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Review

Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates

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Abstract

Acetylcholine and other neurotransmitters play unique trophic roles in brain development. Accordingly, drugs and environmental toxicants that promote or interfere with neurotransmitter function evoke neurodevelopmental abnormalities by disrupting the timing or intensity of neurotrophic actions. The current review discusses three exposure scenarios involving acetylcholine systems: nicotine from maternal smoking during pregnancy, exposure to environmental tobacco smoke (ETS), and exposure to the organophosphate insecticide, chlorpyrifos (CPF). All three have long-term, adverse effects on specific processes involved in brain cell replication and differentiation, synaptic development and function, and ultimately behavioral performance. Many of these effects can be traced to the sequence of cellular events surrounding the trophic role of acetylcholine acting on its specific cellular receptors and associated signaling cascades. However, for chlorpyrifos, additional noncholinergic mechanisms appear to be critical in establishing the period of developmental vulnerability, the sites and type of neural damage, and the eventual outcome. New findings indicate that developmental neurotoxicity extends to late phases of brain maturation including adolescence. Novel in vitro and in vivo exposure models are being developed to uncover heretofore unsuspected mechanisms and targets for developmental neurotoxicants.

Keywords: Tobacco smoke; Neurotoxicity; Neurotransmitters

Beginning in the 1960s, Buznikov et al. (1964, 1970) made the seminal finding that neurotransmitters are present in high concentrations during specific phases of early development of lower organisms, such as sea urchin embryos, and during that period, are unrelated to their function in synaptic signaling. In fact, these small molecules serve instead as morphogens, controlling and coordinating the proper assembly of the embryo, through receptor mechanisms and cell signaling cascades similar to those through which these neurotransmitters act in the mammalian brain (Buznikov, 1990; Buznikov and Rakic, 2000; Buznikov et al., 1996, 2001a). Spurred by these findings, other investigators then identified similar trophic roles for neurotransmitters in the cellular and architectural development of the central nervous system (Lauder, 1985; Whitaker-Azmitia, 1991). In essence, within the appropriate developmental context, activation of a given set of neurotransmitter receptors may: (1) promote neural cell replication, (2) initiate the switch from replication to differentiation, (3) enhance or retard axonogenesis or synaptogenesis, (4) evoke or prevent apoptosis, or (5) enable the appropriate migration and localization of specific cell populations within each brain region. At the same time, these multiple developmental roles of neurotransmitters render the developing brain vulnerable to neuroactive chemicals that elicit or block neurotransmitter responses, with sensitivity extending through all phases of brain assembly, from the early embryonic stage through adolescence (Yanai, 1984). The present review will focus on agents that act through disruption of trophic signaling elicited by one particular neurotransmitter, acetylcholine. This emphasis is particularly appropriate for several reasons. First, prenatal nicotine exposure, in the form of maternal cigarette smoking or exposure to environmental tobacco smoke (ETS), is one of the major health problems of modern society. Second, organophosphate insecticides, which are among the most widely used pesti-

Abbreviations: nAChR, nicotinic acetylcholine receptor; CPF, chlorpyrifos; ETS, environmental tobacco smoke.

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cides in the world, work by inhibiting acetylcholinesterase and thus promoting and prolonging the actions of acetylcholine. Third, the potent warfare nerve gases that are likely to be used in terrorist attacks, sarin, tabun, soman, and VX, are all organophosphates. A focus on cholinergic mechanisms is also appropriate given the critical roles played by acetylcholine in virtually all phases of brain maturation. As recently reviewed (Lauder and Schambra, 1999; Weiss et al., 1998), acetylcholine influences development as early as gastrulation, and initially promotes cell division, at a stage in which acetylcholine formation is "pre-neural." Indeed, nicotinic acetylcholine receptors (nAChRs), which will be the focus of our discussion of adverse effects of nicotine on development, actually become detectable before neurulation (Atluri et al., 2001; Schneider et al., 2002), followed shortly thereafter by the first definitive signs of the cholinergic neuronal phenotype (Lauder and Schambra, 1999). Later on, during terminal neuronal differentiation, acetylcholine promotes the switch from replication to differentiation (Slotkin, 1998a, 1999) and subsequently modulates axonogenesis and synaptogenesis (Audesirk and Cabell, 1999; Chan and Quik, 1993; Dahm and Landmesser, 1991; Navarro et al., 1989a; Quik et al., 1994). Dependent upon the developmental phase, acetylcholine promotes or prevents neuronal apoptosis: when cells are poorly differentiated, the effect is primarily pro-apoptotic, whereas in differentiated cells, it is anti-apoptotic (Berger et al., 1998; Messi et al., 1997; Pugh and Margiotta, 2000; Roy et al., 1998b; Slotkin et al., 1997a; Trauth et al., 1999b; Yamashita and Nakamura, 1996). Accordingly, even relatively late phases of brain development are dependent upon trophic responses to acetylcholine, so that interference with cholinergic signaling disrupts the final architectural assembly of brain regions containing cholinergic target zones (Bachman et al., 1994; Hohmann and Berger-Sweeney, 1998; Hohmann et al., 1988, 1991). Indeed, neuroproliferation, apoptosis, and synaptic rearrangement continue into adolescence (Bayer, 1983; Bayer et al., 1982; Huttenlocher, 1990), especially the critical central cholinergic pathways that control learning, memory, and psychostimulant responses (Matthews et al., 1974; Nadler et al., 1974; Zahalka et al., 1993a). This review will thus summarize the current state of knowledge of the effects of cholinergic disruptors on brain development in stages ranging from early embryogenesis to the adolescent. Finally, we will return to the originating information about neurotransmitters and trophic factors in lower organisms to explore the possibility that these may serve as unique models for the screening of developmental neurotoxicants and for the elucidation of cellular mechanisms operating in disruption of mammalian brain development.

Nicotine as an archetypal developmental neurotoxicant

In terms of the health impact on society, nicotine, in the form of maternal smoking, likely represents the single most important developmental neurotoxicant, involving as much as one-fourth of all pregnancies in the United States (Bardy et al., 1993; DiFranza and Lew, 1995); exposure via ETS adds to that total. Tobacco contributes in a major way to spontaneous abortions, intrauterine growth retardation and perinatal deaths, Sudden Infant Death Syndrome, and, later in life, higher rates of learning disabilities, behavioral problems, and attention deficit and hyperactivity disorder (Bell and Lau, 1995; Butler and Goldstein, 1973; DiFranza and Lew, 1995; Dunn and McBurney, 1977; Naeye, 1978, 1992; Naeye and Peters, 1984). A recent series of papers identified maternal smoking as a significant contributor to disruptive and criminal behaviors in offspring, and to school and career failure (Cornelius et al., 2001; Day et al., 2000; Lefkowitz, 1981; Orlebeke et al., 1999; Piquero et al., 2002; Rasanen et al., 1999; Wakschlag et al., 2002; Weitzman et al., 2002). In light of the role of acetylcholine as a critical factor in all stages of mammalian brain development, and given the fact that nicotine, a primary component of tobacco smoke, is a specific stimulant of nAChRs and is thus an archetypal cholinergic stimulant, it is immediately apparent that the cholinergic–neurotrophic connection is likely to mediate many of these effects. Essentially, by providing excessive cholinergic stimulation throughout fetal life, nicotine from maternal smoking can discoordinate the numerous events in cell replication, differentiation, and synaptic development that are necessary to the proper assembly of the mammalian brain. The findings from human smokers, however, do not obligate an underlying cholinergic mechanism, as cigarette smoke contains thousands of bioactive compounds. Accordingly, isolating the specific role of nicotine needs to be accomplished with appropriate animal models.

The design of animal models of nicotine exposure is less straightforward than one might think. When nicotine is injected repeatedly into pregnant rats throughout gestation, there are indeed behavioral as well as cellular and synaptic abnormalities in the offspring (Martin and Becker, 1970, 1971; Nasrat et al., 1986; Slotkin et al., 1986b, 1987a, 1987b). However, to a large extent, these reflect the consequences of repeated, acute episodes of uteroplacental vasoconstriction and subsequent fetal hypoxia, associated with high peak plasma levels of nicotine achieved by this route of administration (Carlos et al., 1991; Jonsson and Hallman, 1980; McFarland et al., 1991; Seidler and Slotkin, 1990; Seidler et al., 1992; Slotkin et al., 1986a). Beginning about 20 years ago, we and other researchers developed an animal model of nicotine exposure using osmotic minipump delivery of continuous, low-level infusions, a technique that avoids the peak plasma levels and resultant hypoxic episodes (Lichtensteiger et al., 1988; Murrin et al., 1987; Navarro et al., 1988, 1989a, 1989b; Slotkin, 1992; Slotkin et al., 1987b, 1987c). Accordingly, this mode of nicotine administration delivers a fixed dose of drug simulating the steady-state plasma levels seen in smokers or users of transdermal nicotine patches (Lichtensteiger et al., 1988; Murrin et al., 1987). Because the pharmacokinetics are different in rodents as compared to

humans, higher overall doses need to be delivered, but the key element is the matching of plasma concentrations (not total dose) in the rodent to those seen in typical smokers, along with adjustment for the pharmacodynamic differences among species (Barnes and Eltherington, 1973; Lichtensteiger et al., 1988). Thus, in rats, dose rates of 2–6 mg/kg/day are appropriate to reproduce the nicotine plasma levels and CNS effects of nicotine found in moderate (0.5–1 pack/day) to heavy (2 packs/day) smokers (Lichtensteiger et al., 1988; Trauth et al., 2000b).

