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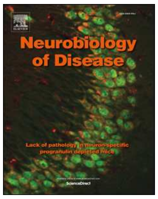

The α3 and α4 nicotinic acetylcholine receptor (nAChR) subunits in the brainstem medulla of sudden infant death syndrome (SIDS)

Atqiya Aishah[a,b], Tina Hinton[a,b], Karen A. Waters[c], Rita Machaalani[b,c,*]

[a] Discipline of Pharmacology, Faculty of Health and Medicine, The University of Sydney, NSW 2006, Australia
[b] The Bosch Institute, Faculty of Health and Medicine, The University of Sydney, NSW 2006, Australia
[c] Central Clinical School of Medicine, Faculty of Health and Medicine, The University of Sydney, NSW 2006, Australia

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ABSTRACT

SIDS occurs in early infancy and predominantly during a sleep period. Abnormalities in nicotine receptor binding and in the expression of the nicotinic acetylcholine receptor (nAChR) subunits α7 and β2 have been reported in the brainstem of SIDS infants. This study focuses on the α3 and α4 nAChR subunits as α3 is important for early postnatal survival while α4 is crucial for nicotine-elicited antinociception and sleep-wake cycle regulation. Tissue from the rostral medulla of infants who died with a known cause of death (eSUDI, $n = 7$), and from SIDS classified as SIDS I ($n = 8$) and SIDS II ($n = 27$), was immunohistochemically stained for the α3 and α4 nAChR subunits and quantified in 9 nuclei comparing amongst these groups. The association with risk factors of sex, cigarette smoke exposure, upper respiratory tract infection (URTI), prone sleeping and bedsharing was also evaluated. Results showed that only α4 changes (increase) were evident in SIDS, occurring in the hypoglossal and cuneate nuclei of SIDS II infants and the nucleus of the spinal trigeminal tract of SIDS I infants. Amongst the SIDS infants, cigarette smoke exposure was only associated with decreased α4 in cribriform fibre tracts, while sex and bedsharing were associated with increases in α3 in the dorsal motor nucleus of the vagus and solitary nucleus, respectively. Combined, these findings suggest that abnormalities in endogenous acetylcholine synthesis and regulation may underlie the altered α3 and α4 nAChR subunit expressions in the SIDS brainstem medulla since the changes were not related to cigarette smoke exposure.

1. Introduction

The definition of Sudden Infant Death Syndrome (SIDS) is widely accepted as “The sudden unexpected death of an infant < 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history” (Krous et al., 2004). There are multiple risk factors that interact in the pathogenesis of SIDS, and as such the precise mechanism(s) is currently unknown. The most prevalent risk factors include cigarette smoke exposure and the prone sleep position (Mitchell et al., 2012).

The main active constitute of cigarette smoke is nicotine which acts by agonising the nicotinic acetylcholine receptors (nAChRs). nAChRs are formed as pentamers with 5 subunits arranged pseudo-symmetrically around a central pore (Lodish et al., 2000). Currently, seventeen subunits are known - α1-10, β1-4, δ, ε and γ. Binding of a ligand such as nicotine or acetylcholine (ACh) enables conformational change in the receptor allowing for the movement of positively charged ions such as Na + , K + and Ca + across the neuron cell membrane, and is dependent on subunit combination, permitting propagation of signals for various responses (Beker et al., 2003; Elgoyhen et al., 2001; Rang et al., 2014). There are two categories of nAChRs in vertebrates; muscle-type, predominantly found at the neuromuscular junction (α1, β1, γ, δ, or ε subunits), and neuronal-type. Neuronal- type nAChRs are homomeric or heteromeric combinations of α2-10 and β2-4 subunits (Colquhoun et al., 2003; Fryer and Lukas, 1999). The most common heteromeric combinations found in the brain are α3β2, α3β4, α3α5β2, α3α5β4, α4β2, α4α6β2, α7β2 and α9α10 (Lips et al., 2005).

In our laboratory, three animal models of SIDS risk factors have been studied: 1- a piglet model of post-natal nicotine exposure (Vivekanandarajah et al., 2015), 2- a mouse model of pre- into post-

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Abbreviations: AN, arcuate nucleus; Cun, cuneate; DMNV, dorsal motor nucleus of the vagus; XII, hypoglossal nucleus; ION, inferior olivary nucleus; nAChRs, nicotinic acetylcholine receptors; NTS, nucleus of the tractus solitarus; SIDS, sudden infant death syndrome; NSTT, nucleus of spinal trigeminal tract; Vest, vestibular

[*] Corresponding author at: Central Clinical School, Room G77, Medical Foundation Building, K25, University of Sydney, NSW 2006, Australia.
E-mail address: rita.machaalani@sydney.edu.au (R. Machaalani).

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natal cigarette smoke exposure (Vivekanandarajah et al., 2016), and 3- a piglet model of intermittent hypercapnic hypoxia (mimicking gaseous conditions associated with the prone sleep position) (Vivekanandarajah et al., 2017). Studying the protein expression of eight nAChR subunits (α2, α3, α4, α5, α7, α9, β1 and β2) using immunohistochemistry, the most prominent changes were seen for the α3 and α4 subunits (Vivekanandarajah et al., 2016, 2015).

