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CHLORPYRIFOS DEVELOPMENTAL NEUROTOXICITY: INTERACTION WITH GLUCOCORTICOIDS IN PC12 CELLS

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Abstract

Prenatal coexposures to glucocorticoids and organophosphate pesticides are widespread. Glucocorticoids are elevated by maternal stress and are commonly given in preterm labor; organophosphate exposures are virtually ubiquitous. We used PC12 cells undergoing neurodifferentiation in order to assess whether dexamethasone enhances the developmental neurotoxicity of chlorpyrifos, focusing on concentrations relevant to human exposures. By themselves, each agent reduced the number of cells and the combined exposure elicited a correspondingly greater effect than with either agent alone. There was no general cytotoxicity, as cell growth was actually enhanced, and again, the combined treatment evoked greater cellular hypertrophy than with the individual compounds. The effects on neurodifferentiation were more complex. Chlorpyrifos alone had a promotional effect on neurogenesis whereas dexamethasone impaired it; combined treatment showed an overall impairment greater than that seen with dexamethasone alone. The effect of chlorpyrifos on differentiation into specific neurotransmitter phenotypes was shifted by dexamethasone. Either agent alone promoted differentiation into the dopaminergic phenotype at the expense of the cholinergic phenotype. However, in dexamethasone-primed cells, chlorpyrifos actually enhanced cholinergic neurodifferentiation instead of suppressing this phenotype. Our results indicate that developmental exposure to glucocorticoids, either in the context of stress or the therapy of preterm labor, could enhance the developmental neurotoxicity of organophosphates and potentially of other neurotoxicants, as well as producing neurobehavioral outcomes distinct from those seen with either individual agent.

Keywords

Chlorpyrifos; Dexamethasone; Glucocorticoids; Organophosphate pesticides; PC12 cells; Preterm delivery

INTRODUCTION

One of the most critical issues in toxicology is to determine the factors that render specific subpopulations vulnerable to a given toxicant. The organophosphate pesticides provide a prime example. This class represents nearly 50% of worldwide insecticide use and exposure of the human population is virtually ubiquitous (Casida and Quistad, 2004); it is increasingly

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clear that individuals that carry genetic polymorphisms that reduce the activity of paraoxonase-1 are at greater risk of adverse effects (Costa et al., 2003; Povey, 2010). Nevertheless, there are other factors that govern susceptibility to organophosphate-induced neurotoxicity. In a number of previous studies, we explored the idea that prior exposures to chemicals from other classes that converge on some of the same neurotoxic endpoints as the organophosphates, could sensitize the brain to these pesticides (Aldridge et al., 2005; Meyer et al., 2005; Rhodes et al., 2004; Slotkin and Seidler, 2007); we focused on development because the major concern for the organophosphates is their propensity to elicit fetal and neonatal brain damage, including cell loss, impaired neurodifferentiation, deficient axonogenesis and synapse formation, and defective synaptic transmission, at exposures below the threshold for inhibition of cholinesterase, the mechanism that triggers systemic toxicity (Colborn, 2006; Rosas and Eskenazi, 2008; Slotkin, 2004). In particular, we evaluated the β -adrenergic agonist, terbutaline, a drug used “off-label” inappropriately, for long-term management of preterm labor (U.S. Food and Drug Administration, 2011). Preterm delivery is the largest contributor worldwide to cognitive loss, followed closely by the organophosphates as one of the most critical toxicologic factors (Bellinger, 2012). In evaluating whether the therapies of preterm labor might contribute to adverse neurodevelopmental outcomes, we found that prior exposure to terbutaline sensitized the developing brain to subsequent injury from chlorpyrifos, thus providing a proof-of-principle that early-life chemical exposures can create a subpopulation that is vulnerable to neurotoxicant injury (Aldridge et al., 2005; Meyer et al., 2005; Rhodes et al., 2004; Slotkin and Seidler, 2007).

