Neuronal cell death in the Sudden Infant Death Syndrome brainstem and associations with risk factors

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Immunoreactive expression of three cell death markers was quantitatively analysed in the human infant brainstem medulla. We assessed active caspase-3, TUNEL and single-stranded DNA (ssDNA) in a cohort of 92 infants, and analysed for: (i) variations in the immunoreactive expression with development; (ii) comparison of infants diagnosed with the Sudden Infant Death Syndrome (SIDS, n = 67) to infants who died suddenly with another diagnosis (non-SIDS, n = 25); and (iii) correlations with known clinical risk factors for SIDS. Five nuclei from the brainstem medulla (caudal and rostral levels) were studied, including the hypoglossal (XII), dorsal motor nucleus of the vagus (DMNV), the dorsal column nuclei (gracile and cuneate) and the arcuate nucleus. Our main hypothesis was that neuronal cell death would be increased in SIDS compared to non-SIDS infants, and the increase would correlate with risk factors such as prone sleeping and cigarette smoke exposure. Comparing SIDS to non-SIDS, there was an increase in caspase-3 in the rostral DMNV (P = 0.01), and a trend to increased TUNEL in the arcuate nucleus (P = 0.1), which was statistically significant when comparing the male SIDS to male non-SIDS cohort (P = 0.04). No major changes for ssDNA immunoreactivity were found. Moreover, TUNEL expression was affected by post-conceptional age, by sleep-related risk factors (predominantly affecting the dorsal column nuclei), and by cigarette smoke exposure in the rostral DMNV and arcuate nucleus. Active caspase-3 was affected by post-conceptual age but only in the XII, while gender-related differences were seen in the arcuate nucleus. This study provides further evidence of increased apoptosis in the brainstem of SIDS infants, but shows for the first time that these changes are also affected by age and gender, and by clinical risk factors such as the sleep position and cigarette smoke exposure.

Keywords: active caspase-3; apoptosis; cigarette smoking; developmental neuropathology; infant medulla

Abbreviations: AN = arcuate nucleus; DCN = dorsal column nuclei; DMNV = dorsal motor nucleus of the vagus; PCA = post-conceptional age; SIDS = Sudden Infant Death Syndrome; ssDNA = single-stranded DNA; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; URTI = upper respiratory tract infection; XII = hypoglossal nucleus.

Received June 26, 2007. Revised and Accepted October 29, 2007

Introduction

Sudden Infant Death Syndrome (SIDS) is the most common cause of post-neonatal deaths in developed countries. The diagnosis of SIDS is one of exclusion and the actual mechanism of death is still unknown, although risk factors have been identified (Mitchell et al., 1997). SIDS deaths commonly occur during a sleep period and a standing hypothesis is that SIDS is due to abnormal brainstem control of cardiac and/or respiratory function (Hunt, 1992). Based on this hypothesis, one line of SIDS research has been to identify brainstem pathology.

One pathology identified has been an increase in neuronal cell death markers in several nuclei of the SIDS brainstem medulla including: increased terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) expression, a marker of DNA fragmentation, predominantly in the dorsal sensory nuclei (Waters et al., 1999) and the nucleus of the solitary tract (NTS) (Biondo et al., 2004), an increase in Alz-50 (a marker of cell degeneration), in nuclei of the dorsal medulla (Sparks et al., 1996; Oehmichen et al., 1998), and recently, as identified by us, an increase in the apoptotic specific marker, active
caspase-3, in the hypoglossal (XII) and dorsal motor nucleus of the vagus (DMNV) (Machaalani et al., 2007a). Collectively, these studies provide compelling evidence that cell death (apoptosis) is increased in the SIDS infant medulla.

None of the previous investigations studied more than one apoptotic marker simultaneously in the same SIDS dataset, so the correlation between these markers is unknown. Furthermore, it is unknown whether increased apoptotic expression is a consequence of the pathophysiological mechanism (aetiology) of SIDS, or whether it is linked to known risk factors. The aims of this study were to investigate the correlation amongst three apoptotic markers including TUNEL, active caspase-3 and single-stranded DNA (ssDNA). In addition, we tested the hypothesis that increased apoptotic expression correlates with the presence of one or more common risk factors for SIDS including sleep position, exposure to cigarette smoke and bed sharing. We focused on five medullary nuclei including the XII, DMNV, gracile, cuneate and raphe nuclei (AN), selected on the basis of our previous findings that TUNEL was predominantly increased in the gracile and cuneate nuclei (Waters et al., 1999), while active caspase-3 was increased in the XII and DMNV (Machaalani et al., 2007a). The AN was included because of reported abnormalities in this nucleus in SIDS infants including decreased serotonergic receptor expression (Kinney et al., 2001; Paterson et al., 2006), and hypoplasia (Filiano and Kinney, 1992; Matturi et al., 2000).

