Nicotine Evokes Cell Death in Embryonic Rat Brain during Neurulation

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Accepted for publication July 7, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Maternal cigarette smoking during pregnancy represents the most prevalent exposure to a suspected neuroteratogen, nicotine. Although animal models have demonstrated brain cell loss and synaptic abnormalities after prenatal nicotine exposure, the multiple effects of nicotine on the maternal-fetal unit make it difficult to prove that nicotine itself is a neuroteratogen. In the current study, whole rat embryo culture was used to study the effects of nicotine at the neural tube stage of development. Beginning on embryonic day 9.5, embryos were exposed to 1, 10 or 100 μM nicotine. After 48 hr, embryos were examined for dysmorphogenesis and were then processed for light microscopic examination of the neuroepithelium. Examination of the forebrain, midbrain and hindbrain regions revealed extensive cytotoxicity, evidenced by cytoplasmic vacuolation, enlargement of intercellular spaces and a sharply increased incidence of pyknotic/apoptotic cells. These alterations were evident in the absence of generalized dysmorphogenesis and were detectable even at the lowest concentration of nicotine. At the highest concentration, abnormalities were present in the majority of cells. Superimposed on cell damage, we found an increase in mitotic figures. Although enhanced mitosis could represent partial compensation for cell loss, the regional selectivity and concentration dependence of the mitogenetic effect differed significantly from that of cell death, suggesting separable mechanisms. The present results support the view that nicotine is a neuroteratogen, specifically targeting brain development at concentrations below the threshold for dysmorphogenesis.

Despite extensive adverse publicity, approximately 25% of all pregnant women in the United States smoke (Bardy et al., 1993; DiFranza and Lew, 1995). The consequences for human development have been well identified in epidemiological studies: tens of thousands of spontaneous abortions and neonatal intensive care unit admissions annually and thousands of perinatal deaths and deaths from sudden infant death syndrome (DiFranza and Lew, 1995). Perhaps of greater long-term impact on society, it is now recognized that maternal smoking substantially increases the risk of learning disabilities, behavioral problems, and attention deficit/hyperactivity disorder in the offspring (Naeye, 1992; Bell and Lau, 1995; DiFranza and Lew, 1995). Although these correlations imply that maternal smoking has the potential to elicit fetal brain damage, the direct proof of a causative relationship has proven elusive because of the many covariables that operate in smoking, such as low socioeconomic status, poor prenatal care or co-abuse of other substances. These problems can be resolved through the use of animal models that incorporate exposures to the separable components present in tobacco but that obviously do not share societal covariables.

Studies of the effects of nicotine on fetal development (review, Slotkin, 1992) have confirmed that nicotine, by itself, is capable of causing fetal brain damage, as evidenced by cell loss, synaptic abnormalities and behavioral deficits. Although cigarette smoke contains thousands of bioactive compounds, the finding that nicotine is injurious to the developing brain provides a mechanistic connection between maternal smoking and adverse fetal/neonatal outcomes. In rats, fetal brain damage is demonstrable at plasma nicotine levels that simulate those found in human smokers and that do not evoke malformations or otherwise compromise general developmental parameters such as growth. Furthermore, regional targeting of adverse effects of nicotine on the developing brain follows the concentration of nicotinic cholinergic receptors, suggesting direct actions on cell development (Slotkin, 1992). Nonetheless, nicotine can exert multiple effects on the maternal-fetal unit, including alterations in cardiovascular or hormonal status, rendering it difficult to label nicotine conclusively as a neuroteratogen. However, in vitro systems have the potential to demonstrate a direct effect of nicotine on neurodevelopment. Studies in cultured PC12...
cells, a cloned cell line that developmentally resembles sympa-
thetic neurons, indicate that nicotine can inhibit DNA
synthesis and neurite outgrowth, but only at concentrations
several orders of magnitude above those found in smokers
(Chan and Quik, 1993; Song et al., 1998). Similarly, cultured
rat embryos show gross morphological disruption by nicotine
exposures at very high concentrations (≥1 mM), sufficiently
high to evoke physicochemical membrane disruption and/or
gross oxidative damage (Joschko et al., 1991). The nicotine-
treated embryos also evince altered development of the neu-
roepithelium, potentially contributing to the elevated rate of
neural tube defects seen with maternal smoking (Joschko
et al., 1991), but given the excessive concentrations, it is not
possible to determine whether these effects are relevant to
clinical exposures. It is not uncommon that the effects of
nicotine on cultured cells require exposure levels above those
experienced in intact systems in order to demonstrate signif-
icant alterations of tightly controlled variables such as cell
replication or differentiation, even when these are known to
be relevant endpoints in vivo (Kono et al., 1991; Chan
and Quik, 1993; Tipton and Dabbous, 1995; Song et al., 1998).
Nevertheless, a definitive demonstration that nicotine spe-
cifically affects neurodevelopment requires an in vitro sys-
tem where effects can be seen at concentrations below those
necessary for gross dysmorphogenesis.