In the current study, we expanded our investigations to the issue of whether glucocorticoids likewise enhance the neurodevelopmental effects of organophosphates. Glucocorticoid exposure is widespread. First, these agents are given as the consensus treatment for preterm labor occurring between 24 and 34 weeks of gestation in order to prevent respiratory distress syndrome (Gilstrap et al., 1995); currently, one of every ten newborns in the U.S. has undergone this treatment (Matthews et al., 2002). Second, endogenous glucocorticoid release from stress can enhance the systemic toxicity of organophosphates or other toxicants (Aisa et al., 2009; Colomina et al., 1995; Pung et al., 2006; Shaikh et al., 2003). By themselves, excess glucocorticoids have an adverse impact on the numbers of neurons, structure and synaptic connectivity in the immature brain, ultimately contributing to a variety of neuroendocrine, behavioral and cardiovascular disorders (Cavalieri and Cohen, 2006; Drake et al., 2007; Meyer, 1985; Moritz et al., 2005; Pryce et al., 2011; Rokyta et al., 2008; Tegethoff et al., 2009). Here, we examined the interaction between dexamethasone and chlorpyrifos using PC12 cells, a well-established model of neurodifferentiation (Costa, 1998; Fujita et al., 1989; Teng and Greene, 1994) that has been used previously to evaluate the mechanisms of developmental neurotoxicity separately for glucocorticoids and organophosphates (Bagchi et al., 1995; Das and Barone, 1999; Ebert et al., 1997; Jameson et al., 2006a, b; Radio et al., 2008; Slotkin et al., 2007; Slotkin and Seidler, 2012; Song et al., 1998). We chose concentrations of each agent based on prior studies showing robust effects on differentiation without cytotoxicity (Jameson et al., 2006b; Qiao et al., 2001; Song et al., 1998): 100 nM dexamethasone and 10 or 50 μ M chlorpyrifos. These concentrations are also appropriate for modeling human exposures. For dexamethasone, the typical levels in preterm infants after direct administration of a relatively low dose of 0.1–0.15 mg/kg is about 600 nM, or 60 nM in the fetus when the treatment is given to the mother (Charles et al., 1993; Osathanondh et al., 1977); as the recommended dose in preterm delivery is about three times higher (Gilstrap et al., 1995), our chosen concentration of 100 nM clearly occupies the therapeutic range. Similarly, our chosen chlorpyrifos concentrations lie at the upper end of levels measured in newborn infants in agricultural communities (Ostrea et al., 2002).

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% horse serum (Sigma Chemical Co., St. Louis, MO), 5% fetal bovine serum (Sigma), and 50 µg/ml penicillin streptomycin (Invitrogen). Incubations were carried out with 5% CO₂ at 37°C, standard conditions for PC12 cells. To initiate neurodifferentiation (Jameson et al., 2006a; Slotkin et al., 2007; Teng and Greene, 1994), the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (NGF; Promega Corporation, Madison, WI); each culture was examined under a microscope to verify the outgrowth of neurites after NGF treatment.

There were three different exposure paradigms, each using final concentrations of 100 nM dexamethasone (Sigma) and 10 or 50 µM chlorpyrifos (Chem Service, West Chester, PA). For studies of simultaneous exposure during neurodifferentiation, both agents were introduced concurrently with NGF, beginning 24 hr after the initial plating, and cells were cultured for an additional six days. To determine whether priming of the cells with dexamethasone had a different effect, we first added the dexamethasone simultaneously with NGF but delayed the chlorpyrifos exposure to begin 24 hr later; determinations were made six days after starting dexamethasone (i.e. 24 hr pretreatment with dexamethasone followed by five days of co-treatment with dexamethasone and chlorpyrifos). In a third set of studies, undifferentiated cells (without NGF) were exposed to dexamethasone starting 24 hr after initial plating; after 24 hr of exposure, chlorpyrifos was then introduced along with NGF and continued for five days of co-exposure with the dexamethasone. For all three exposure protocols, the medium was changed every 48 hr with the continued inclusion of NGF, dexamethasone and chlorpyrifos. Because of its limited water solubility, chlorpyrifos was dissolved in dimethylsulfoxide (Sigma; final concentration 0.1%), which was also added to all the samples regardless of treatment; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation (Qiao et al., 2001; Song et al., 1998).

Assays

Cells were harvested, washed, and the DNA and protein fractions were isolated and analyzed as described previously (Slotkin et al., 2007). Measurements of DNA, total protein and membrane protein were used as biomarkers for cell number, cell growth, and neurite growth (Qiao et al., 2003; Song et al., 1998). Neuronotypic cells contain a single nucleus, so that the DNA content per dish provides a measure of cell number (Winick and Noble, 1965). Since the DNA per cell is constant, cell growth entails an obligatory increase in the total protein per cell (protein/DNA ratio) as well as membrane protein per cell (membrane protein/DNA ratio). If cell growth represents simply an increase in the perikaryal area, then the ratio of membrane to total protein would fall in parallel with the decline in the surface-to-volume ratio (volume increases with the cube of the perikaryal radius, whereas surface area increases with the square of the radius); however, when neurites are formed as a consequence of neurodifferentiation, this produces a specific *rise* in the ratio. Each of these biomarkers has been validated in prior studies by direct measurement of cell number (Powers et al., 2010; Roy et al., 2005), perikaryal area (Roy et al., 2005) and neurite formation (Das and Barone, 1999; Howard et al., 2005; Song et al., 1998).

