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Postnatal nicotine effects on the expression of nicotinic acetylcholine receptors in the developing piglet hippocampus and brainstem

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ABSTRACT

Postnatal exposure to cigarette smoke during infancy is associated with increased number of respiratory illnesses, impaired pulmonary function, and the occurrence of Sudden Infant Death Syndrome (SIDS). It is also associated with reduced cognitive functioning and attention deficits in childhood. Nicotine, the major neurotoxic component of cigarette smoke, induces its actions by binding to nicotinic acetylcholine receptors (nAChR). Using a piglet model of postnatal nicotine exposure, we studied the immunohistochemical expression of nAChR subunits α2, α3, α4, α5, α7, α9, β1 and β2 in the brainstem medulla and the hippocampus, given the role of these structures in cardiorespiratory control and cognition, respectively. We compared piglets exposed postnatally to 2 mg/kg/day nicotine for 14 days ($n = 14$: 7 males: 7 females) to controls ($n = 14$: 7 males: 7 females). In the hippocampus, decreased expression was seen for α3 in CA1 ($p = 0.017$), α9 in CA1 ($p < 0.001$) and CA2 ($p < 0.001$), β1 in CA1 ($p = 0.001$) and CA2 ($p = 0.001$) and β2 in CA3 ($p = 0.036$). In the medulla, the nucleus of the spinal trigeminal tract had increased α2 and α4; vestibular nucleus increased α2 and α3, and decreased α4; hypoglossal decreased α3 and β1; dorsal motor nucleus of the vagus decreased α4 and β1. This is the first demonstration that non-classical nAChR subunits are affected by postnatal nicotine in the developing brain, and the implications are discussed.

1. Introduction

Smoking during pregnancy and postpartum poses a significant threat to a child's health (Committee on Environmental Health, 1997) as does passive cigarette smoke exposure. Both exposures during the infant period are linked to increased incidence of a number of respiratory diseases including middle ear disease (Strachan and Cook, 1998) and otitis media (Owen et al., 1993; Ey et al., 1995), wheeze (Haberg et al., 2007), asthma (Strachan and Cook, 1998; DiFranza and Lew, 1996; Cook and Strachan, 1997), cough, phlegm production, bronchitis, and bronchiolitis (DiFranza et al., 2004). Pulmonary function is impaired (DiFranza et al., 2004) and there is increased risk for Sudden Infant Death Syndrome (SIDS) (Liebrechts-Akkerman et al., 2011). In addition, postnatal smoke exposure reduces cognitive functioning, intelligence, and attention abilities in childhood (Chen et al., 2013).

An estimated 4800 chemicals are identified in cigarette smoke (Green and Rodgman, 1996) with nicotine recognised as a major neurotoxic constituent (Slotkin, 1998). Nicotine induces its actions by binding to its receptor(s) known as nicotinic acetylcholine receptors (nAChR). These nAChRs belong to the family of ligand gated cation channels and are arranged 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, to date. All of the subunits are of mammalian origin with the exception of α8 (avian origin) (Gerzanich et al., 1994; Gotti and Clementi, 2004). They are present as heteropentamers or homopentamers (α7, α9) throughout the Central Nervous System (CNS), autonomic ganglia and at skeletal muscle neuromuscular junctions. The endogenous cholinergic innervation of the nAChRs throughout the CNS regulates processes such as neurotransmitter release, cell excitability, and neuronal integration to name a few, which are vital for important physiological processes to occur (Gotti and Clementi, 2004). However, exogenous activation of these receptors by nicotine (where dose and duration vary), can increase and/or alter the normal regulation

Abbreviations: Cun, cuneate nucleus; DMNV, dorsal motor nucleus of the vagus; IHC, immunohistochemistry; ION, inferior olivary nucleus; NSTT, nucleus of the spinal trigeminal tract; NTS, nucleus of solitary tract; SIDS, Sudden Infant Death Syndrome; Vest, vestibular nuclei; XII, hypoglossal nucleus; CA, Cornu Ammonis; DG, Dentate Gyrus; nAChR, nicotinic acetylcholine receptor.

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Table 1
nAChR subunit primary antibodies used for immunohistochemistry.

Antibody	Host	Company & cat#	Specificity determined by
nAChR α2	Rabbit polyclonal	Santa Cruz; sc-5589	Di Angelantonio et al. (2003)
nAChR α3	Rabbit polyclonal	Santa Cruz; sc-5590	Liu et al. (2011)
nAChR α4	Goat polyclonal	Santa Cruz; sc-1772	Govind et al. (2012), Whiteaker et al. (2006), Martin-Ruiz et al. (2000), Del Mar Arroyo-Jimeínez et al.(1999)
nAChR α5	Goat polyclonal	Santa Cruz; sc-9345	Di Angelantonio et al. (2003), Kurzen et al. (2004), Lang et al. (2003), Oshikawa et al. (2003), Tournier et al. (2006), Yu et al. (2007)
nAChR α7	Rabbit polyclonal	Abcam, ab 10,096	Paulo et al. (2009), Mielke and Mealing (2009)
nAChR α9	Rabbit polyclonal	Abcam, ab 177,119	Abcam and in house[a]
nAChR β1	Rabbit polyclonal	Santa Cruz; sc-11371	Santa Cruz
nAChR β2	Rabbit polyclonal	Santa Cruz; sc-11372	Quitadamo et al. (2005), Pollock et al. (2007), Kabbani and Levenson (2007)

[a] Refer to supplementary data on in house verification.

of these processes and lead to exaggerated/altered physiological activity (Gotti and Clementi, 2004).