Materials and Methods

Data collection

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 1997–2002. All cases were number coded and identifying information (name and address) was removed prior to analysis. In our state, a P534 NSW Police Death Scene Investigation checklist is completed for all sudden infant deaths when trained police arrive at the site of where the death occurred. Clinical and risk factor related information is collected from the parents by the attending police officer. On occasion, because of parental distress, this information is collected a few days after the incident. If it cannot be obtained any other way, information related to birth characteristics is completed by accessing hospital records. This study was conducted with the approval of The Human Ethics Committees of the University of Sydney, and Central Sydney Area Health Service.

To collate the information of relevance to our research, we developed and used a standard questionnaire to profile the infants’ perinatal and clinical history, information concerning common risk factors associated with SIDS such as sleep position and exposure to cigarette smoking and autopsy findings. Additional information relating to tissue handling was obtained because of their potential to impact on histochemical staining results.

Tissue acquisition and immunohistochemistry for apoptotic markers

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 were available. A total of 196 cases were collected over the period of study but only 92 cases were eligible for inclusion. Cases were excluded if they were known to have cause for neurological damage such as head injury or evidence of shaken impact syndrome (n = 7), if a diagnosis of SIDS was doubted [positional asphyxia, bed sharing (sleeping) accident or ‘undetermined/other’; n = 28], or if brain tissue was not available (n = 17). Moreover, only cases for which the brain was fixed in the same fixative, either 10 or 20% neutral-buffered formalin (NBF), were included in this study. This resulted in the exclusion of another 52 cases for which the brain was fixed in a solution of NBF and glacial acetic acid, because this fixation resulted in differential staining for caspase-3 (Machaalani et al., 2007b).

Residual tissue blocks were not always available for study at both levels of the medulla, and as a result, the number of tissue sections differed amongst cases and at different brainstem levels. Serial tissue sections (7µm) were collected from NBF-fixed and paraffin-embedded blocks, mounted on slides coated with 3-aminopropyltriethoxysilane, and then subjected to immunohistochemical staining.

Detailed methodology for TUNEL (Machaalani and Waters, 2003) and active caspase-3 (Machaalani et al., 2007a) staining have been reported by us previously. Staining was performed using a commercial kit for TUNEL (ApopTag kit, Chemicon, S7100), and peroxidase immunohistochemistry applied using a rabbit monoclonal against anti-active caspase-3 antibody (diluted 1:250, BD PharMingen, 559565) that recognizes the active 17 kDa subunit, and a monoclonal antibody against ssDNA (diluted 1:50, Chemicon, MAB3299). Microwave antigen retrieval was applied for all. The ssDNA protocol was applied according to the manufacturers’ instructions but with some modifications. In brief, sections were treated with 50% formamide (v/v distilled H2O) preheated in a water bath to 56–60°C, for 20 min, washed in ice-cold phosphate-buffered saline (PBS), quenched for endogenous peroxidase activity, blocked against non-specific antibody using a commercial kit for TUNEL (Machaalani and Waters, 2007a,b), and counterstained with Harris’ Haematoxylin and then mounted.

Quantitative immunohistochemical analysis

Two levels of the brainstem medulla were studied; the caudal and rostral levels. These were defined with reference to Plates X, XI,
were captured between duplicates (Machaalani and Waters, 2003). 

Scale bar = 30 μm for all panels. 

Fig. 1 Immunohistochemical staining of three apoptotic markers compared. (A, B, D, E, G, H) represent a positive stained section while (C, F, I) represent the negative control counterpart for each stain. The profile of positive-stained neurons (see text for description of profiles) varied amongst the three markers. Positive-stained neurons (black arrows, and black arrow heads) and negative neurons (white arrows, white arrow heads and asterisk). For TUNEL (A, B), positive neurons were a mix of profile B (black arrow) and D (black arrow head). Neurons of profile C were often seen to be slightly positive for TUNEL (asterisk) but were counted as negative. For ssDNA (D, E), positive neurons were typically of profile B (black arrow) and C (thin black arrow head) and to a lesser extent profile D which were more often stained negatively (white arrow head). For active caspase-3 (G, H), positive neurons were predominantly profile D (black arrow heads). Negative controls included sections that (C) had the TdT enzyme replaced with reaction buffer alone, (F) were stained with SI nucleus instead of the ssDNA antibody, and (I) were incubated with 1% normal goat serum instead of the active caspase-3 antibody. Scale bar = 30 μm for all panels.

All quantitative analyses were performed with the observer blinded to the diagnosis. Due to the limited human infant tissue available for study, quantification was performed on one section from each medullary level for each case. Reproducibility of staining was assumed from our previous experience showing <10% difference between duplicates (Machaalani and Waters, 2003).

