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Prenatal cigarette smoke exposure effects on apoptotic and nicotinic acetylcholine receptor expression in the infant mouse brainstem

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ABSTRACT

Infants exposed to cigarette smoked during pregnancy into infancy have increased respiratory and cardiac abnormalities. Nicotine, the major neurotoxic component of cigarette smoke, induces its actions by binding to nicotinic acetylcholine receptors (nAChR), with one downstream effect being increased apoptosis. Using a pre- into post- natal cigarette smoke exposure mouse model (SE), we studied the immunohistochemical expression of nAChR subunits α2, α3, α4, α5, α7, α9, β1 and β2 and two markers of apoptosis, active caspase-3 and TUNEL, in seven nuclei of the medulla and facial nucleus of the pons in male mice. Pups of dams exposed to two cigarettes (nicotine ≤1.2 mg, CO ≤15 mg) twice daily for six weeks prior to mating, during gestation and lactation ($n=5$; SE), were compared to pups exposed to air under the same condition ($n=5$; SHAM) at P20. Results showed that the hypoglossal nucleus had increased α3, α4, α7, α9, Casp-3 and TUNEL, dorsal motor nucleus of the vagus had increased α3, α5, α7, β1 and Casp-3, nucleus of the solitary tract had increased α3 but decreased α4, α5, β1 and apoptosis, cuneate nucleus had increased α3, β2 and Casp- 3, but decreased α5, nucleus of the spinal trigeminal tract had increased α3, α7, β1, lateral reticular nucleus had decreased β1, inferior olivary nucleus had increased β1 but decreased apoptosis, and the facial had increased α2, α3 and α7. This is the first study to demonstrate that nAChR subunits are affected following pre- into post-natal SE and that they simultaneously coincided with changes in apoptotic expression.

1. Introduction

Maternal cigarette smoking during pregnancy is the most preventable risk factor for a complicated pregnancy with negative outcomes for both the mother and the child (Hofhuis et al., 2003). Compelling evidence links maternal smoking to a number of adverse prenatal conditions such as low birth weight (Bernstein et al., 2005), still birth (Wisborg et al., 2001) and preterm delivery (Fantuzzi et al., 2007). Furthermore, a number of epidemiological studies have shown that infants born to mothers who smoked during pregnancy have increased respiratory and cardiac abnormalities including incidence of asthma and wheezing (Gilliland et al., 2001), increased risk for otitis media (Ilicali et al., 2001), impaired pulmonary function (DiFranza et al., 2004) and altered cardiac response during hypoxic conditions (Sovik et al., 2001). In addition, prenatal smoking is a risk factor for the occurrence of Sudden Infant Syndrome (SIDS) (Hoffman et al., 1988; Anderson and Cook, 1997).

Cigarette smoke contains more than 4800 chemicals (Green and Rodgman, 1996), one of which is nicotine, the major neurotoxic constituent (Slotkin, 1998). Nicotine readily crosses the placenta due to its low molecular weight and high lipid solubility hence resulting in 15% higher concentration of nicotine in foetal circulation than maternal circulation (Lambers and Clark, 1996). Nicotine induces its actions by binding to its receptors known as the nicotinic acetylcholine receptors (nAChR). These receptors are ligand gated cation channels that exist as pentamers of subunits around a central pore. Genes encoding a total of 17 subunits (α1-10, β1-4, δ, ε and γ) have been identified, all of which are mammalian origin except for α8 (avian origin) (Gerzanich et al.,

Abbreviations: Cun, cuneate nucleus; Casp-3, active caspase 3; DMNV, dorsal motor nucleus of the vagus; FAC, facial nucleus; IHC, immunohistochemistry; ION, inferior olivary nucleus; LRt, lateral reticular nucleus; NSTT, nucleus of the spinal trigeminal tract; NTS, nucleus of solitary tract; SIDS, sudden infant death syndrome; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; XII, hypoglossal nucleus.

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1994). They are present as either heteropentamers or homopentamers (α7, α9) throughout the central and peripheral nervous system and can be found both at pre and post synaptic membranes (Gotti and Clementi, 2004). nAChRs are important in two stages of brain development: during the perinatal developmental stage and age related cell degeneration (Gotti et al., 2006). The pentameric assembly of the nAChR subunits gives rise to many different combinations that result in a variety of nAChRs regulating processes such as cell excitability, transmitter release and neuronal integration thus influencing many physiological functions such as sleep, arousal, anxiety, central processing of pain and several cognitive functions (Hogg et al., 2003; Gotti and Clementi, 2004).

Maternal cigarette smoke exposure predominantly increases nAChR subunit expressions in various regions of the offspring brain (reviewed in Vivekanandarajah et al., 2015). It also induces neuronal cell death (apoptosis) in the offspring brain as determined in several species: Human (Machaalani and Waters, 2008); monkey (Slotkin et al., 2005); rat embryo (Roy et al., 1998; Slotkin et al., 1987); rat fetus (Onal et al., 2004) and postnatal rat (Tolson et al., 1995). Several previous studies have investigated the relationship between nicotine and apoptosis and found conflicting data (reviewed in Zeidler et al., 2007). Although a strong body of evidence suggests nicotine toward an anti-apoptotic effect, some groups have found conflicting evidence to suggest pro-apoptotic action. Of note, the α7 (Orr-Urtreger et al., 2000; Renshaw et al., 1993; Hory-Lee and Frank, 1995; Dwyer et al., 2009), α4 (reviewed in Gotti and Clementi, 2004; Labarca et al., 2001;), and heterodimers of α3, α4 (West et al., 2003) subunits have been found to directly regulate apoptotic pathways.

