

Developmental Cholinotoxicants: Nicotine and Chlorpyrifos

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The stimulation of cholinergic receptors in target cells during a critical developmental period provides signals that influence cell replication and differentiation. Accordingly, environmental agents that promote cholinergic activity evoke neurodevelopmental damage because of the inappropriate timing or intensity of stimulation. Nicotine evokes mitotic arrest in brain cells possessing high concentrations of nicotinic cholinergic receptors. In addition, the cholinergic overstimulation programs the expression of genes that evoke apoptosis and delayed cell loss. Effects of cholinesterase inhibitors exhibit many similarities to those of nicotine. Chlorpyrifos administered to developing rats in doses that do not evoke signs of overt toxicity decreased DNA synthesis and caused shortfalls in cell numbers in brain regions enriched in cholinergic innervation. In embryo cultures, chlorpyrifos also evoked apoptosis during neurulation. However, chlorpyrifos also evokes noncholinergic disruption of cell development by interfering with cell signaling via adenylyl cyclase, leading to widespread disruption that is not limited to cholinergic systems. We have tested this hypothesis *in vitro* with PC12 cells, which lack the enzymes necessary to produce chlorpyrifos oxon, the metabolite that inhibits cholinesterase. Chlorpyrifos inhibited DNA synthesis in undifferentiated PC12 cells, which have relatively few cholinergic receptors. Furthermore, chlorpyrifos was more effective than nicotine and its effects were not blocked by cholinergic antagonists. When cells were allowed to differentiate in the presence of chlorpyrifos, cell replication was inhibited even more profoundly and cell acquisition was arrested. At higher concentrations, chlorpyrifos also inhibited neuritic outgrowth. Thus, chlorpyrifos elicits damage by both noncholinergic and cholinergic mechanisms extending from early stages of neural cell replication through late stages of axonogenesis and terminal differentiation. Accordingly, the window of developmental vulnerability to chlorpyrifos is likely to extend from the embryonic period into postnatal life. — *Environ Health Perspect* 107(Suppl 1):71–80 (1999). <http://ehpnet1.niehs.nih.gov/docs/1999/Suppl-1/71-80slotkin/abstract.html>

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Neurotransmitters as Trophic Factors

Nearly four decades ago, Buznikov (1,2) demonstrated that neurotransmitters were present in high concentrations during specific phases of early development in sea urchin embryos, unrelated to their function in synaptic communication. Subsequently, transient expression of these substances and their specific receptors has been identified during ontogeny of the mammalian nervous system, and it is now certain that transmitters play essential roles in the cellular and architectural development of the brain (3,4). During

this period, receptor stimulation uniquely communicates with the genes that control cell differentiation, changing the ultimate fate of the cell (Figure 1). As these changes are not typical for the mature nervous system, the ontogenetic state of the target cell is critical in determining whether the outcome of receptor stimulation is an effect on cell replication, differentiation, growth, death (apoptosis), or “learning,” that is, determining the future set-point for responsiveness of the cell. At the same time, these multiple roles create a wide window of vulnerability in which exposure of the brain to neuroactive chemicals that elicit or block neurotransmitter responses can alter development. Thus, unlike classical teratology, in which the first trimester of fetal development is the most sensitive target for adverse effects of drugs or chemicals, brain development is likely to be affected by exposures ranging from the early embryonic stage through adolescence (5).

This review will focus on disruption of brain development elicited by agents targeting cholinergic transmission. Two of

the most widespread chemical assaults on the fetus are cholinergic: nicotine, a direct cholinergic agonist delivered by maternal cigarette smoking, and insecticides, which enhance cholinergic effects through inhibition of cholinesterase, the enzyme that hydrolyzes acetylcholine. A focus on cholinergic mechanisms is also appropriate given the critical role played by acetylcholine in brain maturation. Cholinergic stimulation is essential for establishment of cerebrocortical cytoarchitecture, and even transient interference with cholinergic input during development produces permanent structural and behavioral damage (6–8). Similarly, cholinergic overstimulation at an inappropriate time leads to developmental anomalies. In the rat, the peak of cholinergic tone in the cortex ordinarily occurs during the second postnatal week (9). Administration of cholinergic agonists before that time or dietary alterations that evoke early onset of cholinergic activity result in premature cessation of neuronal mitosis, leading to shortfalls in cell numbers and deficient synaptic activity (9–12). Accordingly, it is important to explore the mechanisms underlying the actions of cholinotoxicants and their impact on the developing brain.

Nicotine: Prototypic Cholinotoxicant

The largest toxic assault on fetal development is provided by maternal cigarette smoking, which involves approximately one-fourth of all pregnancies in the United States (13,14). Epidemiologic studies have established the tragic results: tens of thousands of spontaneous abortions and neonatal intensive care unit admissions annually, thousands of perinatal deaths and deaths from Sudden Infant Death Syndrome (crib death), and substantially increased incidence of learning disabilities, behavioral problems, and attention deficit/hyperactivity disorder (14–20). These findings do not, however, obligate an underlying cholinotoxic mechanism. Cigarette smoke contains thousands of bioactive compounds, including hydrogen cyanide and carbon monoxide. In addition, the smoking “life style” is associated with multiple risk factors including poor prenatal care and low

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Abbreviations used: AChE, acetylcholinesterase; ANOVA, analysis of variance; CPF, chlorpyrifos; NGF, nerve growth factor; NIC, nicotine; ODC, ornithine decarboxylase; PN, postnatal; Rx, treatment.

socioeconomic status. Accordingly, animal models are needed to isolate the role of nicotine from these confounding variables.