The α3nAChR subunit is classified as a predominantly ganglion-type nAChR, where it forms functional heteromeric receptors with the β2, β4 and α5 subunits (Karczmar and Chauhan, 2007), while, the α4 nAChR subunit forms functional receptors with either β2 or β4 in varying stoichiometries with differing nicotine sensitivities. For example, the (α4)2(β2)3 nAChR has higher sensitivity to nicotine in mouse thalamus in comparison to (α4)3(β2)2 (reviewed in Karczmar and Chauhan, 2007). Knock out (KO) of the α3 subunit is lethal, with up to 40% of α3 KO mice dying within 3 days of birth. This is due to a loss of autonomic control of peripheral organs such as bladder and pupil contraction (Xu et al., 1999) where the most common ganglionic postsynaptic membrane receptor combinations are α3β4, α3β2 and α3β4β2 (Nai et al., 2003). Physiologically, the α3-containing nAChRs play important roles in oxygen signalling in carotid bodies; a function not seen for the α4 subunit (Fagerlund et al., 2010). The α3 gene (CHRNA3) is associated with pulmonary diseases where polymorphisms of CHRNA3 predict risks of chronic obstructive pulmonary disease and lung cancer in adults (Yang et al., 2012) and bronchial hyperresponsiveness in children (Torjussen et al., 2012). KO of the α4 subunit leads to a loss of high affinity nicotine binding throughout the brain and reduction in nicotine-elicited antinociception (Marubio et al., 1999). Physiologically, the α4 subunit plays a crucial role in nicotine-induced seizures and mediates changes in sleep-wake cycles as demonstrated in a knock-in mouse model with an amino acid substitution within the α4 subunit in α4β2 nAChRs (Fonck et al., 2005).

Given the predominant changes in the α3 and α4 subunits in our animal models and the roles of these subunits in respiratory control and nicotine sensitivities, this study aimed to: 1- report the cellular and regional distribution of these subunits (at the protein level using immunohistochemistry) in the human infant rostral brainstem medulla, 2- determine any changes in expression in SIDS as compared to non-SIDS infants (with SIDS infants being separated as SIDS I or SIDS II according to Krous et al., 2004 criteria), and 3- determine any changes in expression due to a history of cigarette smoke exposure. This study focuses on the brainstem as it houses nuclei involved in arousal and cardiorespiratory regulation which has been hypothesised to be dysfunctional in SIDS infants (Hunt and Brouillette, 1987). Further analyses were performed to determine any associations with other SIDS risk factors including an upper respiratory tract infection (URTI) and sleep-related factors such as being found in the prone sleep position, and bedsharing.

2. Materials and methods

2.1. Data and tissue collection

Methods of data and tissue collection have recently been outlined in Ambrose et al. (2018). Briefly, the human infant brainstem tissue and data was obtained from the Department of Forensic Medicine in Glebe, NSW, Australia under the approval of the Human Ethics Committees of the University of Sydney and the Central Sydney Area Health Service. The cases were those seen by Forensic Medicine during 2008–2012. Death scene investigation and autopsy records were accessed to obtain information relevant to SIDS. This information includes the sleep position in which the infant was found, and history of cigarette smoke exposure status where the investigating death scene police officer asked the parents if they or any other household member smoked.

Coronal tissue sections of the brainstem medulla (6 μm thin) were obtained from 20% formalin fixed and paraffin embedded (FFPE) blocks that remained after the completion of the autopsy and final diagnosis assigned. Sections were mounted on 3-Aminopropyltriethoxysilane (APTES) coated slides, then dried at 42 °C overnight before storage in a dust free environment at room temperature prior to staining.

A total of 42 cases were included in this study and were a subset of the recently characterised dataset by Ambrose et al. (2018). Following an expert panel assessment applying the Krous et al. (2004) (San Diego) diagnostic criteria, the groups were classified as 31 SIDS (8 SIDS I, 27 SIDS II) and 7 explained sudden unexpected death in infancy (eSUDI) (Ambrose et al., 2018). The cause of death in the eSUDI group included acute pneumonia ($n = 1$), thrombotic occlusion of the Blalock-Taussig shunt ($n = 1$), complications of congenital heart disease ($n = 2$) and myocarditis ($n = 2$), and meningoencephalitis of uncertain aetiology ($n = 1$). The San Diego criteria separate the SIDS group into SIDS I and II, where SIDS I includes cases previously classified as 'classical' SIDS, typically without additional risk factors, and results in a more 'homogeneous' group that facilitates research into the pathogenesis of SIDS and the role of epidemiologically associated risk factors, such as bedsharing and prematurity (Krous et al., 2004).

This study was double blinded with all infant cases coded and the data characteristics de-identified until the time of statistical analysis.

2.2. Immunohistochemistry (IHC)

Immunohistochemistry was performed according to the usual methods of our laboratory as detailed in Machaalani et al. (2011). Briefly, sections were deparaffinized in xylene and rehydrated through a graded series of ethanols to distilled water (dH2O). Cross-linkages formed during formalin fixation were disrupted using heat-induced antigen retrieval (microwaved on 'High'; Homemaker, 900 W, China) in Tris/EDTA buffer (1 mM EDTA, 1 mM sodium citrate, 2 mM Tris, pH 9.0) for 15 mins and then left to cool for a total of 15 mins in a water bath. Sections were washed in dH2O and PBS (0.14 M NaCl, 0.0027 M KCl, 0.01 M Phosphate buffer, pH 7.4), and a hydrophobic barrier applied around the sections before washing in PBS. Endogenous peroxidase was quenched in 3% hydrogen peroxidase (H2O2)/50% methanol in PBS for 25 mins at room temperature. Sections were washed in PBS and incubated in 10% normal horse serum (NHS) made up in PBS for 30 mins. Incubation with rabbit polyclonal primary antibody for α3 nAChR (sc-5590; Santa Cruz) and goat polyclonal antibody for α4 nAChR (sc-1772; Santa-Cruz) diluted in 1% NHS (α3: 1:200, α4: 1:300) was carried out overnight at room temperature. The specificity of the α3 antibody was determined by Liu et al. (2011) and confirmed in our laboratory on human brain tissue comparing it to mRNA staining via non-radioactive in situ hybridisation (Aishah et al., 2017), while the α4 antibody was verified by Govind et al. (2012), Whiteaker et al. (2006), and Martin-Ruiz et al. (2000). Sections were washed in PBS, incubated in anti-rabbit (for α3) or anti-goat (for α4) biotinylated secondary antibody (Vector Laboratories Inc., 1: 200) in 1% NHS for 1 h, followed by PBS washes. Incubation with avidin/biotin horseradish peroxidase enzyme reagent (VERK4000; Vector Laboratories Inc.) was performed for 1 h and colour developed using diaminobenzidine (DAB) (K3468; DakoCytomation, USA). Tissue was counterstained in Haematoxylin (30 s), rinsed in H2O, cleared in acid alcohol, rinsed in H2O and immersed in Scott's Blue solution. After thorough washing in H2O, sections were dehydrated through a graded series of ethanols to xylene, mounted and cover slipped using Di-N-Butyle Phthalate in Xylene (DPX). Negative controls were performed as mentioned above but the primary antibody replaced with 1% NHS.

