Toxicology and Applied Pharmacology 257 (2011) 396–404

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Effects of cigarette smoke exposure on nicotinic acetylcholine receptor subunits α7 and β2 in the sudden infant death syndrome (SIDS) brainstem

Rita Machaalani [a,b,c,*], Meichien Say [a,b], Karen A. Waters [a,b,c]

[a] Department of Medicine, The University of Sydney, NSW 2006, Australia
[b] Bosch Institute, The University of Sydney, NSW 2006, Australia
[c] The Children's Hospital at Westmead, NSW 2145, Australia

ARTICLE INFO

Article history:
Received 3 August 2011
Revised 23 September 2011
Accepted 25 September 2011
Available online 6 October 2011

Keywords:
Infant
nACh
Immunohistochemistry
Smoking
Nicotine
SIDS

ABSTRACT

It is postulated that nicotine, as the main neurotoxic constituent of cigarette smoke, influences SIDS risk through effects on nicotinic acetylcholine receptors (nAChRs) in brainstem nuclei that control respiration and arousal. This study compared α7 and β2 nAChR subunit expression in eight nuclei of the caudal and rostral medulla and seven nuclei of the pons between SIDS (n = 46) and non-SIDS infants (n = 14). Evaluation for associations with known SIDS risk factors included comparison according to whether infants had a history of exposure to cigarette smoke in the home, and stratification for sleep position and gender. Compared to non-SIDS infants, SIDS infants had significantly decreased α7 in the caudal nucleus of the solitary tract (cNTS), gracile and cuneate nuclei, with decreased β2 in the cNTS and increased β2 in the facial. When considering only the SIDS cohort: 1—cigarette smoke exposure was associated with increased α7 in the vestibular nucleus and increased β2 in the rostral dorsal motor nucleus of the vagus, rNTS and Cuneate, 2—there was a gender interaction for α7 in the gracile and cuneate, and β2 in the cNTS and rostral arcuate nucleus, and 3—there was no effect of sleep position on α7, but prone sleep was associated with decreased β2 in three nuclei of the pons. In conclusion, SIDS infants demonstrate differences in expression of α7 and β2 nAChRs within brainstem nuclei that control respiration and arousal, which is independent on prior history of cigarette smoke exposure, especially for the NTS, with additional differences for smoke exposure (β2), gender (α7 and β2) and sleep position (β2) evident.

Introduction

Cigarette smoke exposure is the second major modifiable risk factor for the sudden infant death syndrome (SIDS), after prone sleep position (Mitchell and Milerad, 2006). SIDS is defined as "the sudden unexplained death of an infant less than 1 year of age after a thorough case investigation, including a complete autopsy, examination of a death scene, and review of the clinical history" (Willinger et al., 1991).

Although the exact mechanism by which cigarette smoking increases the risk of SIDS is not known, a credible hypothesis is that nicotine affects neuronal nicotinic acetylcholine receptors (nAChRs) in brainstem nuclei in a manner that diminishes normal respiration and arousal during sleep (Cohen et al., 2005; Lena et al., 2004). In neonatal mice, prenatal nicotine exposure affects the respiratory rhythm pattern generator and causes a decline in central chemoreception during early postnatal life (Eugenin et al., 2008). Nicotine is one of the main constituents of cigarette smoke (Slotkin, 1998) with actions in the brain predominantly mediated through binding to nAChRs. The nAChRs are made up of 12 different subunits including nine α subunits (α2–α10) and three β subunits (β2–β4) (reviewed by Sargent, 1993); they are pentameric structures assembled from homomeric or heteromeric combinations of the 12 different subunits organized around a central pore (reviewed by Gotti and Clementi, 2004).

This study focuses on the α7 and β2 subunits. The α7 subunit predominantly exists as a homomeric structure, and was chosen because it is the most abundant and widespread subtype in the brain (Breese et al., 1997; Dominguez del Toro et al., 1994; Quik et al., 2000). It has many important functions including modulation of glutamatergic and cholinergic neurotransmitter release, synaptic plasticity, regulation of neuronal growth, differentiation and survival, regulation of calcium-dependent gene expression, and mediation of circuit excitability (reviewed by Gotti and Clementi, 2004).