To assess neurodifferentiation into dopamine and acetylcholine phenotypes, we assayed the activities of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively (Jameson et al., 2006a, b). TH activity was measured using [^{14}C]tyrosine as a substrate and trapping the evolved $^{14}\text{CO}_2$ after decarboxylation coupled to L-aromatic amino acid decarboxylase. Each assay contained 55 μM [1- ^{14}C]-tyrosine (Moravsek Biochemicals, Brea, CA; specific activity, 51 mCi/mmol, diluted to 3.3 mCi/mmol with unlabeled tyrosine) as substrate and activity was calculated as pmol synthesized per hour per μg DNA (i.e. activity per cell). ChAT assays were utilized a substrate of 50 μM [^{14}C]acetyl-coenzyme A (specific activity 60 mCi/mmol; PerkinElmer Life Sciences, Waltham, MA). Labeled acetylcholine was extracted and activity calculated on the same basis as for TH.

Data analysis

All studies were performed using 2–3 separate batches of cells, with multiple independent cultures for each treatment in each batch ($n = 8\text{--}12$); each batch of cells comprised a separately prepared, frozen and thawed passage. Results are presented as mean $\pm$ SE, with treatment comparisons carried out by multivariate analysis of variance (ANOVA; data log-transformed when variance was heterogeneous) followed by Fisher's Protected Least Significant Difference Test for post-hoc comparisons of individual treatments. Each treatment paradigm involved an initial three-factor ANOVA: factor 1 = with vs. without dexamethasone; factor 2 = chlorpyrifos concentration (0, 10 μM , 50 μM); factor 3 = cell batch. In each case, we found that the treatment effects were the same across the different batches of cells, although the absolute values differed from batch to batch. Accordingly, we normalized the results across batches prior to combining them for presentation. Significance was assumed at $p < 0.05$.

RESULTS

Exposure of differentiating PC12 cells to chlorpyrifos resulted in a concentration-dependent decline in DNA, indicating a reduction in the number of cells (Fig. 1A). By itself, dexamethasone also produced decrements in cell number of about the same magnitude as chlorpyrifos. The combined exposure produced effects that were significantly less than additive (dexamethasone $\times$ chlorpyrifos interaction, $p < 0.002$), with the effect restricted to the lower chlorpyrifos concentration ($p < 0.002$). At the higher chlorpyrifos concentration, the combined treatment produced effects that were additive (no interaction) and accordingly, the net effect of coexposure was greater than for either agent alone. The total protein/DNA ratio likewise showed significant main effects of both chlorpyrifos and dexamethasone, reflecting overall elevations indicative of increased cell size (Fig. 1B). In this case, the two agents showed no significant interactions; the net effect of the combination was therefore additively greater than either of the two individual effects. For the membrane protein/DNA ratio, each agent caused a significant increase by itself, with a significant interaction between the two treatments (Fig. 1C). Although dexamethasone reduced the effect of the low chlorpyrifos concentration ($p < 0.02$), the combination with the high concentration again showed additively greater effects than with either agent alone. Because the result for membrane protein was different from that from total protein, we examined the membrane protein/total protein ratio to see if neurite formation was affected (Fig. 1D). There was a main treatment effects for dexamethasone, reflecting overall lower ratios in the groups receiving the glucocorticoid, but dexamethasone also altered the response to chlorpyrifos (dexamethasone $\times$ chlorpyrifos interaction, $p < 0.05$), reflected in a switch from slight promotion by the organophosphate to slight inhibition.

We next examined the effects of dexamethasone and chlorpyrifos on neurodifferentiation into dopamine and acetylcholine phenotypes. Both agents evoked significant increases in TH activity but dexamethasone had a much larger effect than chlorpyrifos (Fig. 2A). The

combined exposure evoked increases that were larger than with either agent alone but indistinguishable from simple additive effects (no dexamethasone $\times$ chlorpyrifos interaction). In contrast to the promotional effect on TH, chlorpyrifos and dexamethasone by themselves produced a net decrease in ChAT, again with a substantially greater effect from dexamethasone (Fig. 2B). For ChAT, there was a highly significant dexamethasone $\times$ chlorpyrifos interaction, reflecting a switch from reductions elicited by chlorpyrifos, to a promotional effect in the presence of dexamethasone. This basic difference was further reflected by the TH/ChAT ratio (Fig. 2C). By themselves, both chlorpyrifos and dexamethasone increased the ratio, with a much larger effect from the glucocorticoid; but in the presence of dexamethasone, chlorpyrifos produced a decrement in TH/ChAT.

