NMDA Receptor 1 Expression in the Brainstem of Human Infants and Its Relevance to the Sudden Infant Death Syndrome (SIDS)

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Abstract. The N-methyl-D-aspartate (NMDA) glutamatergic receptor is widely expressed in the brain during the early postnatal period and, among other functions, is involved in cardiorespiratory control and in cell death by excitotoxic mechanisms. This study examined NMDA receptor-1 (NR1) expression in the human infant brainstem and assessed whether expression differed between non-SIDS and SIDS infants. NR1 mRNA was identified using non-radioactive in situ hybridization and quantified by optical density. NR1 protein was identified by immunohistochemistry and quantified by cellular counting. Eight nuclei of the mid-medulla and 2 nuclei of the rostral pons were studied. NR1 mRNA and protein were expressed in all nuclei studied, confirming that the NMDA receptor is widely distributed in the human infant brainstem. Compared to non-SIDS infants (n = 10), SIDS infants (n = 15) had increased mRNA in 6 nuclei of the mid-medulla (p < 0.05 for all) while protein was increased in the dorsal motor nucleus of the vagus (p = 0.04) and decreased in the nucleus of the spinal trigeminal tract (p = 0.03). No differences were observed in the rostral pons. This preliminary study suggests that abnormalities of the glutamatergic system are present in SIDS victims. Further studies will be required to delineate these abnormalities and to investigate potential underlying mechanisms and sequelae.

Key Words: Brainstem; mRNA; N-methyl-D-aspartate (NMDA); NR1 protein; SIDS.

INTRODUCTION

The sudden infant death syndrome (SIDS) is defined as the sudden death of an infant under 1 year of age that remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history (1). One underlying cause of SIDS is postulated to be a developmental disorder of brainstem cardiorespiratory responses, particularly to hypoxic and hypercapnic challenges (2). A number of studies have sought to define neuroreceptor abnormalities that could be responsible for this cardiorespiratory control defect. To date, these studies focused on the muscarinic (2, 3), nicotinic (4), opioid (5), adrenergic (6), serotonergic (7), and kainate (8) receptors.

A previous finding of neuronal apoptosis in SIDS infants (9) has led to our interest in the N-methyl-D-aspartate (NMDA) glutamatergic receptors. The NMDA receptor is involved in cardiorespiratory control and also in cell death by excitotoxic mechanisms. Changes in NMDA receptor expression and neuronal vulnerability to excitotoxic damage during early development (10) would be consistent with NMDA receptor involvement in an age-specific phenomenon such as SIDS.

The NMDA receptor belongs to the glutamatergic neurotransmitter system, which also includes the α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionate (AMPA) and kainate receptors. NMDA receptor binding is increased in the human brainstem between mid-gestation and early infancy, whereas AMPA and kainate receptor binding are elevated during mid-gestation and decrease thereafter (11). It has thus been suggested that in the immature brain, NMDA receptors dominate and are responsible for neuronal maturation and plasticity, while AMPA and kainate receptors are predominantly responsible for neurotransmission (11, 12).

NMDA receptors exist as heteromeric complexes containing NR1 combined with one or more NR2 or NR3 subunits. The NR1 subunit is an obligatory component of functional NMDA receptors and as such, serves as a useful marker for localizing NMDA receptors in brain tissue (13). The NR1 subunit has been shown to be widely distributed in the brainstem of the piglet (14), cat (15), rat (13, 16), and mouse (17). Its wide distribution in the brainstem, a site containing nuclei generally responsible for controlling cardiac activity, respiration, sleep, and arousal suggests that NMDA receptors are important in mediating these functions. Animal physiological studies support this contention (18–20). The distribution of NR1 in the brainstem of the human infant has not been previously determined. The first part of this study reports the distribution of NR1 mRNA and protein in the human infant mid medulla and rostral pons using non-radioactive in situ hybridization (non-RISH) and immunohistochemistry, respectively.

NMDA receptors share a close association with hypoxia, including their role in the hypoxic ventilatory response (21, 22) and changes in their expression and function after hypoxic stimulation (23–25). Moreover,
changes in NMDA receptor activity, either too much or too little, can precipitate cell death. Excessive and abnormal activation of glutamate receptors that leads to cell death is called excitotoxicity (26). During infancy, hypoxic conditions increase neuronal vulnerability to NMDA-mediated excitotoxicity and the neurons in turn become vulnerable to apoptosis (10). Apoptosis, as identified by DNA fragmentation, was found to be increased in hypoxia-sensitive regions of the hippocampus and in the dorsal sensory nuclei of the brainstem of SIDS infants. The conclusion of that study was that hypoxic exposure preceded the death of these infants by at least a few hours (9). This conclusion is further supported by the finding that vascular endothelial growth factor (VEGF), a marker for hypoxia, was significantly increased in the cerebral spinal fluid of a subset of SIDS infants (27).