Unfortunately the design of animal models of nicotine exposure has not proven simple. Injection of nicotine into pregnant rats does produce behavioral (21–23) and cellular (24–26) abnormalities, but many of these effects are caused by vasoconstrictor effects on uteroplacental circulation, evoking episodic hypoxia (27–30). Nicotine injections produce high peak plasma levels of drug, inducing obvious ischemic episodes (blanching, cyanosis) with each dose (10,31). Accordingly, in the mid-1980s, we developed the first animal model of fetal nicotine exposure to make use of continuous infusions delivered by implantable osmotic minipumps (9,26,31–34), a delivery route that avoids hypoxia–ischemia, and that delivers a fixed dose of drug simulating the steady-state plasma levels seen in smokers or users of transdermal nicotine patches (35,36). Pharmacokinetic and pharmacodynamic differences dictate the use of higher overall doses in rats than in humans, so that the critical end point is matching the plasma concentrations and the corresponding pharmacologic effects (36,37). Thus, in rats, dose rates of 2 to 6 mg/kg/day are necessary to reproduce the nicotine plasma levels found in moderate (0.5 to 1 pack/day) to heavy (2 packs/day) smokers.

With the infusion model, we have been able to show definitive damage to developing rat brain by doses of nicotine that reproduce the plasma levels found in heavy smokers (26,31–34). Two indices of these adverse effects are illustrated in Figure 2. In animals exposed prenatally to nicotine, ornithine decarboxylase activity, a marker enzyme for cell damage, is elevated during the postnatal period in both early-developing (forebrain) and late-developing (cerebellum) brain regions even though nicotine exposure terminates at birth. During the same period, deficits in total cell number, as determined by DNA content, worsen. Subsequently, we found that genes associated with programmed cell death (apoptosis) are constitutively activated by prenatal nicotine exposure (38,39), with effects persisting into the period of maximal cell loss; direct morphological assessment of nicotine-exposed embryos confirmed the presence of numerous apoptotic cells (40). Nicotine-induced apoptosis in the developing brain is in direct contrast to the observation that nicotine exerts a neuroprotective effect in

the adult brain (41,42), including protection from injury-induced apoptosis (43,44). Just as with *c-fos* itself (45–47), the developmental context in which nicotine exposure occurs is likely to be critical for determining whether apoptosis is evoked or suppressed. Indeed, cholinergic agonists and antagonists can both elicit apoptosis depending on whether the context involves active or desensitized receptors (48). In the

context of extended exposure to nicotine during fetal development, persistent induction of *c-fos* clearly is associated with enhanced cell death (31), most likely from apoptosis (40).

We also identified a second mechanism for cell deficits caused by nicotine exposure (10): inhibition of DNA synthesis (Figure 3). Administration of even a single dose of nicotine to pregnant or neonatal

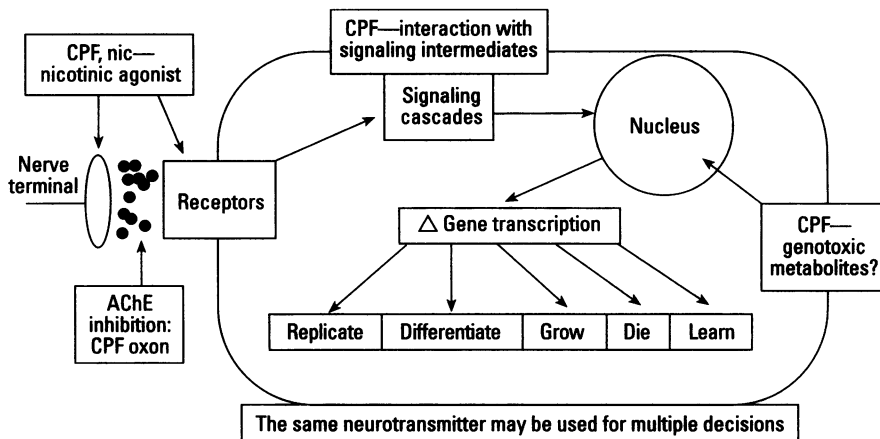


Figure 1. Cholinergic targeting of cell development. Abbreviations: AChE, acetylcholinesterase; CPF, chlorpyrifos; Nic, nicotine. During development, neurotransmitters, through their receptors and associated signaling cascades, control the genes that influence differentiation. Depending on the context in which stimulation occurs, the same neurotransmitter can promote cell replication, can elicit a switch from replication to differentiation, can promote or arrest cell growth, can evoke apoptosis, or can program the genes that determine the future responsiveness of the cell to external stimulation. Nicotine targets nicotinic cholinergic receptors located on target cells, directly evoking changes in gene expression. Presynaptic nicotinic receptors that modulate release of other neurotransmitters produce secondary alterations of target cell development through the actions of these other transmitters on their respective receptors, signaling cascades and gene expression (39). Chlorpyrifos through its active oxon metabolite inhibits acetylcholinesterase, preventing the breakdown of acetylcholine and thus enhancing cholinergic activity. In addition, chlorpyrifos can exhibit agonistlike properties, opening and then desensitizing nicotinic cholinergic receptor/ion channels (81), can interact with signaling intermediates such as G-proteins and adenylyl cyclase (80,82,83), or can produce oxidative damage to DNA (84,85).

