Prenatal Nicotine Exposure Evokes Alterations of Cell Structure in Hippocampus and Somatosensory Cortex

TARA SANKAR ROY, FREDERIC J. SEIDLER, and THEODORE A. SLOTKIN

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina

Received September 4, 2001; accepted October 12, 2001

ABSTRACT

Offspring of women who smoke during pregnancy show behavioral abnormalities, including increased incidence of attentional deficit, learning disabilities, and cognitive dysfunction. Animal models indicate that nicotine elicits changes in neural cell replication and differentiation, leading to deficits in synaptic neurochemistry and behavioral performance, many of which first emerge at adolescence. We evaluated cellular morphology and regional architecture in the juvenile and adolescent hippocampus and the somatosensory cortex in rats exposed to nicotine prenatally. Pregnant rats were given nicotine throughout gestation via minipump infusion of 2 mg/kg/day, a regimen that elicits nicotine plasma levels comparable with those found in smokers. On postnatal days 21 and 30, brains were perfusion-fixed, coronal slices were taken between the anterior commissure and median eminence, and the morphology of the dorsal hippocampus and somatosensory cortex was characterized. 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. Smaller, transient changes were seen in CA1. In layer 5 of the somatosensory cortex, although there was no significant decrement in the average cell size, 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. Taken together with previous work on neurochemical and functional defects, these data demonstrate that prenatal nicotine exposure compromises neuronal maturation, leading to long-lasting alterations in the structure of key brain regions involved in cognition, learning, and memory.

It is now widely recognized that maternal smoking during pregnancy has an adverse effect on fetal outcome, increasing perinatal morbidity and mortality, and evoking long-term neurobehavioral damage (DiFranza and Lew, 1995; Levin and Slotkin, 1998; Slotkin, 1998). The offspring of smokers display attentional and cognitive deficits, impaired learning and memory, lowered IQ, and increased incidence of conduct disorders (Naeye and Peters, 1984; Rantakallio and Koivainen, 1987; DiFranza and Lew, 1995; Wakschlag et al., 1997; Levin and Slotkin, 1998). After correction for confounding variables within the human population, maternal smoking emerges as an unequivocal correlate of these endpoints. Be that as it may, it has been difficult to attribute any of these alterations to nicotine as a specific component because of the presence of thousands of other substances in cigarette smoke, and because smoking elicits substantial fetal hypoxic/ischemic insult (Cole et al., 1972). The importance of separating nicotine itself from the other variables is reinforced by the popularity of nicotine replacement therapy for smoking cessation. If nicotine itself is injurious to the fetal brain then nicotine substitution may not eliminate all of the deleterious effects of maternal smoking.

The implantable osmotic minipump has enabled the development of animal models of continuous exposure to nicotine at doses that simulate plasma levels found in human smokers, but without contributions of hypoxia/ischemia or of other components of cigarette smoke (Lichtensteiger et al., 1988; Slotkin, 1998). A wealth of data now indicates unequivocally that nicotine itself is a neuroteratogen that alters replication and differentiation of neural cells, leading to abnormalities of synaptic biochemistry and behavioral deficits (Levin and Slotkin, 1998; Slotkin, 1998). However, there are a number of important questions that have not yet been answered concerning the mechanisms and specificity of nicotine’s effects on brain development. Are there morphological changes that underlie the anomalies at the level of synaptic function? Do such changes precede the development of synaptic and behavioral deficits? Are specific cell types or brain regions targeted by nicotine? In the current study, we have examined the effects of prenatal nicotine exposure on components of the hippocampus, concentrating on juvenile and adolescent stages in the rat, the period just before the emergence of lasting deficits in synaptic function (Slotkin, 1998). The hippocampus was chosen for several reasons. In vitro studies

ABBREVIATIONS: GD, gestational day; PN, postnatal day; ANOVA, analysis of variance.
suggest that nicotine evokes apoptosis in hippocampal progenitor cells (Berger et al., 1998). With in vivo exposure, nicotine elicits lasting deficits in hippocampal cholinergic function, EEG and hippocampus-related behaviors, with many of the effects first emerging in the postweaning period (Yanai et al., 1992; Zahalka et al., 1992; Levin et al., 1996; Slawecki et al., 2000). Structural correlates of these effects may exist (Roy and Sahberwal, 1998) but have been assessed only with models (injected nicotine) that include episodic hypoxia as a covariate; furthermore, the earlier assessments took place in young adulthood, after the appearance of synaptic and behavioral alterations.