Given the associations between both NMDA receptors and SIDS with hypoxia, we hypothesized that NMDA receptor dysfunction is present in SIDS infants. Therefore, in the second part of this study we compared the distribution and expression of NR1 mRNA and protein in infants who die from SIDS with infants for whom the cause of death was known. Specifically, we postulated that NR1 expression would be increased in the dorsal sensory nuclei of the medulla of SIDS infants relative to controls. Our rationale was that excitotoxic mechanisms could underlie our previous observation of increased neuronal apoptosis in these nuclei in SIDS infants.

MATERIALS AND METHODS
Tissue Collection

Brainstem tissue was available for study from 25 infants who had died at less than 1 year of age. Tissue was collected from routinely processed autopsy blocks at the NSW Institute of Forensic Medicine between 1997 and 1998. This study was approved by The Human Ethics Committees of the University of Sydney and Central Sydney Area Health Service. At the time of autopsy, the brain was removed and fixed in 10% formalin. Tissue blocks from selected brain regions were then paraffin-embedded in an automatic processor. The material used in this study was that remaining from diagnostic tissue blocks after the collection of autopsy, the brain was removed and fixed in 10% formalin. Specimens were then deparaffinized in xylene and taken through a graded series of ethanol and then distilled H2O. Sections were then mounted in a humidiﬁed dark box. The color reaction was terminated by washing in Tris buffer (10 mM Tris, 0.1 mM EDTA, pH 9.0) and microwaved at full power on a rotating turntable, uninterrupted for 12 min. Sections were then washed in the buffer, washed in DEPC H2O and in PBS and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min. After further washing in PBS, sections were prehybridized in 4× standard saline citrate SSC/50% formamide for 2 h at 37°C, rinsed in 2× SSC and 50 ng of antisense probe in 100 μl of hybridization buffer was added (18% formamide, 2× SSC, 1× Denhardt solution, 10% dextran sulfate, 50 mM dithiothreitol (DTT), 250 μg/ml yeast tRNA, 100 μg/ml polyadenylic acid, and 500 μg/ml denatured and sheared salmon sperm DNA). Sections were cooversiled with Parafilm and left to hybridize overnight at 37°C in a humidified chamber. Following hybridization, sections were washed in 2× SSC at room temperature until the Parafilm washed off. Sections were then stringently washed for 15 min each in 2× SSC (twice) and 1× SSC at 37°C, then at room temperature in 1× SSC and 0.5× SSC.

Immunohistological detection for NR1 mRNA was then performed. Sections were washed in digoxigenin buffer (DB) (10 mM maleic acid, 15 mM NaCl, pH 7.5) and blocking solution was added for 1 h at 37°C (10% w/v blocking reagent from Roche Diagnostics, 2.5% normal sheep serum in DB). Anti-DIG-alkaline phosphatase antibody (1:250 dilution in DB, containing 10% blocking reagent and 1% normal sheep serum) was added after blocking and the sections were incubated for 2 h on a rocking platform at room temperature. Sections were then washed in DB and then in digoxigenin detection buffer (DDB) (10 mM Tris, 10 mM NaCl, 50 mM MgCl2, pH 9.5). Alkaline phosphatase color reaction buffer (35 μl nitroblue tetrazolium and 45 μl 5-bromo-4-chloro-3-indolyl-phosphate [BCIP] in 10 ml DDB) was added and sections incubated overnight (16–18 h) in a humidified dark box. The color reaction was terminated by washing in Tris buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and then distilled H2O. Sections were then mounted in Aquamount solution.

Non-speciﬁc hybridization was examined using slides that had been incubated with either sense probe or hybridization solution only, while non-speciﬁc staining was examined using sections without antibody treatment. All cases were included in each run of 2 runs for each brainstem level and uniform conditions were ensured to permit later quantitative comparisons.