Sections were assessed quantitatively using a Zeiss KS 400 image analysis system (Carl Zeiss Vision, Munich, Germany). Images were captured (×10 magnification) using a Sony Progressive CMA-D2 3CCD colour video camera (Sony, Tokyo Japan) mounted on a Zeiss Axioplan microscope. Each nucleus was imaged and analysed in its entirety (2–5 images depending on the size of the nucleus).

Only neuronal staining was analysed. Manual cell counts were made of the positively and negatively stained neurons from each image and the number of positive neurons was then expressed as a per cent of the total neurons present within each nucleus. Results are presented as a percentage rather than kept as the raw counts, to standardize the results amongst the different cases. Positively stained TUNEL (Fig. 1A) and ssDNA (Fig. 1B) neurons were identified as having brown staining in the nucleus, while for active caspase-3 (Fig. 1C) brown staining was predominantly in the cytoplasm.

Data and statistical analyses

Data were collated and exported to SPSS for Windows (V14.0, Chicago, USA) for statistical analysis. Clinical and autopsy characteristics were compared between groups (SIDS and non-SIDS) using Student’s t-test, and all results are presented as mean±SD unless otherwise stated.

The data regarding TUNEL and ssDNA positive neurons were not normally distributed and are therefore presented as median with interquartile range in the text, tables and figures. Statistical analyses were performed using Mann–Whitney U non-parametric tests. For active caspase-3, data were normally distributed and comparisons were made using Student’s t-test. The correlation coefficient between the three stains (caspase-3, TUNEL and ssDNA) was calculated using Kendall’s Tau. Correlation coefficient analysis was also conducted to test the association between each respective stain and clinical characteristics of the patients. To assess changes with development, analyses were undertaken for all infants combined. Subgroup analyses were then undertaken to compare diagnostic groups (SIDS and non-SIDS). We did not attempt to undertake stereological analyses, but we combined data for the nuclei at both brainstem levels to determine whether differences persisted along the rostral–caudal extent of each nucleus when comparing SIDS to non-SIDS. Where a positive correlation was found for a particular variable, analysis of covariance (ANCOVA) was applied to compare the diagnostic groups, but this was only possible for the caspase-3 data which had a normal distribution. No adjustments were made for TUNEL or ssDNA but all significant correlations have been reported in the results. Where more than one risk factor was associated with staining results, associations amongst the risk factors were examined and any positive associations found were reported. A P-value of <0.05 was considered statistically significant.

Results

Infant characteristics

The diagnostic groups included 25 ‘non-SIDS’ and 67 ‘SIDS’. Cases diagnosed as SIDS fitted the definition of Willenger et al. (1991). The diagnoses amongst non-SIDS cases included pneumonia (n = 6), septicamia ± Waterhouse–Friderichsen syndrome (n = 4), pneumococcal meningitis (n = 1), Chiari type II malformation (n = 1), lower respiratory tract infection (RTI) (n = 1), bronchopneumamine toxicity and RTI (n = 1), aspiration of gastric contents (n = 1), myocarditis (n = 3), dilated cardiomyopathy (known as congestive cardiomyopathy; a condition in which the heart becomes weakened and enlarged, and cannot pump blood efficiently) (n = 1), congenital heart disease (n = 2), gastroenteritis (n = 1),
leukaemia (n = 1), drowning (n = 1) and necrotizing enterocolitis (n = 1).

Characteristics of the non-SIDS and SIDS group are provided in Table 1. There were no differences between the two groups for clinical characteristics including gestational age, birth weight, immunization or the presence of a recent upper respiratory tract infection (URTI).

Included amongst the autopsy characteristics, petechiae (thymic, pulmonary, epicardial and/or thoracic) were reported more often in SIDS (79%) than non-SIDS cases (28%) (P < 0.001), as was the presence of blood in the nasal area (13% versus 0%, P = 0.06). However, the presence of pulmonary oedema, aspiration and/or congestion did not differ between diagnostic groups (67% SIDS versus 60% non-SIDS; P = 0.6). Body weight, brain weight, body length and head circumference at death were also not different between diagnostic groups (Table 1).

Comparisons for the sleep-related risk factors were only performed in the SIDS group as these data were often absent for the non-SIDS group. For sleep position, 12% of the SIDS group usually slept prone but 56% were found prone at death. The majority of cases were reported to have died during the night (61%) and 32% of cases were bed sharing at that time of death. A positive history of cigarette smoke exposure was present for a greater proportion of SIDS cases (81%), but this was not statistically different to non-SIDS cases (58%; P = 0.1).

Parameters with the potential to affect staining such as post-mortem interval (PMI) and fixation time were not different between the diagnostic groups (Table 1). Regression analyses also showed no relationship between the quantification of the three apoptotic markers and these parameters, so they were not used as covariates in the analyses.