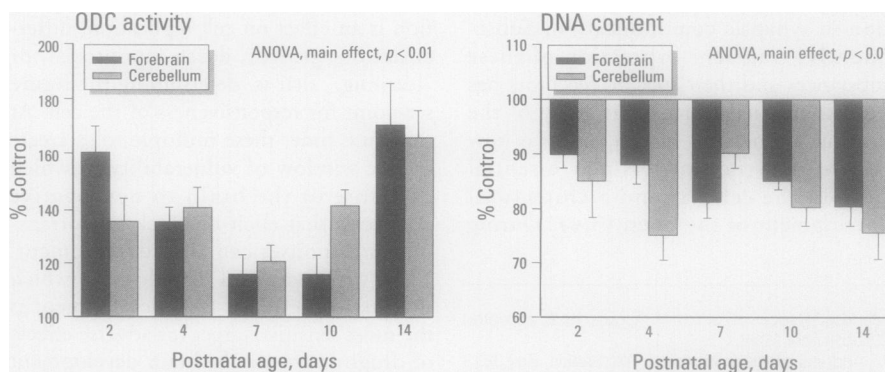


Figure 2. Effects of nicotine on biomarkers of cell damage (ornithine decarboxylase activity) and cell number (DNA content), evaluated in postnatal rat brain (32). Abbreviation: ODC, ornithine decarboxylase. Nicotine exposure elicits persistent damage (elevated ornithine decarboxylase activity) and cell loss (decreased DNA) despite discontinuing nicotine exposure at birth. Effects are discernible in both an early-developing region (forebrain) and late-developing region (cerebellum). Data represent means and standard errors obtained from 8 pups in each group at each age for each determination, with ANOVA main treatment effect indicated within the panels.

rats elicits a precipitous and persistent (several hours long) decline in DNA synthesis, with specific targeting of brain regions with the highest concentrations of nicotinic cholinergic receptors. The same effects are obtained when minute amounts of nicotine are introduced directly into the brain, bypassing any systemic drug effects (10).

Simply losing cells or preventing acquisition of the correct number of cells does not inherently account for neurobehavioral disruption by nicotine exposure; instead it is necessary to demonstrate that synaptic function is affected. Because nicotine works through cholinergic receptors, we first evaluated effects on cholinergic transmission (9,49). Using biochemical indices of neuronal impulse activity, we found that prenatal nicotine exposure blunted the ontogenetic rise of synaptic activity in the forebrain and produced persistent deficits in the hippocampus (Figure 4). However, adverse functional effects are not limited to cholinergic neurotransmission. Nicotinic receptors also play a prominent role in the activity of catecholaminergic systems, and we found that fetal nicotine treatment had adverse effects on these synapses as well, again with the effects appearing well after termination of nicotine exposure. Catecholaminergic function showed two phases of synaptic hypoactivity, one in the immediate postpartum period and another emerging with the onset of puberty (33), accompanied by behavioral anomalies (36,50,51). In the intervening stages, even

though basal activity was within normal limits, the reactivity of noradrenergic systems to acute nicotine challenge was obtunded in the prenatal nicotine group (Figure 4): doses of nicotine that evoked norepinephrine release in brain regions of control animals were unable to do so in the group exposed to nicotine prenatally (52). Thus, fetal exposure to nicotine has lasting adverse effects on synaptic performance, effects that may not emerge fully until adolescence.

We also have identified numerous adverse effects of prenatal nicotine exposure

on postsynaptic signaling mechanisms, all of which are potential participants in neurobehavioral abnormalities. These entail lasting changes in the expression of cell signaling intermediates (53,54), uncoupling of receptors and second messenger systems from downstream cellular events (55,56), and alterations in the expression of receptor proteins themselves (26,53,55–57). Developmental disruption by nicotine thus occurs at numerous loci and ranges from outright cell loss to misprogramming of receptor signaling

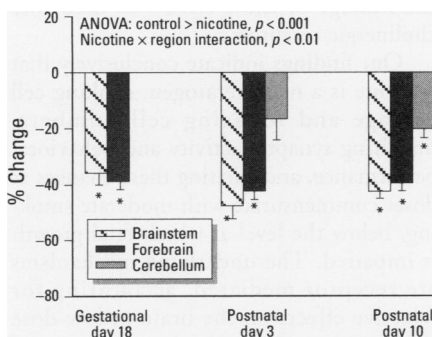


Figure 3. Inhibition of DNA synthesis in rat brain regions after a single dose of nicotine (10,31,106). Measurements of [³H]thymidine incorporation into DNA were made in the first 30 min after nicotine administration. Susceptibility is directly related to the concentration of nicotinic cholinergic receptors in each region, namely brainstem ≥ forebrain > cerebellum. Data represent means and standard errors obtained from 18–30 rats at each age in each treatment group. ANOVA across all ages and regions is shown within the panel; asterisks (*) denote values that differ significantly from the corresponding control.

