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Diverse Neurotoxicants Converge on Gene Expression for Neuropeptides and their Receptors in an In Vitro Model of Neurodifferentiation: Effects of Chlorpyrifos, Diazinon, Dieldrin and Divalent Nickel in PC12 Cells

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Abstract

Unrelated developmental neurotoxicants can produce similar neurobehavioral outcomes. We examined whether disparate agents affect neuromodulators that control numerous neurotransmitters and circuits, employing PC12 cells to explore the targeting of neuroactive peptides by organophosphates (chlorpyrifos, diazinon), an organochlorine (dieldrin) and a metal (Ni^{2+}); we utilized microarrays to profile gene expression for the peptides and their receptors. Chlorpyrifos evoked robust upregulation of cholecystokinin, corticotropin releasing hormone, galanin, neuropeptide Y, neurotensin, preproenkephalin and tachykinin 1; this involved a critical period at the commencement of neurodifferentiation, since the effects were much less notable in undifferentiated PC12 cells. Diazinon targeted a similar but smaller repertoire of neuropeptide genes and the magnitude of the effects was also generally less. Surprisingly, dieldrin shared many of the same neuropeptide targets as the organophosphates and concordance analysis showed significant overlap among all three pesticides. However, dieldrin had more notable effects on neuropeptide receptors, and overlap between diazinon and dieldrin for the receptors led to a stronger resemblance of these two agents than of chlorpyrifos and dieldrin. Ni^{2+} was unique, evoking upregulation of only one of the peptides affected by the other agents, while causing downregulation of several others. Nevertheless, there was still significant concordance between Ni^{2+} and either diazinon or dieldrin, reflecting similarities toward the receptors. Our results show that neuropeptides are likely to be a prominent target for the developmental neurotoxicity of organophosphates and other neurotoxicants, and further, that the convergence of disparate agents on the same genes and pathways may contribute to similar neurobehavioral outcomes.

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Keywords

Chlorpyrifos; Diazinon; Dieldrin; Gene transcription patterns; Microarrays; Neuropeptides; Neuropeptide Receptors; Nickel; Organochlorine insecticides; Organophosphate insecticides; PC12 cells

INTRODUCTION

Exposures of pregnant women and children to neurotoxic chemicals are likely contributors to the “silent pandemic” of neurodevelopmental disorders (Grandjean and Landrigan, 2006), which include learning disabilities, cognitive impairment and autism (Eriksson, 1997; Grandjean and Landrigan, 2006; Landrigan et al., 1994, 1999; Szpir, 2006a, b; Weiss et al., 2004). Behavioral outcomes can display surprising similarities among apparently unrelated agents, whereas there may be disparities between chemicals within the same class (Barone et al., 2000; Monnet-Tschudi et al., 2007; Slotkin, 2004; Slotkin et al., 2006; Slotkin and Seidler, 2007; Slotkin et al., 2007c; Slotkin et al., 2008a; Slotkin et al., 2008b; Slotkin and Seidler, 2008; Slotkin et al., 2010b; Szpir, 2006b; Yanai et al., 2002, 2004). In a series of recent papers, we showed how chlorpyrifos and diazinon, two organophosphate pesticides, share common endpoints for some of their effects on neurodifferentiation (Adigun et al., 2010a, b; Slotkin et al., 2007b; Slotkin et al., 2008c; Slotkin and Seidler, 2009a, b, c; Slotkin et al., 2010b), but also display some notable differences that are likely to account for divergent behavioral endpoints (Aldridge et al., 2004, 2005; Levin et al., 2001; Roegge et al., 2008; Slotkin et al., 2001; Slotkin and Seidler, 2005; Slotkin et al., 2008a; Slotkin et al., 2008b; Timofeeva et al., 2008). At the same time, we found convergent effects of unrelated agents, such as organophosphate insecticides (chlorpyrifos, diazinon) an organochlorine insecticide (dieldrin), as well as a metal (divalent nickel), suggesting that a wide variety of agents may all target common final pathways that influence the emergence of neuronal function (Slotkin et al., 2007b; Slotkin and Seidler, 2009b; Slotkin et al., 2010b).