2.3. Image and quantitative analysis

At the commencement of this project, we had aimed to assess the expression of the two nAChR subunits at both the caudal and rostral medulla levels as was done previously in our laboratory for the α7 and β2 subunits (Machaalani et al., 2011), however, not all cases had a tissue section at an equivalent caudal level (Ambrose et al., 2018). Thus, analysis was only undertaken at the rostral level (as appears in Figs. 8.21–8.25 of the Organisation of Brainstem nuclei by Paxinos et al., 2012). Thus, the data herein, is only representative of functionality of the nuclei at this level which were studied and included the: arcuate (AN), cuneate (Cun), dorsal motor nucleus of the vagus (DMNV), inferior olivary nucleus (ION), vestibular (Vest), nucleus of the spinal trigeminal tract (NSTT), nucleus of the tractus solitarus (NTS), and the hypoglossal (XII) nucleus.

Staining of sections was observed using a Leica Upright MD6000 B Microscope (Leica Microsystems Ltd. Heerbrugg, Switzerland) at 10× magnification for XII, DMNV, Cun, Vest and ION, and 20× magnification for NTS, NSTT and AN, given the neurons in these nuclei are much smaller. Image capture software (LAS v4.2, Leica Microsystems Ltd. Heerbrugg, Switzerland) was used to capture images of these nuclei of interest. For larger nuclei such as the ION, three representative images were taken arbitrarily.

The ‘Cell counter’ feature of Image J (v1.49, National Institute of Health, USA) was used to manually count neurons (not glia or astrocytes) of all imaged nuclei. Summaries of total counts of positive and negative neurons were recorded where nuclei with multiple images were collated in similar fashion. Data of the positively and negatively stained neurons were expressed as a percentage of positively stained neurons to standardize and allow comparison amongst cases, given there may be some variation in neuron numbers in each individual case and that only a maximum of 2 sections per case were analysed.

2.4. Statistical analysis

All data for infant characteristics were exported to SPSS (Statistical Package for Social Sciences) for Windows (V22, SPSS Inc., Illinois, USA) for analysis. Descriptive statistics (mean and standard deviation (SD)) were determined for each characteristic. One-way analysis of variance (ANOVA) was used to test for significances between eSUDI and the SIDS diagnostic groups for numerical values with LSD correction. Categorical data (i.e. sex and smoke exposure) were examined by Chi-square analysis and the Fischer's exact test was used to determine significance. A p value < .05 was considered statistically significant.

Sub group analysis using one-way ANOVA was performed for α3 and α4 nAChR subunit staining quantification expressed as a mean percentage of positively stained neurons ± SD, to compare between SIDS I, SIDS II, and eSUDI (with LSD correction). Comparison for the risk factors was performed via Independent sample t-test.

3. Results

3.1. Infant characteristics

The three groups (eSUDI, SIDSI and SIDS II) were well-matched for clinical and autopsy characteristics including gestational age, age at death, body weight, body length, brain weight (Table 1). Factors which may affect or have the potential to affect the staining of the tissue such as post mortem interval (PMI) and fixation time also did not differ statistically when comparing the groups.

Exposure to cigarette smoke in the SIDS groups was double that of the eSUDI group (40% vs 20%) but the difference was not statistically significant (p = .72). There was also a non-significant trend for higher rates of URTI in the SIDS II group as compared to eSUDI and SIDS I (p = .12) (Table 1).

Regarding SIDS specific risk factors related to sleep, no SIDS infant was reported as being put to sleep prone, but 5 out of 8 (63%) SIDS I and 9 out of 23 (39%) SIDS II were found in the prone position (Table 1). Bed sharing at the time of death was reported in 10/27 SIDS II cases and none from SIDS I as would have been expected based on diagnostic criteria (Krous et al., 2004).

Table 1
Clinical, autopsy and risk factors prevalence characteristics of the study dataset.

	eSUDI	SIDS I	SIDS II	p-value
	(n = 7)	(n = 8)	(n = 27)	
Clinical data				
Male: female	3:4	6:2	18:9	0.39
Gestational age (wks)	38.9 ± 2.0	39.3 ± 1.7	38.4 ± 2.2	0.62
Birth weight (kg)	2.8 ± 0.9	3.0 ± 0.5	3.0 ± 0.8	0.85
Autopsy data				
Age at Death (mths)	4.1 ± 3.3	4.5 ± 2.0	4.2 ± 2.8	0.95
Post-conception age (wks)	51.6 ± 14.2	57.4 ± 7.9	55.1 ± 12.1	0.64
Body weight (kg)	5.7 ± 2.9	6.9 ± 1.7	6.1 ± 2.0	0.51
Brain weight (g)	650 ± 230	765 ± 147	736 ± 166	0.41
Body length (cm)	56.9 ± 12.7	63.9 ± 5.3	61.2 ± 7.8	0.27
Head circumference (cm)	38.9 ± 5.1	41.5 ± 2.4	40.6 ± 3.9	0.42
Fixation duration (days)	7.1 ± 5.8	8.6 ± 5.5	7.1 ± 4.9	0.76
Post-mortem interval (hrs)	25.3 ± 24.7	39.0 ± 11.7	28.4 ± 12.7	0.16
Risk factor prevalence				
Found prone sleeping	n/a	5/8 (63%)	9/23 (39%)	0.25
Co-sleeping/ bed sharing	n/a	0/8 (0%)	10/27 (37%)	0.19
Cigarette smoke exposure	1/5 (20%)	3/8 (38%)	9/23 (39%)	0.72
Upper respiratory tract infection	1/7 (14%)	2/8 (25%)	14/27 (52%)	0.12
Immunised	2/3 (67%)	6/6 (100%)	14/17 (82%)	0.39