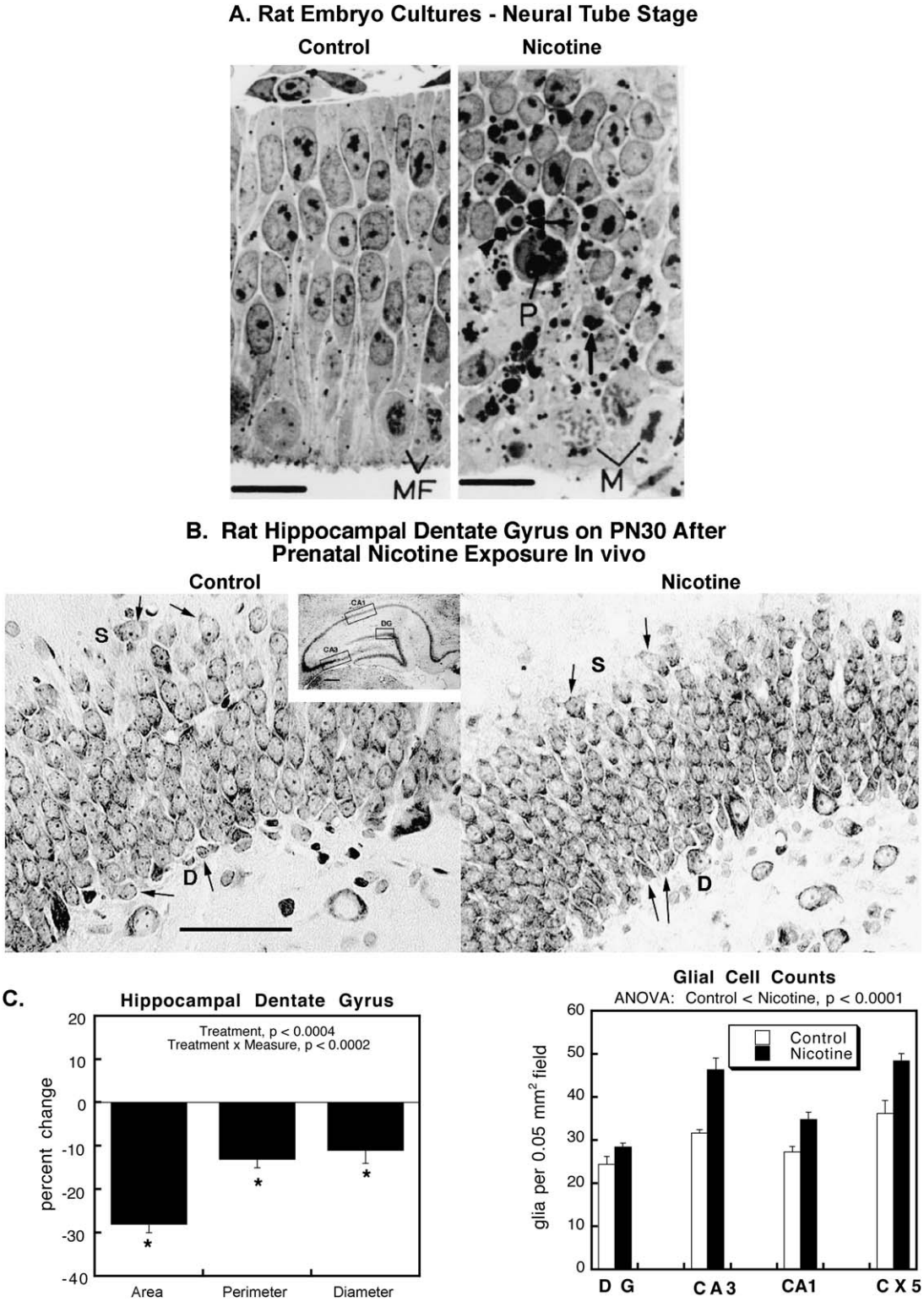
If nicotine disrupts brain development through cholinergic mechanisms, then it would be expected that vulnerability should emerge at the stage at which nAChRs first become detectable. In studies with cultured rat embryos, we were able to show profound disruption of cell development in the brain primordium at around the time of neurulation (Roy et al., 1998b); the notable effects included cytoplasmic vacuolation, enlargement of intercellular spaces, and a sharp increase in apoptotic cells, without general dysmorphogenesis in the rest of the embryo (Fig. 1A). Originally, these findings puzzled us, as the exposure period involved a stage before that at which nAChRs were thought to be expressed, based on ligand binding (Naeff et al., 1992), although the localization of effects did follow the pattern expected from the regions that express nAChRs earliest. This problem has been resolved by recent studies with electrophysiological techniques, which indicate the presence of functional receptors in the early neural tube stage (Atluri et al., 2001; Schneider et al., 2002). What is truly surprising is that, despite the gross dysmorphology in the brain primordium caused by nicotine exposure, substantial recovery occurs even when nicotine administration continues through parturition, so that brain structures are not grossly abnormal when examined later on in adolescence or adulthood (Roy et al., 2002). These differences are not reflective of variant nicotine doses, as the concentrations used in the embryo cultures (as low as 1 μM; Roy et al., 1998b) were quite comparable to those achieved with maternal infusions (0.5 μM; Lichtensteiger et al., 1988), and the exposure period for the culture system was shorter (48 h versus continuous exposure throughout gestation). Quantitative morphology, however, indicates that there are indeed long-lasting alterations caused by gestational nicotine exposure (Fig. 1B). In the hippocampal CA3 region and dentate gyrus, we found a substantial decrease in cell size, with corresponding decrements in cell layer thickness and increments in cell packing density (Fig. 1C). In layer 5 of the somatosensory cortex, there was a reduction in the proportion of medium-sized pyramidal neurons and an increase in the proportion of smaller, nonpyramidal cells. All regions showed elevated numbers of glia. Thus, at the morphological level, prenatal nicotine exposure does evoke long-term alterations in brain development but the effects require quantitative analysis and are not qualitatively obvious.

Morphological examinations do not necessarily give an indication of whether synaptic function is appropriately controlled, so it is necessary to turn to neurochemical examinations to further characterize the effects of nicotine on brain development. Using the nicotine infusion paradigm, we found definitive damage to the developing rat brain at nicotine plasma levels comparable to those in heavy smokers (Navarro et al., 1988, 1989b; Slotkin, 1992; Slotkin et al., 1987b, 1987c). Multiple biomarkers of cell injury indicate that prenatal nicotine exposure damages the developing brain (Fig. 2A). Throughout the brain, ornithine decarboxylase activity is elevated during the postnatal period, despite the discontinuation of exposure at birth. Similarly, c-*fos* is constitutively induced for a prolonged period, a situation known to evoke apoptosis (Slotkin et al., 1997a; Trauth et al., 1999b). DNA content declines by 10–20%; as each brain cell has a single nucleus, decrements in DNA connote an equivalent shortfall in the total number of brain cells. Over the ensuing 2 weeks after birth, deficits in total cell number actually worsen, suggestive of an increase in programmed cell death, which occurs naturally over this period, but which ordinarily comprises a much smaller proportion of cells (Slotkin, 1998a, 1999). Indeed, subsequently, we identified constitutive activation of genes associated with apoptosis, persisting into the period of maximal cell loss (Slotkin, 1998a; Slotkin et al., 1997a). What is particularly notable is that nicotine-induced apoptosis in the developing brain stands in direct contrast to its neuroprotective effect in the mature brain (Janson et al., 1988; Kaneko et al., 1997; Owman et al., 1989; Yamashita and Nakamura, 1996) so that the developmental context in which nicotine exposure

Fig. 1. Effects of prenatal nicotine exposure on brain morphology during exposure and in adolescence. Results were excerpted from primary studies that contain the experimental details (Roy et al., 1998b, 2002). (A) Effects of nicotine on brain development in cultured rat embryos. Exposure occurred for a 48-h period beginning at 9.5 days of gestation. On the left, neuroepithelium from a control embryo, showing closely apposed pseudostratified cells at different phases of mitosis, and their processes. The mitotic figures (MF) are localized to the lumenal surface. On the right, a nicotine-exposed embryo, exhibiting dying cells and debris, in the form of intra- and extracellular bodies, often engulfed by healthy cells, including those undergoing mitosis (M). A large nucleated phagosome (P) contains multiple dark bodies. Scale bar = 20 μm. (B) Morphological changes evident in the ectal limb of the hippocampal dentate gyrus on PN30. Pregnant rats received continuous nicotine infusions throughout gestation. The left panel shows a control tissue whereas the right is from a nicotine-exposed animal. Note the smaller cell size and increased packing density in the nicotine group. For both groups, the early-born large neurons are in the superficial part (S) whereas late-born neurons are in the deep part (D) of the layer; compare cell sizes shown with arrows. Scale bar = 50 μm. Inset shows a photomicrograph of the hippocampus at PN30, showing segments sampled for the pyramidal cell layers of CA1 and CA3, and the granule cell layer of the ectal limb of the dentate gyrus (DG). Scale bar = 300 μm. (C) Quantitative morphometry of the dentate gyrus (left) and glial cell counts (right) in hippocampal dentate gyrus (DG), CA3, and CA1, and in layer 5 of the somatosensory cortex (CX5), from the same study shown in B. Data are shown as means and standard errors. Note the decreases in parameters of cell size and the global increase in glial cells, characteristic of reactive gliosis consequent to cell damage.

occurs is likely to be critical for evoking apoptosis. This has since been confirmed with hippocampal progenitor cells, which similarly show nicotine-induced apoptosis only in early development (Berger et al., 1998).