We contrasted the effects in the hippocampus with those in the somatosensory cortex. The pyramidal neurons of the hippocampus and somatosensory cortex are morphologically distinct and have differing developmental timelines. The pyramidal neurons of layer 5 of the somatosensory cortex appear earliest in gestation, followed by hippocampal CA3 and CA1 pyramidal neurons (Paxinos and Watson, 1998); however, hippocampal CA3 pyramidal cell migration continues into much later developmental periods (Altman and Bayer, 1990b). In contrast, the majority of the granule cells of the hippocampal dentate gyrus appear even later, after birth (Altman and Bayer, 1990a). Accordingly, in this study we compare the vulnerabilities of neuronal populations that are being generated at the time of nicotine exposure (prenatally derived pyramidal neurons of the hippocampus and layer 5 of the somatosensory cortex) with those of populations that complete their generation after birth (postnatally derived granule cells of the hippocampus). At the same time, we explore the vulnerability of similar types of cells located in different regions (hippocampal versus cortical pyramidal neurons), as well as two types of cortical neurons (pyramidal and nonpyramidal) that arise from different germinal zones but migrate to the same region.

Materials and Methods

Timed pregnant Sprague-Dawley Rats (Zivic Laboratories, Pittsburgh, PA) were shipped on gestational day (GD) 2 by climate-controlled truck (total transit time less than 12 h). After arrival, animals were housed individually in breeding cages and allowed free access to food and water. On GD4, before implantation of the embryo in the uterine wall, each animal was lightly anesthetized with ether, a 3- × 6-cm area on the back was shaved, and an incision made to permit s.c. insertion of type 2 ML2 (flow rate 125 μl/day) Alzet osmotic minipumps (Durect, Cupertino, CA). Pumps were prepared with concentrations of nicotine bitartrate (Sigma Chemical, St. Louis, MO) designed to deliver 0.7 mg of nicotine free base daily, dissolved in bacteriostatic water (Abbott Diagnostics, Abbott Park, IL). The average initial weight of the dams was 300 g and the final weight averaged 370 g, so that the dose rate was 2.3 mg/kg/day initially, falling to 1.9 mg/kg/day at the end of the infusion period (Navarro et al., 1989). The incision was closed with wound clips and the animals were permitted to recover in their home cages. Control animals were implanted with minipumps containing only the water and an equivalent concentration of sodium bitartrate. It should be noted that the pump, marketed as a 2-week infusion device, actually repeats every few days and in addition, dams in either treatment group were randomly reassigned to the litters of nursing pups so that any differences in maternal caretaking would be distributed uniformly throughout all treatment groups; because the nicotine pumps are exhausted before birth, none of the groups is being exposed to nicotine postnatally via nursing. Cross-fostering, by itself, has no impact on neurochemical or behavioral effects of nicotine exposure (Rihary and Lichtensteiger, 1989). Treatment groups were sex-matched, using approximately equal proportions of males and females for each experimental point (equal proportions for even numbers, one more male than female for odd numbers), and always taking no more than one animal from a given litter on each experiment day; thus the number of animals represents the number of litters.

Tissue Processing. On PN21 and 30, pups from each treatment group were euthanized under deep ketamine anesthesia and perfused transcardially for at least 20 min with freshly prepared Karnovsky’s fixative (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, 4°C) after which the brains were dissected and preserved in fresh fixative overnight at 4°C. Each brain was separated from the spinal cord by a perpendicular cut at the level of the obex, hemisectioned by a midline incision through the corpus callosum, and then a 4- to 5-mm-thick coronal slab of the right cerebral hemisphere was obtained at the level of the rostral limit of the anterior commissure and the caudal end of the median eminence; each slab thus contained the parietal cortex and the anterior portion of the dorsal hippocampus. Tissues were kept in fresh fixative solution for an additional 48 h with changes of fixative at 12-h intervals. Each coronal slab was dehydrated in ascending concentrations of ethanol and cleared with chloroform, followed by infiltration in paraplast. The slabs were blocked in paraplast, maintaining a coronal orientation for sectioning. Sections were cut on a Reichert Jung rotary microtome by using disposable stainless steel blades. Five-micrometer-thick sections were cut and mounted on glass slides, dried at 60°C overnight, and stained with cresyl violet.

