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DOES MECHANISM MATTER? UNRELATED NEUROTOXICANTS CONVERGE ON CELL CYCLE AND APOPTOSIS DURING NEURODIFFERENTIATION

Theodore A. Slotkin and **Frederic J. Seidler**
Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, North Carolina USA

Abstract

Mechanistically unrelated developmental neurotoxicants often produce neural cell loss culminating in similar functional and behavioral outcomes. We compared an organophosphate pesticide (diazinon), an organochlorine pesticide (dieldrin) and a metal (Ni^{2+}) for effects on the genes regulating cell cycle and apoptosis 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 nearly 400 genes involved in each of the biological processes. All three agents targeted both the cell cycle and apoptosis pathways, evidenced by significant transcriptional changes in 40–45% of the cell cycle-related genes and 30–40% of the apoptosis-related genes. There was also a high degree of overlap as to which specific genes were affected by the diverse agents, with 80 cell cycle genes and 56 apoptosis genes common to all three. Concordance analysis, which assesses stringent matching of the direction, magnitude and timing of the transcriptional changes, showed highly significant correlations for pairwise comparisons of all the agents, for both cell cycle and apoptosis. Our results show that otherwise disparate developmental neurotoxicants converge on common cellular pathways governing the acquisition and programmed death of neural cells, providing a specific link to cell deficits. Our studies suggest that identifying the initial mechanism of action of a developmental neurotoxicant may be strategically less important than focusing on the pathways that converge on common final outcomes such as cell loss.

Keywords

Apoptosis; Cell cycle; Diazinon; Dieldrin; Nickel; PC12 cells

INTRODUCTION

Early-life exposures to many different environmental contaminants or neuroactive drugs can perturb brain development and thus produce lasting behavioral deficits (Boyes, 2001; Grandjean and Landrigan, 2006). Despite the fact that various agents work through completely different mechanisms of action, many of these diverse chemicals often produce

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

quite similar structural and functional deficits (Barone et al., 2000; Eriksson, 1997; Grandjean and Landrigan, 2006; Slotkin, 2004; Weiss et al., 2004; Yanai, 1984). Indeed, the assumption that there are similar outcomes from otherwise unrelated neurotoxicants underlies the implementation of standardized tests for developmental neurotoxicity (Claudio et al., 2000; Crofton et al., 2008). The basic challenge, then, is to establish the cellular events that unite the insults arising from different mechanisms that nevertheless converge on the same eventual deficits.

Many developmental neurotoxicants decrease the numbers of neurons and/or glia, leading to thinning of various brain layers, thus implying a common target on the pathways modulating neural cell acquisition and programmed cell death; in turn, neuronal loss and effects on layer thickness represent major endpoints in standardized testing of developmental neurotoxicity (Claudio et al., 2000; Crofton et al., 2008). Indeed, apoptosis represents a common outcome for toxicant exposures that evoke oxidative stress or excitotoxicity, or that interfere with trophic factors or synaptic connectivity (Hohmann and Berger-Sweeney, 1998; Hohmann et al., 1988; Kuan et al., 2000; Sastry and Rao, 2000; Schwartz, 1991; Slotkin and Seidler, 2009a; Slotkin et al., 2008, 2010b). Accordingly, the current study examines whether toxicants from three otherwise unrelated classes, all with different mechanisms of action, nevertheless converge on the cellular pathways that control cell cycle and apoptosis: an organophosphate pesticide (diazinon), an organochlorine pesticide (dieldrin), and a metal (divalent nickel). These three toxicants are each of importance for human exposures. Organophosphates are used worldwide, representing 50% of all insecticide applications (Casida and Quistad, 2004) and their use has been curtailed in the U.S. specifically because of their propensity to produce developmental neurotoxicity; although their systemic toxicity reflects inhibition of cholinesterase, they interfere with neurodevelopment by targeting cell signaling pathways that mediate neurodifferentiation, unrelated to anticholinesterase actions (Slotkin, 2005). Dieldrin produces fetal neural damage (Uzoukwu and Sleight, 1972) primarily via interaction with $GABA_A$ receptors (Brannen et al., 1998) and by eliciting oxidative stress (Kitazawa et al., 2001, 2003). For nickel, the human fetal concentration from normal dietary and environmental exposure is comparable to that of lead (Casey and Robinson, 1978) and, like lead, nickel interferes with the gating of calcium (Benters et al., 1996) specifically during neurodifferentiation (Nikodijevic and Guroff, 1992).