As already described, acetylcholine, acting as a trophic factor, can either promote mitosis in developing neurons, or at later stages of development, can switch cells from mitosis to differentiation. Examining a later developmental stage for nicotine exposure, at the approach of parturition or in the early neonatal period, we also identified a second mechanism for cell deficits (McFarland et al., 1991): inhibition of DNA synthesis, which compromises the ability of cells to



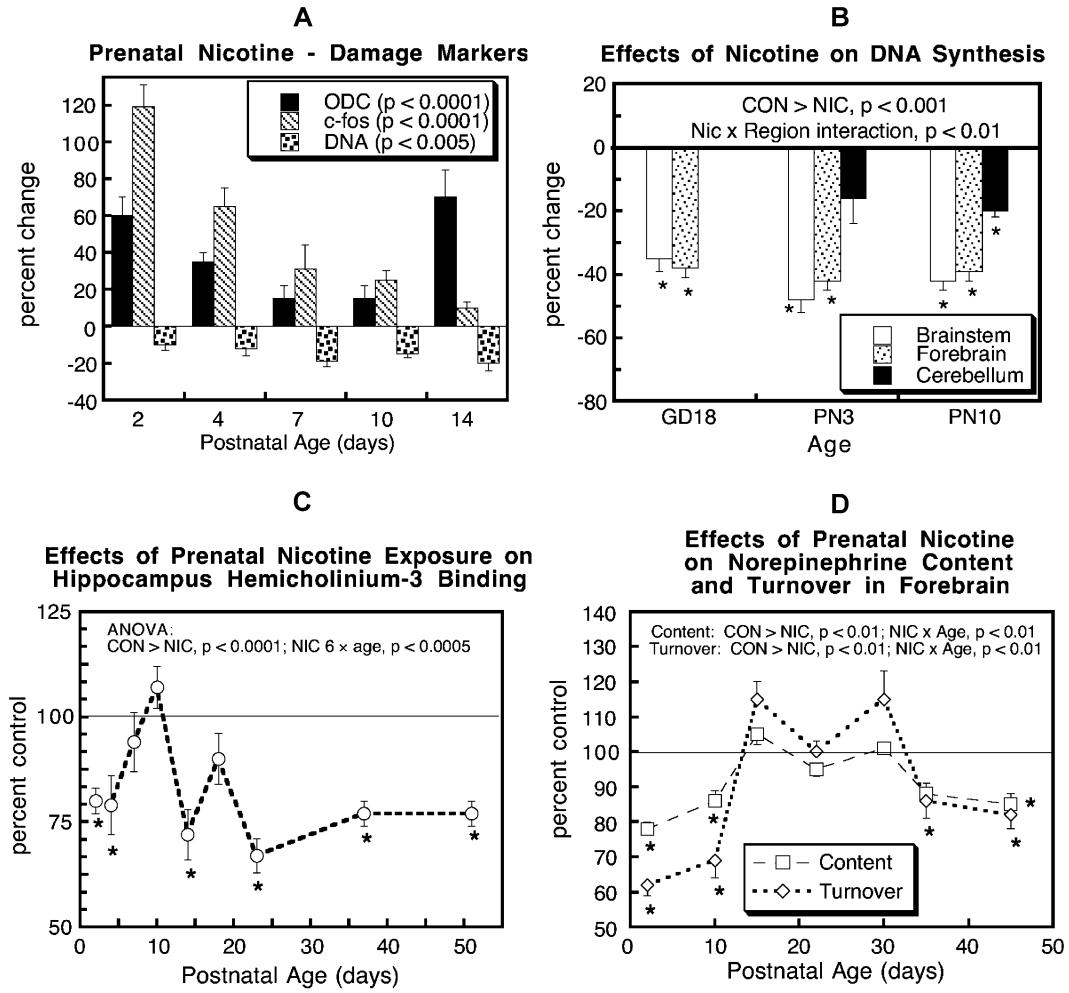


Fig. 2. Prenatal nicotine exposure elicits brain cell damage and loss, and impairs the development of synaptic activity in multiple neurotransmitter pathways. Data were taken from a series of papers that contain the experimental details (McFarland et al., 1991; Navarro et al., 1988; Slotkin, 1998a; Slotkin et al., 1997a; Trauth et al., 1999b; Zahalka et al., 1992). All values are presented as means and standard errors. (A) Cell damage markers in forebrain of rats exposed to nicotine via maternal infusions throughout gestation. Constitutive elevations of ornithine decarboxylase (ODC) and c-*fos* are characteristic of cell injury and apoptosis. Loss of DNA connotes a parallel decline in the number of cells. (B) Acute decline of DNA synthesis in brain regions of rats given a single dose of nicotine on gestational day (GD) 18, postnatal day (PN) 3, or PN10. Note the regional hierarchy paralleling cholinergic innervation and nAChRs. (C) Prenatal nicotine exposure impairs development of presynaptic cholinergic activity, as delineated with hemicholinium-3 binding to the high-affinity choline transporter. Animals were exposed to nicotine via maternal infusions throughout gestation. Note the biphasic effect: initial deficits are corrected for a brief period, only to reappear later in development. (D) Prenatal nicotine exposure impairs development of presynaptic noradrenergic activity, as delineated with norepinephrine levels and utilization rate (turnover). Animals were exposed to nicotine via maternal infusions throughout gestation. Again, note the biphasic effect.

divide. In this phase, even a single administration of nicotine elicits a profound decline in DNA synthesis that lasts for several hours, with specific targeting of brain regions expressing high concentrations of nAChRs (Fig. 2B). The same effects can be elicited when minute amounts of nicotine are introduced directly into the brain, bypassing any systemic effects (McFarland et al., 1991).

Although clearly an adverse effect, deficits in the number cells do not necessarily connote that behavioral performance will be impaired; it is necessary, in addition, to demonstrate that synaptic function is compromised. Because nicotine works through nAChRs, we first evaluated effects on cholinergic neurotransmission (Navarro et al., 1989a; Zahalka et al., 1992). Indices of neuronal impulse activity indicate that prenatal nicotine exposure impairs the ontogenetic rise of synaptic activity and elicits persistent deficits that emerge in adolescence (Fig. 2C). However, in light of the general disruption of cellular development, it is not surprising that the adverse effects of nicotine extend to other neurotransmitters. Nicotinic receptors also play a prominent role in the activity of monoaminergic systems, and we found that fetal nicotine treatment had adverse effects on these synapses as well, again with the effects reappearing well after termination of nicotine exposure (Fig. 2D). For catecholamines (norepinephrine and dopamine), synaptic hypoactivity is evident in the immediate postnatal period and, although values normalize by weaning, deficits reemerge with the onset of puberty (Navarro et al., 1988), accompanied by behavioral anomalies

(Levin and Slotkin, 1998; Lichtensteiger et al., 1988; Ribary and Lichtensteiger, 1989). Even when basal activity is within normal limits, the reactivity of these systems to acute nicotine challenge is desensitized, so that responses that normally are evoked by cholinergic stimulation are ineffective in the animals exposed to prenatal nicotine (Seidler et al., 1992). Similar findings have been obtained for serotonin pathways (Xu et al., 2001). Thus, fetal exposure to nicotine has lasting, adverse effects on synaptic performance, effects that may not emerge fully until adolescence. Again, the findings from animal models thus mimic the delayed appearance of learning and behavioral anomalies in the offspring of women who smoke, as already enumerated.

In addition to its profound effects on patterns of presynaptic neuronal activity, prenatal nicotine exposure alters postsynaptic receptor-mediated signaling mechanisms, effects which are equally likely to elicit neurobehavioral abnormalities. Most interestingly, these actions are exerted at cell signaling proteins downstream from the neurotransmitter receptors themselves, thus eliciting heterologous changes in a wide variety of signals (Slotkin et al., 1990, 1992, 1999; Yanai et al., 2002). Superimposed on the effects on signaling proteins, nicotine exposure leads to diminished responses of signals mediated by a variety of specific receptors (Navarro et al., 1990a; Zahalka et al., 1993b), likely involving changes in the expression of receptor proteins (Navarro et al., 1990a, 1990b; Slotkin et al., 1987b, 1990; Zahalka et al., 1993b). Developmental disruption by nicotine thus occurs at numerous loci, ranging from outright cell loss to specific alterations of neural activity, to misprogramming of receptor signaling mechanisms.

