment × gene, $p < 0.0001$ for treatment × gene × time; dieldrin, $p < 0.004$, $p < 0.007$, $p < 0.0001$ and $p < 0.0001$, respectively; Ni^{2+}, $p < 0.0001$ for all treatments and interactions.

FGF family

Within the *fgf* family and its receptors, 20 genes passed the quality control filters, encoding 16 of the factors and all four receptor subtypes. Of these, 14 showed significant differences vs. predicted false positive rate of one gene ($p < 0.00002$). ANOVA across all treatments and time points indicated a significant main effect ($p < 0.04$) and interactions of treatment × time ($p < 0.01$), treatment × gene ($p < 0.004$) and treatment × gene × time ($p < 0.04$) and accordingly,

data were separated by treatment and gene, and then reexamined for main treatment effects and treatment × time interactions.

Diazinon evoked significant decrements in the expression of 6 members of the *fgf* family, *fgf1, fgf2, fgf12, fgf17, fgf21* and *fgf23* (Figure 1A). There were robust but transient increases in *fgf9, fgf15, fgf16, fgf20* and *fgfr4*, all of which showed significant elevations after 24 hr of exposure that disappeared by 72 hr; only one gene (*fgfr1*) showed a small, but significant increase at 72 hr. The effects of dieldrin bore a striking similarity to those of diazinon, with significant decrements in *fgf1, fgf2, fgf12, fgf17* and *fgf21* (Figure 1B). Dieldrin reduced *fgf23* expression just as did diazinon, but the effect was smaller and thus did not achieve statistical significance compared to control; however, the nonsignificant effect of dieldrin was not itself statistically distinguishable from the significant decrement caused by diazinon. Genes that showed increased expression in response to dieldrin also were quite similar to those affected by diazinon: *fgf9, fgf15, fgfr1* and *fgfr4*. Exposure to Ni^{2+} also elicited significant decreases in the expression of the same set of genes, *fgf1, fgf2, fgf17, fgf21* and *fgf23*, but there were two additional genes showing reductions (*fgf18, fgf22*). Two of the genes that showed the most robust increases with diazinon and dieldrin, *fgf15* and *fgfr4*, also were enhanced by Ni^{2+} exposure, with the same temporal profile of higher values at 24 hr and regression to normal by 72 hr.

Neurotrophins and related receptors

For the family of neurotrophins and related receptors, 11 genes passed the quality control filters, encoding brain-derived neurotrophic factor (*bdnf*), two of the NGF subunits (*ngfb, ngfg*), the low-affinity NGF receptor (*ngfr*), the NGF receptor associated protein 1 (*ngfrap1*), three of the neurotrophins (*ntf2, ntf3, ntf5*), and all three of the neurotrophic tyrosine kinase receptors (*ntrk1, ntrk2, ntrk3*). Of these genes, 7 showed significant treatment-related changes, as opposed to a predicted false positive rate of <1 gene ($p < 0.02$). For this gene grouping, ANOVA across all treatments and time points indicated a significant main effect ($p < 0.007$) and interactions of treatment × time ($p < 0.04$), treatment × gene ($p < 0.0001$) and treatment × gene × time ($p < 0.008$) and accordingly, data were again separated along the same lines as for the *fgf* family.

Diazinon evoked significant decreases in the expression of *ngfb, ntrk2* and *ntrk3*, while causing significant but decidedly smaller increases in *ngfrap1, ntf5* and *ntrk1* (Figure 2A). For this set of genes, some of the effects of dieldrin were distinct, with a minor but significant decrease in *ngfrap1* as well as a small increase in *ntrk2* (Figure 2B), changes in the opposite direction from those seen with diazinon. Nevertheless, three of the genes showed effects that were similar for the two agents: increases in *ntf5* and *ntrk1*, and a decrease in *ntrk3*. Exposure to Ni^{2+} evoked transcriptional changes that were substantially different from the other two agents (Figure 2C). Expression of *ngfb* showed a large but transient increase and *ngfg* displayed a significant decrease in response to Ni^{2+}, effects that were not seen with either diazinon or dieldrin. Additionally, Ni^{2+} evoked a stronger reduction in *ngfrap1* than that seen with dieldrin, and reduced *ntf5* expression whereas the other two agents produced increases. Again, though, there were some genes showing similar changes for all three agents, an increase in *ntrk1* and decrease in *ntrk3*, and Ni^{2+} likewise produced the same decrease in *ntrk2* as had been seen with diazinon.

WNT family and its receptors and modulators

For the *wnt* family, we were able to assess expression of 14 genes, all of which showed significant treatment-related changes, as opposed to a predicted false positive rate of <1 gene ($p < 10^{-6}$). ANOVA indicated a significant main effect ($p < 0.007$) and interactions of treatment × time ($p < 0.0001$), treatment × gene ($p < 0.0002$) and treatment × gene × time ($p < 0.004$).