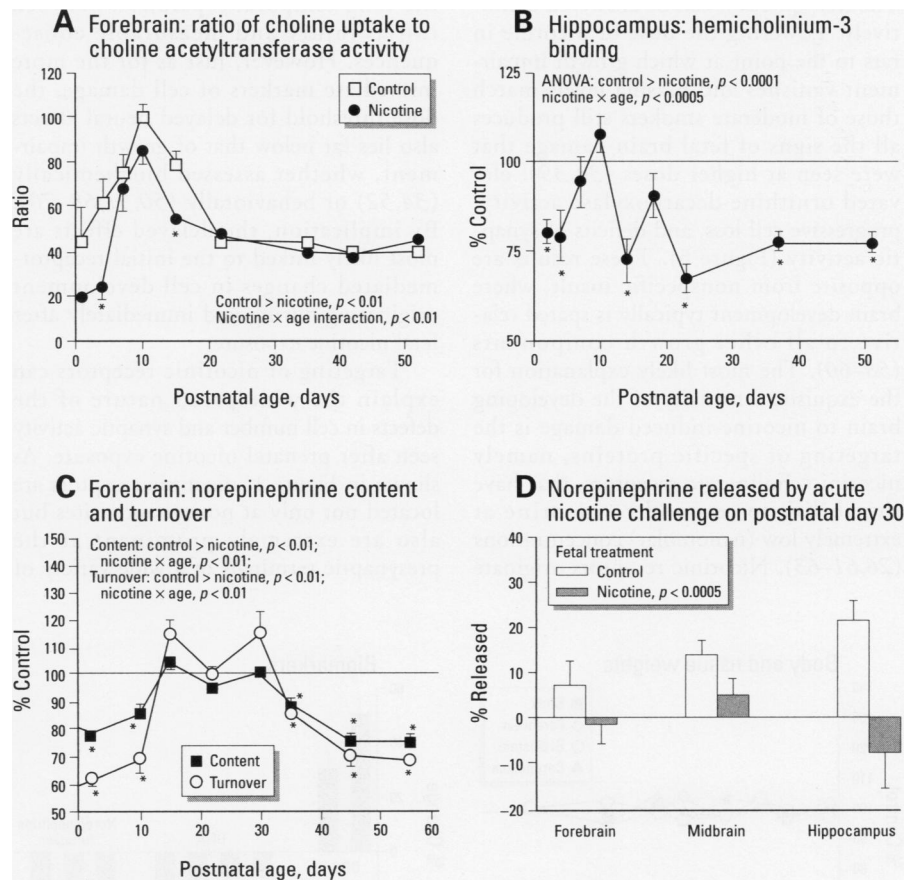


Figure 4. Synaptic hypoactivity elicited by prenatal nicotine exposure. (A) In the forebrain, the ratio of choline uptake to choline acetyltransferase activity (a biochemical marker of impulse activity in cholinergic projections) shows a naturally occurring peak at postnatal day 10; nicotine blunts activity before and during the developmental spike (9). (B) In the hippocampus, [³H]hemicholinium-3 binding to the high-affinity choline transporter, which is regulated by nerve impulse activity, shows both initial postnatal deficits and a later-emerging, permanent deficit in the nicotine group (49). (C) Noradrenergic hypoactivity is also elicited by prenatal nicotine exposure. Norepinephrine content and turnover are suppressed in the forebrain during both the initial postnatal period, and more persistently with the onset of puberty (33). (D) Before the reemergence of deficits in the measures of basal activity, the nicotine group shows a subnormal responsiveness to acute challenges. A single injection of nicotine, which releases norepinephrine in the control group, fails to do so in the nicotine group (52). Data represent means and standard errors obtained from 7–10 animals in each group at each age, for each type of determination. ANOVA is shown within the panels and asterisks (*) denote individual ages at which the nicotine group differs from the corresponding control. Individual tests were not run for acute norepinephrine release because of the absence of a significant interaction of treatment × region.

mechanisms. In trying to determine whether these various outcomes all reflect a similar underlying basic mechanism, two interrelated questions emerge. First, are the effects present at doses corresponding to moderate smoking (one-half pack to one pack per day), where growth impairment, which can lead to nonspecific alterations, is absent? If so, this would imply a specific mechanism targeting the developing brain rather than effects secondary to a more general fetal insult. Selectivity for the developing brain would then raise the second question: Is stimulation of nicotinic cholinergic receptors the underlying target for the effects? The first question can be answered definitively. Lowering the dose of nicotine in rats to the point at which growth impairment vanishes and plasma levels match those of moderate smokers still produces all the signs of fetal brain damage that were seen at higher doses (34,39): elevated ornithine decarboxylase activity, progressive cell loss, and deficits of synaptic activity (Figure 5). These results are opposite from nonspecific insult, where brain development typically is spared relative to all other growth components (58–60). The most likely explanation for the exquisite sensitivity of the developing brain to nicotine-induced damage is the targeting of specific proteins, namely nicotinic cholinergic receptors, that have the ability to respond to nicotine at extremely low (nanomolar) concentrations (26,61–63). Nicotinic receptors originate

in the fetal brain during neurulation and rise dramatically in late gestation and after birth (26,62–65). We have been able to show that these receptors are tonically stimulated by fetal nicotine exposure, as evidenced by receptor upregulation (26), even at doses that do not impair growth (34). Specific tests of each component of fetal brain cell loss evoked by nicotine have verified the involvement of nicotinic receptors, whether for inhibition of DNA synthesis (10), stimulation of damage markers (66), or promotion of apoptosis (38,67). Delayed functional sequelae such as late-appearing reductions in synaptic activity are more problematic because of the long temporal separation between initial injury and measurable consequences. However, just as for the more immediate markers of cell damage, the dose threshold for delayed neural effects also lies far below that of growth impairment, whether assessed biochemically (34,52) or behaviorally (50,51,68–70). By implication, the delayed effects are most likely linked to the initial receptor-mediated changes in cell development originating during and immediately after fetal nicotine exposure.

Targeting of nicotinic receptors can explain the widespread nature of the defects in cell number and synaptic activity seen after prenatal nicotine exposure. As shown in Figure 1, nicotinic receptors are located not only at postsynaptic sites but also are extremely prominent at the presynaptic terminals of a wide variety of

neurotransmitter systems including acetylcholine, catecholamines (norepinephrine, dopamine), and excitatory amino acids, which are themselves potentially neurotoxic. Evoked release of other transmitters that alone exert neurotrophic control of their own targets thus is likely to produce disruption in all the sites “downstream” from nicotinic receptor activation. One issue for further consideration is whether a specific receptor subtype is involved in nicotinic cholinergic neurotrophic actions, and by implication, mediating the disruptive effects of prenatal nicotine exposure. Indeed, based on *in vitro* studies, specific roles have been postulated for control of synaptogenesis by nicotinic receptor subtypes containing the $\alpha 7$ subunit and for adverse effects of nicotine (71). It is also apparent that developing neurons show distinct ontogenetic profiles for expression of the genes encoding the individual subunits of nicotinic receptors (72,73). Nevertheless, some key elements are missing in the current understanding of the role of receptor subtypes in the developmental effects of nicotine, as nearly all studies of subtypes *in vivo* have been conducted at the level of mRNA but not receptor protein. Accordingly, it is unclear as to which subtypes are actually expressed at the cell surface and whether specific subtypes are linked differentially to neurotrophic stimuli. Nevertheless, this absence of knowledge concerning receptor subtypes does not obviate the clear-cut effects of nicotine on cell development and the linkage of these effects to nicotinic cholinergic receptors.