Quantitative In Situ Hybridization

Quantitation was a measure of the signal intensity represented in optical density units (OD) and is restricted to neurons, not glial cells. The method for quantification has been described previously (14) and is a modification of other studies (29, 30).
Briefly, images were viewed using a Nikon Eclipse E800 light microscope (Nikon Cooperation, Tokyo, Japan) and captured with a sensicam slowscan cooled color CCD camera (PCO Computer optics, Kelheim, Germany). At the commencement of each session, prior to image capturing, the camera was white-balanced and exposure time standardized to 0.055 ms. A combination of red, green, and blue channels were used. Images from each nucleus studied were captured at 1,280 × 1,024 pixels using a ×4 objective. Each nucleus studied was captured in its entirety. Thus, the number of images captured per nucleus ranged from 1 (hypoglossal nucleus) to 4 (ION). Images were then analyzed using a Zeiss KS400 system (Carl Zeiss Vision, Munich, Germany). In this program, a grey level of 0 represents black (no transmission) and 255 is white (full transmission). The measured parameters included area, mean grayscale value, and standard deviation of the grayscale value. Three areas were analyzed from each image capture: 1) a non tissue area that contained mounting media and coverslip but no tissue; this provided the measurement of the incident light; 2) connective tissue containing no cells or neurons within the selected nucleus; this provided the measurement for background; and 3) all the individual neurons in the selected nucleus. Note: neurons were only measured if both cytoplasm and nucleus were visible.

The optical density (OD) was derived using the formula:

\[
\text{optical density} = \log_{10} \left( \frac{\text{incident light}}{\text{transmitted light}} \right)
\]

This formula inverts the grey level scaling so that intense (dark) hybridization signal results in a high OD value. Data were normalized by subtracting the background OD value from each neuronal OD value. The neuronal OD values were then averaged for each nucleus and between 2 sections for each subject.

**Immunohistochemistry for NR1 Protein**

The procedure for immunohistochemical staining of NR1 protein has been described previously (14). Briefly, sections were deparaffinized in xylene and rehydrated through a graded series of ethanol to water. Sections were then antigen retrieved by microwaving in Tris/EDTA buffer (1 mM EDTA, 1 mM sodium citrate, 2 mM Tris; pH 9.0) on high for 12 min, left to cool for 15 min, washed in distilled H2O and proceeded to immunostaining. We used a goat polyclonal antibody specifically raised against a peptide mapping at the carboxyl terminus of the NMDA receptor 1 of human origin (sc-1467, Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:200 in 1% NRS for 40 min, washed in PBS, and then incubated in avidin-biotin alkaline phosphatase (Vectastain ABC kit; Vector Laboratories Inc.) in PBS for 40 min. Sections were then rinsed in Tris buffered saline (TBS) pH 7.6 and in 0.1 M Tris pH 9.5, and visualized by reaction with nitroblue tetrazolium and BCIP solution containing 5 μl of 1 M levamisole (Alkaline phosphatase substrate kit, Vector Laboratories Inc.). Sections were then washed in TBS and H2O, taken through a graded series of alcohol, cleared in histoclear, and mounted in vectamount. Negative controls included sections where the primary antibody was replaced with 1% NRS.

**Quantitative Immunohistochemical Analysis**

NR1 protein was quantified by manually counting the number of positively stained neurons and is represented as % positive. Again, quantitation was restricted to neurons and not glial cells. Images were viewed and captured as mentioned above using the Nikon Eclipse E800 light microscope and sensicam CCD camera and were captured at ×10 magnification. Each nucleus studied was captured in its entirety. Thus, the number of images captured per nucleus ranged from 2 (hypoglossal nucleus) to 8 (ION). In each image, neurons were identified and marked manually using the Zeiss KS 400 image analysis system. Positive-stained neurons were marked first and negative-stained neurons second. Positive neurons were identified by a blue stained cytoplasm. Neurons that had no staining in the cytoplasm (white/pale blue in color) or where the staining intensity was equivalent to the background intensity were identified as negative. Neuronal counts were generated automatically by the computer and exported to a spreadsheet (Microsoft Excel 2000). Counts were summed for the 2 to 8 images captured per nucleus and the number of positive neurons were expressed as a percent of the total neurons counted.