Diazinon exposure elicited significant decrements in expression of the majority of the *wnt* genes: *wnt2b, wnt3a, wnt4, wnt5a, wnt7b, wnt8a, wnt9a* and *wnt9b* (Figure 3A). Of the four genes showing significant increases, *wnt4, wnt6, wnt9b* and *wnt10a*, three showed only a transient rise (*wnt4, wnt9b, wnt10a*) and for two of those (*wnt4, wnt9b*), the longer-term effect was a decrease. Dieldrin evoked significant decreases for *wnt2, wnt2b, wnt3a, wnt5a, wnt6, wnt7a* and *wnt9a* (Figure 3B), with transient increases for *wnt5, wnt9a* and *wnt9b*, and a small but more persistent rise for *wnt6*. In general, these patterns were quite similar to those seen for diazinon, differing only to the extent that some of the genes that changed in the same direction were nonsignificant for one agent and significant for the other vs. control; however, as before, the nonsignificant effects for one agent were not themselves distinguishable from the significant differences seen with the other agent.

Exposure to Ni^{2+} produced effects on *wnt* expression that bore some similarities to diazinon and dieldrin, but also showed significant differences (Figure 3C). Ni^{2+} evoked a larger decrease in *wnt1* than was seen with the other two agents, reaching statistical significance whereas the effects had been nonsignificant for diazinon or dieldrin. Likewise, *wnt2* was reduced by Ni^{2+} just as for dieldrin, and the metal also evoked decreases in *wnt2b, wnt3a, wnt8a, wnt9a* and *wnt9b*, as had been seen with one or both of the pesticides; Ni^{2+} also caused the same transient increase in *wnt4* expression as did the other two agents. The notable differences were for *wnt5a* (increased by Ni^{2+}, decreased by diazinon or dieldrin), and for *wnt6, wnt10a* and *wnt11* (all decreased by Ni^{2+}, increased or unaffected by diazinon or dieldrin).

Turning to the genes comprising the receptors and modulators of *wnt* function, the microarrays detected 8 members of the *fzd* family, as well as 3 *dvl* and 2 *dkk* subtypes. Of these 13 genes, 12 showed significant treatment differences, as compared to a false positive rate of <1 gene ($p < 0.00002$). ANOVA identified a main treatment effect ($p < 0.0001$) and interactions of treatment × gene ($p < 0.0001$) and treatment × gene × time ($p < 0.0001$). Diazinon exposure elicited significant decrements in the expression of *fzd5, fzd6, fzd7* and *fzd9*, while evoking increases for *fzd1, fzd3 fzd4, dvl1, dvl3* and *dkk3* (Figure 4A). Some of these genes were very tightly controlled, exhibiting very small coefficients of variation, so that even small changes in expression achieved statistical significance (e.g. *fzd1, dvl1, dkk3*). Dieldrin shared many of these gene changes, namely the decreases in *fzd5, fzd6, fzd7* and *fzd9*, and the increases in *fzd3, fzd4* and *dvl1* (Figure 4B). For *dvl3* and *dkk3*, dieldrin did not produce statistically significant effects, but the changes were in the same direction as those for diazinon and were not themselves statistically distinguishable from the significant increments caused by the organophosphate. Dieldrin did evoke two transcriptional responses that were different from those of diazinon (decreases in *fzd1* and *fzd2*).

Again, treatment with Ni^{2+} elicited transcriptional changes shared by one or both of the insecticides, namely decreases in *fzd1, fzd6* and *fzd9* (Figure 4C). Like diazinon, but unlike dieldrin, Ni^{2+} did not affect *fzd2* expression. For *fzd3, fzd4, fzd7, dvl3* and *dkk1*, the effects of Ni^{2+} resembled those of the other agents, sometimes with one or another treatment achieving statistical significance compared to control yet also indistinguishable from the significant effects of the other two agents (*fzd3, fzd7, dvl3, dkk1*) or with the same temporal pattern (*fzd4*). Notable differences were seen for *fzd5, dvl1* and *dkk3*, where Ni^{2+} elicited transcriptional changes opposite to those of the two insecticides.

DISCUSSION

In our prior work with the PC12 cell model, we found that diazinon, dieldrin and Ni^{2+} all produced a similar set of outcomes, characterized by impairment of cell replication, reductions in cell number but not cell growth, and diversion of differentiation away from the acetylcholine phenotype and towards the dopamine phenotype [49]. In turn, these phenotypic changes are

likely contributors to the deficits in cholinergic synaptic function and related behaviors noted with in vivo exposures to organophosphates [25,41,42,45-47,57]. Underlying these effects, the current results show that these agents also share common patterns of effects on neurotrophins and neurotrophic factors, and on their signaling pathways and modulators. Although there are also some notable differences among the three toxicants, the number of genes affected in each pathway, as well as the direction and magnitude of change, were strikingly similar, well beyond any possibility of statistical chance.

