Published in final edited form as:

Brain Res Bull. 2009 March 30; 78(6): 313–322. doi:10.1016/j.brainresbull.2008.09.020.

Is Fipronil Safer than Chlorpyrifos? Comparative Developmental Neurotoxicity Modeled in PC12 Cells

T. Leon Lassiter, Emiko A. MacKillop, Ian T. Ryde, Frederic J. Seidler, and Theodore A. Slotkin
Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, North Carolina USA

Abstract

Fipronil, a GABA_A receptor antagonist, is replacing many insecticide uses formerly fulfilled by organophosphates like chlorpyrifos. Few studies have addressed the potential for fipronil to produce developmental neurotoxicity. We compared the neurotoxicity of fipronil and chlorpyrifos in undifferentiated and differentiating neuronotypic PC12 cells, evaluating indices of cell replication, cell number, differentiation, and viability for short- and long-term exposures. Fipronil inhibited DNA and protein synthesis in undifferentiated PC12 cells and evoked oxidative stress to a greater extent than did chlorpyrifos, resulting in reduced cell numbers even though cell viability was maintained. In differentiating cells, fipronil displayed an even lower threshold for disruption of development, reducing cell numbers without impairing cell growth, and promoting emergence of neurotransmitter phenotypes; superimposed on this effect, the phenotypic balance was shifted in favor of dopamine as opposed to acetylcholine. Differentiation also enhanced the susceptibility to fipronil-induced oxidative stress, although antioxidant administration failed to provide protection from cell loss. At low concentrations maintained for prolonged periods, fipronil had a biphasic effect on cell numbers, increasing them slightly at low concentrations, implying interference with apoptosis, while nevertheless reducing cell numbers at higher concentrations. Our results suggest that fipronil is inherently a more potent disruptor of neuronal cell development than is chlorpyrifos. The neurodevelopmental effects are not predicated on GABA_A antagonist properties, since PC12 cells lack the GABA_A receptor. If fipronil is intended to provide greater safety than chlorpyrifos, then this will have to entail advantages from factors that are yet unexamined: exposure, persistence, pharmacokinetics.

Keywords

Chlorpyrifos; Fipronil; In vitro; Neurodevelopment; Organophosphate insecticides; PC12 cells; Pesticides; Phenylpyrazole insecticides; Vitamin E

INTRODUCTION

Developmental neurotoxicity represents one of the major health concerns for insecticide exposure of the human population [13,35,45,49,69,81]. A great deal of attention has been paid to the organophosphates, which represent 50% of world-wide insecticide use [11] and the

Correspondence: Dr. T.A. Slotkin, Box 3813 DUMC, Duke Univ. Med. Ctr., Durham, NC 27710, Tel 919 681 8015, Fax 919 684 8197, t.slotkin@duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

adverse effects of these agents on brain development, particularly those of chlorpyrifos (CPF) [15,21,48,50,59,61], have led to increasing restrictions on their application in the U.S. and other countries. Some of the usages vacated by the organophosphates are being filled by agents that act through different mechanisms, notably fipronil (FPN), a phenylpyrazole insecticide that noncompetitively antagonizes GABA_A receptors [12,26] as well as glutamate-activated chloride channels [28,88]. FPN is widely applied in agriculture, termite/fire ant control, and in pet ectoparasite treatment [31,72]. The available findings show that FPN is a potent toxicant affecting environmental species populations and potentially, humans [46,72]. Although there is scant information about the potential for fipronil to elicit developmental neurotoxicity, a recent study in zebrafish showed that the immature nervous system may be especially sensitive, with adverse effects on structure and behavior unrelated to the targeting of GABA_A receptors [67]. In turn, this implies that tests for efficacy or toxicity that depend on the mechanism for adult toxicity or insecticidal activity are not likely to provide adequate prediction of neurodevelopmental effects; the situation with fipronil thus resembles that for organophosphates, where the developmental neurotoxicity involves mechanisms over and above cholinesterase inhibition, the target related to systemic toxicity and on which most regulatory guidelines are based [11,13,15,50,58,59,61].

In the current study, we evaluated the developmental neurotoxicity of FPN in PC12 cells, a neuronotypic cell line derived from rat pheochromocytoma that has been widely used as a model of neuronal development [70]. Because they are transformed cells, the PC12 line enables the delineation of adverse effects aimed at mitotic activity, whereas cultured primary neurons do not maintain cell division and thus cannot detect adverse effects on this likely neurotoxic target. When PC12 cells are treated with nerve growth factor (NGF), they gradually cease dividing and instead undergo neurodifferentiation, with development of neuritic projections, electrical excitability, and neurotransmitter phenotypes for dopamine and acetylcholine [23, 66,70]. In contrast, primary neurons provide a heterogeneous population of cell types, discoordinated staging of differentiation, and a large number of phenotypes. For those reasons, the PC12 model has been used to characterize essential features of the developmental neurotoxicity of organophosphates [5,6,17,18,20,22,30,39,44,51,52,66,76] as well as neurotoxic drugs, metals, organometals and organochlorine pesticides like dieldrin [1,9,16, 41,47,56,64,71]. Hence, our objective here was to evaluate the propensity of fipronil to elicit developmental neurotoxicity, specifically in comparison to CPF.

Our evaluations were based on established protocols for screening of developmental neurotoxicants with PC12 cells [51–53,62–64,66], and in keeping with recent recommendations by the Inspector General of the U.S. Environmental Protection Agency [78]. In undifferentiated cells, we assessed antimitotic activity through measurement of [³H] thymidine incorporation into DNA in comparison to more generalized metabolic effects monitored through [³H]leucine incorporation into protein. Effects on cell number were determined by measuring DNA content, since each neuronotypic cell contains only a single nucleus [83]. Effects on cell viability were monitored by the ability to exclude the dye, trypan blue, and oxidative stress was evaluated by measuring lipid peroxidation through malondialdehyde (MDA) formation [25,52]. In differentiating cells, we evaluated indices of cell number (DNA), cell size (total protein/DNA) and membrane outgrowth associated with the formation of neurites (membrane protein/total protein), along with the same measures of viability and oxidative stress. Finally, we assayed the two enzymes delineating differentiation into the dopamine and acetylcholine phenotypes, tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively.

MATERIALS AND METHODS

All of the techniques used in this study have appeared in previous papers and accordingly, only brief descriptions of procedures will be given.

