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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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Highlights:

The α2, α3, α4, α5, α7, α9, β1 and β2 nAChR protein subunits are expressed in the developing mouse brainstem.

Pre- into post-natal cigarette smoke exposure affected α3 and β1 expression in more regions than any other subunits.

Nuclei predominantly affected by smoke exposure were the XII, DMNV and NTS.

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 ≤1.2mg, CO ≤15mg) 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.

Keywords: Caspase 3, medulla, nAChR, nicotine, smoking chamber, Sudden Infant Death Syndrome (SIDS), TUNEL

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

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 (Di Franza 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 1992). 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., 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 towards 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 et al, 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 $\leq$ 1.2 mg, CO $\leq$ 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 x 27 x 20 cm) at room temperature, and each cigarette was delivered manually for 15 minutes, with a 5-minute interval between the two cigarettes. The SHAM mice were placed in a separate identical Perspex chamber to avoid any contamination and air exposured 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.

2.3.2. Double immunohistochemistry for Casp-3 and TUNEL colocalisation

TUNEL labelling was performed first using a commercial kit (S7100, ApopTag Peroxidase Situ; Apoptosis Detection Kit; LOT: 2528704A, Oncor) following the manufacturers' instructions and then Casp-3 labelling. Following cooling with distilled water after antigen retrieval, endogenous hydrogen peroxidase was quenched (50% PBS, 50% methanol and 3% H_2O_2) for 30 min at room temperature. Sections were then incubated with equilibration buffer

(30s) and then with terminal deoxynucleotidyl transferase (TdT) enzyme for 60 min at 37 °C. Reaction was stopped by placing sections in stop/wash solution for 10 min, washed in PBS, and then incubated with antidigoxigenin–peroxidise for 40 min. After PBS wash, colour was developed with diaminobenzidine (DAB). Sections were then thoroughly washed in PBS, incubated with 10% normal horse serum (NHS) in PBS for 30 min and then in Casp-3 (1:200 dilution in 1% NHS) overnight. After PBS washing, sections were incubated in biotinylated anti-rabbit made in horse (BA-1100 Vector Laboratories Inc.) 1:200 dilution in 1% NHS, for 45 min, washed in PBS and incubated in avidin–biotin alkaline phosphatase (AK 5000, Vectastain ABC kit; Vector Labs.;) in PBS for 45 min. Sections were then rinsed in Tris buffered saline (TBS) pH 7.5, and colour developed by reaction with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (K0598, Dako BCIP/NBT Substrate System; LOT 10096050) in a dark environment for 45 min. After washing in TBS and H_2O, sections were taken quickly through a graded series of alcohol, cleared in histoclear and mounted in vectamount. Negative controls included sections where the TdT enzyme was omitted and/or the primary antibody was replaced with 1% NHS.

2.4. Quantitative Analysis

Seven nuclei at the caudal medulla and the facial nucleus of the pons at the genu of the facial nerve were studied and identified with reference to the mouse brain atlas (Figure 95 and 79, respectively; Paxinos and Franklin 2004). The nuclei included hypoglossal nucleus (XII), dorsal motor nucleus of the vagus (DMNV), nucleus of the solitary tract (NTS), cuneate nucleus (Cun), nucleus of the spinal trigeminal tract (NSTT), lateral reticular nucleus (LRt), inferior olivary nucleus (ION) and the facial nucleus (FAC).

Quantitation was performed blinded to the study group as the slides and tissue sections were coded. Images of the regions were captured using a DCF400 (Leica Microsystems Ltd. Heerbrugg, Switzerland) mounted on Leica DM 6000 Nikon Upright at 10 X magnification. The nuclei of interest were captured entirely using Leica application suite software (LAS V3.8, Leica Microsystems Ltd. Herrbrugg, Switzerland). Neurons were counted using the cell counter function in ImageJ software (National Institutes of Health, USA). For the α2, α3, α4, α5, α7, α9, β1, β2 nAChR subunit that were labeled separately via immunohistochemistry,, neurons with brown colour somata were deemed positive, while neurons with lighter staining similar to the connective tissue staining colour, or blue due to the haematoxylin counter stain, were deemed negative. For Casp-3, neurons with blue colour somata were deemed positive,

while the neurons with brown labeled nucleus (often shrunken in size) were deemed positive for TUNEL. The number of positively and negatively stained neurons was counted manually and calculated to present as a percentage of positively stained neurons per nucleus (% positive).

2.5. Statistical Analysis

Mouse anthropometry and the immunohistochemistry data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) (IBM SPSS Statistics 21 for Windows. IBM Corp., USA) comparing offspring from the maternal SE group to the SHAM controls. A p-value <0.05 was considered significant.

3. Results

3.1. Mouse characteristics

Table 2 presents the mice characteristics. All mice studied were male. As common with offspring from smoking mothers (reviewed in Abbott and Winzer-Sehan, 2012), SE mice had decreased body ($p = 0.013$) and brain weight ($p= 0.003$). Serum cotinine was significantly higher in the pups with maternal cigarette smoke exposure ($p<0.001$; Table 2) with the level being within the range reported in human infants of smoking mothers (5 to 30ng/ml; Luck and Nau 1985). Some cotinine was detected in the SHAM group (2.52 ± 0.35ng/ml). This is unlikely due to contamination since the SHAM chamber was separate to the SE chamber and mice from each group were housed separately. Thus, the levels found in the SHAM offspring are contributed to the nature of background noise of the kit and are of non-significance based on human studies where values <5ng/ml are indicative of non-smoke exposure (Luck and Nau 1985, Benowitz et al., 2009).

