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EFFECTS OF CHRONIC NEONATAL NICOTINE EXPOSURE ON NICOTINIC ACETYLCHOLINE RECEPTOR BINDING, CELL DEATH AND MORPHOLOGY IN HIPPOCAMPUS AND CEREBELLUM

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Abstract—Nicotine, the major psychoactive ingredient in tobacco interacting with nicotinic acetylcholine receptors (nAChR), is believed to have neuroprotective and neurotoxic effects on the developing brain. Neurotoxicity has been attributed to activation of homomeric α7 nAChRs, neuroprotection to heteromeric α4β2 nAChRs. Thus, developmental nicotine could have opposite effects in different brain regions, depending on nAChR subtype expression. Here, we determined if chronic neonatal nicotine exposure (CNN), during a period of brain growth corresponding to the third human trimester, differentially regulates nAChR expression, cell death, and morphological properties in hippocampus and cerebellum, two structures maturing postnatally. Rat pups were orally treated with 6 mg/kg/day nicotine from postnatal day (P)1 to P7. On P8, expression for α4, α7 and β2 mRNA was determined by *in situ* hybridization; nAChR binding sites by receptor autoradiography, dying neurons by TUNEL and Fluoro-Jade staining and morphological properties by analysis of Cresyl Violet–stained sections. In control cerebellum, strong expression of α4, β2 mRNA and heteromeric nAChRs labeled with [^{125}I]-epibatidine was found in granule cells, and α7 mRNA and homomeric nAChRs labeled with [^{125}I]-α-bungarotoxin were in the external germinal layer. In control hippocampus, low expression of α4 mRNA and heteromeric nAChRs and high expression of α7 mRNA and homomeric nAChRs were detected. CNN increased heteromeric nAChR binding in hippocampus but not cerebellum and significantly decreased neuronal soma size and increased packing density in hippocampal principal cells but not in cerebellum. CNN did not increase the number of dying cells in any area, but significantly fewer TUNEL-labeled cells were found in CA3 strata oriens and radiatum and cerebellar granule layer. Thus, the hippocampus seems to be more sensitive than the cerebellum to CNN which could result from different nAChR subtype expression and might explain long-lasting altered cognitive functions correlated with gestational nicotine exposure due to changes in hippocampal cell morphology.

Key words: apoptosis, development, nicotinic, receptor, neuronal density.

Maternal smoking during pregnancy has been linked to decreased cognitive function in children presenting with lower IQ and deficits in learning and memory (Hellstrom-Lindahl and Nordberg, 2002; Batstra et al., 2003; Fried et al., 2003). Although cigarettes deliver hundreds of different compounds, including tars, carbon monoxide and cyanide, nicotine is the major psychoactive ingredient in tobacco. It is rapidly absorbed into the bloodstream and reaches the fetus at concentrations equal to or higher than those in the mother (Ankarberg et al., 2001; Dempsey and Benowitz, 2001). Therefore, studies have focused on the developmental effects of nicotine which is believed to be, at least in part, responsible for the adverse effects of tobacco smoking during pregnancy.

Nicotine activates nicotinic acetylcholine receptors (nAChR), pentameric ion channels widely expressed in the peripheral and CNS (Dani and Bertrand, 2007). In the developing brain, binding sites for nAChRs are detected during embryonic development in both human and animal fetuses (Cairns and Wonnacott, 1988; Hellstrom-Lindahl et al., 1998; Naeff et al., 1992; Tribollet et al., 2004; Adams et al., 2002). In addition, there is evidence that the receptors are functional in prenatal and early postnatal rat pups (O'Leary and Leslie, 2003; Gallardo and Leslie, 1998). Therefore, gestational nicotine exposure could directly influence brain development.

Animal studies have tried to link prenatal nicotine exposure to cognitive deficits, but with limited success (Cutler et al., 1996; Ajarem and Ahmad, 1998; Ankarberg et al., 2001; Abdel-Rahman et al., 2005). Two late developing brain structures, the hippocampus and the cerebellum, are associated with cognitive functions. The hippocampus has been known for a long time to play a key role in learning and memory (Amaral and Witter, 1995), and the cerebellum, better known for controlling motor activity, has now been shown to also contribute to cognitive processing (Dolan, 1998; Schmahmann and Caplan, 2006). In rodents, neurogenesis, migration, and differentiation are still taking place in the hippocampus and cerebellum after birth (Bayer and Altman, 1995). Although the pyramidal cells in the hippocampus and Purkinje cells (PC) in the cerebellum are generate prenatally, granule cells in the dentate gyrus

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Abbreviations: αBTX, alpha-bungarotoxin; CA1, CA3, hippocampal CA1, CA3 regions; CNN, chronic neonatal nicotine treatment; DG, dentate gyrus; EGL, external germinal layer; Epi, epibatidine; FJ, Fluoro-Jade; IGL, internal granule cell layer; LSD, least significant difference; ML, molecular layer of the cerebellum; ml, molecular layer of the dentate gyrus; nAChR, nicotinic acetylcholine receptors; PC, Purkinje cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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(DG) and cerebellum are born during the first postnatal weeks and both structures do not reach maturity until several weeks after birth. Therefore, it might not be surprising that cognitive deficits are hard to find after prenatal nicotine exposure.

However, studies did reveal that prenatal nicotine exposure can result in altered morphological features in hippocampus and cortex (Roy et al., 2002; Roy and Sabherwal, 1998), and can interfere with long-term neuronal survival (Abdel-Rahman et al., 2005). Other studies focusing on receptor expression also showed upregulation of nAChR binding sites, mostly $\alpha4\beta2$* nAChRs (Sugiyama et al., 1985; Cairns and Wonnacott, 1988; van de Kamp and Collins, 1994; Miao et al., 1998; Eriksson et al., 2000; Mugnaini et al., 2002). Thus, morphological and molecular changes in hippocampus and cerebellum in response to developmental nicotine exposure could very well contribute to decreased cognitive performance in the offspring, and could be even more robust after postnatal exposure when hippocampus and cerebellum are still forming.