If all these effects represent the sequelae of inappropriate, mis-timed stimulation of nAChRs controlling neural cell development, then one corollary is that they should be elicited at nicotine exposure levels below the threshold for general fetal growth impairment. The reason this is so important is that intrauterine growth retardation is a well-recognized, major predictor of adverse perinatal outcome in offspring of smokers (DiFranza and Lew, 1995). In animal models of nicotine exposure, lowering the dose of nicotine in rats to the point where growth impairment vanishes, and where plasma levels match those of moderate smokers, still produces all the signs of fetal brain damage that were seen at higher doses (Levin and Slotkin, 1998; Navarro et al., 1989b; Seidler et al., 1992; Slotkin, 1998a, 1999). This outcome thus differs considerably from that seen with nonspecific fetal insult, which typically spares brain development relative to somatic growth (Bell et al., 1987; de Grauw et al., 1986; Dodge et al., 1975). On the other hand, greater sensitivity of brain development relative to growth is entirely commensurate with the specific targeting of nAChRs, which respond to nicotine at nanomolar concentrations (Cairns and Wonnacott, 1988; Hagino and Lee, 1985; Martino-Barrows and Kellar, 1987; Slotkin et al., 1987b). The receptors originate before the neural tube stage (Atluri et al., 2001; Schneider et al., 2002) and rise dramatically in late gestation and after birth (Cairns and Wonnacott, 1988; Hagino and Lee, 1985; Larsson et al., 1985; Lichtensteiger et al., 1987; Slotkin et al., 1987b). Even at low doses of nicotine, insufficient to cause fetal growth retardation, nAChRs are tonically stimulated by fetal nicotine exposure as evidenced by receptor upregulation (Navarro et al., 1989b; Slotkin et al., 1987b). The specific role of nAChRs has been demonstrated for each component of fetal brain cell damage and loss associated with nicotine exposure: inhibition of DNA synthesis (McFarland et al., 1991), stimulation of damage markers (Smith et al., 1991), or promotion of apoptosis (Berger et al., 1998; Roy et al., 1998a; Slotkin et al., 1997a). Even for the later-emerging events, such as altered patterns of synaptic activity, synaptic signaling, and behavioral performance, the dose threshold lies far below that of growth impairment (Cutler et al., 1996; Levin and Slotkin, 1998; Levin et al., 1993a, 1996; Navarro et al., 1989b; Ribary and Lichtensteiger, 1989; Seidler et al., 1992).

If the biochemical and behavioral effects of prenatal nicotine exposure largely reflect drug actions exerted on nAChRs during critical phases of neural cell development, then how can we explain alterations that are present even in brain regions, like the cerebellum, that are relatively sparse in these receptors (Slotkin et al., 1987b)? First, it is important to note that even low levels of receptors may mediate important trophic responses during development. In fact, the cerebellum contains nAChRs that are localized on specific cell populations and that actually undergo peaks of expression during development (Caruncho et al., 1997; Court et al., 1995; Del Toro et al., 1997; Graham et al., 2002; Kawa, 2002; Nakayama et al., 1997; Opanashuk et al., 2001). Second, nAChRs are located not only at postsynaptic sites but also are prominent at presynaptic terminals of other neurotransmitter systems, including acetylcholine, catecholamines (norepinephrine, dopamine), and excitatory amino acids. Because all these transmitters themselves exert trophic actions on the development of their target cells, nAChR-induced release of these transmitters will similarly disrupt development mediated by other transmitters and other circuits; as just one example, nAChR stimulation evokes release of norepinephrine in the developing cerebellum (O'Leary and Leslie, 2003), and in turn, this biogenic amine is a critical factor for cerebellar cell maturation (Podkletnova and Alho, 1998; Sievers et al., 1981; Vernadakis and Gibson, 1974).

These findings leave little doubt that nicotine is a neuroteratogen. As postulated at the outset, the exquisite sensitivity of the developing brain to nicotine is a reflection of the role of acetylcholine as a trophic factor modulating the patterns of brain cell replication and differentiation, synaptic outgrowth, and architectural modeling, all specifically centering around those effects mediated through the activation of nAChRs. One critical implication of these findings is that nicotine replacement therapy, one of the major tools for smoking cessation, may not be appropriate during pregnancy, as the levels of nicotine are likely to exceed the threshold for alterations in fetal brain development (Slotkin, 1998b). Several years ago, we postulated that the first trimester might

represent a relatively safe period for nicotine replacement therapy, as the information available at that time suggested that nAChRs did not emerge until the end of that period (Cairns and Wonnacott, 1988; Hagino and Lee, 1985; Larsson et al., 1985). However, more recent evidence indicates clearly that these receptors are present even before neurulation (Atluri et al., 2001; Schneider et al., 2002), at which stage, excessive cholinergic stimulation leads to apoptosis and mitotic abnormalities (Roy et al., 1998b). Accordingly, in view of the neuroteratogenic effects of nicotine, the most appropriate course for fetal safety would be total maternal abstinence from smoking or nicotine replacement products.

One final issue is to identify how far along in development the brain remains sensitive to disruption by nicotine exposure. Brain development continues into adolescence, specifically encompassing neuroproliferation, apoptosis, and synaptic rearrangement (Bayer, 1983; Bayer et al., 1982; Huttenlocher, 1990). More specifically, the maturation of central cholinergic systems involved in learning, memory, and psychostimulant responses, is consolidated during the periadolescent period (Matthews et al., 1974; Nadler et al., 1974; Zahalka et al., 1993a). Adolescence is also the period in which most life-long smokers begin their habit. In recent years, over 30% of U.S. high school students reported smoking cigarettes, with about 3000 teenagers beginning smoking each day (Centers for Disease Control and Prevention, 1998, 2000; National Institute on Drug Abuse, 1998). Three-quarters of these become daily smokers by the age of 20 (National Institute on Drug Abuse, 1998; Nelson et al., 1995), comprising a cohort for whom smoking will be a long-term addiction (Pierce and Gilpin, 1996) with high daily consumption and a low probability of quitting (Chen and Millar, 1998). Accordingly, the number of deaths attributable to tobacco is expected to more than double worldwide by 2020 (Murray and Lopez, 1997). Recent studies suggest that adolescents are more susceptible than adults to the development of nicotine dependence, often showing signs of loss of autonomy over tobacco consumption and withdrawal symptoms after short-term, episodic cigarette consumption (DiFranza et al., 2000, 2002a, 2002b).

In light of the magnitude of the problem of tobacco use in adolescence, it is surprising that, up until a few years ago, little or no basic research had been done to characterize the neurochemical and behavioral effects of nicotine in the adolescent brain. We recently expanded our rat model of nicotine exposure to encompass the adolescent, designing regimens that produce plasma nicotine levels comparable to those in regular smokers (25 ng/ml) (Slotkin, 2002) as well as the lower levels seen in occasional smokers (Abreu-Villaça et al., 2003a, 2003b). As our findings were recently presented in a comprehensive review (Slotkin, 2002), they will be summarized only briefly here. Salient features of this model include more profound and persistent upregulation of nicotinic acetylcholine receptors (nAChRs) as compared to adults, as well as prolonged suppression of cholinergic activity and, upon nicotine withdrawal, long-term decrements in neural activity and responsiveness (Abreu-Villaça et al., 2003a; Kelley and Middaugh, 1999; Slotkin, 2002; Trauth et al., 2000a, 2000c, 2001; Xu et al., 2001, 2002). In part, these differences reflect continuation of the pattern of developmental neurotoxicity noted for fetal nicotine exposure, albeit to a lesser extent than during the prenatal period (Abreu-Villaça et al., 2003b; Slotkin, 2002; Trauth et al., 1999a, 2000b; Xu et al., 2002). As is true for the fetal exposure model, the effects of adolescent nicotine are accompanied by unique and persistent behavioral alterations that are entirely distinguishable from those seen in adults given nicotine (Levin, 1999; Trauth et al., 2000c). Specifically, the lasting desensitization of cholinergic inputs to monoaminergic systems (Trauth et al., 2001) likely contributes to long-term loss of psychostimulant reward responses (Kelley and Middaugh, 1999) as well as persistent changes in EEG and locomotor activity that have been characterized as an uniquely adolescent "nicotine abstinence syndrome" (Slawecki and Ehlers, 2002). Equally notable, the effects of adolescent nicotine in the rat model duplicate the sex selectivity noted for many aspects of human adolescent tobacco smoking. Female rats show greater degrees of neural cell damage (Abreu-Villaça et al., 2003b; Trauth et al., 1999a, 2000b; Xu et al., in press), greater impairment of synaptic activity of monoaminergic systems (Trauth et al., 2001; Xu et al., 2001, 2002, in press), and greater behavioral deficits (Trauth et al., 2000c). Recent studies indicate that female adolescents show more rapid onset of nicotine dependence, with loss of autonomy and signs of withdrawal after only a few cigarettes (DiFranza et al., 2002a).