For our determinations, we used PC12 cells, a well-established model of neurodifferentiation (Costa, 1998; Fujita et al., 1989; Teng and Greene, 1994). In our earlier work, we showed that diazinon, dieldrin and Ni^{2+} all produce deficits in the numbers of cells and interference with neurodifferentiation at exposures that do not produce overt cytotoxicity (Jameson et al., 2007; Qiao et al., 2001; Slotkin et al., 2007a). Here, we found that all three agents converge on the genes regulating cell cycle and apoptosis in PC12 cells undergoing neurodifferentiation, sharing not only the same pathways, but even the same genes within those pathways, with highly significant correlations for the direction, magnitude and timing of transcriptional changes.

MATERIALS AND METHODS

Cell cultures

Because of the clonal instability of the PC12 cell line (Fujita et al., 1989), the experiments were performed on cells that had undergone fewer than five passages. As described previously (Qiao et al., 2003; Song et al., 1998), PC12 cells (American Type Culture Collection CRL-1721, 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 (Jameson et al., 2006; Slotkin et al., 2007a; Teng and Greene, 1994) twenty-four hours after seeding, the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (Invitrogen). Simultaneously with nerve growth factor, we introduced the neurotoxicants, each at a final concentration of 30 μM: 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 mitotic activity and neurodifferentiation of PC12 cells without outright cytotoxicity for each agent (Slotkin et al., 2007a; Slotkin and Seidler, 2008, 2009c, 2010). Because of their limited water solubility, diazinon and dieldrin were dissolved in dimethylsulfoxide (final concentration 0.1%), which was also added to the control cultures and cultures treated with $NiCl_2$; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation (Qiao et al., 2001, 2003; Song et al., 1998). Cultures were examined 24 and 72 hr after commencing exposure, with 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. The mRNA from each individual culture was run on a separate array.

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 (Slotkin and Seidler, 2007; Slotkin et al., 2007b, 2008). 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. Array normalizations and error detection were also carried out by standard procedures described previously (Slotkin and Seidler, 2007; Slotkin et al., 2007b, 2008). We used type G4131F Agilent Whole Rat Genome Arrays (Agilent Technologies, Palo Alto, CA), selecting the Gene Ontology categories for cell cycle and apoptosis (Ashburner et al., 2000) within the GeneSpring™ platform.

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. In these cases, to avoid artificially inflating the number of genes utilized for statistical analysis, 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 one sample selected from each treatment group (Slotkin and Seidler, 2007; Slotkin et al., 2007b).

Statistical procedures

Results were compiled as means and standard errors of the normalized expression ratios from the arrays, with statistical analyses performed on log-transformed values, since the data are in the form of ratios. Our design involved planned comparisons of four different treatments (control, diazinon, dieldrin, Ni^{2+}) at two different time points, across hundreds of genes in each category. It was therefore important to protect against the increased probability of type 1 errors engendered by repeated testing of the same data base. Accordingly, before looking for effects on individual genes, we performed a global ANOVA

incorporating all the variables in a single comparison. Lower-order ANOVAs on subdivisions of the data set were then carried out as permitted by the interactions of treatment with the other variables; however, where there were no interactions, we report only main treatment effects without further subdivision. Differences for individual treatments were evaluated with Fisher's Protected Least Significant Difference test.

In addition to these parametric tests, we evaluated the incidence of significant differences as compared to the predicted false positive rate, using the χ^2 test. This was necessary because repetitive testing of the large numbers of genes (378 genes for cell cycle, 376 for apoptosis) using a $p < 0.05$ criterion will generate 19 false positive significant differences for each category, i.e. 19 genes will be declared "significant" that are actually random differences; accordingly, an interpretation of a significant impact on either process requires a higher incidence of significant increases and/or decreases in gene expression than predicted from the false positive rate. Unlike the parametric ANOVA, which weights the results according to the magnitude of the effect, this nonparametric approach simply determines whether a given gene shows a significant difference from the corresponding control, regardless of the absolute magnitude of the difference. That way, we can address the important contribution of differences in variability among genes: a large difference for a gene with high variability may not be statistically significant, whereas a smaller difference for a tightly-controlled gene may be both significant and biologically relevant (Slotkin and Seidler, 2007). To classify a given gene as showing a significant increase or decrease, we tested the values using ANOVA with factors of treatment and time. Where a main treatment effect was identified, the result for that gene was classified as an increase or a decrease. Where there was only an interaction of treatment × time, we evaluated each time point for a significant treatment effect, with three possible outcomes. Where the interaction reflected the fact that only one time point showed a significant treatment effect, that gene was classified as "increased" or "decreased" depending on the direction of change for the individual value. Where there was an interaction and both time points showed significant changes, but in opposite directions from each other, that value was tabulated as ½ increase and ½ decrease. Finally, where neither time point showed a significant treatment effect, that gene was classified as "unchanged," despite the significant interaction term (underestimating the number of genes that were changed, and thus making it more difficult to achieve statistical significance from the false positive rate). The χ^2 test was then run for the distribution of observed values in the three categories (increased, decreased, unchanged) as compared to the values expected from a random event: at $p < 0.05$, the false-positive rate corresponds to a statistically significant increase in 2.5% of the genes, a significant decrease in 2.5%, and 95% unchanged.