Data presented as mean ± SD. P values for risk factor prevalence is derived by Pearson Chi-Square.

3.2. Staining pattern

Immunostaining for the α3 subunit was detected predominantly in the neurons of the medulla, with an estimated 10% staining seen in other cell types (glia and astrocytes). In the neurons that were the focus of this study, staining was localized to the cytoplasm (Fig. 1A, C). A similar staining profile was seen for the α4 subunit (Fig. 1B, D), with exception of additional fibre tract staining visible in the Vest, and intensely and extensively detected in the cribriform nucleus (Fig. 2), which lies near the Vest and was identified using the human brainstem atlas (Paxinos et al., 2012). Staining in the cribriform nucleus was quantified by assigning it a score from 0 to 3 where 0 indicates no staining, 1 indicates up to 20% coverage of staining, 2 indicates 21–70% coverage of staining, and 3 indicates > 70%. Examples of images showing these scores are provided in Fig. 2.

Amongst the nuclei, the α3 subunit was highly expressed in the motor nuclei of the XII, DMNV, ION, and AN, with lower expression in the sensory nuclei of the NSTT and NTS (Fig. 3A). A similar distribution was also observed for the α4 subunit (Fig. 3B).

3.3. Quantitative comparison amongst the 3 diagnostic groups

No difference in α3 nAChR subunit expression was seen amongst the 3 groups of eSUDI, SIDS I and SIDS II, (Table 2). For α4, SIDS I had significantly greater expression in the NSTT compared to eSUDI infants (p = .04), and SIDS II infants had greater expression in the XII (p = .03) and Cun (p = .05) compared to eSUDI infants (Table 2). No differences were evident between the SIDS I and SIDS II groups.

3.4. Analysis by sex, cigarette smoke exposure and URTI

Analysis by sex, cigarette smoke exposure and URTI were performed for the entire dataset regardless of diagnosis. There was no difference in expression for either α3 or α4 nAChR subunits comparing the males to females (data not shown). Infants with a positive history of cigarette smoke exposure had lower α4 fibre staining in the cribriform nucleus (p = .04; Table 3). Infants with an URTI up to two weeks prior to death, had higher α4 in the XII (p = .03; Table 3).

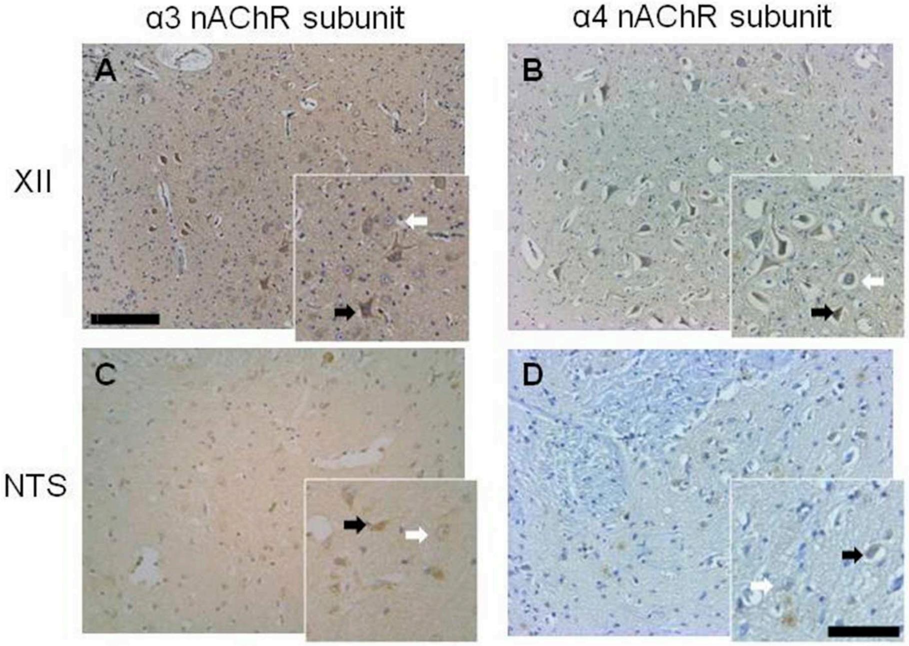

Fig. 1. Protein expression of α3 (A,C) and α4 (B,D) nAChR subunits in the XII (A,B) and NTS (C,D) in infant brainstem medulla. Black arrows indicate positive staining while white arrows indicate negative/ no staining. Scale bar represents 400 μm for all main panels and 120 μm for enlarged inserts.

3.5. Analysis of the SIDS group by risk factors

Within the *SIDS* population (SIDS I and II combined), males had higher α3 expression in the DMNV (males: 54.3 ± 15.8 vs females: 39.6 ± 14.9; $p = .02$). SIDS infants with a positive history of cigarette smoke exposure ($n = 12$) had lower α4 fibre staining in the cribriform nucleus compared to those without exposure ($n = 19$) (0.5 ± 0.2 vs 1.3 ± 0.3; p = .03). SIDS infants with an URTI ($n = 16$) had no difference in expression for either α3 or α4 nAChR subunits compared to those without ($n = 19$) (data not shown).