Our study investigates the effects of nicotine on brain
development at the stage of neurulation in cultured rat em-
bryos, using well-established designs (Joschko et al., 1991;
Andrews et al., 1997). The mammalian central nervous sys-
tem develops from a pseudostratified epithelium with highly
specified patterns of mitosis and cell migration (Sauer, 1935;
Fujita, 1960; Adams, 1996). During this process, it is rare to
find apoptotic cells within the neuroepithelium except in
highly specific locations. Accordingly, the early neuroepithe-
lium can provide a sensitive test system for adverse effects
of nicotine exposure that incorporates targeting of mitosis, cell
migration and laminar organization, and cell death. Accord-
ingly, we have examined the effects of a 48-hr exposure to
nicotine in cultured rat embryos from embryonic day 9.5
through 11.5, a period corresponding to major architectural
change in the neuroepithelium, and to the emergence of
nicotinic cholinergic receptors (Naeff et al., 1992). A key part
of our hypothesis is that, if nicotine is a specific neuroterato-
gen, effects will be demonstrable below the threshold for
general embryonic dysmorphogenesis. Thus, we have used a
range of concentrations well below that shown previously to
elicit gross developmental abnormalities (Joschko et al.,
1991). We have then examined targeting of cell development
by assessing pyknotic and mitotic profiles as well as other
evidence of cell damage.

Methods

Animals. All studies using animals were carried out in accor-
dance with the declaration of Helsinki and with the Guide for the
Care and Use of Laboratory Animals as adopted and promulgated
by the National Institutes of Health. Sprague-Dawley rats (Charles
River Laboratories, Raleigh, NC) were housed in polypolypro-
ylene breeding cages with heat-treated pine savings, and supplied with
standard rat chow and water ad libitum, in a temperature-controlled
facility with a 12-hr light/dark cycle. Each cage contained one male
and two females. Vaginal swabs were examined and the sperm-
positive date was taken as embryonic day zero. At embryonic day 9.5,
the pregnant rats were anesthetized with ether and uteri were
removed to prepare the embryos for culture.

Whole embryo culture and teratology screening. The culture
procedure followed the protocols established in previous work (An-
drews et al., 1997). Excised uteri were dipped immediately in 70%
ethyl alcohol and 2× HBSS solution, and were placed in Waymouth’s
medium. Embryos, along with the decidual tissue, were removed
from the uterus. The conceptus was further dissected from the de-
cidual tissue and Reichert membrane under a stereomicroscope.
Groups of four embryos were placed in 25-ml culture flasks contain-
ing 5 ml of culture medium [medium prepared from male rat serum
preserved at −20°C for not more than 1 mo; heat inactivated at 55°C
for 30 min, sterilized by filtration, supplemented with 50 U/ml of
penicillin G and 50 μg/ml of streptomycin]. The medium was equil-
ibrated for 3 min with 5% O₂, 5% CO₂, 90% N₂ and the flasks were
placed on a rocker platform (19 oscillations/min) and incubated at
37°C. To initiate treatment, nicotine (Sigma Chemical Co., St. Louis,
MO) was dissolved in dimethylsulfoxide and was added to achieve
final concentrations of 1, 10 and 100 μM. An equivalent volume of
dimethylsulfoxide was added to the control group. After 24 and 42 hr
in culture, the medium was reequilibrated with 40% O₂, 5% CO₂,
55% N₂ for 2 min. After 48 hr of total culture time, embryos were
examined under a dissecting microscope for viability by the presence
of yolk sac circulation and heart beat, and for dysmorphogenesis,
after which they were dissected from the membranes and processed
for light microscopy. A standardized morphological scoring system
(Brown and Fabro, 1981) was used to assess embryonic growth
parameters as well as developmental stages.