We further conducted two additional sets of comparisons of progressively increasing stringency from the initial set of tests, which simply tabulated the numbers of genes affected by the treatments. First, to evaluate whether the same genes were affected by the disparate neurotoxicants, we compared the degree to which the gene sets overlapped, examining both pairwise and three-treatment comparisons. Next, we conducted concordance analysis to evaluate whether the magnitude, direction and time course of effects on gene expression were similar for the different toxicants; these were performed by linear regression analysis of pairwise combinations (Slotkin and Seidler, 2009a, b; Slotkin et al., 2010b).

For all tests, treatment effects were considered significant at $p < 0.05$ (two-tailed).

RESULTS

Validation of gene groupings

Transcripts of 378 of the genes designated as "cell cycle-related" were detected and passed the quality-control filters of the GeneSpring platform for samples in the control and toxicant-exposed groups (Supplemental Table 1). Similarly, we identified 376 genes designated as "apoptosis-related" (Supplemental Table 2). These represent approximately 50% more genes than had been detected for each category in our previous study of chlorpyrifos (Slotkin and Seidler, 2012), a consequence of our use in the present work, of a 10-fold more sensitive microarray platform with updated gene annotations. Notably, though, the gene lists generated here included all of the genes identified in the earlier experiments, providing cross-validation of the results. Forty-five of the genes were shared by both the cell cycle and apoptosis categories: *aatf, actn4, ahr, akt1, appbp1, atm, bcl10, brca1, casp3, cdkn1a, cul1, cul2, cul3, dcc, ddit3, dhcr24, e2f1, elk1, espl1, ets1, gadd45b, gadd45g, gml, gsk3b, il18, inha, mnt, mybl2, myc, mycs, notch2, pglyrp1, plagl1, ppp1r13b, pten, sart1, siah2, tcfap2a, tgfa, tnfsf5ip1, tp53, trp53bp2, trp63, ube1c, and vegf.*

To further validate that the gene expression patterns reflected biologically meaningful processes, we compared the control values for cell cycle genes at the two time points (Supplemental Table 1) and found a main effect of time ($p < 0.0001$), reflecting higher gene expression at 24h than at 72h. Similarly, when we evaluated expression of apoptosis-related genes in the control group (Supplemental Table 2), we found a main effect of time ($p < 0.001$) reflecting higher values at 72h than at 24h. These patterns are thus consistent with the gradual fall-off of mitotic activity and rise in apoptosis attendant upon neurodifferentiation initiated by nerve growth factor (Ekshyyan and Aw, 2005; Powers et al., 2010; Song et al., 1998).

Effects on gene expression

Each of the neurotoxicants had highly significant effects on the expression of genes involved in either cell cycle (Supplemental Table 1) or apoptosis (Supplemental Table 2). For both classes of genes, the effects were dependent on time and were gene-selective, as indicated by interactions of treatment with these two variables. Furthermore, robust statistical significance was maintained when the data were subdivided so as to compare each treatment separately to the control group. For cell cycle-related genes (Supplemental Table 1), there were main treatment effects for diazinon ($p < 0.004$) and Ni^{2+} ($p < 0.0001$), reflecting a net decrease in gene expression for each toxicant, along with gene-specific and time-specific effects (significant interactions of treatment with these other variables). For dieldrin, there was no net direction of change (no main treatment effect) but the interaction terms were still highly significant, reflecting time-specific and gene-specific effects. For apoptosis-related genes (Supplemental Table 2), all three neurotoxicants showed significant main treatment effects ($p < 0.0001$ for each treatment vs. control) but again, dieldrin was different from the other two: diazinon and Ni^{2+} evoked significant overall downregulation whereas dieldrin produced a net upregulation. Just as for cell cycle genes, the treatment effects for the apoptosis category showed highly significant interactions with time and gene. The treatment × gene interactions seen in the global ANOVA and in the subtests for each toxicant thus justified examination of treatment effects and treatment × time interactions on a gene-by-gene basis for both the cell cycle and apoptosis categories.