Our findings indicate conclusively that nicotine is a neuroteratogen, evoking cell damage and reducing cell numbers, impairing synaptic activity and behavioral performance, and eliciting these changes at doses commensurate with moderate smoking, below the level at which fetal growth is impaired. The underlying mechanisms are receptor mediated, accounting for selective effects on the brain at low-dose thresholds and for the involvement of brain regions and transmitter systems that have prominent cholinergic inputs. Receptor stimulation leads to two distinct errors in the program of cell development, a premature change from cell replication to differentiation, and after a delay, initiation of the program for cell damage and apoptosis. The next issue, then, is whether other potential cholinotoxicants, especially insecticides, share the same mechanisms and outcomes.

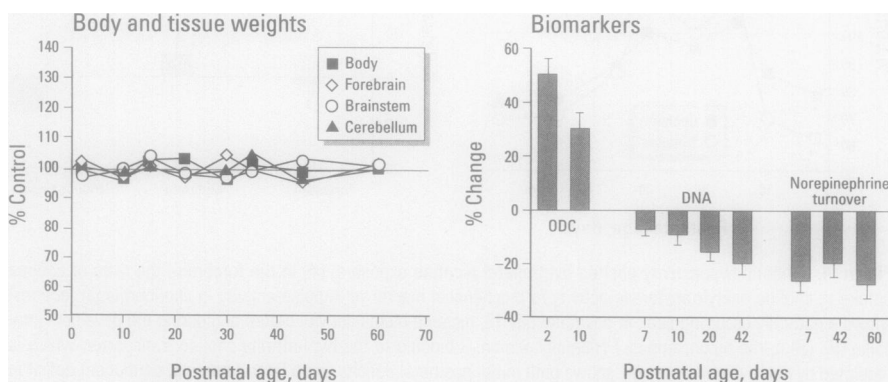


Figure 5. Nicotine damages the developing brain at doses that do not compromise growth (31,34,52). Abbreviation: ODC, ornithine decarboxylase. Administration of 2 mg/kg/day to pregnant rats, which simulates plasma levels of nicotine found in moderate smokers, results in normal body and brain region weights in the offspring. Nevertheless, cell damage (elevated ODC activity), cell loss (reduced DNA content) and synaptic hypoactivity (subnormal norepinephrine turnover) are still fully evident. Data represent means and standard errors obtained from 5–10 animals in each group at each age for each type of determination. Differences for weights are not significant (ANOVA), but effects on biomarkers are (main treatment effect, $p < 0.0001$ across all three biomarkers and for each biomarker taken individually).

Developmental Neurotoxicity of Chlorpyrifos *in Vivo*

Increasing use is being made of the long-lasting organophosphorus insecticide chlorpyrifos, largely because this agent does not elicit organophosphate pesticide-induced persistent neuropathies until the dose is raised above the threshold for lethality (74). Nevertheless, recent concern has arisen over domestic application, which can lead to infant exposures well above acceptable levels (75,76). Animal studies indicate that immature animals are far more susceptible to acute toxicity of chlorpyrifos (77–79) despite the fact that they recover from cholinesterase inhibition more quickly than adults (78–80). As with other organophosphate insecticides, chlorpyrifos, via its reactive metabolite, chlorpyrifos oxon, inhibits cholinesterase and prevents the breakdown of acetylcholine. An initial view of the potential impact of chlorpyrifos on signaling targets in brain development thus could resemble that of nicotine (Figure 1), with promotion of cholinergic signaling as the primary target. However, chlorpyrifos also exhibits direct cholinergic agonistlike properties, opening and then desensitizing nicotinic cholinergic receptor/ion channels (81); it interacts with signaling intermediates such as G-proteins and adenylyl cyclase (80,82,83); and it may produce oxidative damage to DNA (84,85).

If the primary effect of chlorpyrifos on the developing brain is a reflection of its general mode of toxicity as seen in mature animals, namely cholinesterase inhibition, then the net effects during development should bear a strong resemblance to those of nicotine, which also elicits cholinergic hyperstimulation. When we administered chlorpyrifos to neonatal rats (Figure 6), we obtained acute inhibition of DNA synthesis (77). However, at 1 day of age, there was no regional selectivity to the effect: regions with low cholinergic innervation (cerebellum) were affected just as much as cholinergically enriched regions (brainstem, forebrain). Regional selectivity then emerged by the end of the first postnatal week, at which point cholinergic antagonists could block the effect. Thus, chlorpyrifos affects DNA synthesis by at least two different types of mechanisms, an initial, noncholinergic effect, and subsequently, actions mediated through cholinergic activity. In support of the unexpected finding of noncholinergic contributions to effects on DNA synthesis, we obtained the same inhibitory actions when minute amounts of chlorpyrifos were injected directly into the brain, bypassing hepatic activation to chlorpyrifos oxon, the metabolite that inhibits cholinesterase. The contributions of noncholinergic mechanisms to the net adverse effect on brain development are readily demonstrable. With repeated chlorpyrifos administration, we obtained persistent inhibition of DNA synthesis (86), leading to deficits in cell number

(87) and suppression of macromolecular constituents (88). These effects were seen at chlorpyrifos exposure levels that were devoid of any overt toxicity and that reduced cholinesterase activity by only 20% (80), a degree of inhibition insufficient to produce signs of systemic toxicity.