3.2. nAChRs

Positive neuronal staining, as indicated by the brown cytoplasmic staining, was observed for all the subunits in all brainstem nuclei studied. Representative staining of these receptors in some of these nuclei is provided in Fig. 1. For all subunits, staining was predominant to the cytoplasm with the exception of α4 where some nuclear staining was evident in addition to predominant cytoplasmic staining in both motor and sensory nuclei in both SHAM and SE groups. Translocation of staining to other cellular compartments was not observed for any of the subunits studied in the SE pups.

Compared to controls, significantly increased expression of all the subunits was found in various nuclei (Fig. 1 and Fig 2). Specifically, increased expression for α2 in the FAC ($p<0.001$), for α3 in the XII ($p<0.001$), DMNV ($p=0.042$), NTS ($p=0.011$), Cun ($p<0.001$), NSTT ($p=0.019$) and FAC ($p<0.001$), for α4 in the XII ($p<0.001$), for α5 in the XII ($p<0.001$) and DMNV ($p<0.001$), for α7 in the XII ($p=0.025$), DMNV ($p<0.001$), NSTT ($p=0.029$) and FAC ($p=0.005$), for α9 in the XII ($p<0.001$), for β1 in the DMNV ($p=0.016$), NSTT ($p=0.001$) and ION ($p=0.013$) and for β2 in the Cun ($p=0.02$). Significantly decreased expression in the NTS for α4 ($p<0.001$), α5 ($p<0.001$), β1 ($p=0.01$), in the LRT for β1 ($p=0.001$) and FAC for α7 ($p=0.005$).

3.3. Apoptosis

Positive neuronal staining as indicated by blue cytoplasmic staining for Casp-3 and brown labeled nucleus that shrunk in size for TUNEL was observed (Fig 3). Colocalisation was not always evident with some neurons being positive for only Casp-3 (black arrow; Fig 3) or TUNEL (black arrow head; Fig 3), while other neurons were either negative (white arrow head; Fig 3) or positive for both (white arrow; Fig 3).

Compared to controls, Casp-3 was significantly increased in the XII ($p<0.001$), DMNV ($p<0.001$) and Cun ($p=0.007$) but significantly decreased in the ION ($p=0.004$) (Table 3). For TUNEL labelling, a significantly increased expression was seen for the XII ($p<0.001$), while a decreased expression was seen for the ION ($p=0.003$) (Table 3). For colocalisation of both Casp-3 and TUNEL, a significantly decreased expression was seen in the NTS ($p<0.001$) and ION ($p=0.017$) (Table 3)

4. Discussion

4.1. Distribution of the receptors and apoptotic expression in the normal developing mouse brainstem

The protein expression of the nicotinic receptors has been studied extensively in brain regions of various species with the use of immunohistochemistry, ligand binding and autoradiography studies (reviewed in Vivekanandarajah et al., 2015 Supplementary Tables 1 & 2).

Normal protein expression of α2, α3, α4, α5, α7, α9, β1 and β2 nAChR subunits was observed in all of the nuclei studied, which is consistent with our and many other previous studies (Vivekanandarajah et al, 2015; Browne et al, 2010; Machaalani et al, 2010; Centeno et al, 2010; Perry et al, 2002; Marks et al, 1992). Although, we were the first to report α9 and β1 in the piglet brain (Vivekanandarajah et al, 2015), this study has further confirmed its presence in the brainstem nuclei of the mouse species. Furthermore, the widespread expression of these nAChRs in various brainstem nuclei confirms its role in a number of physiological functions including the cardiorespiratory, sleep and arousal control system.

Amongst the nuclei studied, extremes of low and high expression were seen in the NSTT and the NTS. For the NSTT, a high expression of α2, α5, β1 nAChRs and TUNEL and a low expression of α3, α4, α7, α9, β2 nAChRs and Casp-3 were seen. This is in agreement with previous studies where a low level expression for: α7 and β2 was reported in the human infant NSTT (Machaalani et al, 2011), α4 and α7 in adult rabbit NSTT (Centeno et al, 2004) and α4β2 and α3β2 subtypes in adult rat NSTT (Perry et al, 2002). However, the present study is in disagreement with our previous study in that a high level of α7 and a moderate level of β2 expression were observed in the NSTT of developing piglets (Browne et al, 2010). This difference may be attributed solely to species variation and suggests that the NSTT of the piglet may utilize α7 nAChRs more for physiological functions as compared to the other species. Given the role of NSTT in sensing pain, touch and temperature in the ipsilateral face (Woolf., 1993) and since α9α10 nAChRs are known to regulate the pain sensory pathway (McIntosh et al, 2009) proposed to work in conjunction with β1 (Scheffer et al, 2007), it is likely that NSTT regulates the pain sensory pathway in the postnatal mouse via the cholinergic modulation of β1 nAChRs.