Abbreviations: ABD, abducens; AN, arcuate nucleus; Cun, cuneate; DMNV, dorsal motor nucleus of the vagus; DR, dorsal raphe; FAC, facial; Grac, gracile; XII, hypoglossal nucleus; ION, inferior olivary nucleus; LC, locus coeruleus; MR, median raphe; nAChRs, nicotinic acetylcholine receptors; NTS, nucleus of the tractus solitarus; SIDS, sudden infant death syndrome; SpT, spinal trigeminal; SO, superior olivary; Vest, vestibular.

* Corresponding author at: Department of Medicine, Room 206, Blackburn Building D06, University of Sydney, NSW 2006, Australia. Fax: +61 2 9550 3851.
E-mail address: rita.machaalani@sydney.edu.au (R. Machaalani).

0041-008X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2011.09.023

The β2 subunit is commonly seen in the heteromeric α4β2 nAChR subtype. Of particular interest to this study, the β2 subunit is involved in regulation of respiration (Cohen et al., 2005) and sleep (Lena et al., 2004), and mediates the action of nicotine on brainstem nuclei (Cohen et al., 2002). In neonatal rat brainstem slices, activation of α4β2 nAChRs leads to increased respiratory frequency and decreased inspiratory amplitude in the hypoglossal nucleus and pre-Bötzinger complex (Shao and Feldman, 2001). Moreover, β2 knockout mice had depressed breathing and compensatory responses to repetitive hypoxia during sleep (Cohen et al., 2002) compared to wild type mice, and the pups from the knockouts had unstable breathing and impaired arousal (Cohen et al., 2005). Other modulating functions of the β2 nAChRs include neuronal development and plasticity (Champtiaux and Changeux;, 2002), survival and apoptosis (Hejmadi et al., 2003; Mechawar et al., 2004), and protection from excitotoxicity (Stevens et al., 2003).

Our hypothesis was that the immunohistochemical expression of α7 and β2 subunits is changed in brainstem nuclei of SIDS infants compared to non-SIDS infants, and for infants with a history of cigarette smoke exposure compared to non-exposed infants. The clinical history available also permitted examination for associations between changes in brainstem expression of these receptors and two other risk factors for SIDS; gender and sleep position.

Materials and methods

Data and tissue collection. The methods for data and tissue collection have been described in detail previously (Machaalani and Waters, 2008). Briefly, death scene reports, a death scene investigation checklist, and autopsy records were accessed at the Department of Forensic Medicine in Glebe, NSW, Australia, for all cases of sudden death in infants aged 28 days to 1 year for the period 1997 to 2004. All cases were number coded and identifying information (name and address) was removed prior to analysis. This study was approved by The Human Ethics Committees of the University of Sydney, and Central Sydney Area Health Service.

Cases were included if a complete clinical history and the results of the death scene examination were available, a complete autopsy had been performed, the brain had been examined by a neuropathologist after a period of fixation, and paraffin blocks of tissue from the medulla and pons were available. From the total of 196 cases over the period of study, only the 60 cases with information regarding their smoke exposure status could be included in this study. Smoke exposure status was derived from the Police Death Scene Investigation checklist (P534 NSW) where the question asks: “Does anyone in the household smoke? Yes or No”. Infants where the response was “Yes” were considered to have a history of cigarette smoke exposure and were included in the smoke-exposed group.

Serial tissue sections (7 μm) were collected from 10% neutral buffered formalin fixed and paraffin embedded blocks, mounted on slides coated with 3-aminopropyltriethoxysilane, and then subjected to immunohistochemical staining.