Diazinon exposure produced significant alterations in the expression of 40% of the cell cycle genes, with more genes showing downregulation than upregulation (Figure 1A), in keeping with the main treatment effect identified in the ANOVA comparison. Both dieldrin (Figure 1B) and Ni^{2+} (Figure 1C) affected a slightly higher proportion of cell cycle genes (44% for

each) with Ni^{2+} showing a two-to-one preference for downregulation. Similarly, for apoptosis-related genes, diazinon (Figure 1D), dieldrin (Figure 1E) and Ni^{2+} (Figure 1F) each affected approximately 40% of the genes. All the treatment effects for both cell cycle and apoptosis were highly significantly different from the value of 5% (2.5% increased, 2.5% decreased) that would have been seen at random (Figure 1G).

Overlap and concordance of treatment effects

Although the number of genes changed in each category by the diverse toxicants were all similar, we needed to determine if these reflected targeting of the same genes. For cell cycle (Figure 2A), diazinon and dieldrin altered 113 genes in common, out of the 150 genes affected by diazinon and the 165 genes affected by dieldrin. For diazinon and Ni^{2+} (166 genes affected), there was a similar degree of overlap, comprising 101 genes; for the pairing of dieldrin and Ni^{2+}, the overlap was 99 genes. Eighty genes were affected in common by all three toxicants. Thus, the treatment effects of the three neurotoxicants targeted up to 70% of the same genes in pairwise comparisons, and about 50% of the genes were shared by all three. Similar results were seen for overlap directed toward apoptosis-related genes (Figure 2B). Diazinon and dieldrin had shared effects on 86 genes out of the 141 affected by diazinon and 142 affected by dieldrin; diazinon and Ni^{2+} (153 genes affected) shared effects on 85 genes and the pairing of diazinon with Ni^{2+} produced an overlap of 78 genes. Fifty-six genes were affected by all three toxicants. Thus, for apoptosis, the degree of overlap was about 60% for pairwise comparisons and 40% for effects for all three toxicants in common.

Finally, we applied an even more stringent set of criteria, using concordance analysis to examine whether the different toxicants shared their effects across all the genes in each category, regardless of whether a given gene was statistically significantly affected, and comparing the effects for matching of the direction of change, magnitude of change, and time course of change. This produced six pairwise comparisons of the three agents, each of which showed robustly statistically significant positive correlations of the effects across all the genes and time points ($p < 0.0001$ for each, Figure 3). For the cell cycle genes, the strongest correlation was between diazinon and dieldrin (Figure 3A), with lesser relationships for diazinon and Ni^{2+} (Figure 3B) and dieldrin and Ni^{2+} (Figure 3C). The concordance was not as strong for apoptosis-related genes, although each pairwise comparison still showed highly significant correlations; again, the concordance was highest between diazinon and dieldrin (Figure 3D), with slightly lesser agreement for the pairing of diazinon and Ni^{2+} (Figure 3E) and least for dieldrin and Ni^{2+} (Figure 3F).

DISCUSSION

One of the key objectives for analysis of the transcriptome is to be able to predict biological outcomes based on specific patterns of gene expression. Here, we found that three otherwise unrelated developmental neurotoxicants, all of which produce eventual deficits in the number of cells, target the same gene families involved in cell cycle and apoptosis, target the same genes within those families, and further, that the alterations in those genes are similar in their magnitude, direction and time course of effect. These relationships were found despite the fact that diazinon, dieldrin and Ni^{2+} are in totally different classes and act through different initial mechanisms. The conclusion is inescapable that the concept of “mechanism” as a determinant of biologic outcomes may be less important than the sharing of common downstream pathways that define the eventual outcomes for different types of toxicities. Thus, oxidative stress, changes in calcium flux, interference with cell signaling cascades and alterations of neurotransmitter receptor function can all eventually lead to the same net outcome, namely loss of cells through impairment of cell cycle and promotion of apoptosis.