Cell cultures

Because of the clonal instability of the PC12 cell line [23], the experiments were performed on cells that had undergone fewer than five passages. PC12 cells (American Type Culture Collection, 1721-CRL; obtained from the Duke Comprehensive Cancer Center, Durham, NC) were grown as described earlier [17,53,66], in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% inactivated horse serum (Sigma Chemical Co., St. Louis, MO), 5% fetal bovine serum (Sigma Chemical Co.), and 50 µg/ml penicillin streptomycin (Invitrogen); cells were incubated with 7.5% CO₂ at 37°C. For studies in the undifferentiated state, the medium was changed 24h after seeding to include varying concentrations of FPN (98.8% purity; Chem Service) or, for comparison, 30 µM CPF (98.8% purity, Chem Service, West Chester, PA). Because of the limited water solubility of FPN and CPF, all test agents were dissolved in dimethylsulfoxide to achieve a final concentration in the culture medium of 0.1%, which has no effect on replication or differentiation of PC12 cells [51,53,66]; control cultures contained the same concentration of dimethylsulfoxide. For studies in differentiating cells, 24h after seeding, the medium was changed to include 50 ng/mL of 2.5 S murine NGF (Invitrogen) and dimethylsulfoxide with or without the test agents, and were examined for up to 6 days, with medium changes (including test agents) every 48 hr. We chose the CPF concentration to elicit a robust response for each of the effects to be compared to the actions of FPN, inhibition of DNA synthesis, interference with cell acquisition and production of oxidative stress, but below the threshold for outright cytotoxicity or loss of viability [5,18,20,30,51–53,64,66]. For FPN, we evaluated a concentration range spanning two orders of magnitude (1 to 100 µM), bracketing the CPF concentration; although we used only a single CPF concentration for reference in the present studies, we have previously published full concentration-response information for CPF [30,51,63,64,66]. The actual human exposure to FPN is poorly explored, but, as just one example, topical pet treatment preparations contains as much as five orders of magnitude higher FPN concentrations than those used in our assays; with routine “petting” transfers to human skin reach 600 ppm [31].

DNA and protein synthesis

Cells were exposed to the test agents for varying amounts of time and then, for the final hour of exposure, the medium was changed to include the test agents along with 1 µCi/ml of either [³H]thymidine (specific activity, 2 Ci/mmol; PerkinElmer Life Sciences, Waltham, MA) for DNA synthesis, or [³H]leucine (specific activity, 60 Ci/mmol; PerkinElmer) for protein synthesis. After 1 hr, the medium was aspirated and cells were harvested. DNA and protein were precipitated and separated by established procedures [7,65]. We corrected incorporation values to the amount of DNA present in each culture to provide an index of DNA synthesis per cell [83] and the total DNA content was also recorded.

DNA and protein ratios

Cells were harvested, washed, and the DNA and protein fractions isolated and analyzed as described previously [51,53,66], with DNA and total protein analyzed by dye-binding [75]. To prepare the cell membrane fraction, the homogenates were sedimented at 40,000 × g for 10 min and the pellet was washed and resedimented. Aliquots of the final resuspension were then assayed for membrane protein.

Oxidative stress

We evaluated the degree of lipid peroxidation in undifferentiated cells after 24h of exposure to test agents, and in differentiating cells after 4 days of exposure. We measured the concentration of MDA by reaction with thiobarbituric acid using a modification [52] of published procedures [25]. To give the MDA concentration per cell, values were calculated relative to the amount of DNA.

Viability

To assess cell viability, the cell culture medium was changed to include trypan blue (1 volume per 2.5 volumes of medium; Sigma) and cells were examined for staining under 400 $\times$ magnification, counting an average of 100 cells per field in four different fields per culture. Assessments were made after 24h of exposure in undifferentiated cells and after 4 days for differentiating cells.

Enzyme activities

Differentiating cells were harvested after 6 days of exposure as already described, and were disrupted by homogenization in a ground-glass homogenizer fitted with a ground-glass pestle, using a buffer consisting of 154 mM NaCl and 10 mM sodium-potassium phosphate (pH 7.4). Aliquots were withdrawn for measurement of DNA [75]. ChAT assays were conducted by published techniques [36] using a substrate of 50 μ M [14 C]acetyl-coenzyme A (specific activity 60 mCi/mmol; PerkinElmer). Labeled acetylcholine was counted in a liquid scintillation counter and activity calculated as pmol synthesized per hour per μ g DNA. TH activity was measured using [14 C]tyrosine as a substrate and trapping the evolved 14 CO $_2$ after coupled decarboxylation [36,80]. Each assay contained 264 μ M [14 C]tyrosine (Sigma; specific activity, 438 mCi/mmol, diluted to 17.4 mCi/mmol with unlabeled tyrosine) as substrate and activity was calculated on the same basis as for ChAT.

Data analysis

All studies were performed multiple batches of cells, with several independent cultures for each treatment in each batch. Results are presented as mean $\pm$ SE, with treatment comparisons carried out by analysis of variance (ANOVA) followed by Fisher's protected least significant difference test for post hoc comparisons of individual treatments. In the initial test, we evaluated two ANOVA factors (treatment, cell batch) and 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

Effects on undifferentiated cells

Addition of FPN to undifferentiated PC12 cells elicited an immediate reduction in DNA synthesis with a threshold effect between 3 and 10 μ M (Figure 1A). With a 1h exposure to 30 μ M FPN, there was approximately the same inhibition as seen with 30 μ M CPF, and raising the FPN concentration to 100 μ M elicited an even greater decline in DNA synthesis. With more prolonged exposure, FPN became more effective than CPF (Figure 1B). After 24h, the inhibition of DNA synthesis by CPF was no greater than that seen at 1h, whereas even 3 μ M FPN produced a significant reduction equivalent to that of 30 μ M CPF. With the longer exposure, the adverse effect of FPN was enhanced at all concentrations, progressing to >90% arrest of DNA synthesis at 100 μ M. Although 24h of exposure to CPF did not reduce the total number of cells as monitored by DNA content, significant reductions were seen with 30 and 100 μ M FPN (Figure 1C).

To assess whether the binding of FPN to serum proteins provided protection against toxicity, we also determined whether the removal of serum from the medium would enhance the effect on DNA synthesis (Figure 1D). By itself, incubation of cells without serum produced higher [³H]thymidine incorporation than when DNA synthesis was measured in the presence of serum proteins, just as reported previously [51]. The effects of 30 μ M FPN were greatly enhanced (FPN $\times$ serum interaction, $p < 0.0001$) in the absence of serum proteins, showing nearly four times the effect as that obtained in the presence of the proteins.