Morphometry. Morphometry was conducted with NIH Image 1.62f software (http://rsb.info.nih.gov/nih-image/; accessed 20 July 2001). The slides were coded and the examiner was blinded to the animal number and treatment group. Before conducting measurements of cell and layer parameters, we confirmed the validity of the morphometric measurements by evaluating tissue shrinkage at the level of the anterior commissure from both treatments; we evaluated the number of sections and overall thickness of the somatosensory cortex as well as the brain weight and did not find any differences between the two treatment groups (data not shown). Furthermore, we did not observe any gross pathological change such as edema, indicating that cell bodies and neuropil shrunk equally during processing. To ensure uniform sampling, we maintained the septotemporal and mediolateral orientations, and used the positions of blood vessels as landmarks.

Morphometric measurements were carried out using a video camera with a Leitz Diaplan microscope, selecting a random area within the specified cell layer, and counting all the neuronal profiles shown on the monitor. At least 700 cell profiles were evaluated for each region at a given age for each treatment group, an average of 100

Prenatal Nicotine Exposure

Murrin et al., 1987; Lichtensteiger et al., 1988); pregnant women of nicotine transdermal patches generally achieve comparable concentrations to those in smokers (Oncken et al., 1997). Equally important, this dose demonstrably activates central nicotinic receptors (Slotkin et al., 1987; Lichtensteiger et al., 1988; Navarro et al., 1989) and, with gestational exposure, causes behavioral and neurochemical alterations (Slotkin, 1998). Unlike injected nicotine, the infusion of nicotine via osmotic minipumps does not cause any overt signs of hypoxia/ischemia, such as blanching of the skin or cyanosis (Slotkin, 1998).

Parturition occurred in all groups on GD22 (also taken as postnatal day 0). After birth, pups were randomized within treatment groups and litter sizes were culled to 10 to ensure standard nutrition. Randomization was repeated every few days and in addition, dams in either treatment group were randomly reassigned to the litters of nursing pups so that any differences in maternal caretaking would be distributed uniformly throughout all treatment groups; because the nicotine pumps are exhausted before birth, none of the groups is being exposed to nicotine postnatally via nursing. Cross-fostering, by itself, has no impact on neurochemical or behavioral effects of nicotine exposure (Rihary and Lichtensteiger, 1989). Treatment groups were sex-matched, using approximately equal proportions of males and females for each experimental point (equal proportions for even numbers, one more male than female for odd numbers), and always taking no more than one animal from a given litter on each experiment day; thus the number of animals represents the number of litters.

Morphometry. Morphometry was conducted with NIH Image 1.62f software (http://rsb.info.nih.gov/nih-image/; accessed 20 July 2001). The slides were coded and the examiner was blinded to the animal number and treatment group. Before conducting measurements of cell and layer parameters, we confirmed the validity of the morphometric measurements by evaluating tissue shrinkage at the level of the anterior commissure from both treatments; we evaluated the number of sections and overall thickness of the somatosensory cortex as well as the brain weight and did not find any differences between the two treatment groups (data not shown). Furthermore, we did not observe any gross pathological change such as edema, indicating that cell bodies and neuropil shrunk equally during processing. To ensure uniform sampling, we maintained the septotemporal and mediolateral orientations, and used the positions of blood vessels as landmarks.