Some of the postulated, noncholinergic effects of chlorpyrifos involve cell signaling intermediates common to multiple neuronal and hormonal inputs, especially the adenylyl cyclase transduction pathway (82,83,89). Cyclic AMP is universally involved in the control of cell replication and differentiation in virtually all prokaryotic and eukaryotic cells (90–94), so that perturbation of this pathway during development would be expected to have a significant impact on brain cell development. When we examined the effects of otherwise subtoxic doses of chlorpyrifos on adenylyl cyclase activity in the developing brain (80), we found profound effects on G-protein-mediated signaling, including that operating through neurotransmitter receptors known to play neurotrophic roles in cell replication/differentiation patterns (Figure 7). Importantly, low doses of chlorpyrifos administered early in development, with minimal cholinesterase inhibition, had a much greater effect on adenylyl cyclase activity than larger doses given later in development, even though the latter treatment produced much greater inhibition of cholinesterase. Again, this indicates that noncholinergic mechanisms play critical roles in the adverse effects of chlorpyrifos on brain development. Thus,

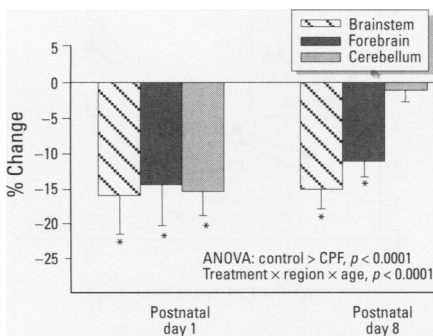


Figure 6. Inhibition of DNA synthesis after a single dose of chlorpyrifos (77). Abbreviation: CPF, chlorpyrifos. Inhibition does not display regional selectivity until the end of the first postnatal week, suggesting two separate mechanisms: an initial, noncholinergic effect, followed by a later-appearing, cholinergic effect. Data represent means and standard errors obtained from 29–63 animals in each treatment group at each age. ANOVA across both ages and all regions appears within the panel. Asterisks (*) denote individual values for which the chlorpyrifos group differs from the corresponding control.

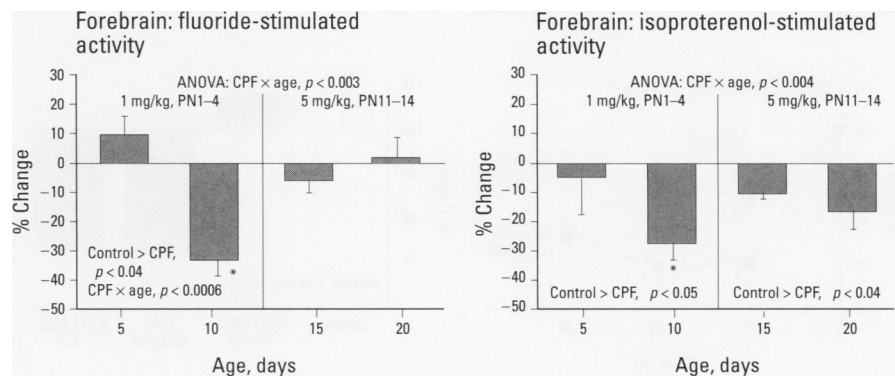


Figure 7. Effects of repeated chlorpyrifos administration on forebrain adenylyl cyclase activity (80). Abbreviations: CPF, chlorpyrifos; PN, postnatal. Treatment with a low dose on postnatal days 1–4 produces a larger deficit than a larger dose given later in development, despite the fact that the later treatment produces greater cholinesterase inhibition. The effect on adenylyl cyclase emerges after a delay of several days after cessation of treatment, at a time when cholinesterase activity has completely recovered to normal. Data represent means and standard errors obtained from 11–12 animals in each treatment group at each age. ANOVA across age is shown within the panels and asterisks (*) indicate individual values that differ significantly from the corresponding control.

conversion of chlorpyrifos to its oxon metabolite and the consequent inhibition of cholinesterase may not be the essential factors in determining neurobehavioral teratology by this compound or potentially for other insecticides as well.

Developmental Neurotoxicity of Chlorpyrifos Modeled *In Vitro*

A definitive demonstration that chlorpyrifos exerts direct effects on neurodevelopment requires control over the cellular environment, as provided by *in vitro* models. We have used PC12 rat pheochromocytoma cells, a cloned cell line that initially resembles sympathetic neuronal precursor cells but that differentiates to resemble sympathetic neurons morphologically, physiologically, and biochemically (95,96). The onset of differentiation is initiated by nerve growth factor after which the cells develop the appearance and function of cholinergic target neurons, including increased expression of cholinergic receptors, choline acetyltransferase, and acetylcholinesterase (97,98). Equally important, these cells lack cytochrome P450 (99), the enzyme that converts chlorpyrifos to its oxon, the metabolite that inhibits cholinesterase. Thus, if the actions of chlorpyrifos seen for brain development *in vivo* are paralleled by similar actions on PC12 cells *in vitro*, the effects cannot be secondary to cholinesterase inhibition, the standard biomarker of organophosphate-induced toxicity.

Using undifferentiated PC12 cells, we obtained immediate (1 hr) inhibition of DNA synthesis (Figure 8); effects on RNA or protein synthesis were much less notable,

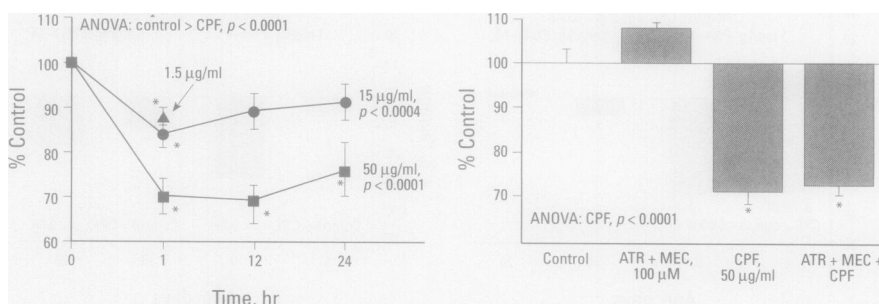


Figure 8. Inhibition of DNA synthesis by chlorpyrifos in undifferentiated PC12 cells (100). Abbreviations: ATR, atropine; CPF, chlorpyrifos; MEC, mecamlamine. Inhibition shows an immediate onset of action and is not mediated by cholinergic hyperstimulation, as receptor blocking agents for muscarinic (atropine) or nicotinic (mecamlamine) receptors do not prevent the effect. Data represent means and standard errors obtained from 6–17 determinations for each treatment and time point. ANOVA across all time points and time points appear within the panels and asterisks (*) denote individual values for which the treated groups differ from the corresponding controls.