An approach using planned comparisons differs in design, purpose and meaning from surveys of the entire genome [50-52]. Here, our objective was to examine a specific set of interrelated pathways because in vivo evaluations already pointed us toward these sets of genes as targets for organophosphates and potentially for other developmental neurotoxicants [50-52]. By restricting our examination to planned comparisons of only 58 genes, we found alterations in over 80% of the genes, as compared to 3 false positives at most. Further, our findings did not rely on changes restricted to one or another gene, but rather reflected multiple changes each pathway, as well as the repetition of the same effects across different treatments. There were also multiple sequences and spots for each gene on the array (see Methods), to ensure that the changes were not random events. Accordingly, there was no chance that most of the gene changes are false positives. Thus, although we did not perform RT-PCR corroboration of the microarray results, this is really required only for array studies of the entire genome, where only a few changes are identified out of thousands of genes, so that the effects need to be distinguished from the large number of false positives. Another major point for planned comparisons is the reliance on statistical significance as the criterion for assigning changes in gene expression: significant differences were compiled regardless of whether they represented an increase or a decrease in expression, and without using a preassigned criterion for magnitude of effect. A decrease in gene expression, for example, could represent a direct effect of toxicant exposure or alternatively could be a rebound response to a prior period of activation (or vice versa). This clearly occurred for a number of genes, as demonstrated by increases at 24 hr followed by suppression at 72 hr. Similarly, it is not appropriate to assign an a priori criterion for magnitude of effect (as distinct from the criterion of statistical significance). A tightly-controlled gene, or one with slow mRNA turnover, will perforce show much smaller changes than a gene that is poorly controlled and therefore has extreme volatility; for a tightly-controlled gene, a small difference may be of equal or greater biological significance than for one that changes greatly in either direction over a short period of time [50].

To compare the degree to which the various agents produce similar outcomes for effects on neurotrophic factor gene expression, we took our primary data and reexamined them for the concordance of effects across pairs of agents using linear regression analysis. Because diazinon's effects on these pathways in the developing brain in vivo have already been validated [51,52], we used diazinon as the primary reference compound for concordance with the other agents. The relationship was high for the *fgf* family: $r=0.83$ ($p < 0.0001$) for diazinon vs. dieldrin (Figure 5A), $r=0.80$ ($p < 0.0001$) for diazinon vs. Ni^{2+} (Figure 5B); similarly, dieldrin and Ni^{2+} showed high concordance ($r=0.76$, $p < 0.0003$, data not shown). In contrast to the strong correlations across compounds for the *fgf* family, the relationships for the neurotrophin group (*bdnf* and all the subtypes for *ngf, ntf and ntrk*) were relatively poor: $r=0.23$ (not significant) for diazinon vs. dieldrin (Figure 5C), $r=0.29$ (not significant) for diazinon vs. Ni^{2+} (Figure 5D), and $r=0.19$ (not significant) for dieldrin vs. Ni^{2+}(data not shown). Although the concordance of effects of the three test compounds was not as strong for the *wnt* family as for the *fgf* group, the relationships were still significant, much more so for diazinon and dieldrin ($r=0.69$, $p < 0.0001$, Figure 5E) than for diazinon and Ni^{2+} ($r=0.40$, $p < 0.04$, Figure 5F) or dieldrin vs. Ni^{2+} ($r=0.39$, $p < 0.04$, data not shown). The same was true for the family of genes comprising the receptors and modulators of *wnt* action, *fzd*, *dvl*, and *dkk*, namely a high correlation between diazinon and dieldrin ($r = 0.85$, $p < 0.0001$, Figure 5G) and a significant, but lower correlation

for diazinon and Ni^{2+} ($r = 0.39$, $p < 0.05$, Figure 5H) or dieldrin and Ni^{2+} ($r=0.49$, $p < 0.01$, data not shown). Accordingly, these results indicate that diazinon and dieldrin are more likely to produce similar types of neurodevelopmental defects than is Ni^{2+}. As a further prediction, those developmental events governed by the *fgf* family of neurotrophic factors are the ones that we predict will show similar effects of all three agents, whereas those connected to *ngf* and *bdnf* actions may differ substantially. For the *wnt* family and its receptors modulators, outcomes should be in between these two extremes: closer for diazinon and dieldrin than for diazinon and Ni^{2+}, but still with some points of convergence for all three agents. Obviously, in vivo studies will be required to verify these predictions, but the main point is that the in vitro findings provide guidance as to what types of events are the likely targets for each agent, to the potential underlying mechanisms, and accordingly, to the types of neurotrophic interventions that might ameliorate or offset neurodevelopmental damage. In our earlier work with microarray studies of neurotrophic factor expression in vivo and in vitro, we detailed how the effects on each gene could contribute to specific neurodevelopmental defects noted for the organophosphates [50-52], so we will not repeat that discussion here.