For sleep-related parameters, those reported as bed-sharing had higher α3 expression in the NTS (24.9 ± 18.4 vs 8.6 ± 12.3; $p = .005$). Meanwhile, only non-significant trends were identified for those found in the prone sleep position compared to others in the groups. These included a trend for increased α3 expression in the ION (56.6 ± 13.8 vs 43.7 ± 19.1; $p = .055$), a trend for decreased α4 in the Cun (29.4 ± 14.0 vs 39.6 ± 14.0; $p = .06$) and a trend to increased fibre staining in the cribriform nucleus (1.4 ± 1.1 vs 0.6 ± 1.0; $p = .056$).

4. Discussion

The main findings of this study are: 1- only α4 nAChR subunit expression changes were evident when comparing SIDS to eSUDI, with increased expression in the NSTT (SIDS I), XII (SIDS II) and Cun (SIDS II), 2- cigarette smoke exposure was only associated with a decrease in α4 expression in the cribriform fibres, while an URTI was associated with higher α4 in the XII for the whole cohort (not specific to SIDS diagnosis), and 3- amongst the SIDS cohort, the sleep parameter of bed sharing was highly associated with an increase in α3 in the NTS, while being found in the prone position resulted in trends of increased α3 in the ION, increased α4 in the cribriform fibres, and decreased α4 in the Cun. Combined, these findings indicate that the increase in α4 in the SIDS infant NSTT and XII is independent of cigarette smoke exposure, yet that in the Cun, may be related to the risk factor of sleep position. The sleep-related risk factors were further independently associated with changes in the NTS, ION and cribriform medullary nuclei.

4.1. Normal expression of the subunits

Our finding that both α3 and α4 nAChR subunits were expressed at higher levels in motor nuclei in comparison to sensory nuclei in normal infant brainstem, is consistent with that reported for mRNA expression of these subunits (Wada et al., 1989; Zoli et al., 1995). This suggests that both these nAChR neuronal subunits may play a greater role in the motor functional systems within the brainstem compared to sensory systems.

The presence of densely packed neuronal fibres has been reported to extend down from the cerebrum and cerebellum to the brainstem. These fibres can contain axons (Stieltjes et al., 2001) and, as such, staining for the receptor α4 subunit in fibres within the cribriform and Vest nuclei may be indicative of axonal transport of the subunit within these nuclei. This conclusion was also made by authors who found fibre staining for the α4 subunit in the human brain using an antibody different to ours (Sparks et al., 1998). The cribriform nucleus has not been extensively studied and was first identified by Paxinos and Huang (1995) and reported to have high acetylcholinesterase (AChE) expression (Paxinos et al., 2012). Thus a deeper look into this nucleus is warranted in future studies of the ACh system.

4.2. SIDS compared to eSUDI and risk factor contribution

To date, three studies of nAChRs in SIDS infant brainstem medulla have been conducted. Radioligand binding to nAChRs using ^{3}H-nicotine was undertaken in the rostral medulla and showed no difference in binding density in SIDS infants compared with controls (Nachmanoff et al., 1998). This was subsequently confirmed in another study (Duncan et al., 2008). In this later study, Duncan et al. (2008) found decreased ^{3}H-nicotine binding in nuclei of the pons regions of the brainstem in response to smoke exposure in the control (non-SIDS) infants; thus a history of smoke exposure in a ‘control’ population of infants led to decreased ^{3}H-nicotine binding but only in the pons. Our laboratory examined immunohistochemical expression of the α7 and β2 nAChR subunits in the medulla and pons of SIDS and non-SIDS (Machaalani et al., 2011) and found changes specific to nuclei of the caudal medulla only (not rostral); a decrease in α7 in the NTS, gracile