Our findings were based on three analytic levels of increasing stringency. First, following the typical mode of pathway-based transcription, we were able to show that diazinon, dieldrin and Ni^{2+} all targeted the gene families for both cell cycle and apoptosis; this was evidenced by significant changes in 30–45% of all the genes in each category, compared to the 5% incidence that would have been seen at random. Thus, all three agents target the same two pathways that ultimately determine the number of neural cells. At the second depth of analysis, we compared the degree of overlap among the toxicants for the genes that showed statistically significant up- or downregulation. For the cell cycle genes, we found up to 70% overlap between pairs of toxicants and about 50% that were affected by all three; for apoptosis, those figures were somewhat lower but were still robust (60% and 40%, respectively). Accordingly, this set of comparisons shows that within the two pathways, the toxicants target the same genes.

Concordance, the third level of analysis, is yet more stringent, comparing effects for magnitude and direction of change, and for temporal course; this approach also includes all genes in each pathway, not just those that were statistically significant. Nevertheless, we still found highly significant relationships among the three toxicants for both cell cycle and apoptosis. The correlation coefficients for each pairwise comparison ranged from 0.24 to 0.60, reflecting the relatively weak relationship for predicting the value of any one gene based on its comparable reading for another toxicant; this is not surprising, given the fact that the pairwise data sets include 10% false positive readings (5% for each for each toxicant). Again, though, given that the three agents are from unrelated classes and act by different initial mechanisms, it seems remarkable that highly significant interrelationships are maintained even with the stringent requirements of concordance analysis.

Superimposed on the strong connections for diazinon, dieldrin and Ni^{2+}, the three levels of analysis also highlight the differences among these agents. The global ANOVA, which considers magnitude and direction of effects across all genes, showed that diazinon and Ni^{2+} both elicited an overall reduction in gene expression for either cell cycle or apoptosis; however, dieldrin had no net direction of change for cell cycle and actually evoked a net increase for genes in the apoptosis pathway. This means that, although all three agents target the same two pathways and the same genes within those pathways, dieldrin, on the whole, raises gene expression whereas the other two produce net suppression. It may then seem puzzling that, in the third level of analysis, we still see concordance among all three for direction, magnitude and time course of effect. The correlational analysis relates the expression for one agent (abscissa) to that of another (ordinate) and the significance of that relationship depends upon the goodness-of-fit of the pairwise points to the regression line. The same correlation is obtained even if the line is displaced upward or downward along the y-axis. Thus, gene expression for dieldrin is highly significantly correlated to the other two agents but with a vertical displacement upward relative to the other two, reflecting the differences in the main effect as identified by ANOVA; for example, in Figure 3, the y-intercept is positive for the pairing of diazinon (abscissa) and dieldrin (ordinate), whereas it is negative for the pairing of diazinon and Ni^{2+}. The three different levels of analysis thus reflect different aspects of the gene expression pattern and their use in combination gives a more complete picture than just looking at a main treatment effect of the genes that are significantly changed on an individual basis.

Two additional questions need to be addressed. First, to what extent is the pattern of triple concordance seen here for cell cycle and apoptosis unique? This issue can be addressed by comparing the present study to our earlier evaluations that focused on gene clusters that define the underlying *mechanisms* of toxicity (as distinct from cell cycle and apoptosis as *outcomes* of toxicity), as well as effects on neurodifferentiation other than those contributing to cell loss. For the first question, we focused on those mechanisms known to be targeted by