For the NTS, a high expression of α7, α9, β1, α3, α4 nAChRs and Casp-3 and a low expression of α2, α5 and TUNEL were seen. This is in agreement with previous studies where a high expression of α7 and α4 was seen in the adult rabbit NTS (Centeno et al, 2004), adult rat NTS (Tribollet et al, 2004) and α4β2 and α3β4 in adult rat NTS (Perry et al, 2002). However, our previous results contradict the present findings where a low expression of α7 was seen in the developing piglet NTS (Browne et al, 2010) and human infant NTS (Machaalani et al, 2011). Given the same experimental technique was employed to study α7 expression in all three studies, it is likely that the difference is attributed to species variation. Given the role of the NTS in controlling blood concentrations of O_2, CO_2, and H^+ via its afferent inputs from the arterial baroreceptors and chemoreceptors from the carotid body and aortic arch and since the α3 subtype mediates the normal functioning of the autonomic nervous system, it is possible that the α3 containing nAChRs may mediate these effects in this region.

Regarding the apoptotic markers, the finding that in our mice highest expression of TUNEL was in the NSTT and lowest in the NTS is in keeping with that seen in the human infant (Stecco et al., 2005); while lowest Casp-3 was in the NSTT but highest in the NTS is also similar to that seen in human infants (Machaalani et al., 2007). This suggests that in the NTS of these mice, early apoptotic mechanisms predominate; while in the NSTT, it is the later stage of apoptosis. Regardless, the findings indicate ongoing neurogenesis/pruning which is common at this age (Nikolaev et al., 2009).

4.2. Effects of pre- into post-natal SE on the expression of the nAChR subunits

Several studies have looked at maternal cigarette or nicotine exposure on nAChR expression in the brainstem (Duncan et al., 2009; Slotkin et al., 2015; Slotkin et al., 2002; Slotkin, 1999; Lv et al., 2008; Hellstrom-Lindahl et al., 1998) although none studied all markers simultaneously nor did they study apoptotic expression. Thus our findings are novel in this respect and as summarized in Table 4, the main findings are that changes for α3 and β1 are more widespread (affect more nuclei) than for the other subunits, and for the nuclei affected, it was the XII, DMNV and NTS that showed changes for a greater number of subunits with apoptotic expression similarly affected.

The α3 subunit is important for early postnatal survival and in mediating the effects of fast

synaptic transmission in the autonomic nervous system (Xu et al., 1999). It commonly co-assembles with α5, β2, β4 subunits to form functional receptors. The autonomic nervous system predominates with a high expression of α3/β4 whereas an abundant expression of α4/β2 is found in the Central Nervous System. Thus, an increase in α3 would suggest that it affects the fast synaptic transmission of the autonomic control of some peripheral organs with obvious phenotypic effects on survival, intracardiac ganglia, pupillary contraction and bladder contraction (Xu et al., 1999).

The β1 subunit is widely expressed in the neuromuscular junctions of muscles and co assembles with α, δ, γ, ε subunits to form functional muscle nAChRs. Its presence has been reported in the upper spinous and granular layers of the skin (Kurzen et al., 2004), cochlea (Scheffer et al., 2007) and placenta (Machaalani et al., 2014). Although its expression in the brain is unexpected, we recently found β1 protein expression in the developing piglet hippocampus and brainstem (Vivekanandarajah et al., 2015). Low level of β1 RNA has also been reported in the human and mouse brain (Su et al., 2002). Since β1 is a muscle type nAChR subunit and since its expression predominates in the muscles, its primary role is inducing excitatory synapses on muscles leading to contraction (reviewed in Albuquerque et al., 2009). Recently, it has also been discovered that novel motifs in the muscle β1/δ subunit cytoplasmic loop mediate Golgi retention signals that regulate surface trafficking of assembled nAChRs thereby helping to prevent surface expression of unassembled nAChR subunits (Rudell et al., 2014). The effects of nicotine exposure on β1 expression are inconsistent. Decreased β1 RNA expression occurs in adult rat cardiomyocytes after chronic nicotine treatment from 5 weeks to 3 months of age (Hu et al., 2002) and β1 protein expression in the developing piglet XII, DMNV and NTS after chronic nicotine treatment from P1-P13 (Vivekanandarajah et al, 2015). However, Ke et al., (1998) reported an increase in β1 nAChRs in TE671 human medullablastoma cell cultures as a result of chronic nicotine exposure. In our mice, β1 expression was increased in the DMNV, NSTT and ION but decreased in the NTS and LRt. Thus it seems the difference in direction of change is sensitive to time of nicotine exposure, with prenatal exposure tending to result in increased expression while postnatal exposure leads to decreased expression.