Morphometric measurements were carried out using a video camera with a Leitz Diaplan microscope, selecting a random area within the specified cell layer, and counting all the neuronal profiles shown on the monitor. At least 700 cell profiles were evaluated for each region at a given age for each treatment group, an average of 100
cells per animal. The values obtained for each parameter in a given animal were then averaged to produce a single number, so that the "%" in each case represents the number of animals, not the number of cells or sections. Hippocampal morphology was evaluated at the level where the hippocampus is most uniform in size, rostrally at the infundibular stem, and caudally at the medial geniculate body (Paxinos and Watson, 1998), choosing sections from a uniform mediolateral and septotemporal level. We took the middle of the ectal limb of the dentate gyrus for granule cell morphometry, as well as the pyramidal cell layer of the CA3b and CA1b regions of Ammon’s horn. We counted the number of neurons in a fixed field size, as well the perikaryal area, perimeter, and diameter (minimum diameter in the case of noncircular cells) of each neuron, using profiles from five consecutive sections.

For the somatosensory cortex, we selected slides at the level of the anterior commissure and analyzed images of layer 5 of the medial one-third of the S1FL and S1HL, containing the primary somatosensory cortex, forelimb, and hindlimb areas (Paxinos and Watson, 1998). We identified layer 5 by the size and packing density of the constituent cells, because this layer contains the larger pyramidal neurons (Paxinos and Watson, 1998).

For all measurements, we selected every fifth section, by using five random frames in the specified area. Because the thickness of each section was 5 μm, this ensures that the same cell was not counted twice, given that the typical cell diameter is smaller than 25 μm. In addition to measurements of cell parameters, at three locations for each section, we measured the thickness of the pyramidal cell layers of hippocampal CA1 and CA3, the granule cell layer of the dentate gyrus, and the somatosensory cortex. Measurements of cell size were restricted to the neuronal cell population, whereas glia were counted separately in each field; glia were readily distinguished from neurons by their size, nuclear shape, cytoplasm, location, and characteristic staining (Kaur et al., 1989).

Statistical Analysis. Data were evaluated as means and standard errors, considering each animal as an experimental subject. For convenience, some results are shown as the percentage of change from control values but statistical evaluations were always conducted on the original data; control values appear in the corresponding figure legends. Treatment effects were first evaluated by a three-factor ANOVA (treatment, region, age), with data log-transformed because of heterogeneous variance. The different variables were categorized as either cell parameters (area, diameter, perimeter) or layer parameters (packing density, thickness), and were first considered as repeated measures, because the multiple measures were evaluated within the same age. Data were then subdivided according to the interactions found in the global test; because these involved interactions of treatment × region (see Results), lower order tests were conducted separately to identify which regions were affected by nicotine treatment, by using the remaining variables of age and the different types of measurement. Where appropriate, individual differences were then evaluated using Fisher’s protected least significant difference. In addition, the frequency distribution of cell sizes in layer 5 of the somatosensory cortex was evaluated using χ² for observed versus expected frequencies. Significance was evaluated at the level of p < 0.05 for all main effects; however, for interactions at p < 0.1, we also examined whether lower order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran, 1967).

Results

In keeping with earlier reports (Navarro et al., 1989; Seidler et al., 1992; Slotkin, 1998), maternal weight gain, and offspring body and brain region weights were unaffected by prenatal nicotine exposure as measured on PN21 or PN30 (data not shown). Nevertheless, quantitative morphology indicated significant overall effects of nicotine on cell and layer parameters in the hippocampus and somatosensory cortex; statistical significance for all parameters is summarized in Table 1. Across the three variables describing cell size (area, perimeter, diameter), global ANOVA indicated a significant main treatment effect of nicotine, representing a net decrease across all regions and ages (F₁,₁₁₂ = 114). The main treatment effect was also present for each of the individual measures (F₁,₈₅ = 113 for area, 119 for perimeter, 68 for diameter), but the magnitude of effect was quantitatively different among parameters (treatment × measure interaction, F₄,₁₇₂ = 47). The nicotine-induced alterations in cell size also were highly selective for brain region (treatment × region interaction, F₃,₁₁₂ = 16) and differed with age (treatment × age interaction, F₁,₁₁₂ = 8). Again, these statistical differences were detected across all three parameters and for each parameter individually: for treatment × region (F₃,₈₅ = 16 for area, 19 for perimeter, 8.6 for diameter); for treatment × age, F₁,₈₅ = 7.8, 6.2, and 6.3, respectively). Across the two parameters describing cell layer characteristics, ANOVA indicated significant treatment effects that differed between the two types of measurements (cell packing density, layer thickness) and among regions, exemplified by significant interactions of treatment × measure (F₁,₈₃ = 9.8) and treatment × region × measure (F₃,₈₃ = 3). Again, significant differences were maintained when the two parameters were examined separately: packing density, main effect of treatment (F₁,₈₃ = 4.1), interaction of treatment × region (F₃,₈₃ = 3.0); layer thickness, main effect of treatment (F₁,₈₃ = 5.5). In light of the dependence of the cell size parameters and the layer parameters on brain region, age, and type of measure-