indicating a selectivity toward replicating cells (100). The effect on DNA synthesis in undifferentiated PC12 cells could not be blocked by cholinergic receptor antagonists, confirming that chlorpyrifos itself produces effect without a requirement for cholinesterase inhibition and its resultant cholinergic hyperstimulation. When PC12 cells were allowed to differentiate in the continuous presence of chlorpyrifos, the inhibition of DNA synthesis intensified and persisted throughout the period of cell development (Figure 9). As a consequence, acquisition of new cells (DNA level) was severely curtailed, or at the highest concentrations, completely arrested, replicating the effects found for chlorpyrifos *in vivo*. In contrast to the profound effects on DNA synthesis and cell acquisition, neurite extension, as measured by the increase in membrane surface area (protein/DNA ratio), was inhibited only at high chlorpyrifos concentrations. These results confirm a targeted, primary effect of chlorpyrifos on cell replication, with other developmental abnormalities requiring higher exposure levels. Just as was found for *in vivo* treatments, the progression of cell differentiation increases the sensitivity to chlorpyrifos, representing emergence of the cholinergic target phenotype; at that point, both direct and cholinergically mediated effects become additive (77), whereas only the direct effects can be expressed in the undifferentiated state.

We have also carried out *in vitro* studies in rat embryo cultures (67). Using chlorpyrifos concentrations that showed no evidence of growth reduction or dysmorphogenesis, we found clear-cut abnormalities of mitosis in the developing brain at the neural tube stage. Embryos were

incubated with chlorpyrifos for 48 hr beginning on embryonic day 9.5 (Figure 10). Examination of the forebrain and hindbrain regions revealed reduced and altered mitotic figures with dispersion and disorientation of the mitotic layer. In

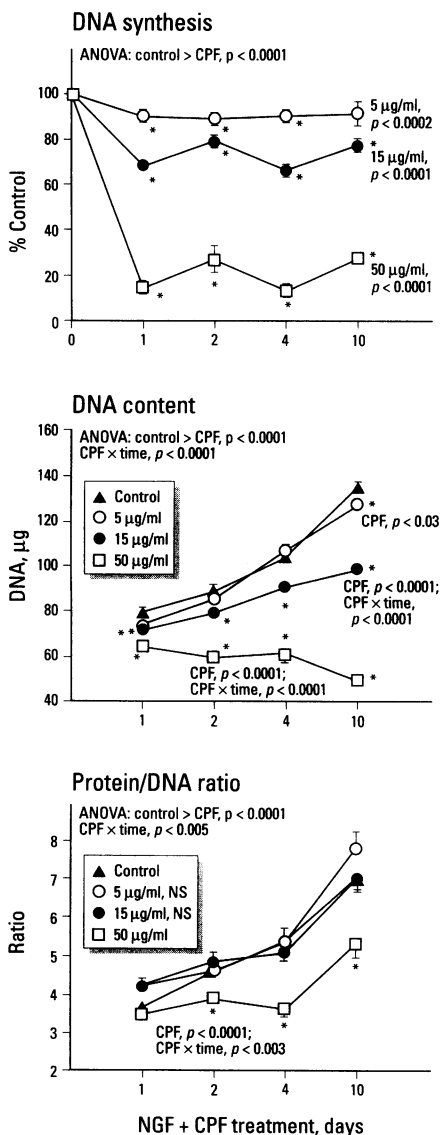


Figure 9. Effects of continuous exposure to chlorpyrifos on PC12 cells during differentiation (100). Abbreviation: CPF, chlorpyrifos. The inhibition of DNA synthesis is intensified and maintained throughout the developmental period, leading to reduced or arrested cell acquisition (DNA level). At the highest concentration, neurite extension (as measured by the protein/DNA ratio) is also inhibited. Data represent means and standard errors obtained from 20–36 determinations in each group at each time point. ANOVA across all time points and treatments appears within each panel and asterisks (*) denote individual values that differ from the corresponding control.