We performed two additional studies to determine if the sequence of exposures to the two agents made a difference. In the first study, cells were exposed to dexamethasone simultaneously with the introduction of NGF, and chlorpyrifos was then introduced 24 hr later, with the cultures maintained for an additional five days. Accordingly, these cells were exposed to dexamethasone for six days but to chlorpyrifos for only five days. Again, chlorpyrifos produced a concentration-dependent decrement in DNA, of about the same magnitude as that evoked by dexamethasone (Fig. 3A). As with the simultaneous exposure model, the combination of dexamethasone with the high concentration of chlorpyrifos produced a larger deficit in DNA than that seen with either agent alone; in this model, dexamethasone did not significantly protect against the low concentration of chlorpyrifos (no dexamethasone $\times$ chlorpyrifos interaction), although the direction of effect was similar. Both dexamethasone and chlorpyrifos again produced increments in the protein/DNA ratio with an additively augmented effect from combined exposure (Fig. 3B). For the membrane protein/DNA ratio, dexamethasone again reduced the effect of chlorpyrifos (Fig. 3C); in this model, although the interaction was not significant by itself, the outcome was also statistically indistinguishable from that seen with simultaneous exposure and was in the same direction, so that a three-factor test (dexamethasone, chlorpyrifos, exposure model) showed a significant dexamethasone $\times$ chlorpyrifos interaction ($p < 0.01$) but no interaction of dexamethasone $\times$ chlorpyrifos $\times$ exposure model. The slightly smaller effect seen with this model obviated the ability to detect any interactions for the membrane protein/total protein ratio (Fig. 3D).

For the third exposure model, we exposed undifferentiated cells to dexamethasone for 24 hr before introducing adding NGF and chlorpyrifos, with exposures then continued for an additional five days. Again, for DNA we observed the same protective effect of dexamethasone against the low concentration of chlorpyrifos but additive worsening of the outcome at the high concentration (Fig. 4A). The total protein/DNA ratio likewise showed additive increments (Fig. 4B) and in this model, the same effect was seen for the membrane protein/DNA ratio (Fig. 4C), a result that was statistically distinct from the effects with the continuous coexposure paradigm (dexamethasone $\times$ chlorpyrifos $\times$ exposure model, $p < 0.04$); accordingly, there was only a main effect of dexamethasone on the membrane protein/total protein ratio, without any interaction with chlorpyrifos (Fig. 4D).

DISCUSSION

Our results show that combined exposure to dexamethasone and chlorpyrifos results in greater neuronal cell loss than with either agent alone, with more complex interactions for specific processes of neurodifferentiation (neurite outgrowth and neurotransmitter phenotype). The augmented cell loss reflects the fact that the two agents reduce cell number through different mechanisms; chlorpyrifos is directly antimitotic and proapoptotic (Qiao et al., 2001; Rush et al., 2010; Slotkin and Seidler, 2012; Song et al., 1998; Tan et al., 2009), whereas dexamethasone enhances the switch from cell replication to neurodifferentiation

(Ebert et al., 1997; Jameson et al., 2006b). Notably, these effects were seen at exposures that are not cytotoxic (Jameson et al., 2006b; Qiao et al., 2001; Song et al., 1998), and indeed, dexamethasone actually promotes cell viability (Jameson et al., 2006b). Furthermore, neither the individual effects nor the combination impaired cell growth but rather, there was significant increase in the total protein/DNA ratio reflecting cellular hypertrophy. The promotion of cell growth was again enhanced by coexposure to both agents. The greater deficits in DNA therefore reflect convergence of dexamethasone and chlorpyrifos on the specific developmental mechanisms that govern cell acquisition and loss, distinct from effects on cell growth.