Since our results were obtained in cell cultures, they share the corresponding limitations of all in vitro systems, namely difficulty in modeling neuronal-glial or other cell-to-cell interactions, a lack of correspondence to more global, architectural aspects of brain development, and problems of correspondence to in vivo properties of dose, pharmacokinetics or bioavailability of the test agents [10,43]. PC12 cells are transformed cells, so they are less sensitive to toxicant injury than are developing neurons in vivo; further, the cell culture treatments involve much shorter exposures than are experienced with environmental exposures extending throughout brain development. Both of these factors operate in the selection of the 30 μM test concentrations studied here. In the case of the organophosphates, this is approximately an order of magnitude higher than the levels in newborn babies after nonsymptomatic environmental exposures in agricultural communities [32] but it should be noted that the cultures contain high concentrations of serum proteins; accordingly, less than 10% of the nominal concentration is actually available to diffuse into the cells [33]. Nevertheless, to offset some of these problems, two different organophosphates, chlorpyrifos and diazinon, have been thoroughly evaluated and found to elicit parallel outcomes in the PC12 model and in developing rat brain [2,3, 11-13,[17],[23],[24],[27],[31],[33],[34],[48],49,52,53,58].

Despite these limitations, the relationship of our findings to in vivo results for organophosphates is striking [50-52]. Further, our results clearly demonstrate a convergence of otherwise disparate developmental neurotoxicants on a common set of neurotrophic pathways known to control neuronal differentiation and brain assembly, thus providing a mechanistic link for the outcomes of exposure to disparate neurotoxicants. Indeed, the fact that the effects can be identified in an in vitro confirms that known and suspected developmental neurotoxicants can exert *direct* effects on the expression of neurotrophins, their receptors and signaling modulators. Finally, our results point out how cell cultures can provide a useful initial screen to identify members of a given class of compounds that may be greater or lesser risks for developmental neurotoxicity, or to provide an indication of agents in different classes that might produce similar outcomes for brain development.

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Abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
FGF	fibroblast growth factor
NGF	nerve growth factor
NTF	neurotrophic factor (neurotrophin)
trk	tyrosine kinase receptor