Our finding that it was the XII, DMNV and NTS that showed changes for a greater number of subunits suggests that these regions are highly sensitive to the effects of nicotine. The XII

controls upper airway patency through an appropriate coordination of a number of events. When XII motoneurons are excited synaptically, they activate the tongue muscle to maintain pharyngeal airway patency, thereby allowing the diaphragm to draw air into the lungs (Remmers et al, 1978; Fregosi and Fuller, 1997). Recently, studies have reported that pre-into post-natal SE, modifies XII motorneuron excitability, intrinsic membrane properties, excitatory synaptic transmission (Pilarski et al., 2011; Jaiswal et al., 2013) and chemoreception (Lei et al., 2015). Moreover, SE modifies spike-timing precision, reliability and spike frequency adaption in response to sinusoidal current injections in the XII from neonatal rat brainstem slices (Powell et al., 2015). This has significant effects on the input-output properties of the XII motoneurons and it was proposed that this may play a role in the altered suckling strength and coordination in nicotine exposed newborn infants (Powell et al., 2015). Our findings would suggest this to be via the α3, α4, α7, and α9 subunits. Physiologically, this would mean infants from smoking mothers would have poor suckling responses which indeed have been reported in human infants (Alm et al., 1998). In relation to SIDS, the use of dummies and therefore suckling is considered protective (Mitchell et al., 1993). Suckling increases the tension of the upper airway muscles, hence keeping the tongue positioned more forward and thereby maintaining the airway free (Alm et al., 2006). Thus changes in the various nAChR subunits in the XII due to SE would be a mechanism for poor suckling response.

The DMNV regulates visceromotor functions by innervating the mucosa of the larynx, pharynx, smooth muscles and glands of the thoracoabdominal viscera through the vagus nerve. At the caudal level, the DMNV innervates the oesophagus, trachea and the heart thus being involved in autonomic cardiac control (Huang et al, 1993). For example, stimulating the DMNV in the pigeon (Cohen and Schnall 1970) and rabbit (Ellenberger et al, 1983) results in bradycardia. A hypoxic challenge to rats exposed to prenatal nicotine, induced excitatory neurotransmission to cardiac vagal neurons in the brainstem that lead to fatal bradycardia (Huang et al., 2005) and suppressed the sinoatrial response of tachycardia which eventually lead to a rapid heart decline (Slotkin et al., 1997a). Similarly, infants of mothers who smoked during pregnancy, had impaired autonomic cardiac control (Sovik et al., 2001; Thirez et al., 2009) and a decline in heart rate (HR) during hypoxic conditions (Sovik et al., 2001). Thus, alterations in the HR response in the SE infants may be mediated via the changes in the nAChR subunits expressed in this region.

The NTS is a major sensory nucleus that receives cardiovascular, respiratory, and gustatory information. The rostral NTS is associated with gustatory processes while the caudal NTS regulates the cardiovascular and respiratory systems. Additionally, NTS is an essential component of the central pathway mediating homeostatic cardiovascular reflex that regulates blood pressure and fluid concentrations of CO_2 and H^+ as mentioned earlier (reviewed in Dampney 1994). Recently, it was reported that prenatal SE directly diminishes central chemoreception in the neonatal rat (Lei et al., 2015). Furthermore, it has been reported that infants born to mothers who smoked during pregnancy, have diminished ventilatory response to hypoxia in both humans (Ueda et al., 1999) and rats (Neff et al., 2004). Thus a change in α3, α4, α5 and β1 expression in the NTS may disturb the homeostatic processes that enable the central chemoreceptors that stimulate the respiratory centre to increase ventilation when CO_2 or H^+ levels rise.

Our results for the other subunits are in accordance with previous studies where an increase in α4, α7 and β2 expression was found following perinatal nicotine treatment in rat fetal forebrain and hindbrain (Lv et al, 2008), human fetal brainstem medulla (Falk et al, 2002; Falk et al, 2005), fetal baboon brain (Duncan et al, 2009), adolescent (P30) rat brainstem (Slotkin et al, 2015) and rhesus monkey infant brainstem (Slotkin et al, 2002): the latter study utilizing a smoking chamber similar to the current study. Regarding the α7 subunit, it is interesting that after pre- into post-natal SE, it is increased but following postnatal nicotine exposure alone, it is decreased (Browne et al., 2010). A similar discordance was evident for α5 where it was increased after pre- into post-natal SE in the DMNV and NTS but not changed following postnatal exposure (Vivekanandarajah et al, 2015). Thus it seems that exposure regimes induce differing mechanisms that could have implications from an apoptotic perspective (see further discussion section). Indeed, cigarette exposure vs nicotine exposure induce differing effects on the cholinergic system (Slotkin et al, 2015), thus it is not unexpected pre- vs post-natal differences would have additional varying mechanisms.

4.3. Effects of pre- into post-natal SE on apoptotic expression

Increased apoptosis in various regions of the brain after cigarette or nicotine exposure is a common finding (reviewed Zeidler et al., 2007). However, only two groups (Simakajornboon et al., 2010; Slotkin et al 1997b; Slotkin et al 2005) have looked at the effects of prenatal exposure on pup brain apoptotic markers. Simakajornboon and colleagues (2010) examined

the effect of prenatal nicotine exposure on PDGF-β receptor activation and its subsequent activation of the anti-apoptotic cascade in the caudal brainstem of 5 day old rat pups. However, they measured cleaved caspase-3 via western blot, thus not allowing for specific nuclei determination. The latter group measured c-fos mRNA in fetal and P2, P8 old rats following fetal and postnatal nicotine exposure in the brainstem and forebrain (Slotkin et al., 1997b) and measured cell loss by reduced DNA and cell loss in fetal rhesus monkey cortex, caudate, hippocampus, and brainstem following gestational (G) nicotine exposure from G30-G160 (Slotkin et al., 2005). Thus, our study is the first to report pre-into post-natal SE effects on active caspase-3 and TUNEL expression in the young offspring brainstem by individual nuclei. We found increased Casp-3 in the XII, DMNV and Cun of SE pups and an increased TUNEL in the XII. This is in agreement with our findings in postnatal- nicotine exposed piglets where greater expression for Casp-3 in the XII and DMNV was observed (Machaalani et al, 2005). Comparing our findings to the only human study that looked at a cohort of infants with reported SE, the DMNV (although at the rostral level) had increased TUNEL (Machaalani et al, 2008). Thus it seems that for the DMNV, both pre- and post-natal exposures can increase apoptosis.