the organophosphates [oxidative stress, excitotoxicity, cell signaling, and effects on neurotrophic factors (Adigun et al., 2010; Slotkin et al., 2010a, b; Slotkin and Seidler, 2009a, b)] as well as groupings representative of neurodifferentiation endpoints (Slotkin and Seidler, 2008, 2009c, 2010, 2011): neurite outgrowth, differentiation into the major (acetylcholine, dopamine) and minor (serotonin, neuropeptides) neurotransmitter phenotypes characteristic of PC12 cells, and genes related to the risk of Parkinson's Disease. Collectively, these comprise a cluster of 336 genes, nearly the same number as those delineating the groupings for cell cycle or apoptosis. Although all three toxicants affected each pathway to varying extents, we did not observe concordance of all three toxicants for any of the groupings with the exception of a subset of genes related to two cell signaling cascades (Adigun et al., 2010; Slotkin and Seidler, 2009b), specifically involving cyclic AMP (G-protein subunits, adenylyl cyclase isoforms and regulators) and protein kinase C (PKC isoforms and regulators). These two pathways are known to be primary regulators of cell cycle and apoptosis in developing neural cells (Ghoumari et al., 2002; Howard et al., 1993; Mantamadiotis et al., 2002; Russell and Acevedo-Duncan, 2005), so the triple concordance for these gene subgroups is entirely consistent with convergent effects leading to overlapping gene targets seen in the current work. The second question is whether the effects on cell cycle and apoptosis are selective for the neuronal phenotype, and therefore are relevant to *neuro*toxicity. Undoubtedly, if concentrations of each agent are raised sufficiently, they would likely compromise cell cycle and activate apoptosis in any cell type; indeed, at doses higher than those necessary to produce developmental neurotoxicity, each of these agents becomes generally embryotoxic (Hamm and Hinton, 2000; Leonard and Jacquet, 1984; Uzoukwu and Sleight, 1972). The PC12 model actually enables a direct demonstration of the targeting of the neuronal phenotype because neurodifferentiation is triggered by the specific addition of nerve growth factor. We compared the effects of another organophosphate, chlorpyrifos, on the gene groupings for cell cycle and apoptosis in the undifferentiated state and after the initiation of neurodifferentiation (Slotkin and Seidler, 2012). Although similar numbers of genes were affected in both states, there was no concordance whatsoever between the genes affected in the undifferentiated state and those with neuronal specification. Thus, the underlying mechanisms, and consequences for impact on cell cycle and apoptosis, are entirely distinct for the neuronal phenotype.

The approach used here is distinct from more traditional uses of microarrays. The typical technique of examining the entire transcriptome to identify a handful of genes that exhibit large effects makes the unsupported assumption that the only the biggest changes are biologically important; it can equally be argued that tightly-controlled genes, which show smaller but more consistent changes, are the most important. Further, inclusion of the entire array in the analysis produces a great number of false-positive results. Using a criterion of $p < 0.05$, there are 2000 false positive readings from an array with 40,000 transcripts, requiring that each positive finding be verified with RT-PCR. Here, we restricted the examination to smaller numbers of genes reflecting specific biological pathways that are known or suspected targets for organophosphates, information that was available from both in vivo and in vitro studies of the specified antimitotic and proapoptotic outcomes. We then used diazinon, a known neurotoxicant, as a "gold standard" against which to compare dieldrin and Ni^{2+}, focusing on overall patterns of gene effects in those pathways so as to characterize the impact of neurotoxicant exposures on the expression *pattern* rather than on a specific gene or small subset of highly-affected genes. The similarities among the different toxicants identified by this approach were clear-cut and statistically robust, confirming the value of this alternative use of microarray data. In the approach taken here, validation of the targeted genes occurs via statistical probabilities. Given clusters of approximately 380 genes, each toxicant will produce 19 false positive "significant" changes for each of the two pathways (i.e. changes that are statistically significant but that are actually not distinguishable from a random incidence). If, however, we add the requirement that a gene

must be significantly altered by at least two toxicants, the chance of its being a double false-positive falls to 1 in 400, so that there would be only one incorrect gene in the group. If, as found here, there is an overlap of all three toxicants, then there is only one chance in 8000 of a false positive; in other words, it is highly unlikely that even a single gene in the cluster defined by triple overlap is in fact a false positive reading for all three toxicants. This approach makes it possible to define groupings that otherwise would contain too many genes to verify by RT-PCR, but that nevertheless are certain to have been affected. As a validation of this interpretation, although we used cell cultures for our comparisons, in a previous study using the same gene families and analytic approach, we showed that the effects of chlorpyrifos in the PC12 cell model, successfully predicted 70% of the genes that were affected in the neonatal rat brain after treatment in vivo (Slotkin and Seidler, 2012).