Tissue processing and quantitative analysis. Embryos were
fixed in Karnovsky’s fixative, dehydrated in ascending concentra-
tions of ethanol and embedded in media containing LX-112 resin and
(2-dodecnyl-1-yl)succinic anhydride. The embryos were placed lon-
gitudinally along the long axis of plastic molds and were blocked in
the same resin, sliced into 1-μm transverse sections with a Reichert
Ultramicrotome, stained with 1% toluidine blue, and examined and
photographed with a Zeiss Axioskop microscope.

Sections were selected for analysis from the neural tube as shown
 schematically in figure 1a. Camera lucida drawings were made at
400× magnification and quantitation was conducted in a 100-μm
portion of the neural tube, 150 μm caudally from the highest point
of the neural tube. For quantitation, the neural tube was divided into
forebrain, midbrain and hindbrain, in each case including portions of
the alar plates of these regions (fig. 1a, inset). In each region, py-
knotic cells and mitotic figures were counted in every tenth section,
for a total of 10 sections per embryo. Healthy cells containing en-
gulfed debris from pyknotic cells were not included in the count but
wherever three or more adjacent clumps of condensed material were
seen between cells, they were considered to represent the remains of
a pyknotic cell. Isolated individual clumps between cells were not
included in the quantitation.

Quantitative analysis was conducted using four embryos from
each treatment group, in each case, selecting embryos that showed
no gross dysmorphology. Data are presented as means and S.E.
Comparisons across different groups were conducted by ANOVA
(data log-transformed whenever variance was heterogeneous), with
factors of treatment, region and section, with the latter two factors
considered to be repeated measures, since multiple regions and sec-
tions were taken from the same embryos. Post hoc evaluations were
conducted with Fisher’s protected least significant difference. For
presentation purposes, the data obtained from the 10 sections of each
embryo were averaged to produce a single value, so that the reported
standard errors consider each treatment group to contain only four
independent values even though each value had ten individual de-
terminations.
Fig. 1. a, Coronal section of control rat embryo, showing neural tube regions studied: alar plates of forebrain (FB), midbrain (MB) and isthmal region of hindbrain (HB); these regions of the neural tube give rise to cerebral cortex, midbrain and pontine region of the adult brain, respectively. Inset shows a camera lucida drawing of a sagittal section, with horizontal lines indicating portions of the neural tube used for quantitation. Subsequent pictures show portions of the neuroepithelium from similar regions of the neural tube. Scale bar = 250 μm. b, Neuroepithelium from a control embryo, showing closely apposed pseudostratified cells at different phases of mitosis, and their processes. The mitotic zone, which contains number of mitotic figures (MF), is located toward the lumen of the neural tube. Scale bar = 20 μm. c, Neuroepithelium from an embryo exposed to 100 μM nicotine, showing massive cell death in the hindbrain region alar plate in the region of neural tube fusion. Scale bar = 100 μm. d, Higher magnification of the necrotic zone inside the box of (c). Dying cells appear at various stages along with their debris, in the form of intra- and extracellular bodies, often engulfed by healthy cells, including those undergoing mitosis (M). Arrowheads and arrows represented pyknotic bodies and dying cells. A large nucleated phagosome (P), containing multiple dark bodies can be seen inside the epithelium. Scale bar = 20 μm.
Results