In our earlier work, we showed that dexamethasone impairs neurite outgrowth (Jameson et al., 2006b); chlorpyrifos, on the other hand, has complex effects on neuritogenesis, retarding the growth of long axons while promoting shorter, dendritic branching (Das and Barone, 1999; Howard et al., 2005; Sachana et al., 2008; Song et al., 1998). Here, we examined the expansion of the cell surface through assessment of membrane proteins. Since both dexamethasone and chlorpyrifos, alone or in combination, reduced the number of cells, the membrane protein/DNA ratio was increased. However, the effects were not parallel to those detailing overall cell growth (total protein/DNA ratio), indicating a specific effect on neurite formation superimposed on general cellular hypertrophy. Accordingly, we examined the proportion of cell proteins associated with the membrane fraction (membrane protein/total protein ratio) as an index of effects on neurite outgrowth. In keeping with their known individual effects, chlorpyrifos produced a small but significant increase in the ratio whereas dexamethasone elicited a decrease. However, with the combined exposure chlorpyrifos produced a decrement in the ratio instead of an increase, indicating a change in the impact of the organophosphate on neurodifferentiation when the cells were primed with glucocorticoids. To confirm this interpretation, we explored differentiation of the cells into the two characteristic neurotransmitter phenotypes, dopamine and acetylcholine. By themselves, dexamethasone or chlorpyrifos promoted the dopaminergic phenotype at the expense of the cholinergic phenotype, as shown by enhanced TH and suppressed ChAT, resulting in a significant rise in the TH/ChAT ratio. With combined exposure, we found a corresponding enhancement of the effect on TH, in other words, a greater effect than with either agent alone. However, for ChAT, when chlorpyrifos was given to the cells that were exposed to dexamethasone, it instead evoked an *increase* in ChAT that was proportionately greater than its effect on TH, thus causing a *decrease* in the TH/ChAT ratio. Accordingly, priming with glucocorticoids renders chlorpyrifos promotional for differentiation into the cholinergic phenotype, whereas by itself it is inhibitory, indicating a fundamental change in the impact of the organophosphate on neurodifferentiation.

We also observed some subtle differences when the exposure conditions were changed to lower chlorpyrifos concentrations or different sequencing of the coexposures. At the lower chlorpyrifos concentration, dexamethasone actually appeared to exert some protection against cell loss, so that the combined exposure produced a decrement in DNA that was indistinguishable from that of dexamethasone alone. This carried over to the membrane protein/DNA ratio but not to the other indices of effects on growth or neurodifferentiation. Glucocorticoids are simultaneously injury-promoting and neuroprotective against neurotoxic injury (Abraham et al., 2001; Chumas et al., 1993; Hassan et al., 1996; Macaya et al., 1998), so this likely accounts for some of the aspects where dexamethasone appeared to reduce or mask some chlorpyrifos effects but not others. Indeed, chlorpyrifos produces neurodevelopmental injury through multiple mechanisms (Gupta, 2004; Slotkin, 2004), so that some can be worsened and others obtunded by dexamethasone. For example, glucocorticoids enhance the damage associated with oxidative stress (Ahlbom et al., 2000; Mulholland et al., 2005), which is one of the known mechanisms of chlorpyrifos-induced injury (Bagchi et al., 1995; Crumpton et al., 2000; Gupta, 2004; Qiao et al., 2005); so where

the effects are increased by the coexposure, this probably reflects participation of that particular mechanism. We also examined whether preexposure to dexamethasone, rather than coexposure, might have different effects, exploring two scenarios where the preexposure occurred in the undifferentiated state or during neurodifferentiation. The results were quite similar to those of the simultaneous exposure model, with only minor variations; notably, though, dexamethasone preexposure in the undifferentiated state appeared to reduce any protective effect directed toward membrane proteins.

The limitations inherent in an in vitro model of developmental neurotoxicity were detailed in our previous work (Qiao et al., 2001; Song et al., 1998) but it is worth repeating the major points here. First, although in vitro models allow for dissection of direct cause-and-effect relationships, they lack the ability to detect many of the important neurodevelopmental events involved in brain assembly, including cell-to-cell interactions and architectural modeling of brain regions. Second, in vitro exposures typically involve treatments over a period of hours, whereas in vivo exposures encompass much more extended exposure periods. Third, transformed cell lines such as PC 12 cells, usually are less sensitive to toxicants than are primary neurons. All these factors mean that it is difficult to extrapolate relevant in vivo concentrations of toxicants from in vitro results alone, and typically, the concentrations required for a given effect in vitro will be substantially higher than those required for parallel effects in vivo. Although our chlorpyrifos concentrations lie at the upper end of prenatal exposures in agricultural settings (Ostrea et al., 2002), much lower exposures can cause clear-cut behavioral and structural damage (Rauh et al., 2011; Rauh et al., 2012). It is thus important to note that the results for lower exposures in humans exactly parallel those predicted from higher-dose exposures in animals or even higher concentrations in vitro (Slotkin, 1999, 2004, 2005). In a similar vein, the chlorpyrifos concentrations used in the present study also exceed the threshold for cholinesterase inhibition (Das and Barone, 1999) but the effects on cell replication and differentiation are not related to anticholinesterase actions (Das and Barone, 1999; Slotkin, 1999, 2004, 2005). Indeed, in animal studies, cell loss and inhibition of axonal outgrowth and synaptogenesis clearly occur independently of cholinesterase effects and below the threshold for inhibition of this enzyme (Slotkin, 1999, 2004, 2005). Accordingly, the noncholinesterase mechanisms are also the ones that are likely to produce the developmental neurotoxicity of low-dose exposures in humans (Rauh et al., 2011; Rauh et al., 2012).