Our studies show that diazinon, dieldrin and Ni^{2+}, three otherwise unrelated neurotoxicants that act by different initial mechanisms, nevertheless converge on gene expression in cell cycle and apoptosis pathways, culminating in their convergence on cell loss as a relevant and meaningful toxic endpoint. Further, they affect the same genes within those pathways and with highly significant concordance for direction, magnitude and timing of the transcriptional changes. Superimposed on this basic similarity, there are also some notable differences that reflect their divergent mechanisms contributing to effects on cell cycle and apoptosis, including oxidative stress, excitotoxicity, and neurotrophic factors (Slotkin et al., 2010a, b; Slotkin and Seidler, 2009a). Nevertheless, the remarkable convergence of all three agents on the same pathways delineating the outcome of reduced numbers of neurons, shows that it may be feasible to develop a transcriptional “fingerprint” that uses defined pathways to identify toxicants that are likely to lead to neural cell loss during development. The approach taken here actually involved <2% of the entire genome, and taken collectively with our earlier work on mechanisms of toxicity and neurodifferentiation outcomes, we have still examined <3%; accordingly, extension to other pathways with specific connections to cell acquisition and death would be likely to identify even more definitive gene clusters that would comprise the “fingerprint.” Our findings thus demonstrates both the power of planned comparisons involving defined gene groupings (ease in identification of detailed changes in a given pathway), while at the same time showing that there is a great deal of valid information still to be examined. Our results indicate that the success of this approach will entail an in-depth analysis of the patterns of gene expression that combines multiple ways of analyzing entire gene groupings, rather than a simple reliance on identifying a small set of particular genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA analysis of variance

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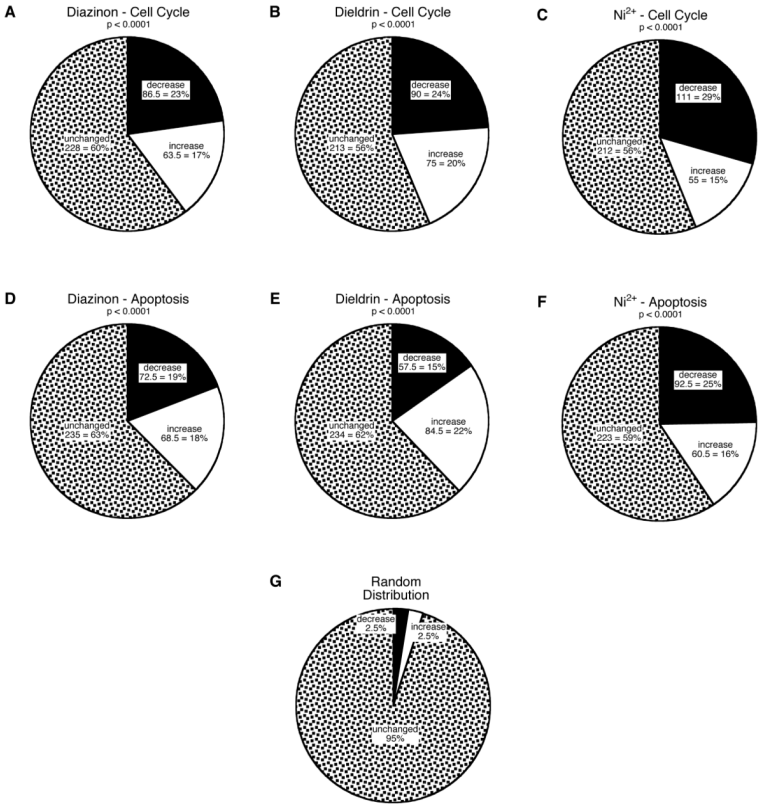

Figure 1.

Effects of neurotoxicants on genes involved in cell cycle (A,B,C) and apoptosis (D,E,F): diazinon (A,D), dieldrin (C,E) and Ni^{2+} (C,F). Charts show the number and percent of genes in each category that were unchanged, or that showed significant increases or decreases from control values. P-values for each chart compare the distribution of values from those expected at random (G).