To evaluate whether DNA synthesis is a specific target for FPN, we then compared the effects on protein synthesis using the same exposure paradigms (Figure 1E). A 1h exposure to FPN elicited declines in protein synthesis with a lower threshold ($< 1 \mu$ M) than that seen for DNA synthesis; extending the exposure to 24h again enhanced the effect of FPN, resulting in much greater inhibition than that seen with an equivalent concentration of CPF.

Despite the robust effects of FPN on DNA and protein synthesis and its ability to reduce the number of cells, a 24h exposure did not cause gross compromise of cell viability as evaluated by trypan blue exclusion (Figure 2A). Although there were significant overall effects of FPN, there was no clear concentration-response relationship and the maximum effect was only a few percent even at the highest concentration. Nevertheless, FPN readily produced oxidative stress, as evidenced by an increase in lipid peroxidation (Figure 2B). Even at 3 μ M FPN, the increase in MDA exceeded that caused by 30 μ M CPF, and raising the FPN concentration further produced correspondingly greater MDA values.

Effects on differentiating cells

Inclusion of FPN during NGF-induced differentiation had an adverse effect on the number of cells, as assessed by DNA content (Figure 3A). At first, the effects became progressively greater with the time of exposure, so that much greater cell loss was evident after 4 days than after 2 days in culture. However, by 6 days, low concentrations of FPN actually produced a small, but statistically significant increase in cell number, whereas the two highest concentrations still elicited reductions that were equivalent or greater than those evoked by 30 μ M CPF. It should be noted that the control values for DNA content cannot be compared for the three time points because different numbers of cells were plated so as to obtain approximately the same number of cells at the time of evaluation, regardless of the culture duration.

The cell loss seen at the two highest FPN concentrations was not associated with generalized growth inhibition, as the total protein/DNA ratio was significantly increased (Figure 3B). Only the highest concentration impaired membrane outgrowth, evidenced by a decrease in the membrane/total protein ratio (Figure 3C). We were puzzled by the slight increase in DNA content seen with prolonged exposure to low concentrations of FPN, and therefore performed an additional study to determine if FPN slowed the NGF-induced decrease in cell replication (Figure 3D). However, the results clearly indicated that FPN maintained its ability to inhibit DNA synthesis during cell differentiation, without showing a biphasic effect that could account for the effect on cell number.

Parallel to the determinations of cell viability and lipid peroxidation in undifferentiated PC12 cells, we performed the same determinations in differentiating cells after 4d of exposure, at the end of the period where all FPN concentrations produced lowering of cell numbers. Up to a threshold concentration of 30 μ M FPN, we did not observe any significant effects on trypan blue exclusion but raising the exposure to 50 or 100 μ M resulted in a progressive and massive loss of viability (Figure 4A), effects that had not been seen in the undifferentiated state. Differentiating cells also showed much greater sensitivity to oxidative stress, reaching MDA levels nearly five times those of controls at the highest FPN concentration (Figure 4B); again,

FPN elicited much greater lipid peroxidation than did CPF. We then used 10 μ M Vitamin E (α -tocopherol; Sigma) to determine whether oxidative stress was responsible for the cell loss caused by exposure to high FPN concentrations. Cotreatment of cells with Vitamin E completely eliminated MDA formation, reducing values in controls and FPN-exposed cells to immeasurably-low levels (Figure 4B). However, the protection against oxidative stress did not prevent cell loss (Figure 4C). We also performed a study to rule out the possibility that any residual Vitamin E in the cell preparation was interfering with the MDA assay, which would have artifactually reduced the values (data not shown).

Finally, we assessed the effects of FPN on differentiation of PC12 cells into their two major neurotransmitter phenotypes. The inclusion of NGF for six days resulted in a doubling of TH activity over that seen in undifferentiated cells (Figure 5A). Addition of either 3 or 30 μ M FPN produced significant, concentration-related increases in TH expression, reaching 3–4 times control values at 30 μ M. For ChAT, differentiation produced an even greater proportional increase over levels seen in undifferentiated PC12 cells (Figure 5B). Again, FPN enhanced the development of neurotransmitter phenotype, producing significant increases in ChAT compared to differentiating control cells. However, the effects on the acetylcholine phenotype were less robust than those seen for the dopamine phenotype, with a nonsignificant rise at 3 μ M FPN, and only a doubling of activity at 30 μ M. Consequently, the TH/ChAT ratio was higher in FPN-treated cells than in controls, connoting a preferential enhancement of the dopamine phenotype as compared to the acetylcholine phenotype (Figure 5C).

DISCUSSION

In earlier work, we and other investigators showed the close connection between the effects of CPF on replication and differentiation of PC12 cells and the corresponding actions on brain development in vivo [5,18,20,30,51–53,63,64,66]. This approach has since been extended successfully to a variety of related and unrelated developmental neurotoxicants [1,16,29,47, 52,62,64,71,87]. Here, we found that FPN disrupts neurodevelopment both during cell replication and differentiation, with greater potency and more widespread effects than those seen with CPF.

In undifferentiated PC12 cells, FPN, like CPF, evoked immediate reductions in DNA synthesis, evident even after only 1h of exposure. However, whereas the effects of CPF remained the same over a more extended, 24h exposure, the adverse effect of FPN intensified to ten-fold higher potency than CPF. Consequently, whereas CPF did not evoke a significant reduction in cell number with a 24h exposure, FPN did. The greater effects of FPN extended to major impairment of protein synthesis, whereas this parameter showed only minor effects in response to CPF, in agreement with earlier work [66]. Finally, FPN elicited much greater oxidative stress than did CPF, as evidenced by a threshold concentration for lipid peroxidation more than an order magnitude lower than that of CPF, and a much greater magnitude of effect at equimolar concentrations. Notably, these were all specifically *developmental* effects, rather than a reflection of generalized cytotoxicity, as the effects were all seen with exposures that did not evoke loss of cell viability.