The XII was the only nucleus to show an increase in both Casp-3 and TUNEL. The fact that DMNV and Cun only showed an increase for Casp-3, suggests that despite recruitment of upstream Casp-3 and its preceding apoptotic pathways, the neurons are not progressing through to DNA fragmentation and subsequent cell death. Our finding of a lack of increase in co-localisation (indicative of the same cell undergoing Casp-3 cell death) is not unexpected since this indicates the time of injury promoting cell death. Since the cell death process is transient, i.e., neuronal populations are affected at different stages due to the insult, the above result indicates that the neurons are undergoing early stages of the apoptotic pathway following SE. In fact, these two nuclei did not show any evidence of increased TUNEL expression further validating the fact that neurons in the early stage of the cell death pathway are detected. Hence, an increase in both Casp-3 and TUNEL in the XII means that the neurons are committed to dying and have passed the point of Casp-3 activation.

Within the ION, we found a significantly decreased expression of Casp-3, TUNEL and Casp-3/TUNEL colocalisation in the ION. This was unexpected and seems to indicate an opposite mechanism which in itself is also detrimental. During development into infancy, there is a

normal rate of apoptosis occurring throughout the brain with growth and differentiation (pruning), as part of the ongoing neurogenesis, and a role of the caspases has been identified in this process (Nikolaev et al., 2009). Our finding that after pre- into post- SE there is a decrease in apoptosis in the ION suggests that there is a disruption in the 'normal' apoptotic pathway required to prune the brain from unwanted neurons and as a result affect the normal functioning of this nucleus. The functions of ION involve motor control, motor learning, movement coordination, balance and sensory processing via its connections to the cerebellum (Bengtsson and Hesslow., 2006; Linas et al., 1975; Rondi-Reig et al., 1997). Hence, a disruption in the normal functioning of the ION, may allude to the fact that infants exposed to maternal SE have a reduced motor ability, which is indeed seen amongst SE infants in subsequent childhood; disrupted balance at 5 years of age (Trasti et al., 1999) and reduced motor competence particularly on the non-dominant side at 11 years of age (Larsson and Montgomery, 2011).

4.4. Proposed mechanisms of the role of nAChR subunit on the expression of apoptotic markers

One hypothesised mechanism by which SE affects apoptosis is via the activation of the pro-apoptotic/anti-apoptotic nAChR subunits: α7 (pro apoptotic in undifferentiated hippocampal progenitor cells; anti-apoptotic in a gain of function model) and α3/α4 (anti-apoptotic causing activation of the Akt pathway that leads to lung tumorigenesis) (West et al., 2003; Berger et al., 1998). In this study, by simultaneously studying the expression of the nAChR subunits with apoptosis, we are able to determine whether any associations are evident between increased apoptosis and change in the subunits.

Previous studies have shown that prenatal nicotine induced apoptosis and cell loss are mediated via nicotinic receptor concentrations (Slotkin et al, 1987) and *in vivo* studies have shown that at developmental stages before the emergence of nicotinic receptors, there is no sign of apoptosis or cell loss as a result of nicotine exposure (Slotkin et al, 1993, 1997b). Nicotinic receptors emerge at gestational age 12 (Naeff et al., 1992) in rats and the persistent effect of nicotine on c-fos expression was absent when nicotine treatment was given prior to this period (Slotkin et al., 1997b).

Our results are interesting in that we see an increase in the anti-apoptotic nAChR subunits

(α3, α4, α7) in nuclei that correspond to an increase in Casp-3 (XII, DMNV, NTS) and TUNEL (XII). It has been reported that activation of Akt through α3/ α4 heterodimers or α7 containing nAChRs by nicotine, increased the normal airway cell survival in cell cultures under condition where cell death is the normal physiological response, hence promoting lung tumorigenesis (West 2003). Furthermore, it has been reported previously that mice generated to express the analogous mutation (L250T) of the α7 nAChR, died within 2-24 hours of birth. Examination of the mouse pup brains revealed a decreased in α7 nAChR protein expression and an immense apoptotic cell death throughout the somatosensory cortex (Orr-Urtreger et al., 2000). Therefore, an increase in the α3, α4 and α7 subunits can be interpreted as a compensatory mechanism against the apoptosis induced by maternal SE. Although, there is evidence for α7 to serve as a pro-apoptotic subunit, this has only been demonstrated in undifferentiated hippocampal progenitor cells but not in differentiated cells using cell culture models after nicotine exposure (Berger et al., 1998). This, together with previous data, provide evidence that α7 regulates developmental programmed cell death in the motor neurons as seen in XII and DMNV (Renshaw et al, 1993) possibly by compensating against cell death, which proceeds to eventually become desensitised.