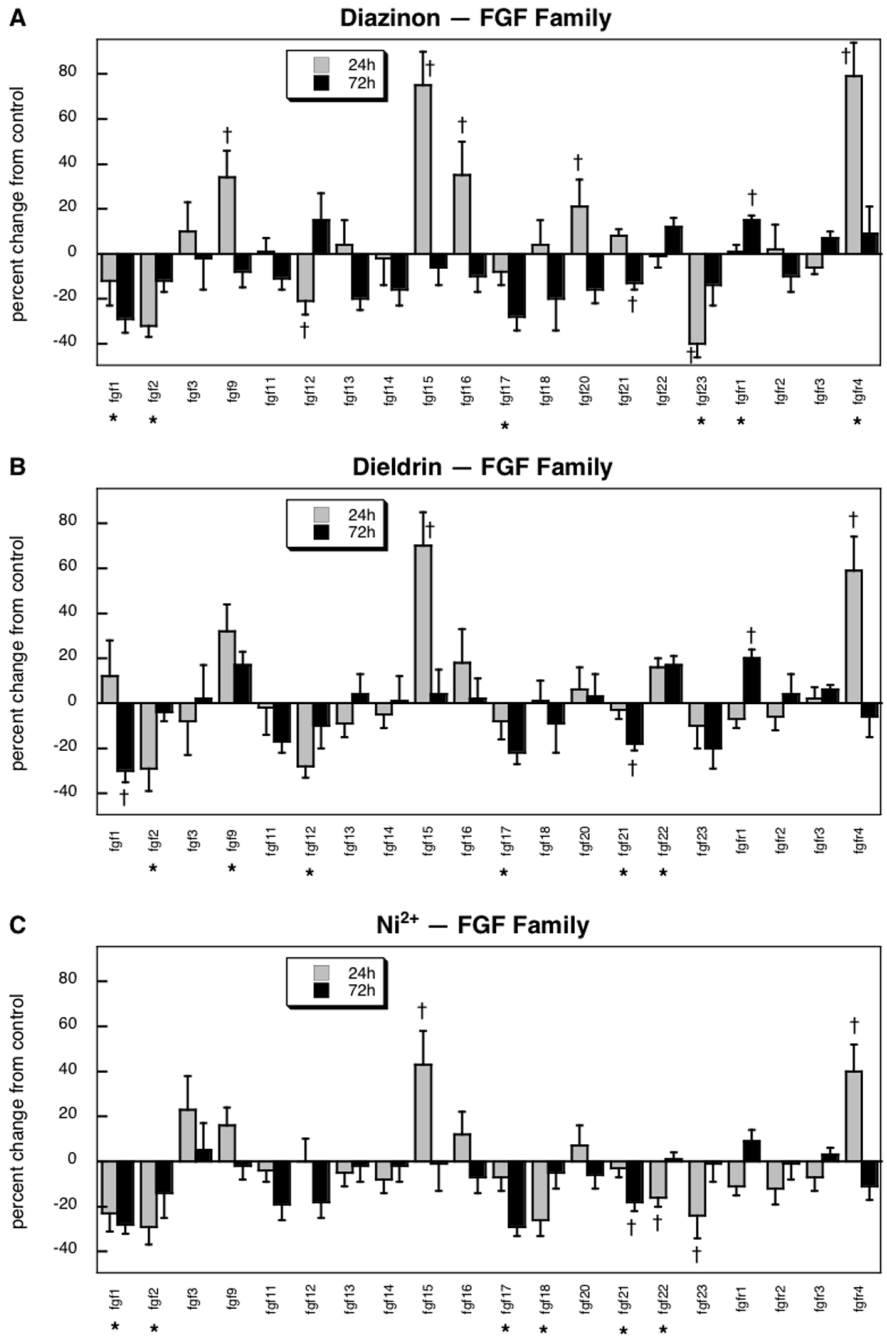

Figure 1.

Effects of 30 μM diazinon (A), dieldrin (B) or Ni^{2+} (C) exposure on expression of genes comprising the *fgf* family and related receptors. Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which a treatment × time interaction was detected and show the individual times for which treatment effects were present. Multivariate ANOVA (all treatments, time) indicates a significant main effect of treatment ($p < 0.04$) and interactions of treatment × time ($p < 0.01$), treatment × gene ($p < 0.004$) and treatment × gene × time ($p < 0.04$).