Growth and morphological development. After 48 hr in culture, the control embryos had completed their neural tube fusion and displayed all the characteristic features of an 11.5-day embryo (data not shown): a well formed optic cup, optic placode and first and second visceral arches; an S-shaped heart tube with structures corresponding to the atrium, a common ventricle and bulbus cordis; and in the distal part of the heart tube, a dorsal aorta connected by arch arteries. Both fore and hind limb buds were distinct and 27 to 28 somites were visible. Only one of a total of 47 control embryos exhibited a structural anomaly (table 1). Similar results were obtained in nearly all the embryos in the nicotine exposure groups, which also showed no growth retardation. There was no significant increase in the incidence of dysmorphogenesis (Fisher’s exact test), whether considered across all three nicotine groups taken together, or as individual treatment groups compared to the control group.

To determine whether nicotine exerts deleterious effects on neuroepithelial cell development, four morphologically normal embryos were selected from each of the treatment groups (table 2). Detailed examination revealed no abnormalities of yolk sac diameter, crown-rump length, developmental scoring scale or numbers of somites.

Neuroepithelial development. In control embryos, gross examination revealed appropriate fusion of the neural folds and a thick neuroepithelium consisting of closely apposed pseudostratified epithelium. The germinal matrix of the cerebral cortex was composed of a single zone, with the great majority of mitotic cells situated in a periventricular position (fig. 1b). The mesenchyme around the neuroepithelium contained stellate shaped cells that made contact with neighboring cells by means of cytoplasmic processes and detergent or fragmented particles. Some of the healthy cells showed darkly stained small areas, fragments of dead cells engulfed by neuroepithelial cells.

In keeping with the observation that nicotine did not cause gross dysmorphogenesis or overall developmental delays, the major brain regions developed on schedule in the treated embryos. However, on further histological examination, it was apparent that nicotine evoked major alterations in neuroepithelial cytoarchitecture. Most notably, the nicotine group showed extensive neuronal cell death throughout the developing neural tube. Qualitatively, the highest concentration of pyknotic figures was found in the hindbrain (fig. 1c) followed by the telencephalic forebrain region, whereas the midbrain was the least affected. Massive damage was found in the alar plate, indicating a particular sensitivity of junctional region neuroblasts (fig. 1, c and d). In contrast, the ventricular zone, where neurons and glia are generated, tended to show fewer dying cells.

These observations were confirmed by quantitative analysis of cells showing pyknotic profiles and debris (fig. 2). There was a strong concentration-dependent effect in all regions of the neuroepithelium, with statistically significant effects achieved even at the lowest nicotine concentration. The regional targeting profile, namely hindbrain > forebrain > midbrain, was confirmed by the presence of a significant treatment × region interaction.

No significant dysmorphogenesis in any individual group or all nicotine groups together vs. control (Fisher’s exact test).

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Embryos</th>
<th>No. Dysmorphogenic</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nicotine 1 μM</td>
<td>47</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Nicotine 10 μM</td>
<td>45</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Nicotine 100 μM</td>
<td>44</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

No significant dysmorphogenesis in any individual group or all nicotine groups together vs. control (Fisher’s exact test).

### TABLE 2

Developmental characteristics

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>Nicotine (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Yolk sac diameter (mm)</td>
<td>4.68 ± 0.07</td>
<td>4.72 ± 0.04</td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td>4.16 ± 0.03</td>
<td>4.14 ± 0.03</td>
</tr>
<tr>
<td>Developmental score</td>
<td>48.0 ± 0.4</td>
<td>47.2 ± 0.4</td>
</tr>
<tr>
<td>No. of somites</td>
<td>27.8 ± 0.4</td>
<td>27.0 ± 0.5</td>
</tr>
</tbody>
</table>