In the NTS, a decrease in the colocalisation of Casp-3 and TUNEL was unexpected. Although this may be linked to many of the nAChR subunit changes in this nucleus, we suggest it is mediated via the β1 subtype, since this is the only subunit that corresponds to the change in the ION (detailed in the next paragraph). A decrease in the Casp-3/TUNEL colocalisation means that the normal neuronal pruning during the developmental stage is affected. Given the role of NTS in controlling blood concentration and fluid pressure, a disruption in the ongoing neurogenesis in this nucleus may result in altered blood pressure mechanisms after birth. Indeed, it has been reported that infants born to smoking mothers have altered blood pressure responses to tilting at 2-3 days and 3 months of age (Browne et al., 2000).

Given that the β1 nAChR subtype was the only one affected in the ION, this would suggest its role in the decreased apoptosis in this nucleus. Recently, it has been reported that δ nAChR subtype (which is a muscle type belonging to the α1, β1, δ, γ nAChRs) is significantly increased in the placenta from smokers and has been proposed to lead to cell death and degradation via increasing Ca^{2+} permeability through ion channels (Machaalani et

al, 2014). Furthermore, Ke and colleagues reported that chronic nicotine increases α1, β1, δ, γ subunit containing muscle type nAChRs and that nicotine treatment induces two phases of functional loss for the muscle type nAChRs despite the increase in receptor number. This functional loss is via a ‘‘persistent inactivation,’’ that is different from the classical desensitisation (Ke et al, 1998). Hence, it can be postulated that the mechanism behind the action in the ION to increase β1 expression is in compensation for the decreased apoptosis in this region.

5. Conclusion

The results of this study show for the first time that the α2, α3, α4, α5, α7, α9, β1, and β2 nAChR subunits are expressed in the developing mouse brainstem with extremes of low and high expression seen in the NSTT and NTS, which also exhibited extremes in levels of apoptotic markers. In pups from SE dams, changes in α3 and β1 were evident in more regions than any other subunits. The smoke exposure effects were more pronounced in the XII, DMNV and NTS nuclei. When considering the nAChR changes in conjunction with apoptotic changes, the results suggest the increase in α7, β1 and β2 subunit expression is a compensatory mechanism to the change in apoptotic expression as a result of cigarette exposure.

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Conflicts of interests

The authors have no conflicts of interest.

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Figure Legends

Fig 1. Micrographs illustrating nAChR staining comparing SE to SHAM in the FAC for α2 (A and B), DMNV for α3 (C and D), XII for α4 (E and F), XII for α5 (G and H), NSTT for α7 (I and J), XII for α9 (K and L), DMNV for β1 (M and N) and Cun for β2 (O and P). Scale bar = 100 μm for A and B; 50 μm for C-P.

Fig. 2: Comparison of nAChR subunit expression between SHAM (white bars) and SE (black bars) mice in 7 nuclei of the caudal medulla and the FAC of the pons. (A) α2; (B) α3; (C) α4; (D) α5; (E) α7; (F) α9; (G) β1; (F) β2. Results presented as mean ± SEM of percent positive neurons. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Fig 3: Double immunostaining for Casp-3 and TUNEL in the Cuneate nucleus of a SHAM (A) compared to SE (B) mouse. Casp-3 positive only neuron (black arrow), TUNEL positive only neuron (black arrow head), TUNEL positive in a Casp- 3 positive neuron (white arrow) and TUNEL negative in a Casp-3 negative neuron (white arrow head). Scale bar = 20 μm.

Tables

Table 1: Primary antibodies used for immunohistochemistry

Antibody	**Host**	**Antibody concentration**	**Company & cat #**	**Specificity determined by**
nAChR α2	Rabbit polyclonal	1:400	Santa Cruz; sc-5589	Di Angelantonio et al. (2003)
nAChR α3	Rabbit polyclonal	1:200	Santa Cruz; sc-5590	Liu et al. (2011)
nAChR α4	Goat polyclonal	1:700	Santa Cruz; sc-1772	Govind et al. (2012), Whiteaker et al. (2006),
nAChR α5	Goat polyclonal	1:175	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	1:300	Abcam, ab 10096	Paulo et al. (2009), Mielke and Mealing (2009)
nAChR α9	Goat polyclonal	1:40	Santa Cruz (sc-13804)	Santa Cruz and in house verification provided in Vivekanandarajah et al. (2015)
nAChR β1	Rabbit polyclonal	1:400	Santa Cruz; sc-11371	Santa Cruz
nAChR β2	Rabbit polyclonal	1:300	Santa Cruz; sc-11372	Quitadamo et al. (2005), Pollock et al. (2007), Kabbani and Levenson (2007)
Active Caspase-3	Rabbit polyclonal	1:200	BD Biosciences 559565	BD Biosciences

Table 2. Characteristic data for the control (SHAM) and cigarette smoke exposed (SE) mouse groups.