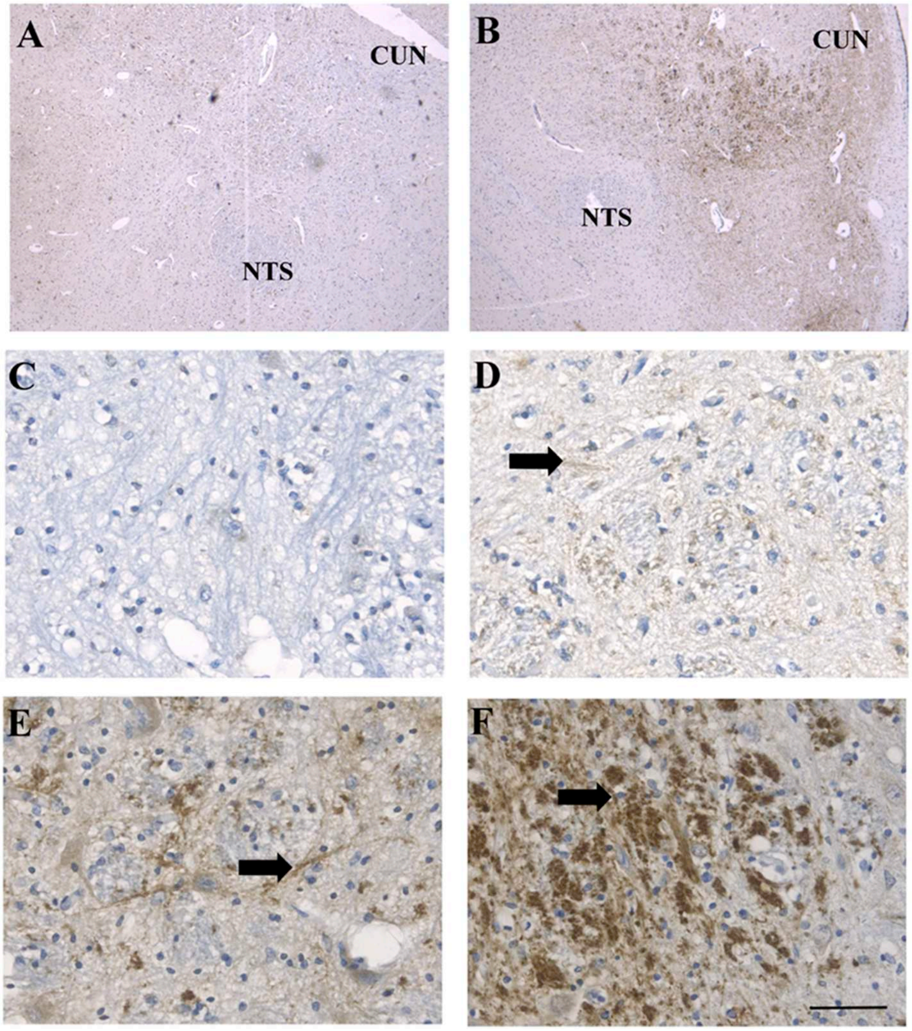

Fig. 2. Fibre staining for α4 nAChR subunit observed in the cribriform located between the NTS and Cun of rostral brainstem medulla of infants where staining observed was graded a score 0 = 0% (A,C), score 1 = 1–20% (D), score 2 = 21–70% (E) and score 3 ≥ 70% (B,D). Scale bar represents 480 μm (A,B) and 50 μm (C−F). Black arrow points to a positive fibre tract.

and cuneate nuclei and decreased β2 in the NTS. Further changes were observed for both subunits stratified for cigarette smoke exposure status (Machaalani et al., 2011).

The study herein adds to the literature and shows that only α4 nAChR expression changes are evident when comparing the SIDS to eSUDI, and not α3. This increase was specific to the NSTT (SIDS I), XII (SIDS II) and Cun (SIDS II). The increases in the Cun being further complicated given it was also found to be affected by the risk factor of sleep position.

It is interesting that higher α4 in the NSTT was only in SIDS I compared to eSUDI and not SIDS II. The SIDS I population differs to SIDS II in that they are predominantly free of the risk factors (Krous et al., 2004). Thus the increased α4 in the NSTT of this group may relate to the cause of death, and not to any of the risk factors analysed. The NSTT contains a population of neurons, the *subnucleus oralis*, that receive information regarding touch, pain and temperature from ipsilateral nasal and oral cavities (Conn, 2008). It also receives information regarding pain from the facial, glossopharyngeal and vagus cranial nerves involved in processing information from skin near the ears, nose and pharynx (Bear et al., 2007). Increased α4 nAChR subunit expression in the SIDS I NSTT suggests altered receptor functionality. Inability of the infant to arouse when faced with changes in temperature, pain or facial proprioception could correlate with increased risk of SIDS in winter months (Mitchell et al., 2012) and in situations where the oral and nasal cavities are compromised. As no confirmatory correlations were found with prone sleep position, the cause versus effects of increased α4 in the SIDS I NSTT is yet to be elucidated.

The XII is involved in motor control of tongue and coordination of swallowing, chewing, and vocalization, and plays a role in specific phases of inspiration via control of the genioglossus (GG) muscle in the tongue (Boone and Aldes, 1984; Cooper, 1981). Activity of the GG is suppressed during sleep via decreased activity of serotonergic and nonadrenergic neurons (Fenik et al., 2005). Yet compensatory mechanisms seem to occur with cholinergic neurons increasing their activity during REM sleep to maintain GG muscle tone (Bellingham and Funk, 2000; Fenik, 2015). Of note, the α4 nAChR subunit has been found to be localized to neurons that project to the GG muscle in the rat (Dehkordi et al., 2005). Although ^{3}H-nicotine preferentially binds to α4β2-containing nAChRs, previous studies found no changes in ^{3}H-nicotine binding in SIDS XII (Duncan et al., 2008; Nachmanoff et al., 1998), nor any change in the α7 or β2 nAChR subunit expression (Machaalani et al., 2011). We hypothesise that this indicates that α4 subunits are specifically affected. Altered expression of selected subunits without overall changes in expression of all receptors may also suggest an altered receptor phenotype in SIDS, with potential for functional outcomes. For example, the α4 nAChR subunit forms functional receptors with either β2 or β4 subunits in varying stoichiometries with differing nicotine sensitivities; the $(\alpha4)_2(\beta2)_3$ nAChR has higher sensitivity to

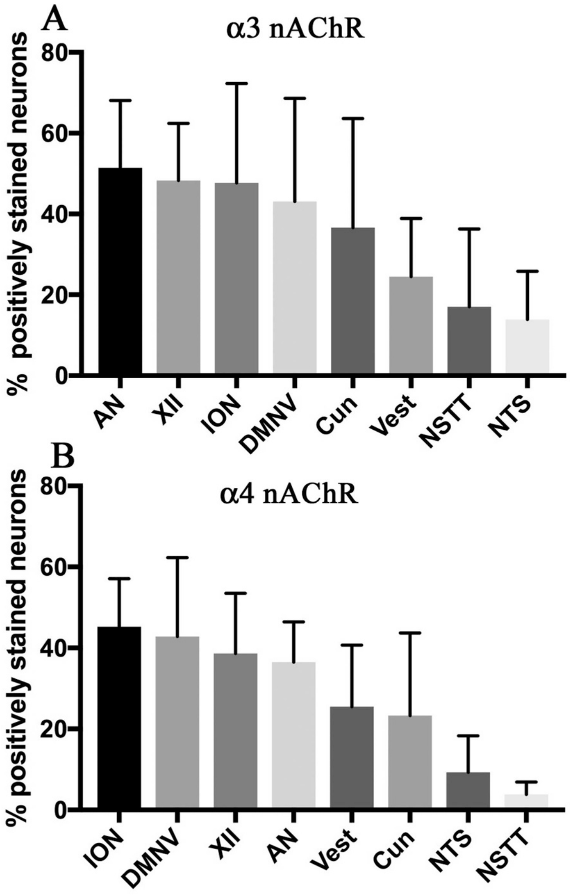

Fig. 3. Expression of the (A) α3 and (B) α4 nAChR subunits in all of the 8 nuclei in the eSUDI (non-SIDS) infants ($n = 7$) at the rostral brainstem medulla from highest to low expression. Data presented as mean ± SD.