Traditionally, most studies of synaptic mechanisms for neuroteratologic effects tend to focus on “classical” neurotransmitters. In our work with organophosphates, we identified mechanisms and consequences directed toward acetylcholine, dopamine, norepinephrine, serotonin and glutamate as prominent targets for these pesticides (Slotkin, 2004, 2005; Slotkin and Seidler, 2009a, b; Slotkin et al., 2010a). Here, we studied the impact on neuropeptides and their receptors. This represents an important extension of neurotoxicity studies, since neuropeptides are often colocalized and released simultaneously with classical transmitters; neuropeptides serve as synaptic modulators, so that neurotoxicants that alter their expression can produce changes over a much wider variety of transmitter systems and circuits than would occur with the targeting of any one transmitter. Almost no work has been done on neuropeptides as a target for neurotoxicants in general, nor for organophosphates in particular. In the adult brain, outright poisoning with a nerve agent can affect neuropeptides consequent to seizures (Kubek et al., 1997) and the organophosphates can bind to acylpeptide hydrolase, an enzyme putatively involved in neuropeptide processing (Richards et al., 2000). Here, we evaluated organophosphate effects on genes encoding neuropeptides and their receptors using PC12 cells, a standard in vitro model for neuronal development (Teng and Greene, 1994) that has already been validated to reproduce the mechanisms and outcomes found after in vivo organophosphate exposures (Bagchi et al., 1995, 1996; Crumpton et al., 2000a, b; Das and Barone, 1999; Flaskos et al., 1994; Jameson et al., 2006, 2007; Li and Casida, 1998; Nagata et al., 1997; Qiao et al., 2001, 2005; Slotkin et al., 2007a; Slotkin et al., 2007b; Slotkin et al., 2008c, 2010b; Song et al., 1998; Tuler et al., 1989). With the addition of nerve growth factor, PC12 cells begin to differentiate, forming neuritic projections and acquiring electrical excitability and neuronal phenotypes

(Fujita et al., 1989; Song et al., 1998; Teng and Greene, 1994). Although this cell line is frequently used for its differentiation into cholinergic and dopaminergic phenotypes, PC12 cells also express a wide variety of neuropeptides and their receptors.

In the present work, we determined the extent to which chlorpyrifos, diazinon, dieldrin and Ni^{2+} show convergent or disparate actions on gene expression in differentiating cells. Further, we assessed whether these changes involve a critical period by comparing the effects of chlorpyrifos on undifferentiated PC12 cells versus cells undergoing neurodifferentiation. We used microarrays in a “planned comparisons” framework, where genes are selected based on a specific pathway and hypothesis prior to examining the microarray data, rather than the other way around; the advantages and limitations of this approach as compared to searches of the entire genome have been presented previously (Adigun et al., 2010b; Slotkin and Seidler, 2007, 2008, 2009b). Here, we focused on a variety of neuropeptides from different classes, all with known neuromodulatory roles: cholecystokinin, corticotropin releasing hormone, galanin, neuropeptide Y, neurotensin, opioids, oxytocin, somatostatin, tachykinins (which include Substance P), vasopressin and vasoactive intestinal polypeptide. 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. Initially, we also included the gene encoding thyrotropin releasing hormone (*trh*) and its receptors but the values for *trh* were highly variable and failed the quality control filters (see Experimental Procedure); accordingly, we did not pursue examination of this neuropeptide or its receptors.

Finally, it is important to note that the test agents employed in this study also have intrinsic interest because of environmental concerns about human exposure and safety. Organophosphates are among the most commonly used pesticides in the world, with nearly-ubiquitous human exposure (Casida and Quistad, 2004), and are undergoing increasing restriction because of their propensity to elicit developmental neurotoxicity (U.S. Environmental Protection Agency, 2000, 2002; Winston, 2006). Dieldrin is also known to produce developmental neurotoxicity (Brannen et al., 1998; Kitazawa et al., 2001, 2003; Liu et al., 1998; Slotkin et al., 2007b; Uzoukwu and Sleight, 1972); nickel shows fetal accumulation similar to that of lead (Casey and Robinson, 1978; Jacobsen et al., 1978) and shares neurotoxic actions with lead and cadmium (Benters et al., 1996).

RESULTS

Effects of differentiation state

We evaluated gene expression for 15 neuroactive peptides and 25 of the corresponding receptors (Table 1). Before examining the effects of toxicant exposures, we first determined whether there were underlying differences in gene expression between undifferentiated and differentiating cells under control conditions. Global statistical analysis for all the genes, comparing expression in the two differentiation states and both time points, revealed significant interactions of state $\times$ gene ($p < 0.0001$) and state $\times$ gene $\times$ time ($p < 0.0001$). We therefore subdivided the genes into two groups, corresponding to those encoding the peptides and those for the receptors, and repeated the multivariate analysis. For the peptides, we found an interaction of state $\times$ gene ($p < 0.004$) and state $\times$ gene $\times$ time ($p < 0.004$); for the receptors, there were interactions of state $\times$ time ($p < 0.05$) and state $\times$ gene $\times$ time ($p < 0.0001$). Accordingly, we examined the values for each individual gene.