---

**TABLE 1**

Global statistical analyses

<table>
<thead>
<tr>
<th>Effect</th>
<th>All Cell Parameters</th>
<th>Area</th>
<th>Perimeter</th>
<th>Minimum Diameter</th>
<th>All Layer Parameters</th>
<th>Packing Density</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>N.S.</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.03</td>
</tr>
<tr>
<td>Treatment × region</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>N.S.</td>
<td>p &lt; 0.04</td>
<td>N.S.</td>
</tr>
<tr>
<td>Treatment × age</td>
<td>p &lt; 0.006</td>
<td>p &lt; 0.06</td>
<td>p &lt; 0.1</td>
<td>p &lt; 0.09</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Treatment × measure</td>
<td>p &lt; 0.0001</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Treatment × region × age</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Treatment × region × measure</td>
<td>p &lt; 0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.04</td>
<td></td>
</tr>
<tr>
<td>Treatment × age × measure</td>
<td>p &lt; 0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Treatment × region × age × measure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>

N.S., not significant.
Hippocampal morphology underwent significant changes during the period from PN21 to PN30. On PN21, cells in the superficial part of the ectal limb of the dentate gyrus were large compared with the deeper layer and a tertiary matrix was observed on the deep aspect of the granule cells. By PN30, the tertiary matrix disappeared, leaving few cells in the hilus. In the CA3 and CA1 regions, pyramidal neurons in the older animals displayed more cytoplasm and contained more Nissl granules than in the younger group. Nicotine exposure did not cause gross morphological alterations in these overall developmental characteristics of the hippocampus. Nevertheless, the nicotine group showed major changes in quantitative aspects of specific cell types and layers. In the dentate gyrus (Fig. 1), neuronal cell size was substantially reduced on PN21, characterized by a 30% deficit in average cell area ($F_{1,9} = 50$) and 15% deficits in diameter ($F_{1,9} = 31$) and perimeter ($F_{1,9} = 46$); the differences in effect size among the individual parameters reflected their expected geometric relationship. Evaluations on PN30 showed essentially the same effect ($F_{1,11} = 26, 11, and 24$, respectively). Measurement of the layer characteristics of the dentate gyrus demonstrated a significant increase in the cell packing density ($F_{1,20} = 4.5$ across both ages), as might be expected from reduced cell size. As a result, the thickness of the dentate gyrus remained unaffected ($F_{1,20} = 0.5$ across both ages), the combined result of smaller, but more numerous cells. These quantitative changes were also obvious from morphological appearance (Fig. 2). Cells in the nicotine-exposed group were distinctly smaller and more numerous.

Pyramidal cells in the CA3 region of the hippocampus also showed a large decrease in size elicited by prenatal nicotine exposure (Fig. 3). On PN21, average cell area was reduced by over 30% ($F_{1,9} = 51$), with parallel deficits in perimeter ($F_{1,9} = 108$) and diameter ($F_{1,9} = 55$). Between PN21 and PN30, there was some improvement in the nicotine group (20–25% deficit in cell area) but the differences remained robust and statistically significant ($F_{1,11} = 51, 36, and 11$, respectively). As in the dentate gyrus, the smaller cells in CA3 of the nicotine group were associated with higher cell packing density within the cell layer, but in this case a distinct increase in the effect was seen between PN21 ($F_{1,9} = 0.5$) and PN30 ($F_{1,11} = 13$). Unlike the dentate gyrus, the change in cell number did not completely offset the reduction in cell size on PN21, so that the actual thickness of the CA3 layer was significantly reduced ($F_{1,9} = 9.2$). The continued increase in cell number in the nicotine group eventually restored layer thickness to control levels by PN30 ($F_{1,11} = 0.06$), at which point the large decrease in cell size was accompanied by the equally large increase in cell number. Again, these characteristics were obvious with examination of representative sections (Fig. 4): cell size was reduced in the nicotine group and cell packing density was increased.