In addition, several nAChR subunits are transiently upregulated in postnatal hippocampus (Adams et al., 2002; Winzer-Serhan and Leslie, 1997, 2005; Son and Winzer-Serhan, 2006), with particularly strong expression of the homomeric $\alpha7$ receptor in hippocampal CA1 and CA3 pyramidal cells but very little expression of heteromeric nAChRs. However, after chronic neonatal nicotine treatment (CNN) increased numbers of heteromeric binding sites composed of $\alpha4$ and $\beta2$ nAChR subunits have been detected (Huang and Winzer-Serhan, 2006b). In the cerebellum the developmental expression patterns are less well documented. Several studies in chick and rodent brain have found transient upregulation of nAChR subunit expression (Torrao et al., 2000; Kaneko et al., 1998; Dominguez del Toro et al., 1997), which is supported by the results from a membrane binding study, documenting transiently increased binding sites for heteromeric and to a lesser extent homomeric nAChRs during early postnatal development (Zhang et al., 1998). However, still missing is a detailed description of cerebellar binding site distribution and subunit mRNA expression in particular for $\alpha4$, $\beta2$, and $\alpha7$ subunits, which make up the majority nAChRs in the brain.

Variations in the expression of particular nAChR subtypes could profoundly influence the outcome of developmental nicotine exposure across different brain regions and in a given tissue. Depending on the receptor subtype expressed, nicotine could exert either neuroprotective or neurotoxic actions. Heteromeric $\alpha4\beta2$ receptors have been shown to promote survival, while $\alpha7$ homomeric receptors mediate neurotoxic effects of nicotine (Berger et al., 1998; Laudenbach et al., 2002). Neurogenesis takes place in the DG and external germinal layer (EGL) of the hippocampus and cerebellum, respectively, where granule cells are born throughout postnatal development with peak numbers of neurons generated around postnatal day (P) 4–P7 (Bayer and Altman, 1995). Previous *in vitro* studies have shown that nicotine could increase apoptosis in immature neurons and undifferentiated hippocampal progenitor cells (Berger et al., 1998; Roy et al., 1998). Thus, nicotine alone could induce widespread cell death, in particular of immature hippocampal and cerebellar neurons during postnatal exposure.

To evaluate the effects of CNN during a developmental period which corresponds to the third trimester in humans and is characterized by rapid brain growth (Dobbing and Sands, 1979), we developed an oral gastric intubation model (Huang et al., 2006). In this study, we compared the effects of CNN on nAChR binding sites, subunit mRNA expression, morphological properties and apoptotic markers in hippocampus and cerebellum.

METHODS AND MATERIALS

Animal and drug administration

Timed-pregnant Sprague–Dawley rat dams were purchased from Harlan (Houston, TX, USA) and arrived on gestational days 14–16. Animals were housed under standard conditions at the College of Medicine's Animal Care Facility in accordance with the guidelines of Texas A&M University Laboratory Animal Care Committee and international guidelines for the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering. The day pups were born was considered as P0. On P1, rat pups were randomly assigned to different treatment groups with four animals per treatment, eight animals per litter. Altogether, seven separate litters were used for the study. To avoid variability due to different litters, controls and nicotine-treated pups were derived from the same litter ($n=4$), in some experiments animals from two or three litters were used ($n=7/8$, $n=11/12$, respectively).

Oral gastric intubation with nicotine was administered as described in Huang et al., 2006. Briefly, (−)-nicotine liquid (Sigma N3876; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in formula (Enfamil® with iron). Pups were treated three times per day with nicotine (2 mg/kg/dose, free-base), 4 h apart, to receive a total dose of 6 mg/kg/day from P1–P7. At P8, pups received the last treatment (2 mg/kg) in the morning and were killed 30 min later. Control animals were intubated without formula to prevent large differences in weight gain between the control and the nicotine treatment group, which result from nicotine's anorexic effect (Huang et al., 2006). The dose used in the present study (6 mg/kg/day) resulted in nicotine blood serum levels of 100–150 ng/ml (L. Z. Huang, J. R. James, U. H. Winzer-Serhan, unpublished observations), roughly equivalent to nicotine levels in heavy smokers using three packs per day (Murrin et al., 1987).

In situ hybridization

In situ hybridization was performed as described (Winzer-Serhan et al., 1999). Briefly, after decapitation the brains were quickly removed, frozen in isopentane and stored at −80 °C. Twenty micrometer thick coronal forebrain or sagittal cerebellar sections were cut on a cryostat, postfixed with 4% paraformaldehyde, dried and stored at −20 °C. pBluescript II SK+ plasmids containing full-length sequences for $\alpha4$, $\alpha7$ and $\beta2$ (2110, 2100, 2196 base pairs, respectively, kindly provided by Dr. Jim Boulter, University of California, Los Angeles, CA, USA) were linearized using appropriate restriction enzymes and antisense probes were reversed transcribed from cDNA templates in the presence of [^{35}S]-UTP using T7 or T3 promoter enzymes (MAXIscript™, Ambion, Austin, TX, USA) as described (Azam et al., 2002). The full-length probes were hydrolyzed into 600 bp fragments. Sections were pre-hybridized with proteinase K, followed by 0.1 M triethanolamine and 0.25% acetic anhydride. Sense probes were used to detect non-specific hybridization. After an overnight hybridization at 60 °C,