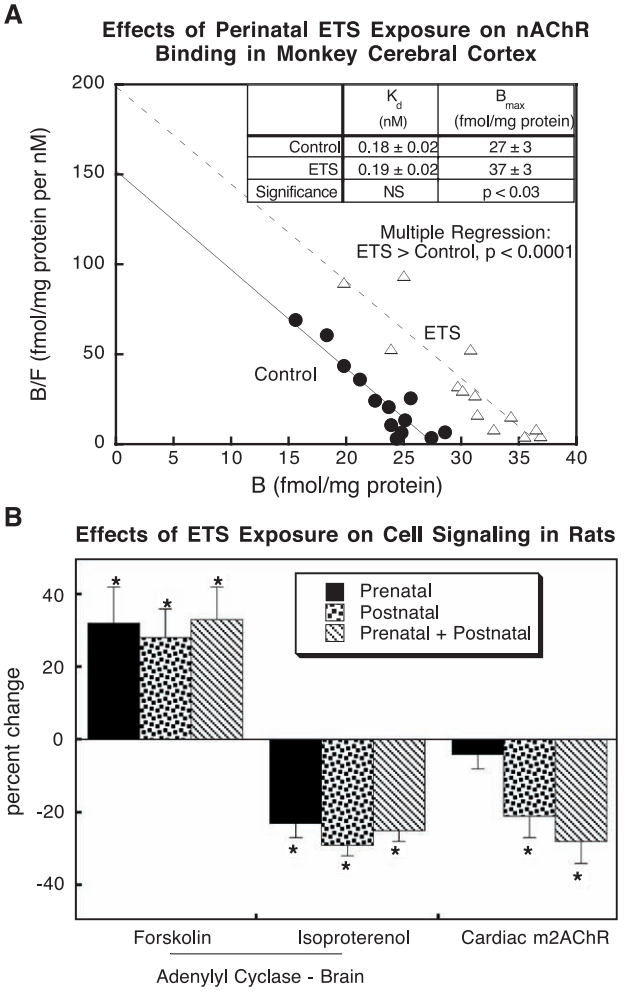
The findings in the adolescent have profound societal implications. Smoking among adolescents is undergoing an unprecedented increase, driven in measure by advertising targeted toward this age group, subtly embedded in films, music videos, youth-oriented magazines, and the Internet, often containing powerful sexual content (Durant et al., 1997; Ginzel, 1998; Hong and Cody, 2002; Sansores et al., 2002). In addition, the medical community has largely failed to recognize the magnitude of the problem or to provide appropriate countermeasures (Thorndike et al., 1999). The fact that nicotine in the adolescent brain, like the fetal brain, elicits neurotoxicity and long-term alterations in synaptic function means that there is likely to be a biological basis for increased susceptibility to nicotine dependence and long-term, adverse consequences during this late developmental stage. In fact, as discussed in the next section, nicotine levels as low as those experienced with typical ETS exposure may be sufficient to cause significant changes both in the fetal and adolescent brain.

Developmental neurotoxicity of environmental tobacco smoke: is nicotine the culprit?

Compared to active maternal smoking, far less is known about the consequences of prenatal ETS exposure, but

nearly all regulatory agencies and independent researchers agree that the extant information indicates significant health risks (Dunn and Zeise, 1997; Witschi et al., 1997). As might be expected, adverse effects of ETS are most prominent for respiratory disorders (Cook and Strachan, 1999; Irvine et al., 1997; Witschi et al., 1997), but additional information indicates that the effects extend to the cardiovascular system and to behavioral outcomes (Eskenazi and Trupin, 1995; Hutchison et al., 1998; Makin et al., 1991). There is a continuum of tobacco effects on neurobehavioral development, with ETS eliciting the same types of damage as active smoking but with a smaller magnitude of effect (Makin et al., 1991). Measurements of the levels of nicotine and its metabolites are particularly instructive in delineating the degree of exposure (Fried et al., 1995): these are readily detectable in amniotic fluid (Jauniaux et al., 1999), meconium (Ostrea et al., 1994) or hair (Eliopoulos et al., 1996) in levels similar to those seen with light active maternal smoking. Similarly, significant nicotine exposure occurs with postnatal ETS, with young children actually accumulating more nicotine than do older children at the same degree of exposure (Fried et al., 1995; Kohler et al., 1999). In terms of developmental liability, nicotine is one of the key components of ETS, because, as described in the preceding section, nicotine itself is a neuroteratogen with adverse effects on brain cell number, synaptic development, and neurobehavioral function (Levin and Slotkin, 1998; Slotkin, 1998a, 1999). As just one example, one of the key endpoints of nicotine-induced perinatal brain damage is interference with central and peripheral autonomic mechanisms that mediate the cardiorespiratory response to hypoxia (Slotkin, 1998b, 1999; Slotkin et al., 1995, 1997b), mechanisms that are thought to underlie SIDS (Fewell and Smith, 1998; Franco et al., 2000; Hafstrom et al., 2000; Harper, 2000; Milerad et al., 1998; Nachmanoff et al., 1998; Sovik et al., 1999; St.-John and Leiter, 1999; Storm et al., 1999; Ueda et al., 1999); ETS exposure, like active maternal smoking, increases the incidence of SIDS (Dybing and Sanner, 1999; Klonoff-Cohen et al., 1995).

In the case of ETS, there are several key questions. First, is there a specific role for nicotine, as opposed to the ancillary neuroteratogenic effects of the myriad substances present in ETS? Second, is there a critical period for effects of ETS? This latter point is particularly important, given potentially high environmental exposures of newborns both in and outside the home (Cook and Strachan, 1999; Dwyer et al., 1999; Kohler et al., 1999; Kulig et al., 1999; Scherer et al., 1999; Tang et al., 1999). In lieu of testing the thousands of compounds in ETS, many investigations from our and other research groups have taken a comparative approach to these questions, using animal models to contrast the effects of ETS to those seen with comparable exposures to nicotine alone. In a study with Rhesus macaque monkeys exposed to ETS throughout pregnancy and into the early neonatal period, we found substantial upregulation of nAChRs in the cerebral cortex and the brainstem (Slotkin et al., 2002a) (Fig. 3A). The magnitude of effect was entirely comparable to that seen with prenatal administration of nicotine itself to rodents (Hagino and Lee, 1985; Slotkin et al., 1987b) and to that seen in the brain of active human smokers (Perry et al., 1999). As already described, this degree of nAChR upregulation represents sufficient nicotine-induced cell stimulation to elicit damage to the developing brain, permanent changes in behavioral performance, and alterations in the expression of nAChRs that last into adulthood (Eriksson et al., 2000; Levin and Slotkin, 1998; Slotkin, 1998a).



	K_d (nM)	B_{max} (fmol/mg protein)
Control	0.18 ± 0.02	27 ± 3
ETS	0.19 ± 0.02	37 ± 3
Significance	NS	p < 0.03

Fig. 3. Neurochemical effects of ETS exposure. Data were taken from primary research papers, to which the reader is referred for experimental details (Slotkin et al., 2000, 2001b, 2002a). (A) Upregulation of nAChRs in cerebral cortex of Rhesus monkeys with episodic, perinatal exposure to ETS. Values for K_d and B_{max} are shown as means and standard errors. (B) Alterations in cell signaling in cell membrane fractions prepared from the brains of rats exposed to ETS prenatally only, postnatally only, or both prenatally and postnatally. Data are shown as means and standard errors. ETS exposure leads to induction of adenylyl cyclase, evidenced by an increase in the direct enzymatic stimulant, forskolin. At the same time, the specific response to β-adrenergic receptor stimulation is impaired, as evidenced by the proportion of total adenylyl cyclase activity that responds to the β-agonist, isoproterenol. In the heart, m2-acetylcholine receptors (m2AChRs) undergo downregulation, but only in the groups exposed to ETS postnatally.

These findings thus indicate that ETS exposes the developing brain to concentrations of nicotine that are likely to be sufficient to elicit neuroteratogenic changes. Indeed, just as noted for prenatal nicotine administration, ETS exposure of developing rats results in a loss of brain cells (Gospe et al., 1996) and alterations in synaptic communication (Slotkin et al., 2001b). The latter effects are of particular interest in that differentiating between actions on signaling proteins and neurotransmitter receptors allows for separation of the relative contributions of prenatal versus postnatal ETS exposure. Prenatal nicotine exposure is characterized by constitutive induction of adenylyl cyclase (AC), in association with, or potentially in reaction to, corresponding deficiencies in receptor inputs (Pennington et al., 1994; Slotkin et al., 1990, 1992, 1999, 2000). Defective signaling persists even when receptors normalize (Navarro et al., 1990a; Slotkin et al., 1990), implying that the problems originate in receptor coupling to response elements. It is therefore of utmost importance that we identified exactly the same pattern with ETS exposure (Slotkin et al., 2002a). In the brain, the response to forskolin, which bypasses receptor activation to stimulate AC directly, is upregulated regardless of whether ETS exposure occurred prenatally, postnatally, or in both periods (Fig. 3B). Additionally, the specific coupling of stimulatory β-adrenergic receptors to AC is reduced by ETS, as evidenced by a decrease in the proportion of AC activity recruited in response to β-receptor stimulation. Thus, ETS exposure recapitulates the same changes in AC signaling seen previously with prenatal nicotine treatment (Pennington et al., 1994; Slotkin et al., 1990, 1992, 1999), but with a critical period extending into postnatal life.

There are also alterations in peripheral reactivity to neural stimulation as a result of ETS exposure. Just as in the brain, ETS evokes induction of AC in the heart in rodent models (Slotkin et al., 2001b) and in the lung in nonhuman primates (Slotkin et al., 2000). In addition, we identified a decrease in cardiac m2-acetylcholine receptors that occurred only with postnatal ETS exposure (Slotkin et al., 2001b) (Fig. 3B). These effects are commensurate with the development of cardiorespiratory abnormalities that are common in children known to be exposed regularly to ETS (Cook and Strachan, 1999; Eskenazi and Trupin, 1995; Hutchison et al., 1998; Irvine et al., 1997; Makin et al., 1991; Pinkerton and Joad, 2000; Witschi et al., 1997).