Of the neuropeptides, over half showed significant differences between the two states (Figure 1). For two of the peptides, *crh* and *npv*, differentiation produced a large increase in expression and one (*pomc2*) showed a smaller increase. Five others showed significantly

lower expression during differentiation as compared to the undifferentiated state, with relatively robust effects for *sst* and *tac1*, along with lesser effects on *avp*, *cck* and *tac2*. For the neuropeptide receptors, 9 of the 25 genes showed significant differences between undifferentiated and differentiating cells (Figure 2). Differentiation produced the largest increase for *opr1*, followed by intermediate increases for *galr2*, *oxtr* and *tacr3*, and small but significant increments for *crhr1*, *crhr2* and *oprs1*; two of the receptors showed modest (*np2r*) or small (*ogfr*) decreases.

Global statistical analysis of neurotoxicant effects

Because only one agent (chlorpyrifos) was tested in both undifferentiated and differentiating cells, we conducted two sets of global statistical tests. For chlorpyrifos, the ANOVA factors were treatment, differentiation state, time and gene, and we found a main treatment effect ($p < 0.0004$) as well as interactions of treatment $\times$ time ($p < 0.02$), treatment $\times$ differentiation state ($p < 0.0004$), treatment $\times$ gene ($p < 0.0001$), treatment $\times$ state $\times$ time ($p < 0.02$), treatment $\times$ gene $\times$ time ($p < 0.0001$), treatment $\times$ gene $\times$ state ($p < 0.0001$) and treatment $\times$ gene $\times$ state $\times$ time ($p < 0.0001$). Accordingly, we subdivided the results for presentation according to differentiation state and into two classes (neuropeptides, neuropeptide receptors) and then identified main treatment effects and interactions of treatment with the remaining variables (gene, time). Diazinon, dieldrin and Ni^{2+} were studied only in differentiating cells, so the ANOVA factors for these agents were treatment, gene and time. The global test identified significant interactions of treatment $\times$ gene ($p < 0.0001$) and treatment $\times$ gene $\times$ time ($p < 0.0001$), so we subdivided the data into the individual treatments and the same two classes for presentation and lower-order examination. The final validation of the significance of the overall changes in gene expression was provided by analysis of the incidence of significant treatment-related differences vs. the false-positive rate; we studied 40 genes, so the expected false-positive rate was a maximum of 2 genes, whereas we found significant treatment effects for 19 genes ($p < 0.00002$ vs. the expected false-positive rate).

Chlorpyrifos

In undifferentiated PC12 cells, chlorpyrifos exposure produced a mixture of increases and decreases in gene expression related to neuropeptides and their receptors. For the neuropeptides (Figure 3A), *np2*, *nts* and *penk* showed significant upregulation whereas downregulation was seen for *cck*, *pdyn*, *sst* and *tac1*. Of the neuropeptide receptors (Figure 3B) *opr1*, *oxtr*, *tacr3* and *vpr1* were increased by chlorpyrifos exposure, while *avpr1a* and *sstr2* were decreased.

The effects of chlorpyrifos were much greater in differentiating cells than in the undifferentiated state and showed a specific overall direction of change instead of a mixed pattern. For the genes encoding the neuropeptides, there was a distinct predominance of upregulation, as indicated by a significant main treatment effect (Figure 4A). Robust increases were seen for *cck*, *crh*, *np2* and *nts*, with lesser but still significant increases for *gal*, *penk* and *tac1*. Only one gene (*tac4*) showed downregulation and this was of smaller magnitude than the incremental responses. Effects for the neuropeptide receptors did not show significant treatment effects in the global ANOVA and hence, lower-order tests for changes in individual genes were not carried out (Figure 4B); however, any differences were clearly of much smaller magnitude than those seen for most of the neuropeptides.

Diazinon

The effects of diazinon exposure were similar to those of chlorpyrifos, with overall upregulation of neuropeptide genes (Figure 5A) and lesser, nonsignificant effects on the neuropeptide receptor genes (Figure 5B). Like chlorpyrifos, diazinon evoked upregulation of

crh, *npv*, *nts* and *tac1* but the effects were generally smaller. Diazinon failed to upregulate two of the genes that were affected by chlorpyrifos (*cck*, *penk*) and increased *tac4* instead of decreasing it.