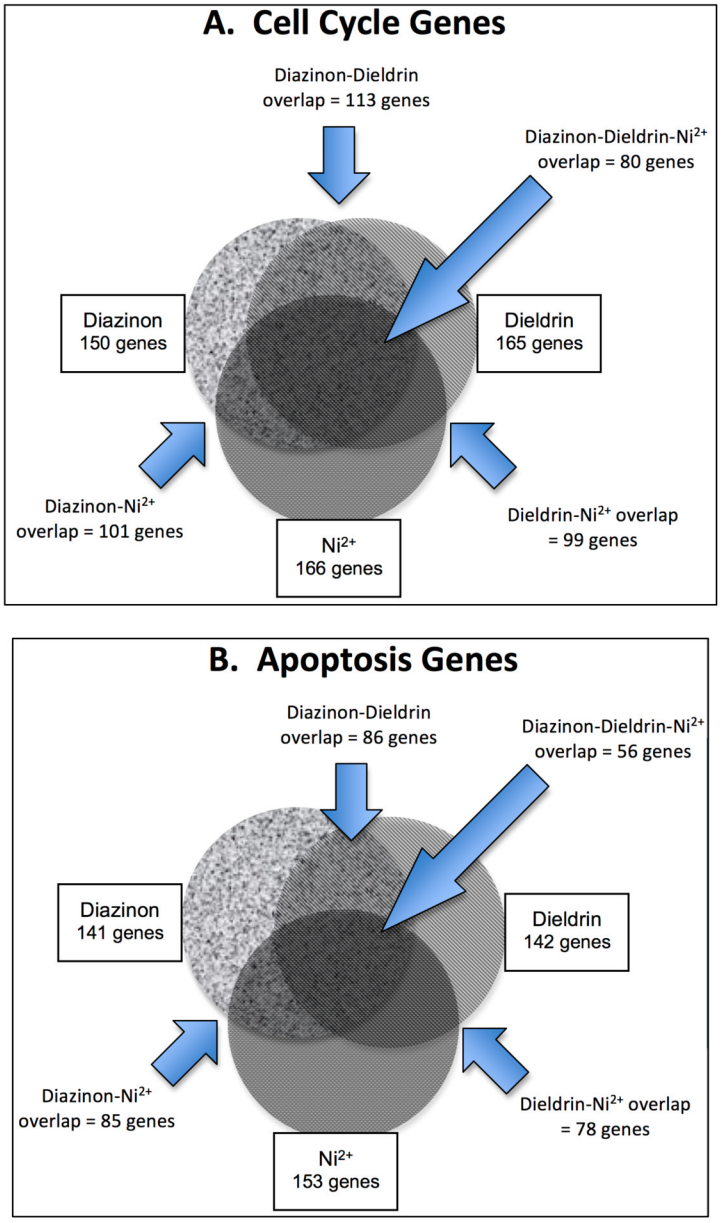

Figure 2.

Venn diagram illustrating the relationships among diazinon, dieldrin and Ni^{2+} for their effects on cell cycle genes (A) and apoptosis genes (B). Boxes show the number of genes affected by each individual treatment. Small arrows designate where the same genes are targeted for each pair of neurotoxicants and the large arrow designates genes that were affected in common by all three toxicants. Overlap is greater for cell cycle genes than for apoptosis genes but for both classes there is a high degree of overlap for pairwise comparisons as well as for all three agents.

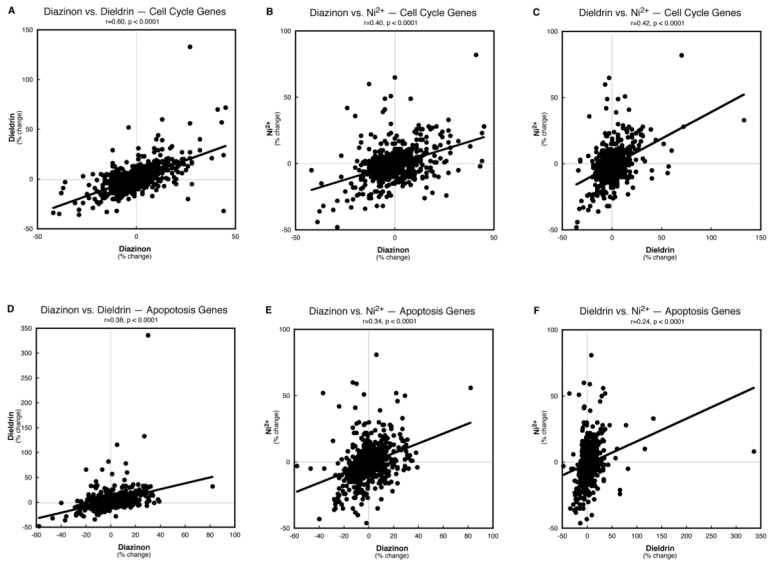

Figure 3.

Concordance analysis of the effects of neurotoxicant treatments on cell cycle genes, (A,B,C) and apoptosis genes (D,E,F), showing pairwise comparisons of diazinon and dieldrin (A,D), diazinon and Ni^{2+} (B,E), and dieldrin and Ni^{2+} (C,F). Data are shown as the percent change from control values for all genes and both time points in each category, regardless of whether the change was statistically significant or not. The linear correlation coefficient and its statistical significance are shown at the top of each panel and the line represents the least-squares fit of the data points.