In the CA1 region of the hippocampus (Fig. 5) on PN21, we also observed a significant reduction in cell size evoked by prenatal nicotine: $F_{1,9} = 22$ for area, 23 for perimeter. However, the effect was significantly smaller than for dentate gyrus (across all three measures, $F_{1,40} = 5.6, p < 0.03$ for comparison of the two regions) or CA3 ($F_{1,40} = 11, p < 0.004$). Furthermore, by PN30, all cell parameters attained control values in CA1 ($F_{1,11} = 0.1$ for area, 0.3 for perimeter, 0.7 for diameter), whereas differences were maintained in dentate gyrus (comparison across all three parameters for CA1 versus dentate gyrus, $F_{1,44} = 17, p < 0.0004$; CA1 versus CA3, $F_{1,44} = 22, p < 0.0001$). The same sparing of CA1 was apparent in the lack of significant differences for cell packing density or layer thickness.

Unlike the hippocampus, there were only minor changes in structure in layer 5 of the somatosensory cortex between PN21 and PN30: on PN30, the pyramidal cells, like those of the CA1 region, contained more cytoplasm and Nissl granules than on PN21. Nicotine treatment had little or no effect on cell size parameters in this cortical layer (Fig. 6). However, in contrast to the fairly uniform neuron populations in each of the hippocampal regions, the somatosensory cortex contains two major classes of neurons, large ($>250 \mu m^2$ perikaryal area) and smaller, nonpyramidal neurons (Miller, 1986), so that differences in the relative distribution of these two types may go undetected in computing average cell sizes. Indeed, although none of the individual cell parameters was statistically significant, every measurement at both ages (area, perimeter, diameter) showed a decrease, a result that is not random; $\chi^2$ analysis for multiple determinations indicated a significant overall difference elicited by nicotine ($p < $...
0.02). We therefore examined the profile of neuronal cell sizes across 1858 cells from control animals, approximately evenly divided between PN21 and PN30, and 1625 cells from nicotine-treated animals across the same ages. Cells were sorted into two categories: those falling within the expected range for pyramidal neurons (>250 μm²) and those representing smaller, nonpyramidal neurons (Miller, 1986). The nicotine group showed a lower proportion of pyramidal neurons (46% compared with 51% in controls) and higher proportion of nonpyramidal neurons (54% compared with 49% in controls);

**Fig. 2.** Ectal limb of the dentate gyrus from control (A) and nicotine-exposed (B) rats on PN30. 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, 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.
Fig. 3. Effects of prenatal nicotine exposure on cell and layer parameters in the CA3 region of the hippocampus. Data represent means and standard errors obtained from seven control animals at each age, and five and six nicotine-treated animals on PN21 and PN30, respectively. Results are presented as the percentage of change from corresponding control values, as follows: area, 314 ± 9 μm² and 309 ± 7 on PN21 and PN30, respectively; perimeter, 68 ± 1 μm and 68 ± 1; minimum diameter, 14.5 ± 0.3 μm and 14.3 ± 0.5; packing density, 36 ± 1 cells/0.05-mm² for field and 37 ± 2, thickness 111 ± 2 μm and 112 ± 4. ANOVA across all variables appears at the top of each panel, along with lower order ANOVAs subdivided by age. N.S., not significant; *p < 0.05 versus control.