In sum, the current state of findings for perinatal ETS exposure indicates that substantial nicotine reaches the developing brain, sufficient to evoke changes in brain cell development and synaptic signaling similar to those associated with prenatal nicotine exposure. Vulnerability clearly extends beyond the prenatal period, as postnatal ETS exposure, at levels mimicking passive smoking, elicits many of the same changes with the same magnitude. Although other tobacco smoke components undoubtedly contribute to many of the effects of developmental ETS exposure, it appears that the nicotine component contributes significantly to adverse outcomes.

To date, there are no basic research studies that specifically address the central nervous system effects of ETS exposure in adolescence. However, in light of the continuation of brain development into this period, we have modeled the effects of nicotine down to the plasma levels typically experienced with ETS exposure, one-tenth of those seen in active smokers (Abreu-Villaça et al., 2003a, 2003b). Upregulation of nAChRs is again evident, along with persistent decrements in indices of cholinergic synaptic activity. These functional changes are also associated with cell loss and damage to neuritic extensions, albeit to a lesser extent than with comparable exposures in the fetus. Equally important, the same effects are seen regardless of whether nicotine exposure is continuous or intermittent, so that repeated episodes of ETS exposure might be sufficient to produce the same types of damage. The exquisite sensitivity of the adolescent brain to nicotine neurotoxicity may thus contribute to lasting neurobehavioral damage even with the lower levels characteristic of ETS.

Developmental neurotoxicity of organophosphates: not just cholinesterase inhibition

Based on nicotine's effects on brain development, one might expect the actions of organophosphate insecticides to be quite similar. Typically, most organophosphates are prototoxicants, requiring metabolic replacement of the P=S bond with P=O, at which point the compounds act as irreversible inhibitors of acetylcholinesterase, forming a covalent linkage with the active site of the enzyme. As a result, acetylcholine breakdown is impaired, resulting in cholinergic hyperstimulation, which, when cholinesterase inhibition exceeds 70–75%, elicits classical signs of "cholinergic storm," salivation, lachrymation, urination, defecation, and eventually, muscle fasciculation, convulsions, and death. If these compounds are administered to developing organisms, then, the resultant cholinergic hyperstimulation should reproduce the types of defects seen with nicotine, a cholinergic agonist. As shall be shown below, this is only partially correct, as it appears that the organophosphates have additional effects on brain development that may overshadow their cholinergic component. By far, chlorpyrifos (CPF) has received the most attention in this regard, and I will summarize studies with this particular compound.

Despite recent regulatory decisions eliminating its use inside the home (U.S. Environmental Protection Agency, 2002), CPF continues to be used worldwide and is responsible for thousands of reported poisonings in the United States annually (Clegg and van Gemert, 1999; Litovitz et al., 1997); many more poisonings, particularly of small children, go unreported because of the absence of overt signs of toxicity (Litovitz et al., 1997). The major use of CPF in agriculture and professional applications such as termite control presents increasing problems of usage,

storage, disposal, and unintended human exposure. In the adult, unlike parathion, CPF evokes delayed neuropathies only with very high exposures (Mileson et al., 1998; Richardson et al., 1993). Nevertheless, there is growing concern that CPF and other organophosphates may produce neurobehavioral damage during development (Landrigan et al., 1999; Pope, 1999; Slotkin, 1999). Indeed, before the decision to ban its domestic use, estimates for pregnant women and children indicated potential CPF exposures above the No Observable Adverse Effect Level (Davis and Ahmed, 1998; Fenske et al., 1990; Gurunathan et al., 1998; U.S. Environmental Protection Agency, 2002), and a recent study of meconium in newborn infants indicates fetal exposure levels well above that threshold (Enrique et al., 2002). Animal studies confirm that CPF has higher systemic toxicity in neonates, with over an order of magnitude lower LD50 values than in adults (Pope and Chakraborti, 1992; Pope et al., 1991; Whitney et al., 1995). However, developing organisms recover more quickly from cholinesterase inhibition than do comparably exposed adults, largely due to the rapid synthesis of new cholinesterase molecules (Pope and Chakraborti, 1992; Pope et al., 1991; Song et al., 1997). This discrepancy means either that cholinesterase inhibition is unrelated to developmental toxicity, or alternatively that even a brief period of cholinesterase inhibition is sufficient to disrupt development. As we shall see, both of these explanations are true.

Parallel to our studies with nicotine (Roy et al., 1998b), we have also carried out studies in rat embryo cultures with CPF (Roy et al., 1998a). Using chlorpyrifos concentrations that bracket the values found in fetal meconium (Enrique et al., 2002), we found clear-cut mitotic abnormalities and evidence of apoptosis in the developing brain after a 48-h exposure at the neural tube stage of development; mitotic figures were dispersed and disoriented and in addition, cytotoxicity was evident from cytoplasmic vacuolation, enlargement of intercellular spaces, and the presence of a significant number of apoptotic figures (Fig. 4A). Significant effects were found even at concentrations more than an order of magnitude below those found in human fetal meconium. There is a striking resemblance of these results to the effects of nicotine in the same embryo culture model (Roy et al., 1998b), a similarity that supports the idea of augmented cholinergic activity underlies the adverse effect of CPF. There is a difference, however, in that nicotine stimulates nAChRs directly, whereas inhibition of cholinesterase resulting from CPF would produce cholinergic stimulation only where acetylcholine is being actively released. Therein lies an interpretive problem. Choline acetyltransferase, the enzyme responsible for acetylcholine biosynthesis, is not detectable in the developing brain until after the neural tube stage (Lauder and Schambra, 1999). Either there is cryptic, non-neuronal enzymatic activity, or else CPF is acting through different mechanisms; some suggestions include direct actions on nAChRs (Katz et al., 1997), interference with nonenzymatic functions of acetylcholinesterase that are involved in neural development (Brimijoin and Koenigsberger, 1999), or effects on cell signaling cascades that are critical for neural cell differentiation (Huff et al., 1994; Olivier et al., 2001; Schuh et al., 2002; Song et al., 1997; Ward and Mundy, 1996). Regardless of the underlying mechanism, even a few days later, in late gestation, there is little biochemical evidence of cell loss or alterations in indices of cell size or neuritic outgrowth (Qiao et al., 2002), a finding very similar to that of nicotine exposure (Fig. 4B). Nevertheless, just as with nicotine, deficits in brain cell numbers, neuritic projections, and synaptic communication emerge in adolescence and continue into adulthood (Qiao et al., 2003a), so that the programming of synaptic development has clearly been altered by CPF exposure in this early gestational period. Comparable, delayed changes are also evident when CPF exposure occurs later in gestation. Again, there are initial morphological changes (Lassiter et al., 2002; White et al., 2002) that either resolve relatively quickly, or at least are difficult to detect with biochemical indices of cell damage (Qiao et al., 2002), only to reemerge later in development, accompanied by behavioral anomalies (Garcia et al., 2002, 2003; Levin et al., 2002; Qiao et al., 2003b). The defects are notable in the same neurotransmitter pathways and regions that are characteristic of nicotine-induced alterations, particularly involving cerebrocortical and hippocampal cholinergic projections (Fig. 4C).

What is particularly important is that the later-appearing deficits elicited after prenatal CPF are fully evident even at exposures that lie below the threshold for detectable cholinesterase inhibition, and certainly below the 70–80% inhibition required for systemic toxicity (Garcia et al., 2002, 2003; Levin et al., 2002; Qiao et al., 2002, 2003b). This reinforces the concept that other mechanisms might be critical to the actions of CPF on the developing brain. This supposition has been tested directly with in vivo models of postnatal CPF exposure as well as in cell cultures. Again, paralleling our studies with nicotine, when we administered chlorpyrifos to 1-day-old rats, we observed acute inhibition of DNA synthesis (Whitney et al., 1995), but there was no regional selectivity to the effect (Fig. 4D): regions with low cholinergic innervation and low nAChR concentrations (cerebellum) were affected just as much as cholinergically enriched regions (brainstem, forebrain), whereas nicotine elicited the expected regional pattern. We obtained the same inhibitory actions when minute amounts of CPF were injected directly into the brain, bypassing hepatic activation to CPF oxon, the metabolite that inhibits cholinesterase. Regional selectivity emerged by the end of the first postnatal week, corresponding to the phase of rapid cholinergic synaptogenesis. Thus, CPF affects DNA synthesis by an initial, noncholinergic effect, and only subsequently through actions mediated by cholinergic hyperactivity. The biological significance of noncholinergic mechanisms is evident from subsequent studies demonstrating persistent inhibition of DNA synthesis with repeated CPF administration (Dam