Dieldrin

Although dieldrin belongs to a different chemical class, its effects on neuropeptide genes nevertheless resembled those of chlorpyrifos and diazinon (Figure 6A), sharing the same, significant upregulation of *crh*, *npv*, *nts* and *tac1*. Like diazinon, but unlike chlorpyrifos, dieldrin increased *tac4* and failed to alter *cck* or *gal*. Uniquely, dieldrin decreased *penk* instead of increasing it (as did chlorpyrifos) or evoking no change (as did diazinon). Dieldrin also evoked increases in neuropeptide receptor expression that were not seen with the organophosphates, involving *avpr1b*, *cckbr*, *smstr28* and *vipr2* (Figure 6B).

Divalent nickel

The effects of Ni^{2+} on neuropeptide gene expression were different from those of the other three toxicants (Figure 7A), sharing only one of the genes that had shown robust significant upregulation (*nts*). Unlike the others, Ni^{2+} downregulated *crh* and *npv* instead of causing upregulation and failed to increase *tac1*; Ni^{2+} was the only agent to evoke an increase in *pomc2*. The other Ni^{2+} -induced effects shared similarities or dissimilarities with one or another of the agents: decreased *gal* (opposite to chlorpyrifos), decreased *penk* (opposite to chlorpyrifos, similar to dieldrin), and decreased *tac4* (similar to chlorpyrifos, opposite to diazinon or dieldrin). Changes in the expression of genes encoding the neuropeptide receptors again did not show overall significance in the global test and therefore were not analyzed for effects on individual genes (Figure 7B).

Concordance analysis

To establish overall similarities and disparities between effects of the different toxicants, we performed pairwise concordance analyses, which includes every gene, regardless of whether individual effects were significant or not. Furthermore, where concordance was affected by the value for a single gene, we made sure that the conclusions did not depend solely on that measure. We made three comparisons for each pair: all genes in the planned comparisons (neuropeptides and receptors), genes for just the neuropeptides, and genes for just the receptors. The effects of chlorpyrifos in differentiating cells were only poorly related to those in the undifferentiated state, with no significant correlation across all the genes (Figure 8A), a weak and nonsignificant correlation for the neuropeptides (Figure 8B), and no apparent relationship for the receptors (Figure 8C). In contrast, the effects of chlorpyrifos and diazinon in differentiating cells were highly concordant overall (Figure 8D), a relationship that was driven almost exclusively by the similarities in patterns of effects on the neuropeptides (Figure 8E) rather than the neuropeptide receptors (Figure 8F). Similarly, the effects of chlorpyrifos were highly concordant with those of dieldrin (Figure 9A), again primarily by the resemblance because of the neuropeptide genes (Figure 9B) rather than the receptor genes (Figure 9C). The effects were totally different for chlorpyrifos and Ni^{2+} , where there was no significant concordance whatsoever (Figure 9D,E,F).

Diazinon and dieldrin also showed strong concordance across all genes (Figure 10A) and the neuropeptide genes (Figure 10B); however, in this case, they also showed concordance for the receptor genes (Figure 10C). Indeed, relationships were even stronger when a single outlying value was deleted (Figures 10A,B). To further ensure that the positive concordance between diazinon and dieldrin did not depend on a few, large individual effects, we also evaluated their concordance nonparametrically using Spearman's rank correlation, without discarding any of the data points; the correlations remained highly significant for all genes ($p < 0.0001$), neuropeptide genes ($p < 0.0003$) and receptor genes ($p < 0.004$).

Diazinon did not show a significant correlation with the effects of Ni^{2+} across all genes (Figure 10D) or for neuropeptides (Figure 10E); however, there was a significant relationship in their effects on neuropeptide receptors (Figure 10F). Although dieldrin and Ni^{2+} showed significant concordance across all genes (Figure 10A) and the neuropeptides genes (Figure 10B), these relationships both depended entirely on a single value, without which the correlations were poor and nonsignificant. However, as with the pairing of diazinon and Ni^{2+} , dieldrin and Ni^{2+} showed slight but significant concordance for their effects on the neuropeptide receptors.

It was notable that, even for the nonsignificant regression relationships, all the correlations were positive, a highly nonrandom finding. Accordingly, besides the pairwise comparisons, we established the overall significance of correlations across all four agents using the χ^2 test for combining p-values (Table 2). There was high overall concordance among the four agents toward the neuropeptide genes as well as toward the neuropeptide receptors, albeit the relationship was stronger for the peptides, as shown by the higher overall χ^2 value. Across both classes of genes, relationships were strong for all pairings except chlorpyrifos and Ni^{2+} ; however, the strength of the relationship of dieldrin to Ni^{2+} remained highly dependent on a single value (arrow in Figure 10H), without which the χ^2 value was too low to achieve significance.