In conclusion, our results show that combined exposure of developing neuronotypic cells to dexamethasone and chlorpyrifos results in enhanced cell loss, greater impairment of neurite formation, and enhanced neurodifferentiation into the dopamine phenotype. For the cholinergic phenotype, however, dexamethasone entirely changed the response to chlorpyrifos: whereas chlorpyrifos by itself reduces cholinergic differentiation, in the presence of dexamethasone, it was promotional. Accordingly, if these effects occur in vivo, we would expect to see worsened neurobehavioral outcomes reflecting neuronal cell deficits and impaired connectivity, as well as more subtle alterations evoked by shifts in specific neurotransmitter systems. Because we observed protective effects of dexamethasone against some aspects of chlorpyrifos-induced toxicity at lower organophosphate concentrations, the outcomes may also show some differences for low vs. high exposures. Our results point to the likelihood that developmental exposure to glucocorticoids, either in the context of stress or pharmacologic management of preterm labor, leads to a subpopulation that is likely to show enhanced vulnerability to environmental neurotoxicants. We are currently conducting parallel studies in vivo in developing rats to confirm these predictions.

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Abbreviations

ANOVA	analysis of variance
ChAT	choline acetyltransferase
NGF	nerve growth factor
TH	tyrosine hydroxylase

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Figure 1.

Indices of cell number, cell growth and neurite formation in PC12 cells exposed simultaneously to 100 nM dexamethasone, with and without 10 or 50 μ M chlorpyrifos during neurodifferentiation: (A) DNA, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane/total protein ratio. Bars represent mean $\pm$ SE obtained from 8–12 independent cultures for each condition. ANOVA for main treatment effects and interactions appears at the top of each panel and asterisks denote significant differences evoked by chlorpyrifos compared to the corresponding control condition. Abbreviation: CPF = chlorpyrifos.

Figure 2.

Differentiation into dopamine and acetylcholine neuronal phenotypes in PC12 cells exposed simultaneously to 100 nM dexamethasone, with and without 10 or 50 μ M chlorpyrifos during neurodifferentiation: (A) TH activity, (B), ChAT activity, (C) TH/ChAT ratio. Bars represent mean $\pm$ SE obtained from 8–12 independent cultures for each condition. ANOVA for main treatment effects and interactions appears at the top of each panel and asterisks denote significant differences evoked by chlorpyrifos compared to the corresponding control condition. Abbreviation: CPF = chlorpyrifos.

Figure 3.

Indices of cell number, cell growth and neurite formation in PC12 cells exposed to 100 nM dexamethasone for the first 24 hr of neurodifferentiation, followed by treatment with and without 10 or 50 μM chlorpyrifos for an additional five days: (A) DNA, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane/total protein ratio. Bars represent mean $\pm$ SE obtained from 8–12 independent cultures for each condition. ANOVA for main treatment effects and interactions appears at the top of each panel and asterisks denote significant differences evoked by chlorpyrifos compared to the corresponding control condition. Abbreviation: CPF = chlorpyrifos.

Figure 4.

Indices of cell number, cell growth and neurite formation in PC12 cells exposed to 100 nM dexamethasone for 24 hr in the undifferentiated state, followed by addition of NGF and treatment with and without 10 or 50 μM chlorpyrifos for an additional five days: (A) DNA, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane/total protein ratio. Bars represent mean $\pm$ SE obtained from 8–12 independent cultures for each condition. ANOVA for main treatment effects and interactions appears at the top of each panel and asterisks denote significant differences evoked by chlorpyrifos compared to the corresponding control condition. Abbreviation: CPF = chlorpyrifos.