With toxicant exposure in vivo, the net effect on the developing brain depends highly upon the binding of the agent to serum proteins, chiefly albumin, which reduce the diffusable, bioactive concentration [10,33,51,84,85]. This aspect also can be modeled in undifferentiated PC12 cells, which can be maintained without serum for brief periods of time, whereas differentiating cells undergo apoptosis when serum is withdrawn [24,73,74]. In our previous work, we showed that deletion of serum from the medium evokes a large increase in the antimitotic activity of CPF, consistent with high serum protein binding [51]. In the current study, we similarly found that the effects of FPN were markedly enhanced in the absence of serum. This may have important

implications for the developmental neurotoxicity of FPN in vivo. In the fetus and neonate, the concentration of serum proteins is lower than in adults, so that at the same toxicant concentration, a greater proportion is diffusible into the brain [85]. This could also contribute to increased sensitivity of subpopulations with reduced circulating plasma proteins as a result of malnutrition [42,54,82], especially in developing countries where exposures may be higher because protective measures against pesticide exposure are poorly enforced.

The progressive temporal course of the decline in DNA and protein synthesis, reduction in cell numbers, and lipid peroxidation evoked by FPN exposure in undifferentiated PC12 cells, combined with little evidence for loss of viability, suggested to us that FPN might be evoking these changes in part by enhancing the transition from neural cell replication to cell differentiation. Indeed, oxidative stress itself plays a key role as an endogenous pro-differentiation signal [32]. When we examined the effects of FPN on differentiating PC12 cells, we obtained clear evidence for such promotional actions. First, initiation of differentiation with NGF produced an enhanced sensitivity to FPN, as would be expected from convergence of the two agents on a common set of endpoints. Thus, the reductions in cell numbers became progressively greater with exposure during differentiation. Importantly, this was not associated with impaired cell growth, as would be expected from cytotoxicity or other, generalized adverse effects on development; instead, the protein/DNA ratio was increased by FPN, reflecting *enhanced* cell growth at the expense of cell numbers (decreased DNA), precisely what would be expected from accelerated differentiation. The membrane/total protein ratio, which reflects elaboration of neuritic projections, was unaffected until the FPN concentration was raised to 100 μ M, implying little or no compromise of this aspect of neurodevelopment despite the reduction in cell numbers. We also found an augmented sensitivity to FPN-induced oxidative stress in NGF-treated cells, consistent with convergent effects of the pesticide with the changes accompanying differentiation [52]. As with the undifferentiated cells, these effects could all be elicited with exposures that did not produce loss of viability as monitored with trypan blue, although raising the concentration even higher, to 50–100 μ M did eventually reduce viability.

However, our findings with TH and ChAT provide the most convincing proof of a prodifferentiation effect of FPN: we found increased expression of both enzymes with FPN exposure, consistent with promotion of both the dopamine and acetylcholine phenotypes that are characteristic of neurodifferentiation in this cell line. In this regard, FPN differs substantially from the effects of CPF, which enhances differentiation into the dopamine phenotype but suppresses the acetylcholine phenotype [30,64]. Thus, although both fipronil and CPF evoke an increase in the TH/ChAT ratio, they do so through different fundamental mechanisms, since FPN causes enhancement of both phenotypes, but more so for dopamine than for acetylcholine. Regardless of the difference in mechanism, both agents produce a switch in transmitter phenotype that is likely to produce “miswiring” of neural circuits, where nerve terminals containing a specific neurotransmitter are juxtaposed to postsynaptic sites expressing inappropriate receptors. These kinds of effects have been clearly demonstrated for CPF [2–4, 19,38], and our results in PC12 cells point to the need to perform similar in vivo evaluations for FPN.

We performed two additional tests to distinguish whether the decline in cell number evoked by FPN represented a toxic effect as opposed to a switch from cell replication to differentiation. First, we examined the decline in DNA synthesis accompanying differentiation by assessing [3 H]thymidine incorporation after a 2 day coexposure to NGF and FPN. Since our measurement corrects for the impact on the number of cells (incorporation per μ g DNA), simple cell loss would leave the ratio unaffected, whereas an acceleration of differentiation would reduce the ratio. We found a clear decrease even at 1 μ M FPN, with progressively greater effects up to 30 μ M, a concentration that still did not reduce cell viability. Second, we attempted to “rescue” the cells from the decline in DNA content by cotreatment with an antioxidant, Vitamin E. If

the lipid peroxidation is sufficiently high to produce cell loss through outright toxicity, then preventing this effect with Vitamin E should likewise obtund the decrease in DNA content. However, although the antioxidant clearly averted oxidative stress, it did not protect the cells from the decrease in DNA evoked by FPN. Again, this is consistent with a prodifferentiation effect of the pesticide, rather than cytotoxicity. At the same time, though, it effectively rules out the possibility that enhanced differentiation is solely a result of an increase in oxidative stress.

One puzzling feature was that, although FPN uniformly reduced cell numbers after 2 to 4 days of differentiation, by 6 days, we found a biphasic effect, viz. a significant *increase* in DNA content for 1–10 μ M FPN but reductions at higher concentrations. This did not reflect a slowing of the transition from cell replication to differentiation, especially as we did not see an elevated rate of DNA synthesis that would be expected from such an effect; indeed, we found the opposite. Further, a slowed transition would produce the effect at all time points, whereas this anomaly emerged between 4 and 6 days of differentiation. One possible explanation is a slowing of the cell loss that occurs through apoptosis as a normal consequence of neurodifferentiation [24,73]. Stimulation of nicotinic acetylcholine receptors provides an effective antiapoptotic signal, both in PC12 cells [40,68,79,86] and in the brain in vivo [8,37,43,57]. Here, we found that FPN enhanced PC12 cell differentiation into the acetylcholine phenotype, which might thus provide an antiapoptotic signal that results in maintenance of cells that otherwise would be fated to die. Clearly, further work is required to see if this is the case, but if these effects also occur with FPN exposure in vivo, they would have potentially devastating consequences. Apoptotic elimination of excess neurons is a critical feature of brain development, allowing for pruning of unutilized pathways, neural plasticity and learning, and architectural modeling of virtually all brain structures [34,55]. Interference with the normal elimination of superfluous neurons could thus evoke widespread disruption of brain circuitry. Notably, these effects are *not* a characteristic of CPF and may thus represent a major divergence between the consequences of exposure to FPN as opposed to the older pesticide.