DISCUSSION

To our knowledge, this is the first study to show that neuropeptides are likely to be a prominent target in the developmental neurotoxicity of organophosphates and other neurotoxicants. Indeed, the changes seen here after exposure to chlorpyrifos or diazinon were substantially larger than those for the genes related to cholinergic and dopaminergic neurotransmitter phenotypes, the two major classical transmitters found in PC12 cells (Slotkin and Seidler, 2009b). Further, our studies indicate unexpected similarities elicited by exposure to organophosphates, an organochlorine and a heavy metal, pointing to neuropeptides as a common target for disparate agents affecting neuronal development.

Of the agents studied, chlorpyrifos evoked the largest and most widespread increases in neuropeptide gene expression. Although the effects spanned multiple peptides, there was distinct specificity, since not all peptides were affected to the same degree nor in the same direction; the most prominent effects, though, involved upregulation, especially for *cck*, *crh*, *gal*, *npv*, *nts* and *tac1*. The effects of chlorpyrifos could not be explained simply as an indirect consequence of an enhancement of neurodifferentiation. Although two of the peptides affected by chlorpyrifos (*crh*, *npv*) were among those that also showed a large increase with normal differentiation, *nts*, which did not change with differentiation, was nevertheless highly induced by chlorpyrifos, and *tac1*, which was decreased by differentiation, was also enhanced by the organophosphate. Chlorpyrifos thus targets the expression of neuropeptides in a selective manner. Furthermore, we were able to identify a specific critical period for these effects, since exposure during differentiation had a much greater effect than in undifferentiated cells; as with many of our other findings for organophosphate effects on neuronal development, the period at the initiation of neurodifferentiation appears to be particularly sensitive to disruption (Adigun et al., 2010a; Jameson et al., 2006; Qiao et al., 2005; Slotkin and Seidler, 2008, 2009b, c; Slotkin et al., 2010b).

On the whole, the effects of diazinon were quite similar to those of chlorpyrifos, with large effects on many of the same peptides, and the same, lower impact on neuropeptide receptor genes. Indeed, the two organophosphates showed high overall concordance of their effects, indicating a substantial overlap in the specific genes targeted by the two agents (Fig. 11A).

At the same time, there were also notable differences, with chlorpyrifos affecting a larger repertoire of neuropeptides, and with different directions of change for one peptide (*tac4*, increased by chlorpyrifos, decreased by diazinon). Given the modulatory role of neuropeptides in neurotransmission, these differences could explain some of the divergent neurobehavioral outcomes of the two agents (Aldridge et al., 2005; Levin et al., 2001; Roegge et al., 2008; Timofeeva et al., 2008). Our studies thus point to the need to examine the extent to which the targeting of neuropeptide function contributes to adverse outcomes after developmental exposure to organophosphates in vivo.

The effects of dieldrin showed unexpectedly strong concordance with chlorpyrifos, and even more so with diazinon. The similarities largely reflected the induction of many of the same neuropeptide genes as those stimulated by the organophosphates but in addition, dieldrin had more substantial effects on neuropeptide receptor genes. Further, the overall pattern of dieldrin's effects on the receptors was concordant with evoked by diazinon, even though the effects of diazinon on each gene was not statistically significant on an individual basis. The neuropeptide receptor genes thus account for a greater degree of overlap between dieldrin and diazinon as compared to dieldrin and chlorpyrifos (Figure 11A). Indeed, Ni^{2+} , a toxicant that is clearly structurally unrelated to either organophosphates or organochlorines, affected some of the same neuropeptide receptor genes as diazinon or dieldrin; the effects on the receptors thus appear to involve a different mechanism from that responsible for those on the neuropeptides themselves. In turn, this explains why chlorpyrifos can be concordant with the effects of diazinon, and diazinon with those of Ni^{2+} , and yet chlorpyrifos and Ni^{2+} are unrelated to each other (Figure 11B); the relationship between chlorpyrifos and diazinon reflects their common actions on neuropeptides, whereas that between diazinon and Ni^{2+} depends on the receptors. Since the changes in the genes encoding the neuropeptides were substantially greater than those for the receptors, it is likely that the concordance for the former genes will be relatively more important in determining the net outcome of toxicant exposure.