### Staining

Four neuronal profiles were identified. These were: (A) typical and ‘normal’, where the neuron was intact with the
nucleus and nucleolus centrally positioned in a well-defined and full cytoplasm, and atypical including; (B) shrunken neuron where the cytoplasm is barely noticeable but nucleus and nucleolus clearly visible and still centrally positioned; (C) nucleus laterally positioned in a plump cytoplasm; and (D) morphologically characteristic of apoptosis, where the neuron is shrunken with condensation of the nucleus (pyknotic).

TUNEL positive staining was specifically localized to the nucleus of neurons which were one of the two profiles (B) and (D) (Fig. 1A, black arrow and black arrow head, respectively). Neurons fitting profile (C) that were slightly stained for TUNEL (Fig. 1A, white arrow head) were counted as negative.

Positive staining for ssDNA was localized to the nucleus of neurons. Positive ssDNA neurons were mostly of profiles (B) and (C) (>90%), and less often profile (D) (<10%) (Fig. 1B). Staining seen in the cytoplasm was considered non-specific staining due to the secondary IgM antibody. This was confirmed by pre-treating sections with S1 nuclease which showed that nuclear staining of ssDNA was abolished but slight cytoplasmic staining was still evident (Fig. 1E).

Almost all positive neurons for active caspase-3 were of profile (D) (showing morphological features of apoptosis), including shrinkage of the neuron and condensation of the nucleus (pyknosis) (Fig. 1C, black arrow). Pyknotic neurons were rarely negative for caspase-3, but neurons of normal appearance occasionally stained positive (<10%).

Correlation between the three markers
Amongst the nuclei, Grac and Cun consistently showed greater expression of all three markers. Consequently, most of the observed positive correlations between the markers were in the Grac and Cun nuclei. There was a good correlation between TUNEL and ssDNA in XII, Cun (Fig. 2A) and Grac (P ≤ 0.01 for all) in both diagnostic groups. For TUNEL and caspase-3, in the non-SIDS group, there was a positive correlation in the caudal Cun (Fig. 2B), and rostral AN (P = 0.05), but these were lost in the SIDS population. For caspase-3 and ssDNA, a correlation was only observed in the Cun nucleus and this was for both diagnostic groups, but was much stronger at the rostral medulla level (P < 0.01) compared to the caudal (P = 0.02) (Fig. 2C).

Analysis for developmental characteristics, SIDS versus non-SIDS, and risk factors
TUNEL
Analysis of TUNEL data for the entire dataset showed a positive correlation between TUNEL staining and post-conceptional age (PCA) at the rostral Cun (P = 0.05) and rostral AN (P = 0.02). Sub-group analysis according to diagnosis showed a positive correlation with PCA for the rostral DMNV only in the non-SIDS group (P = 0.03); there were no correlations with PCA within the SIDS group. No significant relationships were observed between growth parameters and TUNEL expression for the non-SIDS group. But for the SIDS group, there was a significant positive correlation between growth characteristics and TUNEL in the rostral Cun including body length ($R^2 = 0.124$, $P = 0.01$), head circumference ($R^2 = 0.132$, $P < 0.01$), brain weight ($R^2 = 0.113$, $P = 0.04$) and body weight at death ($R^2 = 0.07$, $P = 0.04$). Of these, head circumference was the most significant contributor (P < 0.01).
Comparing the diagnostic groups, SIDS versus non-SIDS (Table 2), there was no statistically significant difference in the proportion of cases with positive staining or in the median values for TUNEL. Comparison between diagnostic groups, stratified for gender, showed that SIDS males had a significant increase in rostral AN compared with non-SIDS males (non-SIDS 0/C6 46.5 versus SIDS 54.7/C6 66.8; \( P = 0.04 \)).

Categorical variables included the presence of petechiae, history of cigarette smoke exposure, recent URTI, immunization, blood in the nasal area and death during the night. Amongst the whole study population, infants with the presence of petechiae had greater TUNEL staining in the caudal AN (0/C6 24.6 versus 47.6/C6 65.7; \( P = 0.02 \) without vs. with petechiae, respectively) and the rostral DMNV (0/C6 25.5 versus 22.6/C6 50.3; \( P = 0.046 \) without vs. with petechiae, respectively). For the SIDS group this difference was apparent in the rostral DMNV (0/C6 0.0 versus 24.9/C6 77.9; \( P = 0.03 \) without vs. with petechiae, respectively). For the whole group, infants exposed to cigarette smoke had significantly greater TUNEL immunoreactivity compared to non-exposed infants in the rostral DMNV (\( P = 0.046 \)) and rostral AN (\( P = 0.01 \)) (Table 3). There were no associations between staining and recent URTI or immunization. SIDS infants found with blood in the nasal area had greater TUNEL levels in the caudal Cun compared to those with no blood in the nasal area (\( P = 0.001 \)), and infants found dead during the night had greater TUNEL in rostral DMNV (\( P = 0.02 \)). SIDS infants who did not bed share had greater TUNEL in the caudal Grac (10.4/C6 67.6 versus 67.9/C6 31.1; \( P = 0.02 \) bed sharing versus no-bed sharing) and Cun (16.9/C6 68.5 versus 65.0/C6 31.2; \( P = 0.006 \)), and infants found in the prone position had a non-significant trend towards increased TUNEL in the caudal Grac (54.3/C6 77.3 versus 72.2/C6 22.4; \( P = 0.08 \)).