Data represent mean and standard error from four morphologically normal embryos in each group. None of the values in the treatment groups differs significantly from control (one-factor ANOVA).
Fig. 3. a. Pyknotic cells in the forebrain region of the neural tube from an embryo exposed to 100 μM nicotine. Arrowheads indicate pyknotic cells and arrows indicate cells at earlier phases of degeneration. Note the extensive intercellular spaces in the upper part of the neuroepithelium. Scale bar = 20 μm. b. Hindbrain brain neuroepithelium showing phagosomes with dark included bodies (P). Frequently, pyknotic bodies (arrowheads) and degenerating cells (arrow) are present, along with a large number of mitotic figures (MF) in the germinal zone. Scale bar = 20 μm. c. Forebrain neuroepithelium from an embryo exposed to 10 μM nicotine. Note the extensive cytoplasmic vacuolation. Pyknotic bodies are seen even in the mitotic zone (M). Scale bar = 20 μm. d. Hindbrain neuroepithelium from an embryo exposed to 10 μM nicotine. Vacuoles (arrowheads) are present in almost all cells, including those undergoing mitosis (M). Scale bar = 20 μm.
Morphology and fate of dying cells. The dying cells recognizable at the light microscopic level were marked by a darkly stained nucleus, sometimes with condensation of chromatin at one side of the nucleus, but with the other cellular constituents generally appearing relatively normal. Darkly stained (pyknotic) spherical granules of various sizes, and spherical vesicles with a dense core were also common (fig. 3a and b). Most of the debris was found within the cytoplasm of otherwise healthy-looking cells, with few cells showing signs of cytoplasmic degeneration, suggesting a rapid engulfment of the dead cell cytoplasm. Large pyknotic granules were usually found very close to the host nucleus, often partially embedded in the healthy nucleus; smaller pyknotic granules were also found but these tended to be separated from the nucleus. Taken together, these observations indicate that “healthy” nondying cells may engulf the fragments of adjacent degenerated cells. Accordingly, the initial stages of neuroepithelial apoptosis, which were rarely observed in the embryos after 48 hr of nicotine exposure, are unlikely to be detected because of the subsequent degeneration and engulfment of debris by adjacent cells.

The most common form of cell death after embryonic chemical injury is apoptosis (Zakeri and Ahuja, 1997), and we therefore chose to examine whether the intracellular and intercellular debris showed apoptotic characteristics. The light microscopic appearance of apoptotic bodies is quite diverse, round or roughly oval, varying in size from nearly that of the parent cell to barely discernible (Clarke, 1990). Those derived from cells with a high nuclear cytoplasmic ratio usually comprise single masses of pyknotic chromatin surrounded by narrow cytoplasmic rims and we were able to identify such figures readily in the nicotine group (fig. 3a and b). In contrast, where the process affects cells with copious cytoplasm, many of the resting bodies contain only tiny specks of pyknotic chromatin and some are devoid of a nuclear component. These are obviously much more difficult to quantify, but were also clearly present in higher concentration in the nicotine group as compared to controls (figs. 3a and b).

Other types of damage, indicative of either necrosis or apoptosis, were readily demonstrable in the nicotine exposed embryos. Phagosomes, large cells with four to eight round intracellular bodies, were frequently found in the nicotine group in the fusion region the alar plate and were never found in the control embryos (figs. 1d and 3a and b). Within the phagosome, apoptotic bodies undergo degeneration and in these cells, chromatin was clearly present as coarse masses of densely staining material. We also found a large number of cells with extensive cytoplasmic vacuolation (fig. 3c, c and d). At the highest dose of nicotine, the majority of cells displayed vacuoles, which were distributed throughout the cytoplasm and its processes, and were even seen bulging from the cell surface. The latter may represent portions of the cell membrane budding off prior to disintegration into a series of membrane bound fragments, each containing a portion of cell organelles (Clarke, 1990). Accompanying cell death, intercellular spaces were enlarged in the nicotine group (fig. 3a).