Our results point to four key consequences of FPN exposure during neuronal cell development, several of which are likely to be interlinked: promotion of differentiation and a resultant shortfall of cell number, eliciting of oxidative stress, fostering of the dopamine phenotype over the acetylcholine phenotype, and delaying of cell death. Although we do not yet know the cellular mechanism(s) by which FPN produces these effects, they certainly do *not* depend on antagonism of GABA_A receptors, the mechanism that underlies FPN's insecticidal effects, since PC12 cells lack functional GABA_A receptors [27,77]. In that regard, our results reinforce findings in developing zebrafish, which likewise show neurodevelopmental toxicity of FPN unrelated to GABA_A antagonism [67]. Further, this outcome resembles an important lesson learned from the organophosphates, which likewise produce their developmental neurotoxicity unrelated to cholinesterase inhibition, the mechanism that underlies their insecticidal activity and systemic toxicity in adult animals [11,50,59,61].

Most importantly, though, our studies provide some of the first evidence to show that FPN can directly disrupt neuronal cell replication and differentiation, effects that if they occur in vivo, would likely produce developmental neurotoxicity and neurobehavioral anomalies. Indeed, in direct comparison to CPF, FPN appears to be more potent as a neurodevelopmental disruptor and to produce a wider spectrum of alterations, particularly during neurodifferentiation. If the global objective is to replace more dangerous pesticides like the organophosphates with supposedly safer alternatives like FPN, then our results point to the need to perform much more extensive examinations of the consequences of fetal and neonatal FPN exposure in vivo. Further, the fact that we found worse outcomes for FPN than for CPF indicates that, if there is indeed an improvement to be engendered by FPN use, it will have to reside in factors like

exposure, persistence and pharmacokinetics, features which can not readily be evaluated with in vitro models [14,60].

Acknowledgements

Acknowledgments/disclaimers: Research was supported by NIH ES10356 and the Leon Golberg Postdoctoral Fellowship. The authors do not have any conflicts of interest, but TAS and FJS have provided past expert witness testimony on behalf of government agencies, corporations and/or individuals.

Abbreviations

ANOVA	analysis of variance
ChAT	choline acetyltransferase
CPF	chlorpyrifos
FPN	fipronil
MDA	malondialdehyde
NGF	nerve growth factor
TH	tyrosine hydroxylase

References

1. Abreu-Villaça Y, Seidler FJ, Qiao D, Slotkin TA. Modeling the developmental neurotoxicity of nicotine in vitro: cell acquisition, growth and viability in PC12 cells. *Dev Brain Res* 2005;154:239–246. [PubMed: 15707677]
2. Aldridge JE, Levin ED, Seidler FJ, Slotkin TA. Developmental exposure of rats to chlorpyrifos leads to behavioral alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression. *Environ Health Perspect* 2005;113:527–531. [PubMed: 15866758]
3. Aldridge JE, Meyer A, Seidler FJ, Slotkin TA. Alterations in central nervous system serotonergic and dopaminergic synaptic activity in adulthood after prenatal or neonatal chlorpyrifos exposure. *Environ Health Perspect* 2005;113:1027–1031. [PubMed: 16079074]
4. Aldridge JE, Seidler FJ, Slotkin TA. Developmental exposure to chlorpyrifos elicits sex-selective alterations of serotonergic synaptic function in adulthood: critical periods and regional selectivity for effects on the serotonin transporter, receptor subtypes, and cell signaling. *Environ Health Perspect* 2004;112:148–155. [PubMed: 14754568]
5. Bagchi D, Bagchi M, Hassoun EA, Stohs SJ. In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicology* 1995;104:129–140. [PubMed: 8560491]
6. Bagchi D, Bhattacharya G, Stohs SJ. In vitro and in vivo induction of heat shock (stress) protein (Hsp) gene expression by selected pesticides. *Toxicology* 1996;112:57–68. [PubMed: 8792849]
7. Bell JM, Whitmore WL, Slotkin TA. Effects of α -difluoromethylornithine, a specific irreversible inhibitor of ornithine decarboxylase, on nucleic acids and proteins in developing rat brain: critical perinatal periods for regional selectivity. *Neuroscience* 1986;17:399–407. [PubMed: 2422585]

8. Belluardo N, Mudo G, Blum M, Fuxe K. Central nicotinic receptors, neurotrophic factors and neuroprotection. *Behav Brain Res* 2000;113:21–34. [PubMed: 10942029]
9. Benters J, Schafer T, Beyersmann D, Hechtenberg S. Agonist-stimulated calcium transients in PC12 cells are affected differentially by cadmium and nickel. *Cell Calcium* 1996;20:441–446. [PubMed: 8955559]
10. Braeckman RA, Audenaert F, Willems JL, Belpaire FM, Bogaert MG. Toxicokinetics of methyl parathion and parathion in the dog after intravenous and oral administration. *Arch Toxicol* 1983;54:71–82. [PubMed: 6639354]
11. Casida JE, Quistad GB. Organophosphate toxicology: safety aspects of nonacetylcholinesterase secondary targets. *Chem Res Toxicol* 2004;17:983–998. [PubMed: 15310231]
12. Chen L, Durkin KA, Casida JE. Structural model for gamma-aminobutyric acid receptor noncompetitive antagonist binding: widely diverse structures fit the same site. *Proc Natl Acad Sci* 2006;103:5185–5190. [PubMed: 16537435]
13. Colborn T. A case for revisiting the safety of pesticides: a closer look at neurodevelopment. *Environ Health Perspect* 2006;114:10–17. [PubMed: 16393651]
14. Costa LG. Neurotoxicity testing: a discussion of in vitro alternatives. *Environ Health Perspect* 1998;106(Suppl 2):505–510. [PubMed: 9599699]
15. Costa LG. Current issues in organophosphate toxicology. *Clin Chim Acta* 2006;366:1–13. [PubMed: 16337171]
16. Crumpton T, Atkins DS, Zawia NH, Barone S Jr. Lead exposure in pheochromocytoma (PC12) cells alters neural differentiation and Sp1 DNA-binding. *Neurotoxicology* 2001;22:49–62. [PubMed: 11307851]
17. Crumpton TL, Seidler FJ, Slotkin TA. Developmental neurotoxicity of chlorpyrifos in vivo and in vitro: effects on nuclear transcription factor involved in cell replication and differentiation. *Brain Res* 2000;857:87–98. [PubMed: 10700556]
18. Crumpton TL, Seidler FJ, Slotkin TA. Is oxidative stress involved in the developmental neurotoxicity of chlorpyrifos? *Dev. Brain Res* 2000;121:189–195.
19. Dam K, Garcia SJ, Seidler FJ, Slotkin TA. Neonatal chlorpyrifos exposure alters synaptic development and neuronal activity in cholinergic and catecholaminergic pathways. *Dev Brain Res* 1999;116:9–20. [PubMed: 10446342]
20. Das KP, Barone S. Neuronal differentiation in PC12 cells is inhibited by chlorpyrifos and its metabolites: is acetylcholinesterase inhibition the site of action? *Toxicol. Appl Pharmacol* 1999;160:217–230.
21. Eskenazi B, Rosas LG, Marks AR, Bradman A, Harley K, Holland N, Johnson C, Fenster L, Barr DB. Pesticide toxicity and the developing brain. *Basic Clin Pharmacol Toxicol* 2008;102:228–236. [PubMed: 18226078]
22. Flaskos J, McLean WG, Hargreaves AJ. The toxicity of organophosphate compounds towards cultured PC12 cells. *Toxicol Lett* 1994;70:71–76. [PubMed: 8310459]
23. Fujita K, Lazarovici P, Guroff G. Regulation of the differentiation of PC12 pheochromocytoma cells. *Environ Health Perspect* 1989;80:127–142. [PubMed: 2647474]
24. Fulle S, Mariggio MA, Belia S, Nicoletti I, Fano G. Nerve growth factor inhibits apoptosis induced by S-100 binding in neuronal PC12 cells. *Neuroscience* 1997;76:159–166. [PubMed: 8971768]
25. Guan ZZ, Yu WF, Nordberg A. Dual effects of nicotine on oxidative stress and neuroprotection in PC12 cells. *Neurochem Intl* 2003;43:243–249.
26. Hainzl D, Casida JE. Fipronil insecticide: novel photochemical desulfinylation with retention of neurotoxicity. *Proc Natl Acad Sci* 1996;93:12764–12767. [PubMed: 8917493]
27. Hales TG, Tyndale RF. Few cell lines with GABA_A mRNAs have functional receptors. *J Neurosci* 1994;14:5429–5436. [PubMed: 8083746]
28. Horoszok L, Raymond V, Sattelle DB, Wolstenholme AJ. GLC-3: a novel fipronil and BIDN-sensitive, but picrotoxinin-insensitive, L-glutamate-gated chloride channel subunit from *Caenorhabditis elegans*. *Br J Pharmacol* 2001;132
29. Jameson RR, Seidler FJ, Qiao D, Slotkin TA. Adverse neurodevelopmental effects of dexamethasone modeled in PC12 cells: identifying the critical stages and concentration thresholds for the targeting