Immunohistochemistry. Immunostaining was performed as previously detailed (Machaalani et al., 2010) using commercial antibodies for α7 (ab10096, Abcam Ltd), a rabbit polyclonal synthetic peptide conjugated to KLH which corresponds to amino acids 28–42 of human nicotinic acetylcholine receptor, and β2 (AChRβ2 (H-92): sc-11372, Santa Cruz Biotechnology, Inc), a rabbit polyclonal raised against amino acids 342–433 mapping near the C-terminus of the subunit of human origin. Although tests using knockout (KO) tissue demonstrate that several antibodies against the nicotinic AChRs are non-specific to their targets (Moser et al., 2007), the α7 antibody used in this study did not demonstrate this characteristic; although not tested in KO tissue by the Moser group it was tested in the proteomics study by Paulo et al. (2009). Paulo et al. (2009) used α-bungarotoxin isolated and bead supported immunoprecipitation Western blot, with corresponding Coomassie stained gel, and found no band for α7 in KO tissue compared to wild type that revealed a band at 56 kDa. Additional evidence from the Paulo group regarding the specificity of this antibody included testing its presence in untransfected human embryonic kidney (HEK) cells versus cells transfected with the α7-5HT$_{3A}$ chimeric construct (personal communication). Additional confirmation of the antibody's specificity has recently come from a different group (Mielke and Mealing, 2009). The specificity of the β2 antibody we used has been demonstrated in several studies including (1) Quitadamo et al. (2005) who applied it in immunofluorescent IHC on hippocampal tissue from β2 KOs and found no staining compared to the wild type mice, (2) Pollock et al. (2007) who used two β2 antibodies (MAb270 and sc-11372) and found similar results in immunoblots of brain cells, and (3) Kabbani and Levenson (2007) who performed a similar proteomic study to Paulo et al. (2009) and found no β2 band in the Western blot or the corresponding Coomassie stained gel within the KO homogenized brain tissue compared to wild type tissue.

Detailed methodology of our laboratory's standard immunohistochemical protocol using the above mentioned antibodies has been provided previously (Browne et al., 2010; Machaalani et al., 2010). Briefly, all steps were carried out at room temperature unless otherwise noted. After deparaffinization and microwave antigen retrieval in Tris-EDTA buffer (1 mM EDTA, 1 mM Na citrate, 2 mM; pH 9) on high for 14 min (Black and Decker 700W, USA), sections were quenched for endogenous peroxidase activity, incubated in 10% normal horse serum (NHS) in phosphate buffered saline (PBS) for 30 min, and then incubated in each of the respective primary antibodies (α7 diluted 1:500 and β2 diluted 1:300 in 1% NHS) overnight at 4 °C. Sections were then incubated in the secondary antibody (biotinylated horse anti-mouse IgG/anti rabbit IgG, Vector Laboratories Inc.) for 40 min, then with the avidin-biotin peroxidase reagent (Vectastain ABC kit; Vector laboratories Inc.) for 40 min. Color was developed with DAB, counterstained with Harris' hematoxylin, and sections were mounted in DPX. Negative control sections were immunostained as above, but the primary antibody was replaced with 1% NHS.

Nuclei studied. Our analyses were confined to 4 levels from the pons and medulla: the rostral pons at the isthmus; the caudal pons at the genu of the facial nerve; and the rostral and caudal medulla. The anatomical levels and nuclei were identified with reference to Plates X–XIII, XXVII, XXV, and XXIII of the atlas Cytoarchitecture of the Human Brainstem (Olszewski and Baxter, 1954) and Fig. 17 (caudal) and Fig. 23 (rostral) of the Atlas of the Human Brainstem (Paxinos and Huang, 1995).

The nuclei studied from the pons included: locus coeruleus (LC), dorsal raphe (DR), median raphe (MR), abducens (ABD), facial (FAC), superior olivary (SO), and the spinal trigeminal (SpT). The nuclei from the medulla included: arcuate (AN), cuneate (Cun), dorsal motor nucleus of the vagus (DMNV), gracile (Grac), inferior olivary nucleus (ION), vestibular (Vest), nucleus of the tractus solitarus (NTS), and the hypoglossal (XII) nucleus. Adjacent H&E stained sections were used to define the anatomic boundaries of the brainstem nuclei being studied.

Quantification. All quantification was performed with the use of computerized image analysis systems at the Australian Key Centre for Microscopy and Microanalysis, University of Sydney. Images were captured at ×10 magnification using the Nikon Eclipse E800 light microscope and sensicam CCD camera. The number of images captured per nucleus ranged from 1 to 6, depending on the size of each nucleus. All slides were coded so that analysis was performed blinded to the diagnosis.

Only neuronal staining was analyzed. A manual count of neurons was undertaken, identifying the positive and the negative stained