Our results point to neuropeptides and their receptors as prominent targets for the organophosphates as well as toxicants from other chemical classes, in the specific context of neurodifferentiation. Further, the convergence on the same genes and pathways points to mechanisms that may underlie the similarities in outcomes from early-life exposure to disparate developmental neurotoxicants. Indeed, three particular neuropeptides (*crh*, *npv*, *nts*) appeared to be the most susceptible across all the different agents, raising the prospect that these neuromodulators may be particularly vulnerable to toxicant insult. Finally, the approach taken here shows how a combination of an in vitro model, microarray analysis, and an approach using planned comparisons for defined target pathways, can provide novel information to guide future in vivo work cementing the synaptic mechanisms underlying neurobehavioral teratology.

EXPERIMENTAL PROCEDURE

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% horse serum (Sigma Chemical Co., St. Louis, MO), 5% fetal bovine serum (Sigma), and 50 $\mu\text{g}/\text{ml}$ penicillin streptomycin (Invitrogen). Incubations were carried out with 7.5% CO_2 at 37°C, standard conditions for PC12 cells. To initiate neurodifferentiation (Jameson et al., 2006; Slotkin et al., 2007b; 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). Along with the nerve growth factor, we added 30 μ M 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 (Jameson et al., 2007; Qiao et al., 2001; Slotkin et al., 2007b; Slotkin et al., 2008c). Because of the limited water solubility of the three insecticides, these agents were dissolved in dimethylsulfoxide (final concentration 0.1%), which was also added to the control cultures and to cultures containing NiCl_2 ; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation (Qiao et al., 2001, 2003; Song et al., 1998). Regardless of whether cells were undifferentiated or differentiating, exposure to the test agents commenced 24 hr after the initial plating; we then made measurements 24 and 72 hr after the start of the exposure, with 5–8 independent cultures evaluated for each treatment at each time point; mRNA from each culture was run on a separate array. For chlorpyrifos, we evaluated the effects both on undifferentiated cells (without addition of nerve growth factor) and during differentiation, whereas for the other agents, we studied the effects only during differentiation.

In vitro systems are problematic for comparisons of toxicant concentrations to those achieved in vivo, since cultures cannot reproduce the pharmacokinetic, metabolic and other factors that influence the concentration delivered to target tissues. Also, PC12 cells are transformed cells, so they are less sensitive to toxicant injury than are developing neurons in vivo; and further, the cell culture treatments involve much shorter exposures than are experienced with environmental exposures extending throughout brain development. Both of these factors operated in our selection of the 30 μ M 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 (Ostrea et al., 2002) 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 (Qiao et al., 2001). For the same reasons, we chose to evaluate equivalent concentrations of all the toxicants rather than trying to account for factors that may or may not be relevant for in vivo exposures.

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., 2007c, 2008c). 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., 2007c, 2008c). We used Agilent Whole Rat Genome Arrays (Agilent Technologies, Palo Alto, CA), type G4131A for the studies of chlorpyrifos in undifferentiated and differentiating cells, whereas type G4131F was used for the studies of diazinon, dieldrin and Ni^{2+} in differentiating cells. The two chips contain exactly the same gene sequences but the latter has a lower detection threshold.

For several 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 genes utilized for 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., 2007c).

Statistical procedures

Because of the requirement to normalize the data across arrays, 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, and the corresponding control values are shown in Figures 1 and 2.

Our design involved planned comparisons of four agents at two time points, as well as the effects of one agent (chlorpyrifos) in undifferentiated vs. differentiating states. It was therefore important to 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 involved in the planned comparisons. Lower-order ANOVAs on subdivisions of the data set were then carried out as permitted by the interactions of treatment with the other variables. 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.

Transcriptional responses can show different degrees of variability for each gene, so that even where two genes display the same magnitude of biologic effect, one may fail to be statistically significant because of higher variability. Therefore, we also evaluated concordance between treatments by plotting the percentage change from control and calculating the linear correlation coefficient between pairs of agents, incorporating all the genes in the planned comparisons, regardless of whether the individual effects were statistically significant (Adigun et al., 2010b; Slotkin and Seidler, 2009a; Slotkin et al., 2010b). In addition to pairwise concordance comparisons of the various agents, we also evaluated groupings of correlations by gene class and treatment, using the χ^2 test for combining p-values: $\chi^2 = \Sigma[-2 \ln(p_i)]$, with degrees of freedom corresponding to twice the number of p-values used in the calculation.

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Figure 1.

Effects of neurodifferentiation on neuropeptide gene expression. Each panel shows the expression ratio at 24h and 72h in undifferentiated cells differentiating cells. Values represent means and standard errors, normalized by setting the value for all genes on the array to 1.0. Asterisks below each panel denote genes showing a significant difference between undifferentiated and differentiating cells. Note the different scale for *crh* and *npy*.