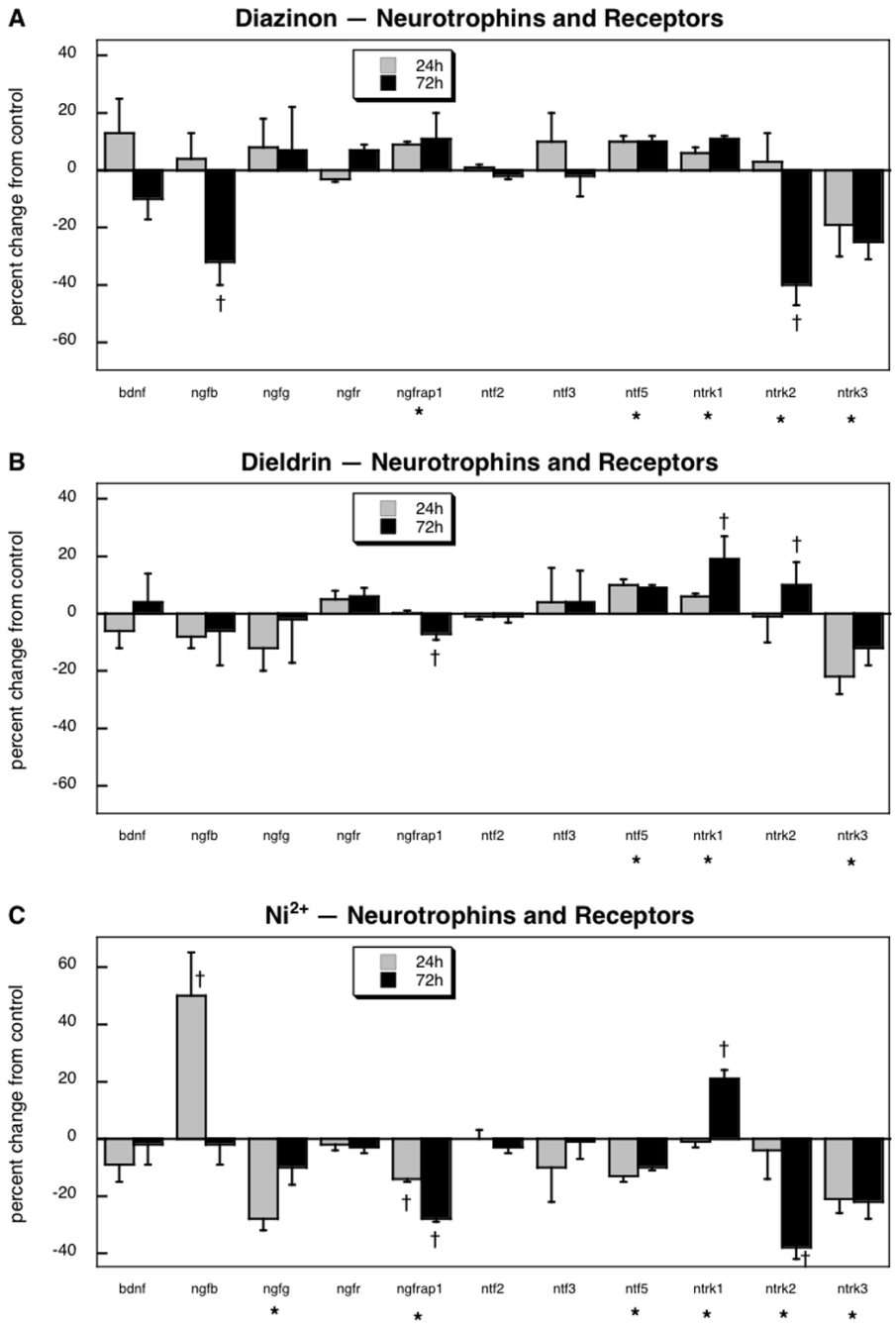

Figure 2.

Effects of 30 μM diazinon (A), dieldrin (B) or Ni^{2+} (C) exposure on expression of genes comprising the neurotrophins and their receptors. Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which a treatment × time interaction was detected and show the individual times for which treatment effects were present. Multivariate ANOVA (all treatments, time) indicates a significant main effect of treatment ($p < 0.007$) and interactions of treatment × time ($p < 0.04$), treatment × gene ($p < 0.0001$) and treatment × gene × time ($p < 0.008$).