- of cell acquisition, differentiation and viability. *Neuropsychopharmacology* 2006;31:1647–1658. [PubMed: 16319912]
30. Jameson RR, Seidler FJ, Qiao D, Slotkin TA. Chlorpyrifos affects phenotypic outcomes in a model of mammalian neurodevelopment: critical stages targeting differentiation in PC12 cells. *Environ Health Perspect* 2006;114:667–672. [PubMed: 16675418]
 31. Jennings KA, Canerdy TD, Keller RJ, Atieh BH, Doss RB, Gupta RC. Human exposure to fipronil from dogs treated with frontline. *Vet Hum Toxicol* 2002;44:301–303. [PubMed: 12361121]
 32. Katoh S, Mitsui Y, Kitani K, Suzuki T. Hyperoxia induces the differentiated neuronal phenotype of PC12 cells by producing reactive oxygen species. *Biochem Biophys Res Commun* 1997;241:347–351. [PubMed: 9425274]
 33. Krishna RB, Levitz M, Dancis J. Transfer of cocaine by the perfused human placenta: the effect of binding to serum proteins. *Am J Obstet Gynecol* 1993;169:1418–1423. [PubMed: 8267039]
 34. Kuan CY, Roth KA, Flavell RA, Rakic P. Mechanisms of programmed cell death in the developing brain. *Trends Neurosci* 2000;23:291–297. [PubMed: 10856938]
 35. Landrigan PJ. Pesticides and polychlorinated biphenyls (PCBs): an analysis of the evidence that they impair children's neurobehavioral development. *Mol Genet Metab* 2001;73:11–17. [PubMed: 11350178]
 36. Lau C, Seidler FJ, Cameron AM, Navarro HA, Bell JM, Bartolome J, Slotkin TA. Nutritional influences on adrenal chromaffin cell development: comparison with central neurons. *Pediatr Res* 1988;24:583–587. [PubMed: 2905035]
 37. Lee HK, Choi SS, Han EJ, Lee JY, Kwon MS, Shim EJ, Seo YJ, Suh HW. Role of nicotinic acetylcholine receptors in the regulation of kainic acid-induced hippocampal cell death in mice. *Brain Res Bull* 2004;64:309–317. [PubMed: 15561465]
 38. Levin ED, Addy N, Christopher NC, Seidler FJ, Slotkin TA. Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. *Dev Brain Res* 2001;130:83–89. [PubMed: 11557096]
 39. Li WW, Casida JE. Organophosphorus neuropathy target esterase inhibitors selectively block outgrowth of neurite-like and cell processes in cultured cells. *Toxicol Lett* 1998;98:139–146. [PubMed: 9788582]
 40. Li YX, King MA, Meyer EM. $\alpha 7$ Nicotinic receptor-mediated protection against ethanol-induced oxidative stress and cytotoxicity in PC12 cells. *Brain Res* 2000;861:165–167. [PubMed: 10751577]
 41. Matsuoka M, Igisu H. Induction of *c-fos* expression by tributyltin in PC12 cells: involvement of intracellular Ca^{2+} . *Environ Toxicol Pharmacol* 1996;2:373–380.
 42. Morgane PJ, Miller M, Kemper T, Stern W, Forbes W, Hall R, Bronzino J, Kissane J, Hawrylewicz E, Resnick O. The effects of protein malnutrition on the developing central nervous system in the rat. *Neurosci Biobehav Rev* 1978;2:137–230.
 43. Mudo G, Belluardo N, Fuxe K. Nicotinic receptor agonists as neuroprotective/neurotrophic drugs.: progress in molecular mechanisms. *J Neural Transm* 2007;114:135–147. [PubMed: 16906354]
 44. Nagata K, Huang CS, Song JH, Narahashi T. Direct actions of anticholinesterases on the neuronal nicotinic acetylcholine receptor channels. *Brain Res* 1997;769:211–218. [PubMed: 9374188]
 45. National Research Council. Pesticides in the diets of infants and children. National Academy Press; Washington D.C: 1993.
 46. Overmyer JP, Rouse DR, Avants JK, Garrison AW, Delorenzo ME, Chung KW, Key PB, Wilson WA, Black MC. Toxicity of fipronil and its enantiomers to marine and freshwater non-targets. *J Environ Sci Health B* 2007;42:471–480. [PubMed: 17562454]
 47. Parran DK, Barone S Jr, Mundy WR. Methylmercury decreases NGF-induced TrkA autophosphorylation and neurite outgrowth in PC12 cells. *Dev Brain Res* 2003;141:71–81. [PubMed: 12644250]
 48. Perera FP, Rauh V, Whyatt RM, Tang D, Tsai WY, Bernert JT, Tu YH, Andrews H, Barr DB, Camann DE, Diaz D, Dietrich J, Reyes A, Kinney PL. A summary of recent findings on birth outcomes and developmental effects of prenatal ETS, PAH, and pesticide exposures. *Neurotoxicology* 2005;26:573–587. [PubMed: 16112323]
 49. Physicians for Social Responsibility. Pesticides and Children. Physicians for Social Responsibility; Washington DC: 1995.