Although various studies with animal models of nicotine exposure exist in the literature, those examining nAChR expression are limited to prenatal, adolescent, and adult brain and only examine mRNA and receptor binding (summarized in Supplementary Tables 1 and 2). Our laboratory is unique in that it has a piglet model of early postnatal nicotine exposure to mimic the clinical condition of nicotine intake through passive cigarette smoke exposure or via breast milk from a smoking mother (Machaalani et al., 2005). Using this model, we previously studied the expression of α7 and β2 nAChRs in the brainstem medulla and found α7 to be decreased in the dorsal motor nucleus of the vagus (DMNV) while β2 was increased in the DMNV, hypoglossal and nucleus of the spinal trigeminal tract, although specific to the male gender (Browne et al., 2010). In this study, we extend our research to other nAChR subunits, specifically α2, α3, α4, α5, α9, and β1 since they are important for stoichiometries of functioning nAChRs (reviewed Gotti and Clementi, 2004; Gotti et al., 2006). These subunits have been found to be expressed in the brain (Han et al., 2000; Quik et al., 2000) with the exception of the α9 and β1 subunits which have not yet been studied in brain tissue. We hypothesize that nicotine exposure increases the expression of the neuronal subtypes α2, α3, α4, α5, α7, α9 and β2 while having no effect on the non-neuronal subtype β1 The study focuses on the brainstem medulla as it contains vital nuclei that control cardiac and respiratory systems, which are affected by postnatal cigarette smoke exposure. The hippocampus is additionally studied as the activity of nAChRs within the hippocampus regulates cognitive functions such as learning and memory formation (Jones et al., 1999; Levin 1992), both of which are impaired by nicotine exposure (Gray et al., 1996; McGehee and Role, 1996).

2. Experimental procedures

2.1. Animal model

Piglets were chosen as the animal model based on the similarities they share with the human infant with regards to brain development, peak development being at birth as compared to rats which is at 7 days postnatal while for sheep it is 1–2 months prenatal (Dobbing and Sands, 1979), functional maturation and structure of the pulmonary vasculature (Hall and Haworth, 1986) and the cardiorespiratory system (Scott et al., 1990).

The live piglet work was performed previously and detailed in Machaalani et al., 2005. In brief, mixed breed miniature piglets aged 0–2 days were randomly allocated to either the control or nicotine group. At 0–2 days after birth, both groups underwent aseptic surgery for the intraperitoneal insertion of an osmotic minipump (Alzet; Alza Corporation, USA, Model 2ML2) that delivered 2.0 mg/kg/day of nicotine (−) hydrogen tartrate salt (Sigma; N5260) dissolved in sterile water to the nicotine group while the control group received sterile water only, for 14 days continuously after which they were immediately euthanised with an overdose of pentobarbital (200 mg/kg piglet body weight). This age was chosen since 13–14 days of the piglet corresponds to postnatal 2–4 month age in humans from a brain development perspective (Dobbing and Sands, 1979), which is the peak incidence age for the occurrence of SIDS (Hunt 1992) and corresponds to the developmental stage where exposure to passive smoke results in reduced cognitive functioning subsequently in childhood (>8 years) (Cho et al., 2010; Bauman et al., 1991). Nicotine treated piglets were caged separately to control piglets to prevent cross-contamination of nicotine from urine and/or other body fluids. The level of nicotine exposure was determined by analysis of cotinine (metabolite of nicotine) in blood and urine samples collected at the time of euthanasia, confirming that levels were equivalent to infants exposed to cigarette smoke. Ethical approval was obtained from the University of Sydney Animal Ethics Committee, and all experiments conformed to the international guidelines on the ethical use of animals.

2.2. Brain tissue collection and fixation

The whole brain was removed down to the spino-medullary junction, weighed and fixed in 10% buffered formalin for 14 days. The hippocampus and the brainstem were sectioned into 4 mm slices and returned to 10% buffered formalin for a further 5 days, then processed to paraffin.

Tissue blocks at the rostral level of the medulla and the hippocampus, were sectioned at 6 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. Although blocks at the caudal medulla level were also available, the tissue at this level was scarce given the use of this tissue in our laboratory over the past 10 years. Thus, we were only able to study the rostral medulla level in this paper.

2.3. Immunohistochemistry (IHC)

IHC for nAChR subunits was performed using eight commercially available antibodies that are well characterised and have had their specificity determined as described in Table 1. Care was taken in choosing these antibodies ensuring where available, they were tested in knockout tissue or in studies where complementary mRNA data was performed and showed results to be consistent.

The IHC protocol applied is standard for our laboratory (Machaalani and Waters, 2003). Briefly, all steps were performed at room temperature unless otherwise stated. Sections were deparaffinised in xylene, rehydrated through a graded series of ethanol to distilled water and subjected to heat induced epitope retrieval by