ssDNA

There were no statistically significant correlations found between ssDNA immunoreactivity and developmental parameters or between SIDS and non-SIDS groups (Table 4). Moreover, there was no association with any of
Caspase-3

For the entire group, caspase-3 immunoreactivity in the XII, at both levels, increased with post-conceptional age (both levels \( P = 0.03 \)), but when diagnostic groups were separated, this finding only persisted for the non-SIDS infants (Fig. 3). Within the non-SIDS group, female infants had greater caspase-3 compared to the males in the caudal Grac (\( P = 0.04 \)) and rostral AN (\( P = 0.01 \)). In the SIDS group, female SIDS had less caspase-3 compared to male SIDS in the rostral AN (\( P = 0.008 \)) (Table 5). There were no significant interactions with growth.

The % caspase-3 positive neurons was greater in SIDS than non-SIDS infants for the combined DMNV levels (\( P = 0.02 \)) although when levels were separated, this significance was only maintained in the rostral DMNV (\( P = 0.01 \), Table 5). Stratifying for gender, the increase in the DMNV was predominantly due to the male cohort (non-SIDS males versus SIDS males combined DMNV \( P = 0.02 \), rostral DMNV \( P = 0.01 \), Table 5). A non-significant trend towards increased staining was present in the rostral AN for SIDS males compared to non-SIDS males (\( P = 0.06 \)), and was opposite to the decrease seen in female SIDS compared to female non-SIDS (\( P < 0.001 \), Table 5). Adjustment for post-conceptional age produced no major changes to these results.

When examining for associations between staining and the presence of petechiae, history of cigarette smoke exposure, recent URTI and immunization, no significant interactions were observed. Analysis of the SIDS group for the risk factors including sleep position and bed sharing also showed no significant correlations.

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>All dataset</th>
<th>Non-SIDS</th>
<th>SIDS</th>
<th>P value</th>
</tr>
</thead>
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<td></td>
<td>No smoking</td>
<td>Smoking</td>
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<td>n = 34</td>
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<td>n = 3</td>
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<td>579 (0–61)</td>
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<tr>
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</table>

All data is presented as median (range).

Table 4 Quantitation of ssDNA-positive neurons in the studied nuclei at both the caudal and rostral medulla levels

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>All data set</th>
<th>Non-SIDS</th>
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<th>P value</th>
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<tr>
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<td>0 (0–22)</td>
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<tr>
<td>DMNV</td>
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<td>32.2 (0–64)</td>
<td>0 (0–29)</td>
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<tr>
<td>Gracile</td>
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<tr>
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<td>44.9 (9–81)</td>
<td>0 (0–4)</td>
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<tr>
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</table>

All data is presented as median (range).
Neuronal apoptosis in SIDS medulla

Table 5 Active caspase-3 quantitative comparison between SIDS and non-SIDS, with additional analysis by gender.

<table>
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<tr>
<th>Nucleus</th>
<th>Non-SIDS</th>
<th>SIDS</th>
<th>P-value</th>
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<th>SIDS</th>
<th>P-value</th>
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<th>SIDS</th>
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<td>Females</td>
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<td>Females</td>
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<tr>
<td>Caudal medulla</td>
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<td></td>
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</tr>
<tr>
<td>XII</td>
<td>491 ± 12.4</td>
<td>63.0 ± 3.4</td>
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<td>547 ± 40.4</td>
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<td>50.2 ± 3.5</td>
<td>0.33</td>
<td>38.5 ± 9.4</td>
<td>576 ± 9.2</td>
<td>0.37</td>
<td>52.3 ± 5.1</td>
<td>472 ± 4.6</td>
<td>0.47</td>
</tr>
<tr>
<td>Gracile</td>
<td>40.3 ± 6.9</td>
<td>40.6 ± 4.0</td>
<td>0.97</td>
<td>35.6 ± 8.5</td>
<td>559 ± 0.4</td>
<td>0.04</td>
<td>39.1 ± 5.5</td>
<td>429 ± 5.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Cuneate</td>
<td>41.4 ± 6.6</td>
<td>43.3 ± 3.9</td>
<td>0.81</td>
<td>37.1 ± 8.1</td>
<td>558 ± 4.0</td>
<td>0.25</td>
<td>42.5 ± 5.3</td>
<td>446 ± 6.0</td>
<td>0.45</td>
</tr>
<tr>
<td>AN</td>
<td>60.3 ± 6.0</td>
<td>592 ± 3.4</td>
<td>0.88</td>
<td>63.1 ± 5.9</td>
<td>537 ± 16.2</td>
<td>0.50</td>
<td>594 ± 5.2</td>
<td>590 ± 3.3</td>
<td>0.70</td>
</tr>
</tbody>
</table>