Effects on mitosis. In examining the regional distribution of pyknotic cells, we noted that, curiously, cell death was also present in the mitotic zone (fig. 3c). Darkly stained granules, indicative of engulfed pyknotic material were readily detectable in mitotic cells (fig. 1d), indicating that some of the damage to neuroepithelial cells is likely to involve the zone of proliferation. Quantitation of mitotic figures within each zone showed a strong, dose-dependent effect of nicotine on mitosis, but surprisingly, nicotine enhanced the number of mitotic figures (fig. 4). The effect differed from that seen for cell death in three respects. First, the lowest concentration of nicotine did not evoke significant changes in two of the three regions. Second, regional selectivity was not readily apparent for the mitogenic effect of nicotine, as equivalent stimulation was seen in all three regions (no significant interaction of treatment × region). Third, the effect was of much smaller magnitude, with 25 to 50% changes at the highest nicotine concentration, as opposed to 3-fold increases in cell death.

Discussion

Our results indicate that, in concentrations below the threshold for general dysmorphogenesis, nicotine damages the developing neuroepithelium, findings that provide a basic mechanism for the long-term alterations in cell number, synaptic development and function, and behavior seen with prenatal nicotine exposure in vivo (Slotkin, 1992). Clearly, in extreme cases, such changes can also contribute to the minor increase in the incidence of neural tube defects seen in the offspring of smokers (Evans et al., 1979). Most prominently, we found evidence of concentration-dependent apoptosis and cell loss throughout the neuroepithelium, characterized by extensive vacuolation, numerous pyknotic cells and the presence of apoptotic condensations. As a result of extensive cell loss, intercellular spaces were expanded in the areas of maximal damage. It should be emphasized that these changes are selective for the neuroepithelium, as general developmental markers were unaffected even at the highest concentration of nicotine. Previous work with nicotine concentrations several orders of magnitude above those used here also found exten-

![Fig. 4. Quantitation of mitotic cells in the neuroepithelium. Data represent means and standard errors obtained from four embryos in each treatment group, determined from values averaged across 10 sections (repeated measure) in each embryo, and shown as number of cells per 100-µm segment of neuroepithelium. ANOVA across all regions appears shown within the panel, along with subdivision by dose and region. Asterisks denote individual values that differ from the corresponding control. Comparing the relative effects on mitosis vs. pyknosis, ANOVA indicates a main effect of treatment across both measures (p < .0001) but a selectively greater pyknotic effect as compared to mitotic effect (interaction of treatment × variable, p < .0001).](image-url)
sive neuroepithelial cell death in association with dysmorphogenesis (Joschko et al., 1991) but the two effects obviously can be differentiated from each other, with neuroepithelial cells targeted at otherwise subteratogenic concentrations. The ability of nicotine to elicit cell loss in the developing brain in vivo continues into late gestation and even into the postnatal period, as evidenced by persistently elevated expression of genes involved in apoptosis (Slotkin et al., 1997) and by further cell loss occurring after the termination of nicotine exposure (Slotkin et al., 1987b; Slotkin, 1992).