nicotine in mouse thalamus in comparison to $(\alpha4)_3(\beta2)_2$ (reviewed in (Karczmar and Chauhan, 2007). Higher expression of α4 subunits and dominant expression of the $(\alpha4)_3(\beta2)_2$ phenotype would confer lower affinity for nicotine. Our finding of similar correlation with the presence of an URTI suggests this could precipitate similar changes.

The Cun is integral to processing information from deep muscle and joint tissue of the forelimb (Li et al., 2012). Awareness and movement of the forelimb may form part of the activity in clearing the airway if prone sleeping or covering of the face cause breathing difficulties during sleep (Kinney and Thach, 2009). Our findings were apparently inconsistent in this regard, since SIDS II infants had increased α4 in the Cun, but it was decreased in infants found in the prone position. Thus, a more detailed explanation involving receptor regulation (translation, endocytosis, desensitization) may be required to cohesively explain our findings (Vivekanandarajah et al., 2019).

The increase in α4 subunit in SIDS cases (NSTT, XII, and Cun) was independent of smoke exposure status. This may be compensation for reduced choline acetyltransferase (ChAT), an enzyme responsible for the synthesis of ACh, with reduced expression identified in several structures of the SIDS infant brain including the XII and DMNV (Mallard et al., 1999), the hypothalamus (Sparks and Hunsaker, 1991), and basal ganglia (Kalaria et al., 1993). We hypothesise that decreased ChAT results in decreased ACh levels in the SIDS infant brain, thus leading to a compensatory upregulation of α4 subunit-containing nAChRs. This proposal is supported by a study where ablation of the nucleus basalis magnocellularis (a site of ChAT production) in the rat resulted in increased α4 subunit RNA in the cerebral cortex but no change in the α3 subunit (Miyai et al., 1990). Hypoxic events are thought to preceded SIDS and have been shown to decrease ACh synthesis in rat brain (Gibson and Duffy, 1981) as well as in in vitro studies conducted in mice (Gibson and Blass, 1976; Gibson et al., 1981). The ChAT enzyme was also shown (via immunohistochemistry) to be decreased in the pons of rats exposed to hypoxic conditions (Ray et al., 2011).

Table 2

Expression of the α3 and α4 nAChR subunits in the nuclei of the rostral brainstem medulla between the three diagnostic groups.

Nucleus	eSIDS	SIDS I	SIDS II
	$n = 7$	$n = 8$	$n = 23$
α3 nAChR subunit			
XII	48.3 ± 14.1	57.3 ± 27.1	59.2 ± 18.8
DMNV	43.1 ± 25.5	47.6 ± 15.9	49.5 ± 17.4
NTS	13.9 ± 11.9	5.1 ± 6.0	16.8 ± 17.6
Vest	24.5 ± 14.4	32.4 ± 19.9	32.5 ± 18.7
Cun	36.6 ± 27.0	31.7 ± 7.8	38.6 ± 18.1
NSTT	17.0 ± 19.3	16.4 ± 16.6	14.3 ± 12.4
ION	47.7 ± 24.6	42.1 ± 17.2	52.1 ± 16.8
AN	51.4 ± 16.7	50.3 ± 17.2	45.9 ± 13.2
α4 nAChR subunit			
XII	38.6 ± 14.9	45.0 ± 27.0	60.1 ± 19.9*
DMNV	42.8 ± 19.5	30.1 ± 17.2	40.1 ± 15.3
NTS	9.3 ± 9.0	8.0 ± 3.4	7.7 ± 14.9
VEST	25.5 ± 15.2	38.6 ± 21.5	36.8 ± 14.5
Cun	23.3 ± 20.4	28.3 ± 14.7	38.0 ± 15.3*
NSTT	3.8 ± 3.1	16.3 ± 18.8*	10.5 ± 8.8
ION	45.2 ± 11.9	54.0 ± 21.5	55.7 ± 10.6
AN	36.5 ± 9.9	45.3 ± 22.9	47.1 ± 12.9
Cribriform$	1.5 ± 1.2	0.9 ± 1.1	0.9 ± 1.1

Data presented as mean % immuno-positive neurons ± SD. $ Value is the mean score ± SD.

* $p < .05$ compared to eSUDI as determined by LSD post-hoc one way ANOVA.

4.3. *Effects of cigarette smoke exposure on nAChRs*

Smoke exposure was associated with reduced α4 expression in the cribriform nucleus of our cohort. No additional changes were seen when analysing the SIDS cases alone. This isolated finding was unexpected given that other studies found more widespread changes, including our study in the mouse model of pre- into post-natal cigarette smoke exposure (Vivekanandarajah et al., 2016), studies of mRNA in the rat with similar exposure (Eppolito et al., 2010), and studies of receptor binding and expression (reviewed in Vivekanandarajah et al., 2019). Desensitization due to reduced numbers of receptors (Reitstetter et al., 1999; Wang and Sun, 2005) is thought to reduce or eliminate biological responses after prolonged or repetitive stimulation by an agonist. Acute nicotine injected in rats resulted in cholinergic receptor desensitization for up to 6 h (Hulihan-Giblin et al., 1990a; Sharp and Beyer, 1986) while chronic nicotine exposure resulted in a loss of receptor function for up to 8 days (Hulihan-Giblin et al., 1990b). Several questions remain unanswered, including whether different receptor subtypes have different time sequences for desensitization, or the α4 containing nAChRs lose function. Study results conflict in this regard since one study found increased functional activation of α4β2 nAChRs after long-term exposure to nicotine (Buisson and Bertrand, 2001), while another found marked variance in the reduction of ACh-mediated responses; 90% reduction in α4β2 nAChRs for > 24 h compared to 10% reduction in α3β2β4α5 nAChRs (Olale et al., 1997).