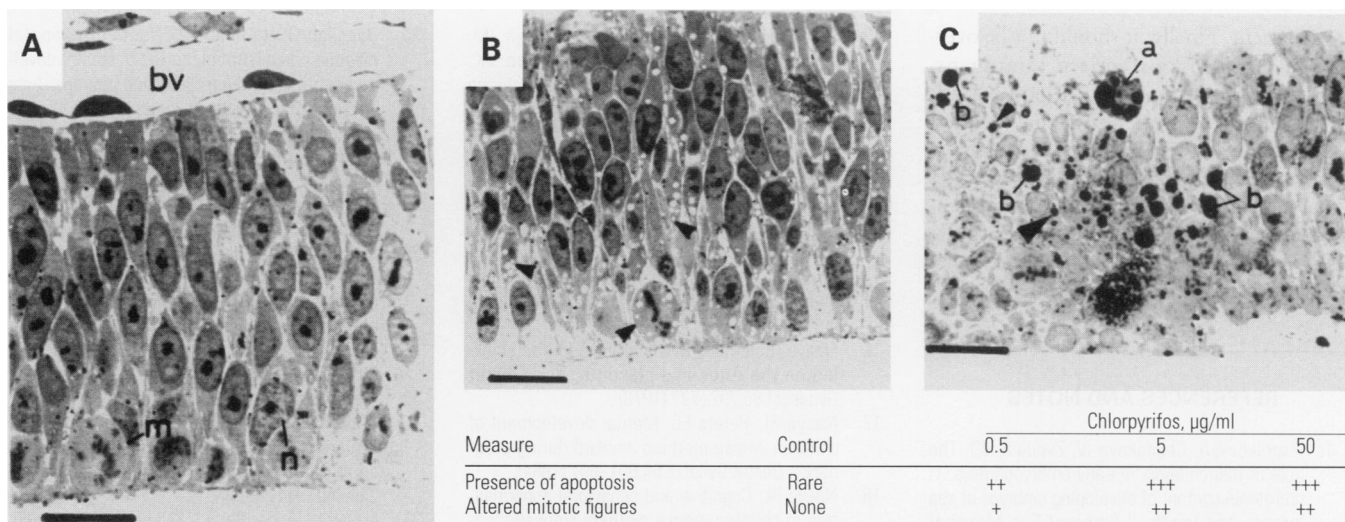


Figure 10. Effects of chlorpyrifos on brain development in cultured rat embryos (67). Abbreviations: bv, blood vessel; CPF, chlorpyrifos; m, mitotic figure; n, inactive heterochromatin. (A) Forebrain neuroepithelium in control embryos at embryonic day 11.5, showing a bipolar pseudostratified epithelium: apical and basal processes contain a granular nucleus and inactive heterochromatin. Mitotic figure can be seen toward the internal limiting membrane. Mesenchyme around the germinal epithelium shows blood vessel. (B) Neuroepithelium from an embryo exposed to chlorpyrifos (50 µg/ml). Note the extensive vacuolation of the cytoplasm of the epithelial cells (arrowheads). (C) Forebrain neuroepithelium from a chlorpyrifos-exposed embryo showing extensive cell death (b) and extracellular bodies (arrowheads). A large cell (a) with multiple apoptotic condensations is also visible. Scale bar = 20 µm. For semi-quantitative measurements (table at bottom of figure), evaluations were made in numerous sections obtained from four otherwise morphologically normal embryos in each treatment group. Over a much larger cohort (>40 embryos per treatment), there was no evidence of gross dysmorphogenesis or of changes in developmental landmarks aside from the disruption of cell development in the neuroepithelium.

addition, cytotoxicity was evidenced by cytoplasmic vacuolation, enlargement of intercellular spaces, and the presence of a significant number of apoptotic figures. Significant effects were found even at concentrations as low as 0.5 µg/ml.

Our results with PC12 cells or rat embryo cultures support the idea that chlorpyrifos specifically targets brain development. However, a major problem is how to compare exposures *in vitro* with those likely to be experienced with environmental contamination. Certainly, the concentration and exposure period necessary to affect brain cell development *in vitro* lie well below those necessary for dysmorphogenesis, for chromosome damage (101) or for general cytotoxicity (101,102). Although scant information is available concerning the actual levels of chlorpyrifos achieved in fetal brain, we have already demonstrated that doses that cause only 20% cholinesterase inhibition nevertheless depress mitosis in neonatal rat brain *in vivo* (77,80,86), leading to deficiencies in cell numbers (87). A preliminary report on pregnant rats (103) found that a comparable degree of cholinesterase inhibition, which is well below the threshold for any observable signs of cholinergic hyperstimulation, produces peak fetal brain concentrations of the major metabolite of chlorpyrifos of approximately 0.25 µg/g,

which on a molar basis, corresponds to the lowest concentration of chlorpyrifos used in our studies with embryos *in vitro* (67). On a body weight basis, the doses of chlorpyrifos needed for adult or developmental toxicity in rats range up to tens to hundreds of mg/kg (78,79,104,105) and certainly no lower than 2 mg/kg (77). Mitotic arrest *in vivo* occurs with brain concentrations of 2 µg/g (77), again well within the concentration range needed for *in vitro* effects. The likely acute exposure level for infants after home application of chlorpyrifos is also above this range: 350 µg/kg/day for a 2-week period, for a total of 5 mg/kg (76). Although there are clear limitations of extrapolation across species and between cultures and intact systems, *in vitro* evaluations nevertheless can point the way to likely mechanisms and adverse outcomes, and are likely to be within the range of relevant exposure levels *in vivo*.

Conclusions and Future Directions

Drugs or chemicals that target cholinergic neurotransmission probably represent the largest source of neurobehavioral teratogenesis. Nicotine exposure involves one-fourth of all pregnancies in the United States, and exposure to insecticides that target cholinesterase is ubiquitous. Establishing the underlying mechanisms, and

hence safety thresholds, for these compounds must represent a major focus of future work. We have shown that nicotine damages the developing brain at concentrations achieved in moderate smokers or with nicotine replacement therapies such as the transdermal patch. The sequelae of maternal smoking are already well established (14) and include high rates of miscarriage, fetal death, intrauterine growth retardation, deaths in the postnatal period, and behavioral and learning disturbances. The finding that a specific substance in tobacco (nicotine) is a major contributor to adverse outcomes provides the first definitive proof that tobacco is a direct cause of these problems, not simply a covariable with other components of the smoking life style. In the case of chlorpyrifos, our findings indicate that inhibition of cholinesterase, the standard biomarker for organophosphate toxicity, is inadequate to explain the effects of this compound on brain development. The uncovering of alternative mechanisms indicates the need for research on screening methods that emphasize unique attributes of developing systems such as DNA synthesis, cell acquisition, apoptosis, and cytoarchitectural modeling of specific brain regions. *In vitro* systems such as neural cell lines or embryo cultures can play key roles in elaborating these mechanisms and in establishing new safety thresholds for insecticide exposure during

development. Finally, it should not be overlooked that unlike standard teratogens, agents that target specific cell populations in the nervous system rather than general organogenesis, can be expected to have adverse effects that extend to the final stages of development: childhood and adolescence. In the future, we will need to acquire new ways of evaluating potential postnatal effects of environmental contaminants.

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