A critical issue addressed by our findings is the underlying mechanism by which nicotine affects brain development. In vivo models of prenatal nicotine exposure are invariably confounded by the multiple effects of the drug on the maternal-fetal unit, including alterations in fetal nutritional status and cardiovascular function, largely attributable to the vascular constrictor actions of nicotine (Slotkin, 1992). However, the use of an in vitro system obviates the participation of utero-placental circulation; furthermore, within the embryo itself at this developmental stage, the vasculature is neither adequately developed nor functionally innervated, so that nicotine cannot evoke altered perfusion. The yolk sac is the main nutritive organ during organogenesis and we found no abnormalities in this structure in any of the nicotine-treated embryos. Indeed, instead of a generalized insult to the developing brain, nicotine appears to target selective cell populations within the neuroepithelium, as evidenced by the regional selectivity of cell death (hindbrain > forebrain > midbrain). Although it is not possible at this time to demarcate the reasons for the vulnerability of particular cells, the potential role of the specific cellular elements with which nicotine interacts, nicotinic cholinergic receptors, stands out prominently. Nicotinic receptors are first detectable by the end of the period of nicotine exposure used here (Naeff et al., 1992). In the fetal brain, nicotine induces the formation of its own receptors (Slotkin et al., 1987a), so it is possible that the receptors are present in even higher concentration in the nicotine group. Studies conducted at later stages of development have shown that the regional selectivity for nicotine-induced apoptosis and cell loss are determined primarily by the nicotinic receptor concentration and that receptor stimulation evokes immediate evidence of cell damage (Slotkin et al., 1987a; Smith et al., 1991; Slotkin, 1992). Similarly, in vitro exposures at developmental stages before the emergence of the receptors do not evoke signs of cell damage or apoptosis (Slotkin et al., 1993, 1997), nor does nicotine elicit mitotic abnormalities in neuronal cell lines that lack nicotinic receptors (Song et al., 1998). With embryonic exposure, we found the greatest concentration of pyknotic cells in the hindbrain, the region in which receptors emerge first (Naeff et al., 1992). Furthermore, we found lower concentrations of pyknotic cells in the midbrain zones, the areas showing lowest receptor concentrations (Naeff et al., 1992). The similarities of distributions of receptors and pyknotic cells, along with the ability of low concentrations of nicotine to elicit cell damage, all suggest that the emergence and distribution of nicotinic receptors are critical in determining the pattern of neuroepithelial damage. A definitive proof of this hypothesis will require detailed receptor mapping in both control and nicotine-exposed embryos. However, earlier work indicates that the point at which nicotinic receptors can be detected and quantified may lag significantly behind the actual emergence of the receptor-positive phenotype (Naeff et al., 1992; Broide et al., 1995; Ostermann et al., 1995). Unfortunately, a simpler, alternative approach using nicotinic receptor blocking agents does not provide an unequivocal tool with which to demonstrate receptor-mediated mechanisms of nicotine on neuroepithelial development: cholinergic input is required for proper assembly of cortical cytoarchitecture (Hohmann et al., 1988; Navarro et al., 1989; Slotkin, 1992) and cholinergic antagonists themselves interfere with cell replication in the developing brain (Whitney et al., 1995). A receptor-mediated mechanism does not rule out additional participation of other processes; e.g., the E11.5 embryo possesses metabolic enzymes capable of forming cotinine, which is also found in the fetus with maternal smoking. To date, no information is available concerning the potential developmental neurotoxicity of cotinine or other nicotine metabolites.