Offspring	SHAM	SE	P value
Number	5	5	
Body weight (g)	11.44 ± 0.28	9.96 ± 0.44	0.01
Brain weight (g)	0.29 ± 0.00	0.27 ± 0.00	<0.01
Brain to body weight ratio (%)	0.03 ± 0.00	0.03 ± 0.00	0.05
Serum cotinine (ng/mL)	2.52 ± 0.35	8.93 ± 0.87	<0.001

Results presented as mean ± S.E.M. n=5.

Table 3: Comparison of Casp-3, Casp-3/TUNEL colocalisation and TUNEL expression between SHAM and SE mice in 7 nuclei of the caudal medulla and the FAC of the pons.

	Casp-3		Casp-3/TUNEL		TUNEL	
	SHAM	SE	SHAM	SE	SHAM	SE
XII	30.2±1.1	38.5±0.2***	21.7±1.1	26.3±3.0	24.4±1.1	36.7±0.9***
DMNV	26.1±1.0	42.7±0.9***	22.3±0.7	19.6±1.4	33.1±1.1	32.2±2
NTS	31.1±0.8	30.8±0.5	27.4±0.7	18.2±0.6***	34.0±0.2	35.0±0.8
Cun	25.3±1.2	30.4±0.4**	23.6±0.8	23.3±0.2	37.5±1.2	35.7±0.6
NSTT	14.5±1.1	18.4±1.6	13.1±1.0	18.9±2.3	43.4±2.5	40.3±2.0
LRt	39.4±0.7	45.4±6.4	30.6±0.4	32.5±2.5	45.1±1.2	42.2±1.2
ION	32.6±0.8	25.2±1.0**	26.8±0.9	23.0±0.7*	36.8±0.7	29.2±1.1**
FAC	23.7±0.5	24.3±0.5	20.8±0.4	23.3±1.2	33.9±1.4	30.4±1.3

Results presented as mean ± SEM of percent positive neurons. *p<0.05, **p<0.01, ***p<0.001

Table 4: Summary of findings of SE effects on the nAChR subunits and apoptotic expression in the mice brainstem.

	nAchR subunit								Apoptosis		
	α2	α3	α4	α5	α7	α9	β1	β2	Casp-3	Casp-3/ TUNEL	TUNEL
XII		↑***	↑***		↑*	↑***			↑***		↑***
DMNV		↑*		↑***	↑***		↑*		↑***		
NTS		↑*	↓***	↑***			↓*			↓***	
CUN		↑***		↓***				↑*	↑**		
NSTT		↑*			↑*		↑*				
LRt							↓*				
ION							↑*		↓**	↓*	↓**
FAC	↑***	↑***			↑**						

Results presented as increased (↑) or decreased (↓) expression. *p<0.05; **p<0.01; ***p<0.001 in SE compared to SHAM.

Figure

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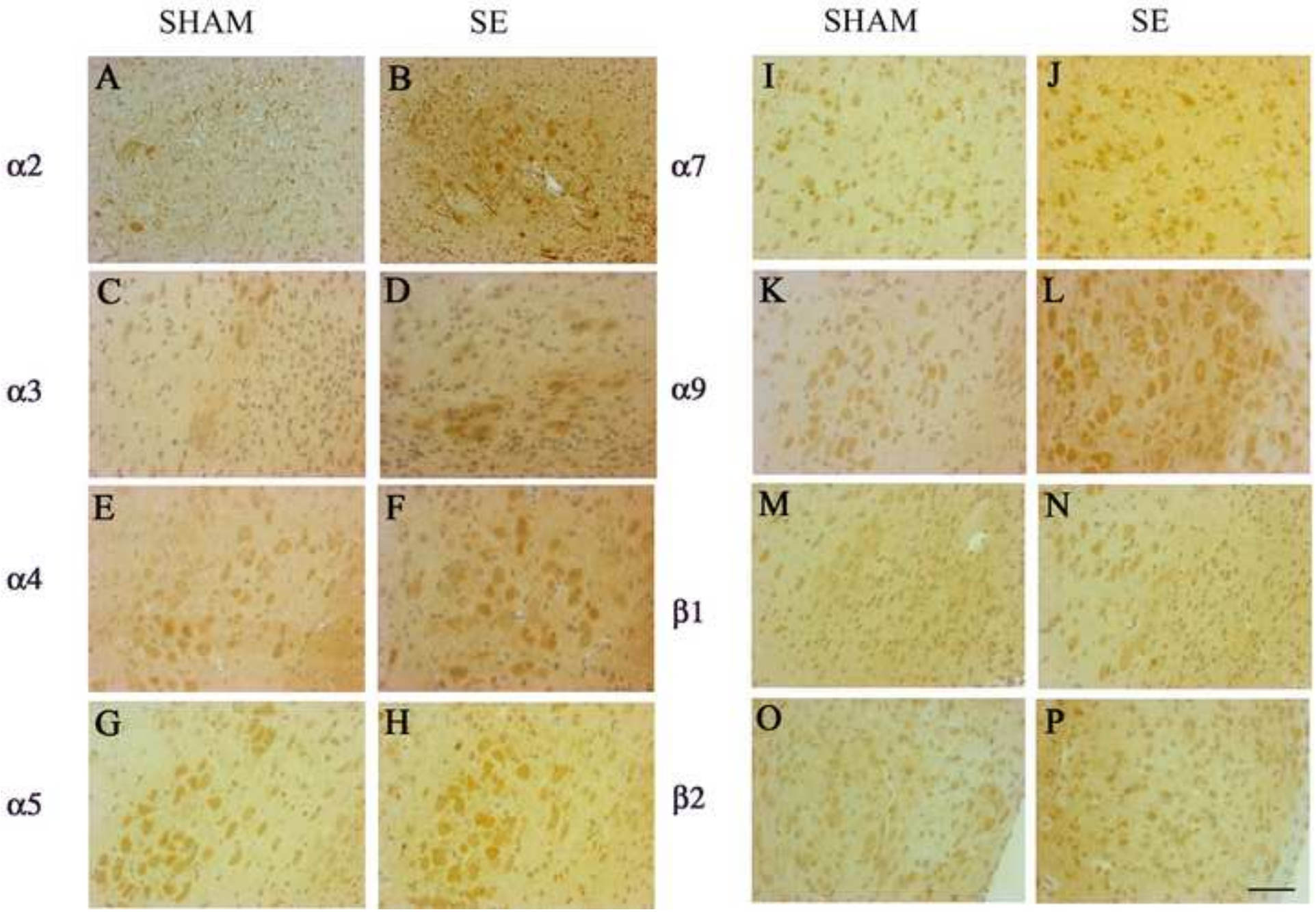