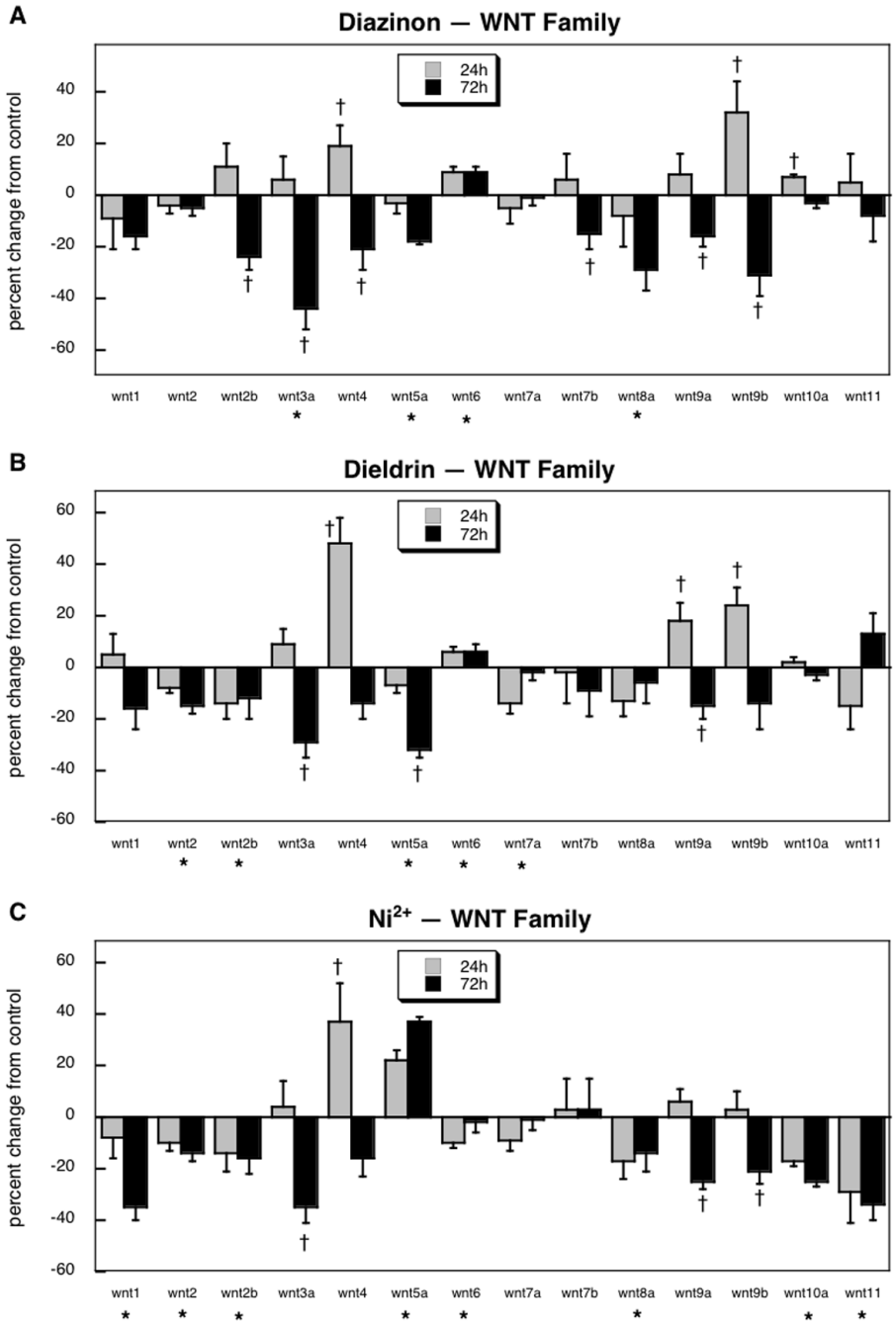

Figure 3.

Effects of 30 μM diazinon (A), dieldrin (B) or Ni^{2+} (C) exposure on expression of genes comprising the *wnt* family. Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which a treatment × time interaction was detected and show the individual times for which treatment effects were present. Multivariate ANOVA (all treatments, time) indicates a significant main effect of treatment ($p < 0.007$) and interactions of treatment × time ($p < 0.0001$), treatment × gene ($p < 0.0002$) and treatment gene × time ($p < 0.004$).