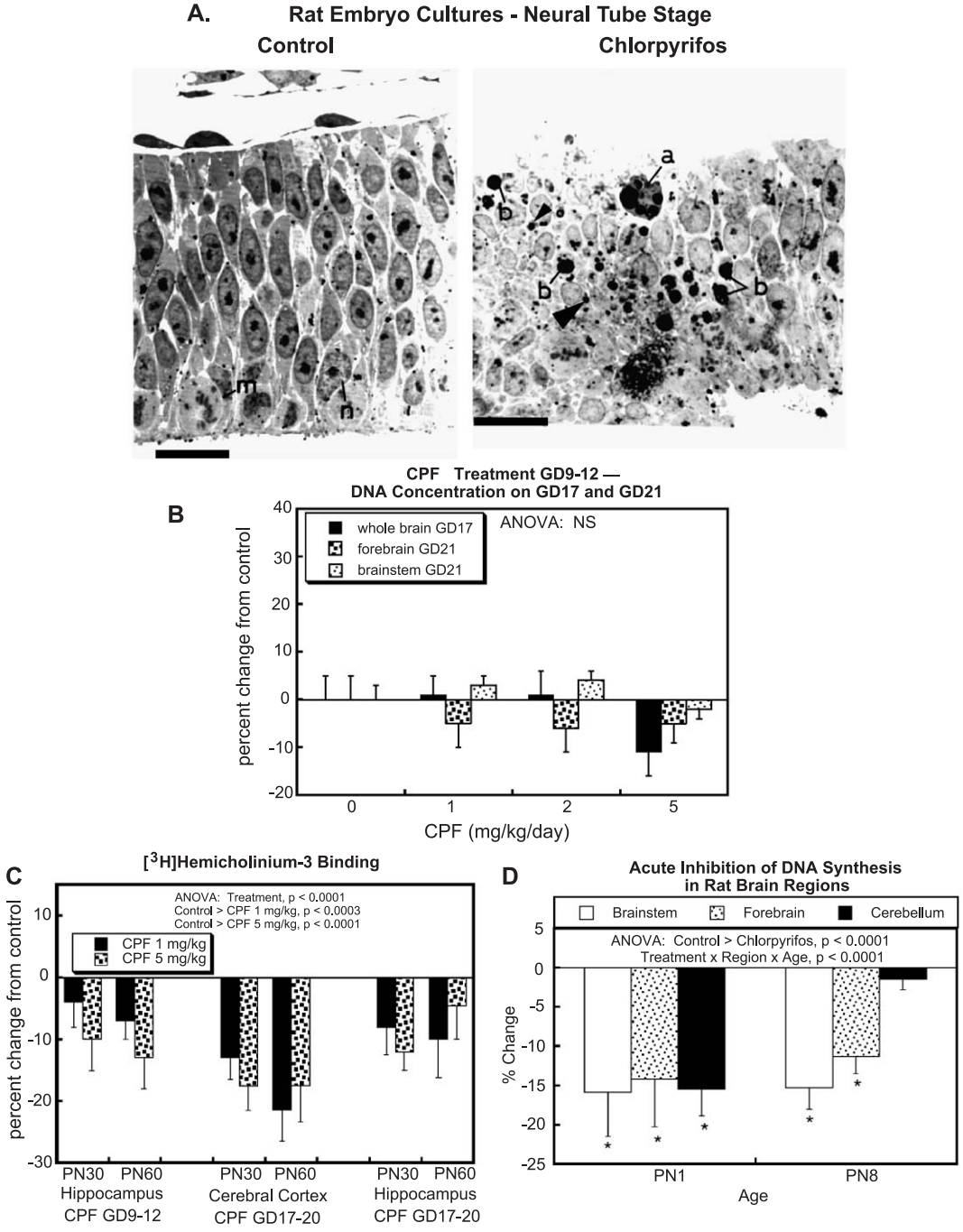


Fig. 4. Developmental neurotoxicity of CPF; experimental details for each panel are available in the original publications (Qiao et al., 2002, 2003a, 2003b; Roy et al., 1998a; Whitney et al., 1995). Quantitative results are shown as means and standard errors. (A) Effects of CPF on brain development in cultured rat embryos. Exposure occurred for a 48-h period beginning at 9.5 days of gestation. The left panel shows forebrain neuroepithelium in control embryos at embryonic day 11.5, displaying a bipolar pseudostratified epithelium, apical and basal processes containing a granular nucleus and inactive heterochromatin (n), as well as normal mitotic figures (m) located towards the internal limiting membrane. The right panel shows neuroepithelium from a chlorpyrifos-exposed embryo, exhibiting extensive cell death (b) and extracellular bodies (arrowheads). A large cell (a) with multiple apoptotic condensations is also visible. Scale bar = 20 μm. Over a much larger cohort (>40 embryos per treatment), there was no evidence of gross dysmorphogenesis nor changes in developmental landmarks, aside from the disruption of cell development in the neuroepithelium. (B) After CPF exposure in vivo by maternal administration on GD9–12, there is no biochemical evidence of cell loss in the fetal brain by GD17 or GD21, as evaluated by measurements of DNA. (C) Deficits in hemicholinium-3 binding to the presynaptic choline transporter, a biomarker for cholinergic synaptic activity, emerge in adolescence (postnatal day 30) and adulthood (PN60), shown for the hippocampus after CPF exposure on gestational days (GD) 9–12, and for the cerebral cortex and hippocampus after CPF exposure on GD17–20. (D) Acute inhibition of DNA synthesis in brain regions of rats given a single dose of CPF at 1 or 8 days of age. Note the lack of a regional hierarchy on postnatal day (PN) 1, whereas by 8 days of age, CPF does evoke regionally selective effects that parallel cholinergic innervation and nAChRs.

et al., 1998), eventual deficits in the number of neural cells (Campbell et al., 1997), suppression of gene expression and macromolecular constituents involved in cell differentiation (Dam et al., 2003; Johnson et al., 1998), and consequent abnormalities of synaptic function and behavioral performance (Dam et al., 2000; Levin et al., 2001; Slotkin et al., 2001a, 2002b). Again, these effects are seen at CPF exposure levels that are devoid of any overt toxicity and that reduce cholinesterase activity by only 20% (Song et al., 1997), a degree of inhibition insufficient to produce signs of systemic toxicity.

Further characterization of developmental neurotoxicity of CPF comprising mechanisms over and above those related to cholinesterase inhibition has been provided by several in vitro models. PC12 rat pheochromocytoma cells are transformed neural precursor cells that, upon initiation of differentiation with nerve growth factor, develop neuritic projections and resemble neurons morphologically, physiologically, and biochemically, including increased expression of cholinergic receptors, choline acetyltransferase and acetylcholinesterase (Berse and Blusztajn, 1997; Greene and Rukenstein, 1981; Greene and Tischler, 1976; Tischler and Greene, 1975). Using undifferentiated PC12 cells, we were able to reproduce the ability of CPF to inhibit DNA synthesis by mechanisms unrelated to cholinergic activation (Song et al., 1998). When PC12 cells were allowed to differentiate, in the continuous presence of CPF, the inhibition of DNA synthesis intensified and persisted throughout the period of cell development and consequently, acquisition of new cells was severely curtailed, reproducing the effects found for chlorpyrifos in vivo. Other laboratories have found potent inhibition of neurite outgrowth, again unrelated to cholinesterase inhibition (Das and Barone, 1999; Li and Casida, 1998), and we (Crumpton et al., 2000; Garcia et al., 2001; Song et al., 1998) and others (Schuh et al., 2002) identified specific, noncholinergic interference with nuclear transcription factors that control cell differentiation. Although the noncholinergic mechanisms participating in the developmental neurotoxicity of CPF are just beginning to be understood, there are many likely candidates, all concerning signaling cascades that are common to multiple neuronal and hormonal inputs. These include the AC-cyclic AMP-protein kinase A cascade (Garcia et al., 2001; Huff et al., 1994; Olivier et al., 2001; Schuh et al., 2002; Song et al., 1997; Ward and Mundy, 1996; Yanai et al., 2002; Zhang et al., 2002), receptor signaling operating through protein kinase C (Bomser et al., 2002; Buznikov et al., 2001b; Yanai et al., 2002), as well as direct actions on the expression and function of nuclear transcription factors that mediate the switch from cell replication to differentiation and neuritic outgrowth, including c-*fos*, p53, AP-1, Sp1, and CREB (Crumpton et al., 2000; Dam et al., 2003; Garcia et al., 2001; Schuh et al., 2002).

What is critical about noncholinergic targets for CPF is that they are all involved in signaling elements that represent the convergent, final pathways for multiple receptor types; these kinds of heterologous alterations might therefore explain the widespread and delayed-onset nature of many of the effects of developmental exposure to CPF. In any case, the fact that these are unrelated to cholinesterase inhibition is likely to have a significant impact on our view of organophosphate-induced developmental neurotoxicity. In the past, these compounds, along with other cholinesterase inhibitor insecticides such as carbamates, have been grouped together because of their purported common mechanism of cholinesterase inhibition (Mileson et al., 1998). Now that it is evident that there are other mechanisms for disruption of brain development, the underlying assumption of a common mechanism and summation of effects of different compounds is no longer tenable. Unfortunately, we may therefore have to consider each individual compound as a separate entity.