Although pyknotic figures were less prominent in the ventricular zone, where neurons and glia are generated, we nevertheless found pyknotic cells in close association with the mitotic region of the alar plate; there was also a significant number of mitotic cells with engulfed remnants of damaged cells, implying either that mitotic cells are among those being damaged, or that cells immediately surrounding the mitotic zone are targets for nicotine. However, the cells in this region also displayed an elevated rate of mitosis. Obviously, given the enhancement of cell death, the induction of mitotic figures by nicotine could represent compensatory mitogenesis. However, two features argue against this explanation. First, the concentration-response relationships differ for cell death and mitogenesis: cell death is enhanced 3-fold, whereas mitosis is stimulated only 25 to 50% by nicotine exposure; cell death continues to increase as the concentration is raised above 10 μM, whereas mitosis is stimulated maximally by 10 μM nicotine. Perhaps more importantly, however, the regional selectivity seen for cell death (hindbrain > forebrain > midbrain) is not shared by enhanced mitosis, which instead shows a virtually identical stimulation in all three regions. These data suggest that nicotine exerts two distinct effects, one evoking cell death, and the other evoking mitosis, with two separable concentration relationships and discrete cell targets. Indeed, there is ample prior evidence for nicotine to act as a direct mitogen in a variety of isolated cell lines (Quik et al., 1994; Maritz and Thomas, 1995), with totally independent actions mediating cell damage or death (Konno et al., 1991; Tomek et al., 1994; Tipton and Dabbous, 1995). For the developing brain, it is particularly perplexing that, in late gestation or after birth, nicotine elicits mitotic arrest through the very same nicotinic receptors that we presume mediate promotional effects on cell division in the neural tube stage (Slotkin, 1992). The actions of nicotine are thus very strongly dependent on the developmental context in which exposure occurs. This is not unexpected, as neurotransmitters, acting as trophic substances, typically elicit biphasic developmental actions, promoting cell division at early stages but fostering the switch from replication to differentiation later in development (Buznikov et al., 1970; Lauder, 1985; Slotkin et al., 1987c, 1988a,b; Whitaker-Azmitia, 1991). Accordingly, acetylcholine and by extension nicotine, both influence cell formation and cytoarchitectural organization of the brain through a combination of promotional and inhibitory actions (Hohmann et al., 1988; Navarro et al., 1989; Slotkin, 1992). The differ-
ence is that, in the setting of maternal smoking or nicotine administration, nicotine exposure elicits the effects prematurely and with excessive intensity relative to the normal developmental signal. Such overstimulation is likely to enhance and discoordinate naturally occurring processes in the modeling of the developing brain, including mitosis and apoptosis. Regardless of the actual mechanism underlying enhanced mitotic figures in the nicotine-exposed embryos, it is evident that this effect does not offset the much larger cell loss caused by nicotine, as shown by the much greater magnitude of cell death found here and by the eventual deficits in the total number of cortical cells seen with *in vivo* exposure models (Slotkin et al., 1987b; Slotkin, 1992). Indeed, accumulated mitotic figures could even represent mitotic arrest at S-phase, rather than enhanced cell division.

In our study, we were able to detect significant effects of nicotine on neuroepithelial development at 1 mM, a concentration well below that for dysmorphogenesis (Joschko et al., 1991) or for cell damage in culture systems other than developing embryos (Konno et al., 1991; Tomek et al., 1994; Tipton and Dabbous, 1995). The average venous plasma concentration well below that for dysmorphogenesis (Joschko et al., 1991) decay of plasma levels as is seen with smoking; although we recommend removal of the patch at night-time should allow for a daily increment, continuous exposure paradigms (transdermal patch) may be less desirable than episodic dose delivery (nicotine-lozenges, chewing gum, nicotine inhaler). At the very least, nicotine replacement for smoking cessation in pregnant women is most akin to continuous infusions. Thus, in recommending nicotine replacement therapy for smoking cessation, issues such as total dose and delivery formulation are likely to be critical in avoiding adverse effects. Although, with acute exposures, the placental barrier can be expected to protect the fetus from nicotine to some extent, smokers tend to maintain a steady-state plasma level that enables equilibration of the fetal compartment with maternal plasma. Similarly, continuous exposure paradigms such as transdermal nicotine patches in man, or osmotic minipump infusions in rats, maintain a constant drug level in mother and fetus (Lichtensteiger et al., 1988), and the exposure paradigm used in our study is obviously most akin to continuous infusions. Thus, in recommending nicotine replacement for smoking cessation in pregnancy, continuous exposure paradigms (transdermal patch) may be less desirable than episodic dose delivery (nicotine-containing chewing gum, nicotine inhaler). At the very least, removal of the patch at night-time should allow for a daily decay of plasma levels as is seen with smoking; although we did not model fluctuating exposures in rat embryos, it would be worthwhile to determine if episodic recovery periods could reduce the net adverse effects of nicotine on brain development. Finally, if nicotine receptors underlie the loss of neural cells, then the ontogenetic emergence of these receptors can be used to identify the critical period and specific sites for which damage is most probable.

**Acknowledgments**

The authors thank Dr. B. D. Abbott and H. P. Nichols for their technical assistance. This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, and mention of trade names of commercial products does not constitute endorsement or recommendation for use.

**References**


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