Discussion

Prenatal exposure to nicotine disrupts synaptic function and behavioral performance in two distinct phases (Lichtensteiger and Schlumpf, 1985; Lichtensteiger et al., 1988; Levin and Slotkin, 1998; Slotkin, 1998): initial deficiencies are present in the immediate postnatal period but are often made up by weaning, only to reappear in adolescence. The current study indicates that structural abnormalities are present in the hippocampus and somatosensory cortex before the re-emergence of functional deficits. With the emergence of nicotinic cholinergic receptors toward the end of neurulation (Naeff et al., 1992), nicotine exposure elicits widespread apoptosis and disruption of mitotic organization in the embryonic brain (Roy et al., 1998). Indeed, these early effects are likely to account for the selectively greater effects on the hippocampus as seen here; nicotine-induced apoptosis is especially notable for hippocampal progenitor cells (Berger et al., 1998). Nevertheless, it is equally clear that the profound damage seen in the immediate period of gestational nicotine exposure (Roy et al., 1998) is largely repaired in later stages, because the effects we observed in the juvenile and adolescent brain were far more subtle than otherwise expected. In fact, our results point to a later “mis-programming” of neural cell development within the hippocampus, a region in which architectural modeling continues into young adulthood. Despite the cessation of nicotine exposure at birth, changes were still occurring between PN21 and PN30 in the nicotine-exposed group. In CA3, for example, the deficits in layer thickness seen on PN21 were rectified by PN30, but by an abnormal mechanism: cells were smaller but more numerous than in controls.

Our results address the issue of whether prenatal nicotine targets specific cell types as opposed to selective brain regions, because we compared effects on granule cells versus pyramidal cells in the hippocampus, on pyramidal cells in the hippocampus versus the same cell type in the somatosensory cortex, and on pyramidal cells versus nonpyramidal cells contained in the same region (somatosensory cortex). Within the hippocampus, granule cells and pyramidal cells have widely disparate birth dates, with the majority of pyramidal cells generated prenatally and most of the granule cells postnatally (Altman and Bayer, 1990a,b). Nevertheless, granule cells in the dentate gyrus and pyramidal cells in CA3 were the most affected populations, showing profound and persistent decreases in average cell size; in both regions, cell packing density was increased, denoting filling of the intervening space with additional cells. Accordingly, it is unlikely that the effects represent a direct action of nicotine on neurogenesis of these particular cells; rather, effects on earlier events, such as genesis or death of progenitor cells (Berger et al., 1998; Roy et al., 1998), or on postmitotic cell migration and connectivity, are likely to underlie the structural anomalies. In support of the latter hypothesis, pyramidal cells within the CA1 area were less affected than the same cell type in CA3 and indeed, cell and layer properties in CA1 were entirely restored to normal values by PN30. The pyramidal cells in both CA3 and CA1 are generated prenatally, during the period of nicotine exposure; the same is true for pyramidal neurons in the somatosensory cortex, yet we found even less effect in that region. Accordingly, postmitotic events are likely to be critical in establishing the morphological abnormalities associated with prenatal nicotine exposure, and consequently with the later emerging neurobehavioral deficits.

There are a number of ways in which prenatal nicotine exposure could elicit later appearing structural changes. Granule cells of the dentate granule cells project to the CA3 pyramidal neurons, so that effects on granule cells may in turn elicit alterations in pyramidal cells. This could explain why pyramidal cells in CA1 and in the somatosensory cortex,
which do not receive dentate granule cell projections, are spared relative to the same cell population in CA3, despite the fact that the pyramidal cells in all three are generated prenatally. Alternatively, circuitry arising in other areas may evoke changes in the hippocampus. Prenatal nicotine exposure up-regulates the expression of nicotinic acetylcholine receptors (Hagino and Lee, 1985) and the effects persist in the hippocampus through juvenile stages (Van de Kamp and Collins, 1994). These receptors in turn modulate the release of trophic factors in response to cholinergic input (Maggio et al., 1997), and the hippocampus receives prominent cholinergic innervation from the septal nucleus. Stimulation of nicotinic receptors also has been shown to reduce neuritic extension (Pugh and Berg, 1994) and to increase

Fig. 4. Pyramidal cell layer of the CA3 region of the hippocampus at PN30 in control (A) and nicotine-exposed (B) rats. Note the smaller cell size and increased packing density in the nicotine group. The nicotine group also shows increased numbers of neuroglia (arrows). Scale bar, 50 μm.
neuronal survival (Pugh and Margiotta, 2000), which may lead to more numerous, smaller cells filling the space ordinarily occupied by neuropil. Additionally, formation of synaptic connections is likely to be affected by prenatal nicotine exposure, because nicotinic receptors influence neuronal pathfinding and target selection (Zheng et al., 1994). Although the mature CA3 region expresses a fairly low concentration of nicotinic receptors, this does not obviate its vulnerability. Nicotine exposure compromises cholinergic control over the activity of other neurotransmitter systems (Slotkin, 1998), so that changes in cholinergic input outside of the CA3 region may in turn affect neurotrophic control by noncholinergic inputs. As just one example, CA3 subfields receive prominent noradrenergic inputs from the locus coeruleus, and prenatal nicotine exposure produces a profound impairment of noradrenergic tonic activity (Slotkin, 1998) and of noradrenergic responsiveness to cholinergic stimulation (Seidler et al., 1992).