**Analysis**

Two adjacent tissue sections from each of the 2 brainstem levels (mid medulla and rostral pons) were stained for NR1 mRNA and NR1 protein for each infant (total 4 sections). Eight nuclei of the mid medulla and 2 nuclei of the rostral pons were analyzed. Nuclei at these 2 levels of the brainstem were selected for study on the basis of their functional relevance to cardiorespiratory, sleep, and arousal control. All 25 cases had a section at the mid medulla level but only 15 had sections available from the rostral pons. Slides were coded so that analysis was performed blinded to the study groups. To define the anatomic boundaries of brainstem nuclei, the sections were stained with hematoxylin and eosin and compared to the adjacent sections stained for NR1 mRNA and protein. The anatomical level of the mid medulla was defined with reference to Figure 23 from the atlas of the human brainstem by Paxinos and Huang (31). The following 8 nuclei were studied: hypoglossal (XII), dorsal motor nucleus of the vagus (DMNV), the principal inferior olivary nucleus (ION), arcuate nucleus (AN), the nucleus of the solitary tract (NTS), vestibular (Vest), cuneate (Cun), and nucleus of the spinal trigeminal tract (NSTT). Figure 41 from Paxinos and Huang (27) was used as a reference to define the anatomical level of the rostral pons and the following 2 nuclei were studied: locus coeruleus (LC) and pontine nuclei. Photomicrographs from a non-SIDS infant case representative of the mid-medulla and rostral pons levels studied and the location of their respective nuclei are presented in Figure 1A and 1D.

**Statistical Analysis**

Statistical analyses were undertaken using SPPS for Windows (V11.5; SPPS, Chicago, IL). Clinical and autopsy characteristics were compared between groups (15 SIDS and 10 non-SIDS) using Student t-test and are expressed as mean ± SD. Analysis of covariance (ANCOVA) was used to evaluate...
CHANGES IN NMDA RECEPTOR 1 IN SIDS BRAINSTEM

Fig. 1. Photomicrographs illustrating NR1 mRNA staining by nonradioactive in situ hybridization in the brainstem of 1 non-SIDS infant at the level of (A) mid medulla with enlargement of (B) XII and (C) NTS; (D) rostral pons with enlargement of (E) LC and (F) pontine nucleus. Calibration bar: A, D = 0.11 cm; B, C = 125 μm; E, F = 100 μm.

Fig. 2. Photomicrographs illustrating NR1 protein staining by immunohistochemistry in the brainstem of 1 non-SIDS infant at the level of (A) mid medulla with enlargement of (B) DMNV and (C) ION. Calibration bar: A = 0.11 cm; B, C = 80 μm.

differences in NR1 mRNA and protein quantification by diagnosis (SIDS vs non-SIDS), adjusted for age and gender. When there was a significant difference in quantification by case diagnosis across groups with ANCOVA, the Bonferroni procedure was employed to evaluate pairwise comparison of the means. Staining results are presented as age-adjusted mean ± SEM. A 2-sided p value of <0.05 was considered statistically significant.

TABLE 1
Causes of Death in the non-SIDS Group

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brompheniramine toxicity</td>
<td>1</td>
</tr>
<tr>
<td>Bronchopneumonia</td>
<td>5</td>
</tr>
<tr>
<td>Gastroenteritis</td>
<td>1</td>
</tr>
<tr>
<td>Intraabdominal teratoma</td>
<td>1</td>
</tr>
<tr>
<td>Septicemia</td>
<td>1</td>
</tr>
<tr>
<td>Waterhouse-Friedrichson syndrome</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

RESULTS

Dataset Characteristics

The dataset comprised 25 cases (10 non-SIDS and 15 SIDS). Causes of death in the non-SIDS group are given in Table 1. All of these non-SIDS cases died suddenly and none were admitted to hospital or ventilated prior to death. Thus, the infants were included because their history supports exposure to minimal or only terminal hypoxia. Comparison of the non-SIDS and SIDS groups showed no significant difference between gender, gestational age, age at death, birth weight, body weight, body length, and head circumference at death (Table 2). There was a nonsignificant trend towards higher brain weight in the non-SIDS group (p = 0.07). Information regarding sleep position was only available for 5 of the 10 non-SIDS cases, but was available for all 15 SIDS cases, for whom standardized questionnaire data had been collected. Of the 5 non-SIDS cases only 1 (20%) was found in the prone position compared with 6 out of the 15 (40%)
NR1 mRNA and Protein Staining

NR1 mRNA showed a similar pattern of distribution amongst the non-SIDS and the SIDS groups. Positive staining of neurons for NR1 mRNA was identified by a blue color reaction in the cytoplasm. The general staining pattern and cellular distribution of NR1 protein in a non-SIDS infant is presented in the mid medulla (Fig. 2A–C). Although NR1 protein was present in all nuclei studied, the number of neurons positive for NR1 protein varied among the nuclei. Thus, regardless of diagnosis the number of NR1 protein positive neurons was greatest in the XII and lowest in the NTS at the level of the mid medulla (Table 3) with a greater expression in the LC and rostral pontine nuclei at the level of the rostral pons (Table 4).