Figure 2.

Effects of neurodifferentiation on neuropeptide receptor gene expression. Each panel shows the expression ratio at 24h and 72h in undifferentiated cells and differentiating cells. Values represent means and standard errors, normalized by setting the value for all genes on the array to 1.0. Asterisks below each panel denote genes showing a significant difference between undifferentiated and differentiating cells.

Figure 3.

Effects of chlorpyrifos exposure on expression of genes for neuropeptides (A) and neuropeptide receptors (B) in undifferentiated PC12 cells. Multivariate ANOVA appears at the top of each panel and asterisks shown below each gene denote a significant main treatment effect; daggers denote genes for which a treatment $\times$ time interaction was detected and show the individual times for which treatment effects were present.

Figure 4.

Effects of chlorpyrifos exposure on expression of genes for neuropeptides (A) and neuropeptide receptors (B) in differentiating PC12 cells. Multivariate ANOVA appears at the top of each panel and asterisks shown below each gene denote a significant main treatment effect; daggers denote genes for which a treatment $\times$ time interaction was detected and show the individual times for which treatment effects were present. Testing for individual gene effects were not carried out for neuropeptide receptors because the ANOVA failed to show significant treatment effects or interactions of treatment with other variables.

Figure 5.

Effects of diazinon exposure on expression of genes for neuropeptides (A) and neuropeptide receptors (B) in differentiating PC12 cells. Multivariate ANOVA appears at the top of each panel and asterisks shown below each gene denote a significant main treatment effect; daggers denote genes for which a treatment $\times$ time interaction was detected and show the individual times for which treatment effects were present. Testing for individual gene effects were not carried out for neuropeptide receptors because the ANOVA failed to show significant treatment effects or interactions of treatment with other variables.

Figure 6.

Effects of dieldrin exposure on expression of genes for neuropeptides (A) and neuropeptide receptors (B) in differentiating PC12 cells. Multivariate ANOVA appears at the top of each panel and asterisks shown below each gene denote a significant main treatment effect; daggers denote genes for which a treatment $\times$ time interaction was detected and show the individual times for which treatment effects were present.

Figure 7.

Effects of Ni^{2+} exposure on expression of genes for neuropeptides (A) and neuropeptide receptors (B) in differentiating PC12 cells. Multivariate ANOVA appears at the top of each panel and asterisks shown below each gene denote a significant main treatment effect; daggers denote genes for which a treatment $\times$ time interaction was detected and show the individual times for which treatment effects were present. Testing for individual gene effects were not carried out for neuropeptide receptors because the ANOVA failed to show significant treatment effects or interactions of treatment with other variables.

Figure 8.

Pairwise correlations of the effects of chlorpyrifos in undifferentiated vs. differentiating cells (A,B,C), and in differentiating cells for chlorpyrifos vs. diazinon (D,E,F), including all the genes in the study (A,D), just the neuropeptide genes (B,E) or just the neuropeptide receptor genes (C,F). Values were taken from Figures 3, 4 and 5. The line in each panel shows the least-squares fit of the data. The corresponding equation and correlation coefficient are shown at the top of each panel, along with the significance of the correlation.

Figure 9.

Pairwise correlations in differentiating cells of the effects of chlorpyrifos vs. dieldrin (A,B,C), and chlorpyrifos vs. Ni^{2+} (D,E,F), including all the genes in the study (A,D), just the neuropeptide genes (B,E) or just the neuropeptide receptor genes (C,F). Values were taken from Figures 4, 6 and 7. The line in each panel shows the least-squares fit of the data. The corresponding equation and correlation coefficient are shown at the top of each panel, along with the significance of the correlation.

Figure 10.

Pairwise correlations in differentiating cells of the effects of diazinon vs. dieldrin (A,B,C), diazinon vs. Ni^{2+} (D,E,F) and dieldrin vs. Ni^{2+} (G,H,I), including all genes in the study (A,D,G), just the neuropeptide genes (B,E,H) or just the neuropeptide receptor genes (C,F,I). Values were taken from Figures 5, 6 and 7. The line in each panel shows the least-squares fit of the data. The corresponding equation and correlation coefficient are shown at the top of each panel, along with the significance of the correlation.

Figure 11.