50. Pope CN. Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J. Toxicol Environ Health* 1999;2:161–181.
51. Qiao D, Seidler FJ, Slotkin TA. Developmental neurotoxicity of chlorpyrifos modeled *in vitro*: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells. *Environ Health Perspect* 2001;109:909–913. [PubMed: 11673119]
52. Qiao D, Seidler FJ, Slotkin TA. Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. *Toxicol Appl Pharmacol* 2005;206:17–26. [PubMed: 15963341]
53. Qiao D, Seidler FJ, Violin JD, Slotkin TA. Nicotine is a developmental neurotoxicant and neuroprotectant: stage-selective inhibition of DNA synthesis coincident with shielding from effects of chlorpyrifos. *Dev Brain Res* 2003;147:183–190. [PubMed: 14741763]
54. Resnick O, Miller M, Forbes W, Hall R, Kemper T, Bronzino J, Morgane PJ. Developmental protein malnutrition: influences on the central nervous system of the rat. *Neurosci Biobehav Rev* 1979;3:233–246. [PubMed: 44355]
55. Sastry PS, Rao KS. Apoptosis and the nervous system. *J Neurochem* 2000;74:1–20. [PubMed: 10617101]
56. Shafer TJ. Effects of Cd^{2+} , Pb^{2+} and CH_3Hg^+ on high voltage-activated calcium currents in pheochromocytoma (PC12) cells: potency, reversibility, interactions with extracellular Ca^{2+} and mechanisms of block. *Toxicol Lett* 1998;99:207–221. [PubMed: 9862287]
57. Shimohama S, Greenwald DL, Shafron DH, Akaika A, Maeda T, Kaneko S, Kimura J, Simpkins CE, Day AL, Meyer EM. Nicotinic $\alpha 7$ receptors protect against glutamate neurotoxicity and neuronal ischemic damage. *Brain Res* 1998;779:359–363. [PubMed: 9473725]
58. Slotkin TA. Developmental cholinotoxicants: nicotine and chlorpyrifos. *Environ Health Perspect* 1999;107(suppl 1):71–80. [PubMed: 10229709]
59. Slotkin TA. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol Appl Pharmacol* 2004;198:132–151. [PubMed: 15236950]
60. Slotkin TA. Guidelines for developmental neurotoxicity and their impact on organophosphate pesticides: a personal view from an academic perspective. *NeuroToxicology* 2004;25:631–640. [PubMed: 15183016]
61. Slotkin, TA. Developmental neurotoxicity of organophosphates: a case study of chlorpyrifos. In: Gupta, RC., editor. *Toxicity of Organophosphate and Carbamate Pesticides*. Elsevier Academic Press; San Diego: 2005. p. 293–314.
62. Slotkin TA, MacKillop EA, Melnick RL, Thayer KA, Seidler FJ. Developmental neurotoxicity of perfluorinated chemicals modeled *in vitro*. *Environ Health Perspect* 2008;116:716–722. [PubMed: 18560525]
63. Slotkin TA, MacKillop EA, Ryde IT, Seidler FJ. Ameliorating the developmental neurotoxicity of chlorpyrifos: a mechanisms-based approach in PC12 cells. *Environ Health Perspect* 2007;115:1306–1313. [PubMed: 17805420]
64. Slotkin TA, MacKillop EA, Ryde IT, Tate CA, Seidler FJ. Screening for developmental neurotoxicity using PC12 cells: comparisons of organophosphates with a carbamate, an organochlorine and divalent nickel. *Environ Health Perspect* 2007;115:93–101. [PubMed: 17366826]
65. Slotkin TA, Persons D, Slepatis RJ, Taylor D, Bartolome J. Control of nucleic acid and protein synthesis in developing brain, kidney, and heart of the neonatal rat: effects of α -difluoromethylornithine, a specific, irreversible inhibitor of ornithine decarboxylase. *Teratology* 1984;30:211–224. [PubMed: 6208628]
66. Song X, Violin JD, Seidler FJ, Slotkin TA. Modeling the developmental neurotoxicity of chlorpyrifos *in vitro*: macromolecule synthesis in PC12 cells. *Toxicol Appl Pharmacol* 1998;151:182–191. [PubMed: 9705902]
67. Stehr CM, Linbo TL, Incardona JP, Scholz NL. The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. *Toxicol Sci* 2006;92:270–278. [PubMed: 16611622]
68. Sun X, Liu Y, Hu G, Wang H. Protective effects of nicotine against glutamate-induced neurotoxicity in PC12 cells. *Cell Mol Biol Lett* 2004;9:409–422. [PubMed: 15332118]