The existence of multiple mechanisms underlying the developmental neurotoxicity of CPF has additional implications for identification of critical windows of vulnerability. If there are multiple mechanisms, then vulnerability is likely to extend over a broad developmental period and to display shifting cellular and regional targets, depending on phases of brain development and the specific mechanism at each phase. Indeed, neurobehavioral anomalies can be elicited by CPF exposure even relatively late in brain development, during the second and third postnatal weeks in the rat (Dam et al., 2000; Levin et al., 2001; Moser, 2000; Moser and Padilla, 1998). This period lies well outside of the major phase of neurogenesis in most brain regions but is within the peak period of gliogenesis and synaptogenesis. It is therefore important to note that we have found greater sensitivity of developing glia to CPF as compared to neurons, studies that have been conducted both with in vivo exposure models and with cultures of gliotypic cells (Garcia et al., 2001, 2002; Qiao et al., 2001). Glia provide nutritional, structural, and homeostatic support that are essential to architectural modeling of the brain (Aschner et al., 1999; Barone et al., 2000; Guerri and Renau-Piqueras, 1997; Morita et al., 1999; Tacconi, 1998), and because glial development continues well into the postnatal period, glial targeting implies a prolonged vulnerability, extending into childhood. In keeping with this interpretation, CPF administration in vivo inhibits DNA synthesis and causes loss of brain cells during gliogenesis (Campbell et al., 1997; Dam et al., 1998; Whitney et al., 1995), with maximal effects on neural function appearing during peaks of glial development (Campbell et al., 1997; Dam et al., 1999; Monnet-Tschudi et al., 2000; Slotkin, 1999; Song et al., 1997). In aggregating brain cell cultures, CPF affects glial markers, again unrelated to cholinesterase inhibition (Monnet-Tschudi et al., 2000).

Thus, although many aspects of the developmental neurotoxicity of CPF resemble those of nicotine, and hence appear to be consistent with the predicated role of acetylcholine as a trophic factor in nervous system maturation, there are a host of other mechanisms that operate as well. The consequences of CPF exposure represent a shifting

target in terms of mechanisms, types of cells involved, developmental processes that are compromised, and eventual behavioral outcomes. However, the main point is that vulnerability to CPF involves periods as early as the neural tube stage and as late as childhood or adolescence.

Where do we go from here?

The multiple and critical roles of acetylcholine as a trophic factor controlling the development of the brain from its primordial origins, through the final stages of plasticity and the programming of synaptic activity, mean that drugs or chemicals that target cholinergic neurotransmission represent a large and varied source of neurobehavioral anomalies. In the cases of maternal or adolescent smoking, or organophosphate insecticides, these exposures likely comprise a major proportion of the human population. However, it is a mistake to suppose that all compounds exhibiting cholinergic activity act only through that mechanism, and CPF provides a major example where other processes need to be taken into account. Establishing the underlying mechanisms, and hence safety thresholds, for these compounds must therefore represent a major focus of future work, but therein lies a serious problem: the uncovering of unexpected, alternative mechanisms that are particular to the immature organism will require new screening methods that emphasize unique attributes of developing systems. As shown here, and reviewed elsewhere (Slotkin, in press), in vitro systems, such as neural cell lines or embryo cultures, can play a key role in elaborating these mechanisms. An additional possibility is to return to the evolutionary origin of the role of neurotransmitters in brain development, namely, their role as morphogens in lower organisms. The best-studied case is the sea urchin, where acetylcholine, norepinephrine, serotonin, and other neurotransmitters are required for assembly of the embryo (Buznikov, 1990; Buznikov and Rakic, 2000; Buznikov et al., 1996, 2001a), using similar receptors and, importantly, intracellular signaling cascades that are likely to be the secondary targets of neurodevelopmental disruptors. In a recent study, we used the sea urchin model to demonstrate its ability to discriminate between the cholinergic and noncholinergic components of CPF-induced developmental alterations (Buznikov et al., 2001b), as these organisms appear to represent potential biosensors for a wider variety of toxicants (Buznikov, 1983; Buznikov et al., 1997), including nicotine (Buznikov et al., in press). CPF, but not CPF oxon, the active metabolite that inhibits cholinesterase, evokes profound structural abnormalities, appearing only when the embryonic genome is turned on and the maternal genome is turned off, as would be expected from interference with gene transcription involved in cell differentiation, precisely the proposed mechanism for adverse effects on brain development. Unlike the elaborate procedures required to detect abnormalities of synaptic function in the mammalian brain, the morphological abnormalities in the sea urchin embryo are readily visualized in the live organism under light microscopy (Fig. 5), and of course, the sea urchin produces thousands of virtually identical embryos, which can be used for screening of developmental neurotoxicants. There are similar prospects for nonmammalian, vertebrate models, such as the zebrafish (Grunwald and Eisen, 2002; Moens and Prince, 2002). Interfacing these newer models with the standard Developmental Neurotoxicity Protocol will represent a worthwhile challenge.

The findings reviewed here have many implications for future work in developmental neurotoxicity. The first of these concerns exposure scenarios. In the case of nicotine, although we have a reasonable conception of the likely fetal exposure levels from active maternal smoking, there are remaining issues about levels achieved in the fetus, especially as nicotine pharmacokinetics change during pregnancy (Dempsey et al., 2002). Additional issues are raised for quantitation of ETS exposure, or for the actual fetal levels achieved with the use of the transdermal nicotine patch or other nicotine replacement therapies for smoking cessation

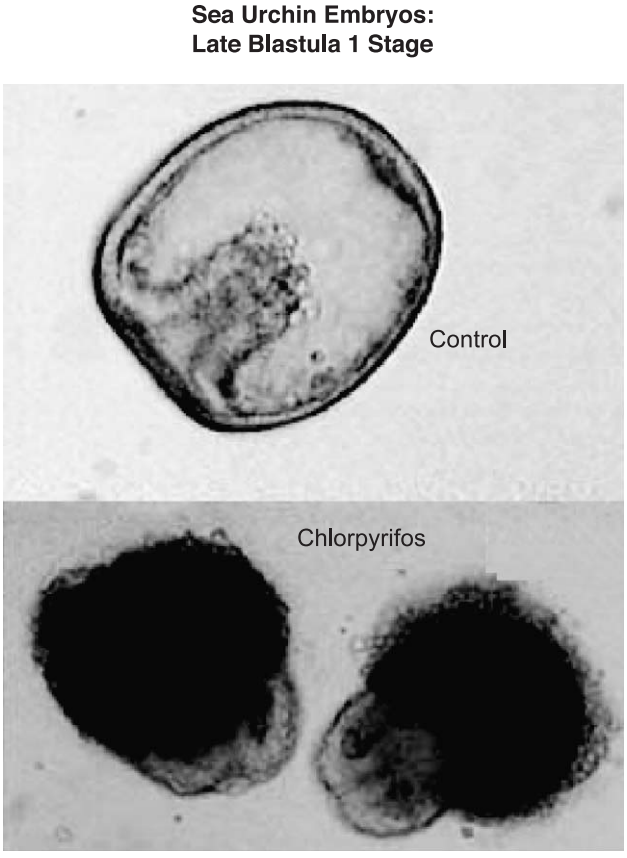


Fig. 5. Effects of CPF on morphogenesis in sea urchin embryos at the late blastula 1 stage. The top panel shows a control embryo and the bottom panel shows the effects of CPF. Note the abnormal pattern of cell differentiation, resulting in pigmented cells forming a "mushroom-like" extralarval cap. CPF oxon, non-organophosphate cholinesterase inhibitors, and the organochlorine insecticide, dieldrin, were all ineffective. Experimental details can be obtained from the primary publication (Buznikov et al., 2001b).

(Slotkin, 1998a). In the case of organophosphates, whereas nearly all investigations have concerned relatively high exposures typical of insecticide application in the home or agriculture, there is little or no information about the consequences of long-term intake of the much lower levels typical of food and water supplies; children consume much higher amounts of food and water per unit body weight, so that their exposures are considerably higher than those of adults, factors that may contribute to childhood vulnerability (Landrigan, 2001; Landrigan et al., 1999; May, 2000; National Research Council, 1993; Physicians for Social Responsibility, 1995; Ray and Richards, 2001). Nor should we overlook the potential exposures that may occur in a terrorist incident, involving organophosphate nerve gases, such as that carried out several years ago in the Tokyo subway: What are the long-term consequences of fetal or childhood exposures below the threshold at which toxic symptoms are evident? Another new concept emerges from the fact that the period of vulnerability of the developing brain to both nicotine and CPF extends to the period of synaptic modeling, continuing into childhood and adolescence. Although the guidelines for the Developmental Neurotoxicology Protocol have recently been extended to incorporate periods of brain development corresponding to the early neonatal period, it is evident that a complete picture of adverse effects may require extension to much later phases (Slotkin, in press). There is every reason to suspect that, unlike standard teratogens, many more agents that target brain development will exert adverse effects extending into adolescence. Finally, the identification of specific cellular and synaptic mechanisms underlying the actions of developmental neurotoxicants, and the equally important task of specifying the pathways and regions whose functions are ultimately compromised, hold the promise of enabling the design of therapies that might reverse or offset the adverse effects. To date, this has been attempted successfully in at least two different models with strategies of fetal cell transplants (Yanai and Pick, 1988) and pharmacologic manipulations (Levin et al., 1993b). Future work may thus reveal intervention strategies that allow us to do something about developmental neurotoxicants other than just identifying their ability to damage the developing brain.

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