Venn diagram showing overall concordance among neurotoxicants. (A) Chlorpyrifos, diazinon and dieldrin are concordant with each other but the relationship between dieldrin and diazinon is stronger than that between chlorpyrifos and diazinon or between chlorpyrifos and dieldrin; this reflects the greater correlation of neuropeptide receptor genes for diazinon and dieldrin. (B) Chlorpyrifos is concordant with diazinon and diazinon with Ni²⁺; nevertheless, chlorpyrifos and Ni²⁺ are dissimilar because the genes accounting for the relationship between chlorpyrifos and diazinon differ from those accounting for the similarities of diazinon to Ni²⁺.

TABLE 1

Gene List

Gene	Genbank Accession Number	Corresponding Peptide or Receptor
<i>avp</i>	NM_016992	arginine vasopressin
<i>cck</i>	NM_012829	cholecystokinin
<i>crh</i>	NM_031019	corticotropin releasing hormone
<i>gal</i>	NM_033237	galanin
<i>npv</i>	NM_012614	neuropeptide Y
<i>nts</i>	XM_216884	neurotensin
<i>oxy</i>	NM_012996	oxytocin
<i>pdyn</i>	NM_019374	prodynorphin
<i>penk</i>	NM_017139	preproenkephalin
<i>pomc2</i>	NM_139326	β -proopiomelanocortin (β -endorphin)
<i>sst</i>	NM_012659	somatostatin
<i>tac1</i>	NM_012666	tachykinin 1
<i>tac2</i>	NM_019162	tachykinin 2
<i>tac4</i>	NM_172328	tachykinin 4
<i>vip</i>	XM_217838	vasoactive intestinal polypeptide
<i>avpr1a</i>	NM_053019	arginine vasopressin receptor 1A
<i>avpr1b</i>	NM_017205	arginine vasopressin receptor 1B
<i>avpr2</i>	NM_019136	arginine vasopressin receptor 2
<i>cckbr</i>	NM_013165	cholecystokinin B receptor
<i>crhr1</i>	NM_030999	corticotropin releasing hormone receptor 1
<i>crhr2</i>	NM_022714	corticotropin releasing hormone receptor 2
<i>galr2</i>	NM_019172	galanin receptor 2
<i>galr3</i>	NM_019173	galanin receptor 3
<i>npv2r</i>	NM_023968	neuropeptide Y receptor Y2
<i>ntsr</i>	XM_345484	neurotensin receptor
<i>ntsr2</i>	NM_022695	neurotensin receptor 2
<i>ogfr</i>	NM_053340	opioid growth factor receptor
<i>opr1</i>	NM_012617	opioid receptor δ 1
<i>opr1</i>	NM_017167	opioid receptor κ 1
<i>opr1</i>	NM_013071	opioid receptor μ 1
<i>opr1</i>	NM_030996	opioid receptor σ 1
<i>oxyr</i>	NM_012871	oxytocin receptor
<i>ssr2</i>	NM_019348	somatostatin receptor 2
<i>ssr28</i>	NM_133522	somatostatin receptor 3
<i>ssr4</i>	NM_013036	somatostatin receptor 4
<i>ssr5</i>	NM_012882	somatostatin receptor 5
<i>tacr2</i>	NM_080768	tachykinin receptor 2

Gene	Genbank Accession Number	Corresponding Peptide or Receptor
<i>tacr3</i>	NM_017053	tachykinin receptor 3
<i>vipr1</i>	NM_012685	vasoactive intestinal peptide receptor 1
<i>vipr2</i>	NM_017238	vasoactive intestinal peptide receptor 2

Table 2

Comparisons Across Gene Classes and Treatments

	Neuropeptides	Neuropeptide Receptors	Row χ^2 , p-value
Chlorpyrifos vs. Diazinon	0.0005	0.10	$\chi^2=20$ p < 0.001
Chlorpyrifos vs. Dieldrin	0.0001	0.11	$\chi^2=23$ p < 0.001
Chlorpyrifos vs. Ni^{2+}	0.10	0.50	$\chi^2=6$ p > 0.4
Diazinon vs. Dieldrin	0.0001	0.0006	$\chi^2=33$ p < 0.0001
Diazinon versus Ni^{2+}	0.10	0.0008	$\chi^2=19$ p < 0.001
Dieldrin vs. Ni^{2+}	0.0001 *	0.03	$\chi^2=25$ p < 0.0005
Column χ^2 , p-values	$\chi^2=80$ p < 0.0001	$\chi^2=47$ p < 0.0001	$\chi^2 = 127$ p < 0.0001

Values shown are the individual p-values for each comparison, along with the corresponding χ^2 test evaluating the effects for each row and column.

* p < 0.9 without arrowed value in Figure 10H; corresponding row $\chi^2 = 7$ (not significant).