Similar factors may influence cell development in layer 5 of the somatosensory cortex. Nonpyramidal interneurons contain the inhibitory transmitter γ-aminobutyric acid, whereas the pyramidal neurons contain the excitatory transmitter glutamate (DeFelipe, 1999). Although the effects of prenatal nicotine in this region were more subtle than in the hippocampus, we observed an increase in nonpyramidal interneurons at the expense of pyramidal neurons. Again, this is likely to represent a postmitotic effect, because the two cell types arise with different timetables and in different areas of the embryonic brain, but eventually migrate to the same region. Alterations in the number, morphology and function of the interneurons produce corresponding changes in cell excitability (DeFelipe, 1999), and it is especially notable that reductions in the activity of cortical catecholaminergic projections emerge in adolescence after prenatal nicotine exposure (Slotkin, 1998). Further studies are warranted to examine the specific interrelationships between interneurons and pyramidal neurons in the somatosensory cortex so as to characterize the exact mechanism by which the changes in cell distribution contribute to the neurochemical and behavioral deficits seen after prenatal nicotine exposure (Levin and Slotkin, 1998; Slotkin, 1998). In any case, our additional finding of increased glial cell proliferation in the somatosensory cortex indicates that this region is not totally spared from nicotine-induced damage.

Finally, our results provide insight into the relative contributions of nicotine as a neuroteratogen, compared with the...
effects of fetal hypoxia/ischemia, such as is found in nicotine injection models or in maternal cigarette smoking (Slotkin, 1998). Previous work with maternal nicotine injections identified changes in hippocampal morphology in adulthood, with at least some of the effects resembling those seen here (Roy and Sabherwal, 1998). Accordingly, nicotine, by itself appears to be sufficient to elicit the defects. On the other hand, much larger changes were seen in the somatosensory cortex after maternal nicotine injections (Roy and Sabherwal, 1994) as opposed to the more subtle effects seen here with nicotine infusions, implying that a hypoxic/ischemic component contributes to the effects in that region. However, it must be noted that changes in synaptic activity are prominent in the cerebral cortex even with the nicotine infusion model (Slotkin, 1998), so that adverse effects may involve functional deficits unaccompanied by morphological alterations, or alternatively, that other regions of the cerebral cortex may be more affected than the specific layer examined here. In light of the reactive gliosis typically seen in response to neuronal injury (O'Callaghan, 1988) our observation of increases in neuroglia after prenatal nicotine exposure, throughout all regions, including the somatosensory cortex, suggests that damage may be far more widespread than just the areas examined here. Again, future work will need to delineate these possibilities.

The present results indicate that prenatal nicotine exposure, at blood levels comparable with those seen in human smokers or in users of transdermal nicotine patches, elicits structural changes in the hippocampus and somatosensory cortex that precede the reemergence of neurochemical and behavioral deficits. Nicotine appears to target specific subregions and cell types, including cells with postnatal birth dates, indicating that exposure alters the program for brain
cell development and for the architectural assembly of critical regions involved in learning and memory. These morphological changes are likely to underlie many of the neural and behavioral deficits evoked by nicotine in animal studies (Levin and Slotkin, 1998; Slotkin, 1998), and in turn may account for adverse neurobehavioral outcomes of maternal smoking during pregnancy (DiFranza and Lew, 1995).

Acknowledgments

We thank Dr. R. D. Schwartz-Bloom for assistance with imaging.

References


Address correspondence to: Dr. T. A. Slotkin, Box 3813 Duke University Medical Center, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710. E mail: t.slotkin@duke.edu