There is limited information regarding the role of the cribriform nucleus, although it is known to have high AChE, which is important in hydrolyzing ACh, and presumably inhibits post-synaptic signal transmission. Decreased α4 expression along the fibre tracts (presumably axons and dendrites), suggests that the presence of exogenous nicotine disrupts interplay between the α4 subunit and ACh formation. Indeed, nicotine has been shown to directly decrease AChE levels (Chang et al., 1973) in a dose-dependent manner (Ijomone and Nwoha, 2015).

Table 3
Analysis of α3 and α4 nAChR subunits according to history of cigarette smoke exposure and upper respiratory tract infection amongst the *whole cohort*.

	Cigarette smoke exposed		p-value	Upper Respiratory Tract Infection		
	No (*n* = 23)	Yes (*n* = 13)		No (*n* = 24)	Yes (*n* = 17)	p-value
	α3 nAChR subunit			α3 nAChR subunit		
XII	56.3 ± 19.5	64.9 ± 19.4	0.23	57.5 ± 20.2	56.7 ± 19.3	0.90
DMNV	52.0 ± 19.9	47.9 ± 13.5	0.53	48.0 ± 20.0	48.3 ± 15.8	0.96
NTS	15.6 ± 17.9	12.2 ± 13.3	0.56	15.2 ± 13.6	12.5 ± 18.7	0.60
Vest	28.8 ± 16.7	35.0 ± 20.9	0.39	27.4 ± 18.3	36.7 ± 16.5	0.14
Cun	38.1 ± 20.0	36.9 ± 18.2	0.86	34.7 ± 18.2	40.2 ± 18.1	0.36
NSTT	18.0 ± 16.1	14.2 ± 12.2	0.49	12.4 ± 15.5	18.7 ± 11.6	0.19
ION	48.0 ± 20.9	52.2 ± 14.1	0.54	46.8 ± 18.9	53.4 ± 17.4	0.27
AN	48.0 ± 20.9	46.4 ± 13.0	0.79	48.7 ± 15.0	45.8 ± 13.3	0.59
	α4 nAChR subunit			α4 nAChR subunit		
XII	55.1 ± 23.1	59.1 ± 21.1	0.61	47.9 ± 20.4	62.9 ± 22.2	***0.03***
DMNV	39.9 ± 8.3	38.6 ± 17.3	0.84	36.9 ± 17.3	40.5 ± 15.7	0.52
NTS	7.5 ± 6.9	4.4 ± 3.8	0.16	9.3 ± 15.7	6.3 ± 5.4	0.48
Vest	33.6 ± 15.9	37.4 ± 10.2	0.47	35.6 ± 18.3	34.5 ± 13.4	0.56
Cun	36.7 ± 17.9	30.7 ± 15.2	0.34	29.9 ± 15.7	38.6 ± 17.0	0.11
NSTT	11.8 ± 13.8	10.0 ± 7.4	0.69	9.7 ± 12.2	11.23 ± 9.3	0.68
ION	54.1 ± 15.9	56.4 ± 10.7	0.66	51.0 ± 15.8	57.0 ± 9.9	0.18
AN	42.4 ± 17.1	49.7 ± 11.9	0.26	45.8 ± 16.4	44.4 ± 13.4	0.79
Cribriform$^{\$}$	1.3 ± 1.2	0.6 ± 0.7	***0.04***	1.0 ± 1.1	0.9 ± 1.1	0.78

Data presented as mean % immuno-positive neurons ± SD. $^{\$}$ Value is the mean score ± SD. Bold italics indicate significant p value.

4.4. *Risk factors of sex and bed sharing*

Male gender and bed sharing were associated with increases in the α3 nAChR subunit in the DMNV and NTS, respectively, amongst the SIDS cohort. Male infants are known to be more susceptible to respiratory related pathophysiologies including SIDS (Mage and Donner, 2014), and the rostral DMNV is involved in parasympathetic control of the lungs (Getz and Sirnes, 1949; Katz and Karten, 1985; Bejjani et al., 2013). Moreover, α3-containing nAChRs play important roles in oxygen signalling in carotid bodies which are intricately linked to the DMNV (Finley and Katz, 1992). The NTS, with increased α3 linked to bed-sharing, may further contribute to respiratory vulnerability of these infants.

5. Conclusion

In conclusion, the only finding in this study that was isolated to SIDS cases, was of increased α4 in the NSTT of SIDS I infants, and in the XII of SIDS II cases. Cigarette smoke exposure had a minor effect on α4 subunit expression in the cribriform nucleus (both amongst the whole cohort and in SIDS alone). The presence of an URTI was associated with increased α4 (independent of diagnostic group).

Changes in the α3 subunit affected respiratory-related nuclei (DMNV and NTS) and were higher in male infants and in those with the SIDS risk of bed sharing. Together these findings add to evidence for abnormalities in the nAChR in the brainstem of infants dying from SIDS, and show independent effects of sleep-related risk factors and the presence of an URTI. We suggest that this points to an underlying common stimulus (hypoxia) that could contribute via abnormalities in production and regulation of acetylcholine.

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