| Rostral medulla | | | | | | | | | |
| XII     | 53.8 ± 6.8 | 62.4 ± 3.1 | 0.25 | 478 ± 8.0 | 697 ± 11.2 | 0.15 | 61.8 ± 4.1 | 632 ± 4.9 | 0.09 |
| DMNV    | 43.2 ± 3.6 | 54.9 ± 2.5 | 0.01 | 42.6 ± 4.7 | 449 ± 4.9 | 0.78 | 58.1 ± 3.6 | 513 ± 3.2 | 0.01 |
| Cuneate | 37.3 ± 6.1 | 38.1 ± 3.3 | 0.91 | 35.1 ± 7.4 | 43.3 ± 10.1 | 0.56 | 41.8 ± 4.5 | 34.0 ± 3.2 | 0.22 |
| AN      | 52.0 ± 4.1 | 55.1 ± 3.0 | 0.57 | 49.2 ± 4.8 | 63.2 ± 1.1 | 0.01 | 61.9 ± 4.2 | 473 ± 3.6 | <0.01 |

Table represents the mean percentage positive neurons ± SEM.

Discussion

No previous investigation has studied more than one apoptotic marker simultaneously in the same SIDS dataset and no studies have performed correlatative analyses between apoptotic expression and postnatal developmental and risk factor parameters. Our ability to provide such an analysis was made possible due to our large infant dataset. In agreement with previous findings, we report an increase in apoptotic expression and postnatal developmental and risk factor parameters. Our ability to provide such an analysis was made possible due to our large infant dataset. In agreement with previous findings, we report an increase in apoptotic expression and postnatal developmental and risk factor parameters. Our ability to provide such an analysis was made possible due to our large infant dataset. In agreement with previous findings, we report an increase in apoptotic expression and postnatal developmental and risk factor parameters. Our ability to provide such an analysis was made possible due to our large infant dataset. In agreement with previous findings, we report an increase in apoptotic expression and postnatal developmental and risk factor parameters. Our ability to provide such an analysis was made possible due to our large infant dataset.

Neurons that had the typical apoptotic morphology of cellular shrinkage and condensation of nucleus were predominantly (>90%) active caspase-3 positive and to a lesser extent TUNEL (~50%), but there was no consistent association with ssDNA (10%) positive staining. Of the three markers, active caspase-3 is considered to be a more specific marker of morphological apoptosis than the other two (Zhu et al., 2000, Arai et al., 2005). The profile of positive active caspase-3 neurons has been shown to change with time after an insult. For example, 3 h after a hypoxic-ischaemic (HI) insult, active caspase-3 staining was largely cytoplasmic whereas 24 h after HI, the immunopositive cells were predominantly condensed, and the staining was primarily around the shrunken nuclei (Zhu et al., 2000). Based on these observations and the fact that 90% of our active caspase-3 neurons were condensed and shrunken, we can extrapolate that the majority of neurons stained for active caspase-3, have been activated between 8 and ~24 h previously.

This timing of insult prior to death could also explain the difference in the correlation between the markers and their regional variations. To our knowledge, this is the first study to have investigated the correlation between caspase-3 activation and other histochemical markers of DNA damage (TUNEL and ssDNA) in the developing human infant brainstem. As expected, the strongest correlation was between TUNEL and ssDNA, but this was restricted to the DCN and XII nuclei. A lesser correlation existed between caspase-3 and TUNEL and caspase-3 and ssDNA. This is not surprising considering the findings by Zhu et al., 2000, where co-localization of caspase-3 and ssDNA was reported to be <10% and only visible 72 h after insult, compared with caspase-3 and TUNEL that showed 20% co-localization 3 h after insult, but increased to a maximum of about 40–50% 24 h after insult (Zhu et al., 2000).