69. Szpir M. New thinking on neurodevelopment. *Environ Health Perspect* 2006;114:A101–A107.
70. Teng, KK.; Greene, LA. Cultured PC12 cells: a model for neuronal function and differentiation. In: Celis, JE., editor. *Cell Biology: A Laboratory Handbook*. Academic Press; San Diego: 1994. p. 218-224.
71. Tian X, Sun X, Suszkiw JB. Upregulation of tyrosine hydroxylase and downregulation of choline acetyltransferase in lead-exposed PC12 cells: the role of PKC activation. *Toxicol Appl Pharmacol* 2000;167:246–252. [PubMed: 10986016]
72. Tingle CC, Rother JA, Dewhurst CF, Lauer S, King WJ. Fipronil: environmental fate, ecotoxicology, and human health concerns. *Rev Environ Contam Toxicol* 2003;176:1–66. [PubMed: 12442503]
73. Tong L, Perez-Polo JR. Effect of nerve growth factor on AP-1, NF- κ B, and Oct DNA binding activity in apoptotic PC12 cells: extrinsic and intrinsic elements. *J Neurosci Res* 1996;45:1–12. [PubMed: 8811508]
74. Tong LQ, Werrbach-Perez K, Perez-Polo JR. Prolonged activation of transcription factor AP-1 during NGF-mediated rescue from apoptotic cell death in PC12 cells. *Neurochem Res* 1999;24:1431–1441. [PubMed: 10555784]
75. Trauth JA, Seidler FJ, Slotkin TA. An animal model of adolescent nicotine exposure: effects on gene expression and macromolecular constituents in rat brain regions. *Brain Res* 2000;867:29–39. [PubMed: 10837795]
76. Tuler SM, Hazen AA, Bowen JM. Release and metabolism of dopamine in a clonal line of pheochromocytoma (PC12) cells exposed to fenthion. *Fund Appl Toxicol* 1989;13:484–492.
77. Tyndale RF, Hales TG, Olsen RW, Tobin AJ. Distinctive patterns of GABA_A receptor subunit mRNAs in 13 cell lines. *J Neurosci* 1994;14:5417–5428. [PubMed: 8083745]
78. U.S. Environmental Protection Agency, Opportunities to Improve Data Quality and Children's Health Through the Food Quality Protection Act. [[accessed 7 July 2006]. Report no. 2006-P-00009, 2006: Available: <http://www.epa.gov/oig/reports/2006/20060110-2006-P-00009.pdf>
79. Utsugisawa K, Nagane Y, Obara D, Tohgi H. Overexpression of $\alpha 7$ nicotinic acetylcholine receptor prevents G1-arrest and DNA fragmentation in PC12 cells after hypoxia. *J Neurochem* 2002;81:497–505. [PubMed: 12065658]
80. Waymire JC, Bjur R, Weiner N. Assay of tyrosine hydroxylase by coupled decarboxylation of dopa formed from 1-¹⁴C-L-tyrosine. *Anal Biochem* 1971;43:588–600. [PubMed: 4400965]
81. Weiss B, Amler S, Amler RW. Pesticides. *Pediatrics* 2004;113:1030–1036. [PubMed: 15060196]
82. West CD, Kemper TL. The effect of a low protein diet on the anatomical development of the rat brain. *Brain Res* 1976;107:221–237. [PubMed: 817786]
83. Winick M, Noble A. Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in the rat. *Dev Biol* 1965;12:451–466. [PubMed: 5884354]
84. Wu HX, Evreu-Gros C, Descotes J. Diazinon toxicokinetics, tissue distribution and anticholinesterase activity in the rat. *Biomed Environ Sci* 1996;9:359–369. [PubMed: 8988804]
85. Yaffe, SJ.; Stern, L. Clinical implications of perinatal pharmacology. In: Mirkin, BL., editor. *Perinatal Pharmacology and Therapeutics*. Academic Press; New York: 1976. p. 355-428.
86. Yamashita H, Nakamura S. Nicotine rescues PC12 cells from death induced by nerve growth factor deprivation. *Neurosci Lett* 1996;213:145–147. [PubMed: 8858629]
87. Zachor DA, Moore JF, Brezausk C, Theibert A, Percy AK. Cocaine inhibits NGF-induced PC12 cells differentiation through D₁-type dopamine receptors. *Brain Res* 2000;869:85–97. [PubMed: 10865062]
88. Zhao X, Yeh JZ, Salgado VL, Narahashi T. Sulfone metabolite of fipronil blocks gamma-aminobutyric acid- and glutamate-activated chloride channels in mammalian and insect neurons. *J Pharmacol Exp Ther* 2005;314:363–373. [PubMed: 15701711]

Figure 1.

Effects of fipronil (FPN) in comparison to chlorpyrifos (CPF) in undifferentiated PC12 cells: (A) DNA synthesis after a 1h exposure, (B) DNA synthesis after a 24h exposure, (C) DNA content after a 24h exposure, (D) DNA synthesis after a 2h exposure with and without inclusion of serum proteins, and (E) protein synthesis after a 1h or 24h exposure. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments appears at the top of each panel and asterisks denote values that differ significantly from the corresponding control. #Control with serum significantly different from control without serum.

Figure 2.

Effects of fipronil (FPN) in comparison to chlorpyrifos (CPF) in undifferentiated PC12 cells: (A) viability assessed with trypan blue after a 24h exposure, (B) lipid peroxidation after a 24h exposure. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments appears at the top of each panel and asterisks denote values that differ significantly from the corresponding control.

Figure 3.

Effects of fipronil (FPN) in comparison to chlorpyrifos (CPF) in differentiating PC12 cells: (A) DNA content measured after 2, 4, and 6 days of exposure; (B) total protein/DNA ratio after 6 days of exposure; (C) membrane/total protein ratio after 6 days of exposure; (D) DNA synthesis after a 48h exposure. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments appears at the top of each panel and asterisks denote values that differ significantly from the corresponding control. Absolute values in panel A cannot be compared for the three time points because different numbers of cells were plated so as to obtain approximately the same number of cells at the time of evaluation, regardless of the culture duration.

Figure 4.

Effects of fipronil (FPN) in comparison to chlorpyrifos (CPF) in differentiating PC12 cells: (A) viability assessed with trypan blue after a 4d exposure; (B) lipid peroxidation after a 4d exposure, with or without inclusion of 10 μ M Vitamin E; (C) DNA content after a 4d exposure, with or without inclusion of 10 μ M Vitamin E. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments appears at the top of each panel and asterisks denote values that differ significantly from the corresponding control. In panel B, the effect of CPF is actually marginally significant ($p = 0.052$) and would achieve statistical significance if compared to control by itself, without the inclusion of the FPN values showing greater effects.

Figure 5.

Effects of fipronil (FPN) on differentiation into dopamine and acetylcholine phenotypes, comparing undifferentiated cells to cells after 6 days of NGF-induced differentiation: (A) tyrosine hydroxylase activity, (B) choline acetyltransferase activity, (C) TH/ChAT ratio. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments appears at the top of each panel and asterisks denote values that differ significantly from the differentiating control.