Utilizing a maternal cigarette smoke exposure model (via a smoking chamber) six weeks prior to mating, during gestation and lactation, the present study is unique in that it measures the protein expression of the α2, α3, α4, α5, α7, α9, β1, β2 nAChR subunits, as well as the two common markers of apoptosis (active caspase-3 (Casp-3) and DNA fragmentation via the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)) in seven nuclei of the brainstem medulla and the facial nucleus of the pons in the male mice. We chose the male sex since it has been shown previously that the nicotine effects are more pronounced in males and further exacerbated by the presence of other tobacco smoke components in males than females (Slotkin et al., 2015). We hypothesize that maternal cigarette smoke exposure increases the expression of the neuronal nAChR subtypes α2, α3, α4, α5, α7, α9 and β2 while having no effect on the non-neuronal subtype β1, and that apoptotic expression (Casp-3 and TUNEL) is also increased. The study focuses on the brainstem as it contains vital nuclei that control cardiac and respiratory systems, which can be affected by continuous maternal cigarette smoke exposure.

2. Methods

2.1. Maternal cigarette smoke exposure

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A). All protocols were performed according to the Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Virgin Balb/c mice (6 weeks) were obtained from Animal Resources Center (Perth, Australia). The mice were housed at 20 ± 2 °C and maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h) with ad libitum access to standard laboratory chow and water. The mice were randomly assigned to sham exposure (SHAM) or cigarette smoke exposure (SE) group. SE group was exposed to two cigarettes (Winfield Red, nicotine ≤ 1.2 mg, CO ≤ 15 mg, Philip Morris, VIC, Australia) twice daily for six weeks prior to mating, during gestation and lactation. Exposure occurred by placing mice in a Perspex chamber of 15L (40 × 27 × 20 cm) at room temperature, and each cigarette was delivered manually for 15 min, with a 5-min interval between the two cigarettes. The SHAM mice were placed in a separate identical Perspex chamber to avoid any contamination and air exposure delivered under the same condition. All females were mated with male Balb/c mice (8 weeks) from the same source, which were not exposed to cigarette smoke.

2.2. Tissue collection

Male offspring were sacrificed by decapitation at postnatal day 20 (P20) (normal weaning age) after anesthetized with 4% isofluorane. The brain stem was collected and fixed with 10% formalin and then stored in 70% ethanol for paraffin embedding. Blood was collected by cardiac puncture and plasma was stored at −20 °C for measurement of cotinine concentration using a cotinine ELISA kit (Abnova, Taipei, Taiwan) as per manufacturer's instructions.

Tissue blocks at the caudal level of the medulla were sectioned at 4 μm by a rotary microtome (Shandon Finesse 325, Thermo Fisher Scientific Inc., Massachusetts, USA), mounted onto silanized slides, dried overnight at 45 °C and stored at room temperature in a dust-free environment for a minimum of one week prior to immunohistochemical staining.

2.3. Immunohistochemistry

Sections from all cases were stained within the same experimental run for each respective antibody, hence avoiding day-to-day variation. Furthermore, 20% of cases were stained in duplicate to verify the reproducibility of results. For the nAChR subunits, single immunohistochemistry was performed. For Casp-3 and TUNEL, this was double immunohistochemistry.

2.3.1. Immunohistochemistry for nAChR subunits

Separate labeling of α2, α3, α4, α5, α7, α9, β1, β2 nAChR subunit via IHC is standard for our laboratory and is detailed in Machaalani et al. (2014) and Vivekanandarajah et al. (2015).

All steps were performed at room temperature unless otherwise noted. Tissue sections were first deparaffinised to distilled H_2O. Heat-induced epitope retrieval was applied by microwaving on 'high' (Homemaker; EM925ENV; 900W) in 10% TRIS-EDTA antigen retrieval buffer (1 mM EDTA, 1 mM sodium citrate, 2 mM Tris, pH 9.0) for 14 min. After cooling to room temperature and rinsing with distilled water, sections were washed with phosphate buffered saline (PBS), a hydrophobic barrier was drawn surrounding the sections and endogenous hydrogen peroxidase quenched in 50% PBS, 50% methanol and 3% H_2O_2 for 25 min at room temperature, followed by two 3-min washes in PBS. Sections were blocked by 10% normal horse serum (NHS) in PBS for 30 min then incubated with primary antibodies (Table 1) overnight at room temperature. Negative controls were incubated with 1% NHS only. Details of these primary antibodies and their specificities are provided in Table 1.

Two 3-min PBS washes were undertaken prior to 45-min incubation with biotinylated secondary antibodies made in horse (1:200 dilution; Vector laboratories Inc., California, USA). Following two 3-min PBS washes, the sections were incubated with avidin–biotin complex (ABC) (VEPH4000, Vector Laboratories Inc., California, USA) for 30 min. The sections were then color-labelled with 3,3′-diaminobenzidine (DAB) (K346811, DAKO; Lot: 10092996 USA), followed by counterstaining with Harris's Haematoxylin, dehydration through graded ethanol to xylene, and coverslipped with DPX.