Of the nuclei we studied, the dorsal column nuclei (giracile and cuneate), consistently showed greater immunoreactivity for all three markers. This reflects the distribution reported in a Canadian infant population (Waters et al, 1999) and in an adult population (Stecco et al., 2005). Possible reasons for this differential apoptotic expression amongst the nuclei of the medulla include differences in vulnerability or timing of death in response to hypoxic injuries, characteristics of blood supply to the nuclei, metabolic demand (Stecco et al., 2005) as well as differences in the expression of neurotransmitters, receptors or growth factors. This may also have important functional implications. The majority of the projections of the DCN are to the thalamus, with minimal projections to the cerebellum, mostly arising from the rostral cuneate (Ostapoff et al., 1988; Kemplay and Webster, 1989). The main role of the DCN with regards to thalamic projections is transmitting touch and kinaesthesia information, as well as visceral pain signals (Berkley and Hubscher, 1995). A secondary role of the DCN, with regards to cerebellar projections, is in controlling orientation and balance. The high amount of apoptosis seen in these nuclei suggest that these pathways may have been activated and/or damaged long before the pathways involved with the XII, DMNV or AN.
A limitation of this study was that the quantitative analyses were based on only one tissue section at each medullary level. Thus, we were not fully able to assess the changes in a nucleus throughout its rostro-caudal length, nor were we able to determine whether different measurements achieved were due to either the structural heterogeneity of the cellular organization of the nucleus, or to random variability. This limitation of the study was predominantly due to the limited tissue available, in addition to the fact that not all cases had tissue sections at both levels. So, to maximize the number of cases studied, we maintained the separate level analysis with the anticipation that for the majority of the nuclei, similar quantitative outcomes would be evident. An exception was the DMNV given it has a different topographic localization of functions (Huang et al., 1993). As anticipated, most of the results were consistent for each nucleus at both levels, except the DMNV for which changes were often found to be significant only in the rostral DMNV.

One of the strongest findings in this study was the increase in active caspase-3 in the rostral DMNV of SIDS, consistent with our recent semi-quantitative analysis of active caspase-3 in the infant brainstem (Machaalani et al., 2007). We showed that this increase was independent of birth characteristics and risk factors, suggesting a SIDS-specific factor is the predominant variable associated with increased caspase-3 in the DMNV. The involvement of the DMNV is not unexpected given reports of other abnormalities found in this nucleus in SIDS infants including increased gliosis (Yamanouchi et al., 1993), delayed neuronal maturation (Quattrochi et al., 1985) and decreased neuronal density (Konrat et al., 1992). Furthermore, our finding that increased caspase-3 was specific to the rostral DMNV, and not found in the caudal DMNV, is consistent with the findings of Lavezzi et al., (2003) who found abnormal c-fos expression only in the rostral DMNV of SIDS infants. Functionally, this suggests SIDS infants are more likely to have abnormal respiratory rather than abnormal cardiac control given that the rostral DMNV predominantly innervates the lung and abdominal organs whereas the caudal DMNV innervates the heart (Huang et al., 1993).

Throughout the brain, there is a normal rate of apoptosis with growth and differentiation. Increases in staining were associated with increasing PCA for TUNEL and caspase-3. Sub-group analyses showed that PCA correlated best in the non-SIDS group, with the greatest changes seen for caspase-3 in the XII, and TUNEL in the rostral DMNV. A similar correlation with PCA in the XII has been reported for serotoninergic receptor 1A (5-HT1A) activity (Paterson et al., 2004). These findings suggest that some 'normal' developmental processes (e.g. active apoptotic changes) are still occurring during infancy in the XII and DMNV. However, acute changes relating to the death insult in SIDS infants may have negated the usual age-related effects.

Difference between genders in the SIDS cohort would not be unexpected given that SIDS predominantly affects male infants. Our hypothesis was males would show more evidence of cell death than females. However, females within the non-SIDS cohort had greater caspase-3 immunoreactivity in the Grac and rostral AN compared to the males and within the SIDS cohort staining was greater for males in the rostral AN. Studies in animals provide a plausible explanation for our findings in the non-SIDS population. Young female mice (Zhu et al., 2006) and piglets (Machaalani and Waters 2006) were shown to have an increase in neuronal caspase-3 expression, and not for any other cell death marker, in several regions of the brain 24h after a HI, which was not evident in males. Those studies concluded that females seem to have the caspase-3 cycle turned on earlier or faster than males and as such, caspase-3 activation follows different paths according to gender. Again, it seems possible that pathological changes in the SIDS cohort may result in failure to reflect these patterns.

We hypothesized that TUNEL would be more highly expressed, and present in a greater proportion of infants in the SIDS than the non-SIDS diagnostic group, especially in the DCN, based on previous TUNEL findings from a Canadian SIDS cohort (Waters et al., 1999). However, those findings were not replicated in this study. Possible reasons for the difference in findings include a difference in: (i) the composition of the non-SIDS cohort, which is a major problem in SIDS neuropathology studies in general; (ii) infant birth characteristics; this seems especially evident for the rostral cuneate where many associations were found with infant growth; (iii) in the prevalence of the risk factors; this information was not available for the Canadian cohort which consisted of cases from 1993 to 1996, a time where risk reduction campaigns were in their infancy compared with the cohort of this study which consisted of cases from 1997 to 2002, a time where risk reduction campaigns were well underway and would presumably have resulted in a lesser number of prone sleeping infants. Indeed, one of the novel findings of this study was a positive association between TUNEL in the DCN of SIDS infants and the risk factors of prone sleeping and bed sharing; and (iv) a difference in tissue fixation; tissue in this study was fixed in 10% neutral-buffered formalin whereas tissue in the Canadian study were fixed in an acidified formalin solution.