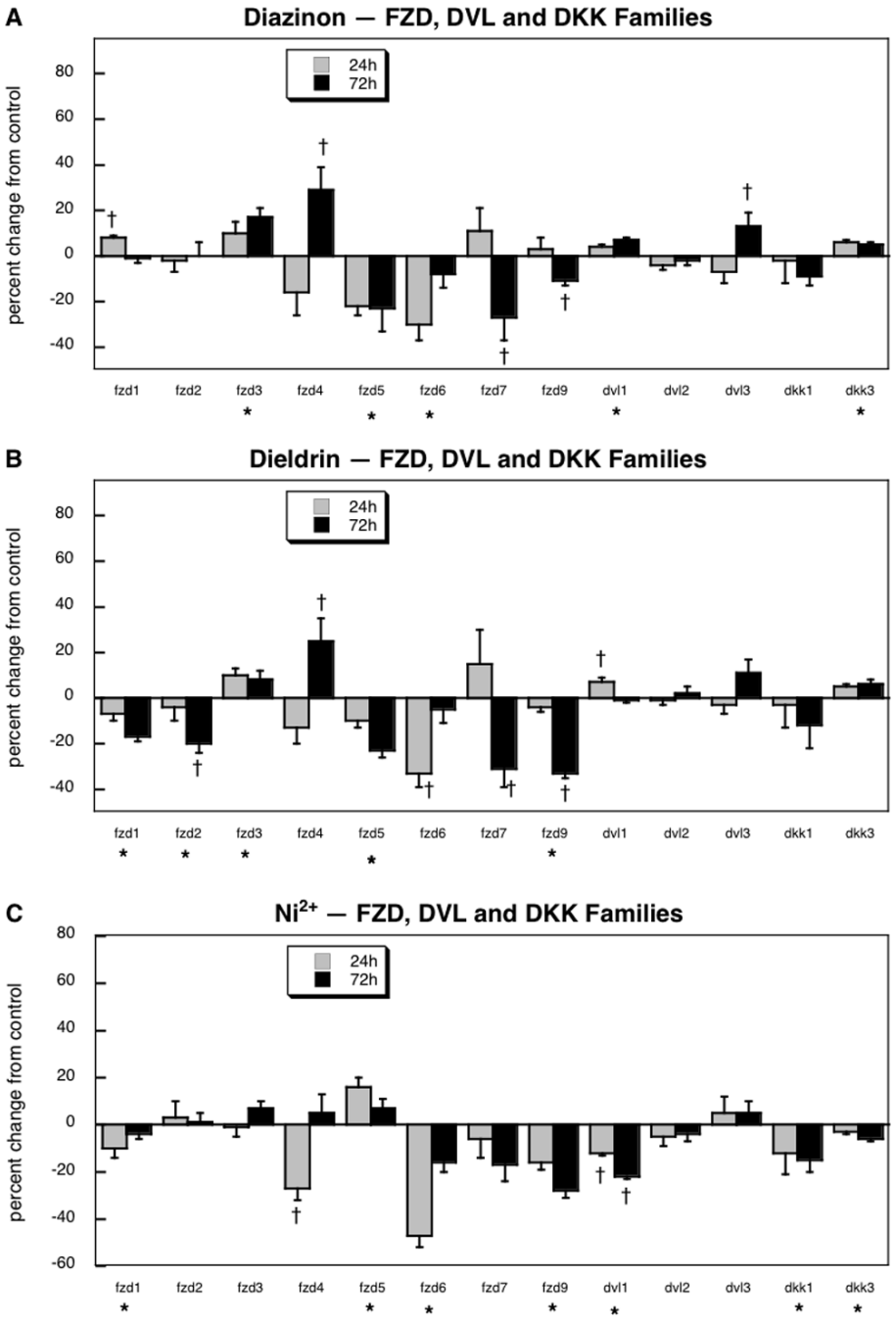

Figure 4.

Effects of 30 μM diazinon (A), dieldrin (B) or Ni^{2+} (C) exposure on expression of genes encoding the receptors and modulators of the *wnt* family: *fzd*, *dvl* and *dkk*. Asterisks shown below each gene denote a significant main treatment effect. Daggers denote genes for which a treatment × time interaction was detected and show the individual times for which treatment effects were present. Multivariate ANOVA (all treatments, time) indicates a significant main effect of treatment ($p < 0.0001$) and interactions of treatment × gene ($p < 0.0001$) and treatment gene × time ($p < 0.0001$).

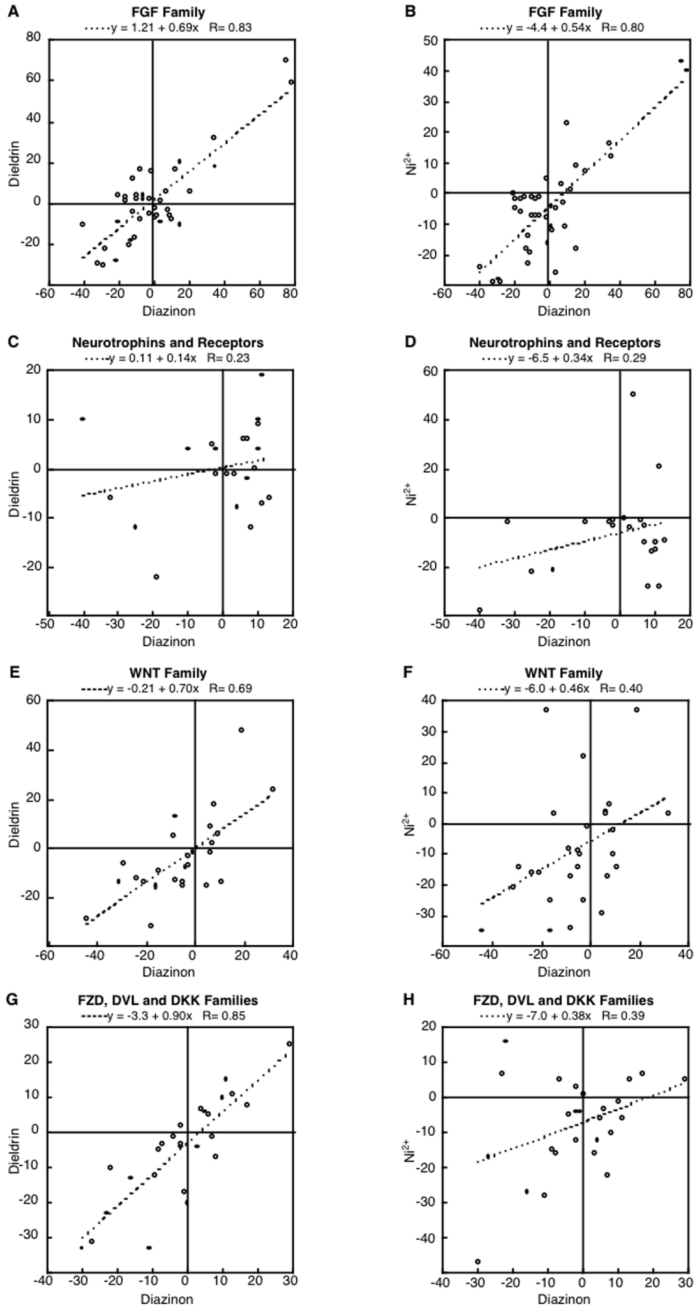

Figure 5.

Correlations between the effects of diazinon vs. dieldrin (A, C, E, G) and diazinon vs. Ni^{2+} (B, D, F, H), evaluated for the FGF family (A, B), neurotrophins and their receptors (C, D), the WNT family (E, F), and the FZD, DVL and DKK families (G,H). Data were taken from the values shown in figures 1-4.