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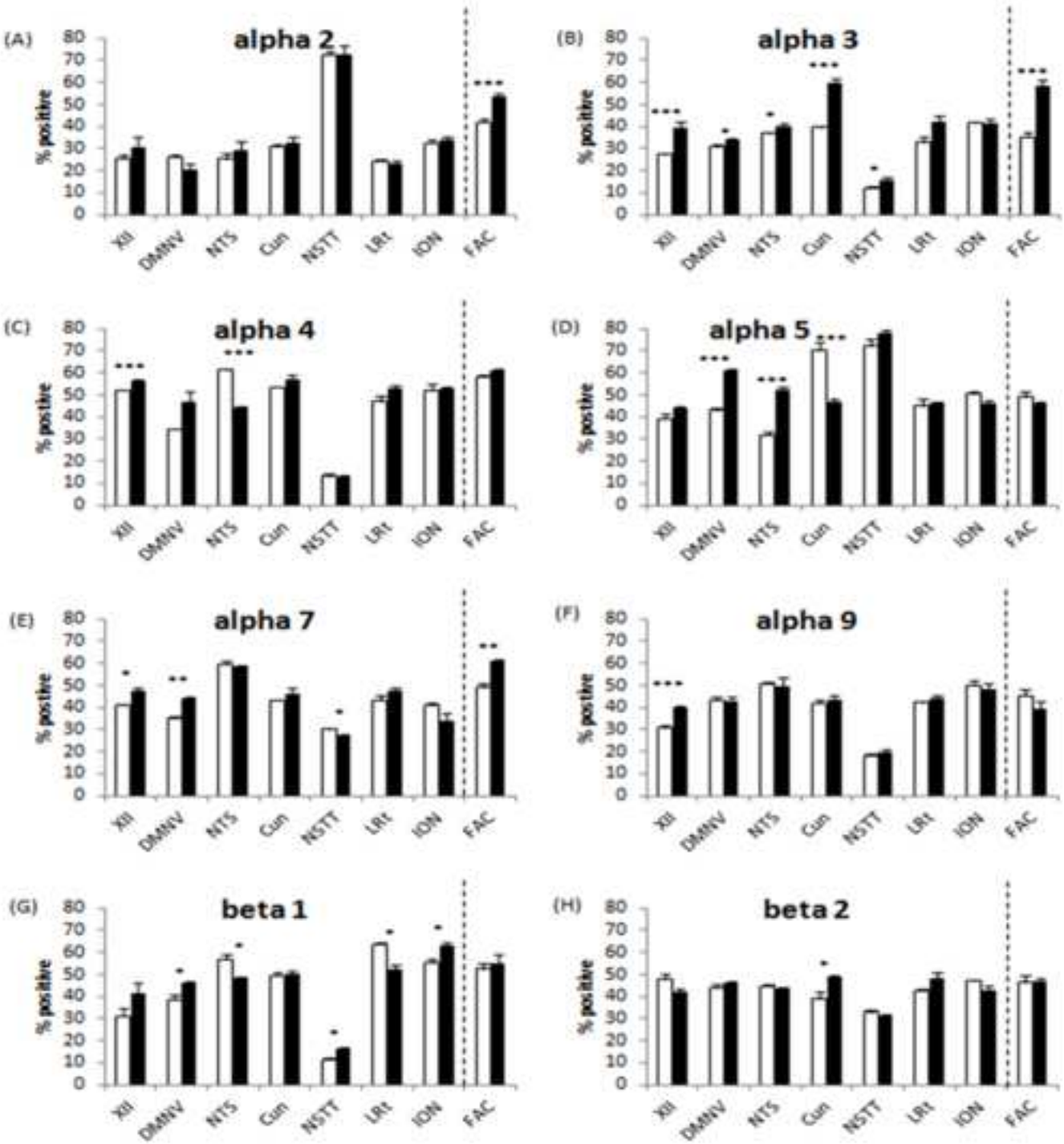

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