An additional finding in this study which was not investigated in the Canadian study was increased TUNEL in the AN, that was relatively specific to males. This corresponded with greater changes in active caspase-3 expression in the male AN. Other reported abnormalities in the AN in SIDS infants include decreased 5-HT1A receptor-binding density (Paterson et al., 2006), and hypoplasia (Filiano and Kinney, 1992; Matturi et al., 2000). Combined, these findings suggest a possible mechanistic link, particularly amongst male SIDS infants,
where the loss of inhibitory function provided by the 5-HT\textsubscript{1A} receptors could predispose the infants to increased cell death (Azmitia, 2001).

Positive associations with abnormal pathology and modifiable SIDS risk factors were only observed for TUNEL staining. These included the presence of petechiae, blood in or around the nasal area, a history of cigarette smoke exposure and the sleep-related parameters of bed sharing and prone sleeping. The presence of petechiae is a common pathological finding amongst SIDS infants but is not conclusive to all SIDS cases, and is observed in infants dying from other causes (Becroft et al., 1998). The cause of petechiae is still unknown, but there is some evidence that its distribution and frequency is affected by the age, ethnicity, parity, exposure to cigarette smoke and the sleep position of the infant (Becroft et al., 1998, Krous et al., 2001), the latter leading to the suggestion that the petechiae arise from an obstruction of the airways (Krous et al., 2001). Our data appear to support the association with an obstructed airway because of the positive association between the presence of petechiae and increased TUNEL in the rostral DMNV of SIDS infants.

One of the limitations of our data pertaining to cigarette smoke exposure is that we could not differentiate whether the changes were due to active or passive exposure because this information was not always present. As such, infants with any exposure to cigarette smoke (be it via the mother, father or any other person in the household) were analysed as having a positive history of smoke exposure. Our finding of a positive association between TUNEL in the rostral DMNV and AN with a history of cigarette smoke exposure, regardless of diagnosis, is an important and novel finding. This finding implies that smoke exposure to any infant during early postnatal development makes the rostral DMNV and AN vulnerable to apoptotic mechanisms and could result in unfavourable physiological outcomes such as impaired respiratory responses (due to the change in the DMNV) and CO\textsubscript{2} chemosensitivity (AN). We postulate that the mechanism via which cigarette smoke exposure induces the increase in TUNEL is most likely via nicotine acting on the nicotinic acetylcholine receptors (nAchR), predominantly the \(\alpha7\) subunit. Activation of \(\alpha7\) nAchR has been shown to result in excitotoxic mechanisms where increased calcium influx, triggered the expression of p53, a cell cycle related protein, and lead to the subsequent increase in TUNEL in undifferentiated hippocampal progenitor cells (Berger et al., 1998).

The association between TUNEL in the DCN of SIDS infants and the prone sleep position has important implications because it provides a biological mechanism that may explain how the prone position increases the risk for SIDS. The prone sleeping position is associated with a hypercapnic hypoxic environment due to re-breathing of expired gases (Waters et al., 1996). Our piglet model of intermittent hypercapnic hypoxia (IHH) provides evidence that increased TUNEL is induced in the DCN (Machaalani and Waters, 2006), so repeated exposure of infants to this gaseous environment could result in increased TUNEL expression. Although we hypothesized prone sleeping would also be associated with increased active caspase-3, our findings did not support this. That result is also consistent with findings in our piglet model of IHH, where TUNEL changes were more evident and of a greater magnitude in the DCN than changes in active caspase-3. Combined, the data suggest that the cell death process in the DCN, activated by the prone sleeping position, is well underway and in the final stages of the cell death pathway. This increased cell death in the DCN could subsequently result in dysfunction of these nuclei with the consequence being a dampening or loss in relay of touch, proprioception, orientation and balance. Within this scenario, this could be detrimental in the face of the hypercapnic hypoxic challenge that could arise when the infant is in the prone position, and attempts to turn over to the supine position fail.

In conclusion, we present a more detailed analysis of the pattern of increased neuronal apoptosis in the brainstem of SIDS compared to non-SIDS infant deaths, and provide novel evidence that clarify links with developmental, environmental and risk factor parameters, with the majority of the associations evident only for the expression of TUNEL staining. These findings advance our knowledge related to SIDS neuropathology and provide evidence for the first time that active apoptotic mechanisms are associated with age, gender, prone sleeping and cigarette smoke exposure.

**Acknowledgements**

The authors acknowledge the co-operation of the Department of Forensic Medicine, Glebe, NSW, Australia, in particular, Dr Michael Rodriguez for his work in the collection and routine histological assessment of the tissue, and helpful discussions concerning the data of this manuscript. The study was funded by the National Health and Medical Research Council of Australia (#302006), the Sudden Infant Death Syndrome Foundation of South Australia, and K.A.W. is supported by a National Health and Medical Research Council of Australia Practitioner Fellowship (#206507).

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