Staining Quantitation

For the staining quantitation, postmortem interval (non-SIDS: 17.4 ± 10.4 h vs SIDS: 19.5 ± 15.2 h) and fixation time (non-SIDS: 6.7 ± 1.5 vs SIDS: 7.6 ± 3.0 weeks) were not used as additional covariates because they did not differ significantly between the 2 groups. All

TABLE 2
Characteristics of the Dataset*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-SIDS (n = 10)</th>
<th>SIDS (n = 15)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:female</td>
<td>7:3</td>
<td>8:7</td>
<td>---</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>38.3 ± 2.63</td>
<td>37.6 ± 3.41</td>
<td>0.41</td>
</tr>
<tr>
<td>Age at death (months)</td>
<td>3.23 ± 1.22</td>
<td>2.83 ± 1.01</td>
<td>0.66</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.29 ± 0.99</td>
<td>2.71 ± 0.60</td>
<td>0.20</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>5.42 ± 1.34</td>
<td>5.03 ± 1.44</td>
<td>0.50</td>
</tr>
<tr>
<td>Brain weight (kg)</td>
<td>0.68 ± 0.11</td>
<td>0.59 ± 0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>Body length at death (cm)</td>
<td>58.2 ± 4.42</td>
<td>57.4 ± 5.61</td>
<td>0.73</td>
</tr>
<tr>
<td>Head circumference at death (cm)</td>
<td>39.9 ± 2.71</td>
<td>38.4 ± 1.94</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* The values are expressed as mean ± SD.

TABLE 3
NR1 mRNA and Protein in 8 Nuclei at the Level of the Mid Medulla. Comparison between non-SIDS (n = 10) and SIDS (n = 15)*

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Non-SIDS</th>
<th>SIDS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>XII</td>
<td>0.33 ± 0.02</td>
<td>0.45 ± 0.02</td>
<td>0.001</td>
</tr>
<tr>
<td>ION</td>
<td>0.26 ± 0.02</td>
<td>0.33 ± 0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>AN</td>
<td>0.26 ± 0.03</td>
<td>0.31 ± 0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>Cun</td>
<td>0.22 ± 0.02</td>
<td>0.31 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vest</td>
<td>0.20 ± 0.02</td>
<td>0.27 ± 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>DMNV</td>
<td>0.17 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>NSTT</td>
<td>0.14 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>NTS</td>
<td>0.14 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Non-SIDS</th>
<th>SIDS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>XII</td>
<td>81.3 ± 3.2</td>
<td>80.7 ± 2.6</td>
<td>0.89</td>
</tr>
<tr>
<td>ION</td>
<td>75.5 ± 1.9</td>
<td>77.1 ± 1.5</td>
<td>0.51</td>
</tr>
<tr>
<td>AN</td>
<td>74.4 ± 3.1</td>
<td>76.3 ± 2.6</td>
<td>0.63</td>
</tr>
<tr>
<td>Cun</td>
<td>72.8 ± 3.4</td>
<td>71.1 ± 2.8</td>
<td>0.70</td>
</tr>
<tr>
<td>Vest</td>
<td>62.3 ± 3.7</td>
<td>58.2 ± 3.1</td>
<td>0.40</td>
</tr>
<tr>
<td>DMNV</td>
<td>67.8 ± 2.2</td>
<td>74.1 ± 1.8</td>
<td>0.04</td>
</tr>
<tr>
<td>NSTT</td>
<td>46.3 ± 3.6</td>
<td>35.8 ± 2.9</td>
<td>0.03</td>
</tr>
<tr>
<td>NTS</td>
<td>42.5 ± 3.3</td>
<td>35.8 ± 2.7</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* The values are expressed as age-adjusted mean OD ± SEM for NR1 mRNA and age-adjusted mean % positive ± SEM for NR1 protein. Abbreviations: XII, hypoglossal; ION, principal inferior olivary nucleus; AN, arcuate nucleus; Cun, cuneate; Vest, vestibular; DMNV, dorsal motor nucleus of the vagus; NSTT, nucleus of the spinal trigeminal tract; and NTS, nucleus of the solitary tract.
NR1 mRNA and Protein in 2 Nuclei at the Level of the Rostral Pons. Comparison between non-SIDS (n = 7) and SIDS (n = 8)*

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Non-SIDS</th>
<th>SIDS</th>
<th>p value</th>
<th>Non-SIDS</th>
<th>SIDS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>0.17 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>0.23</td>
<td>81.1 ± 3.0</td>
<td>79.4 ± 2.8</td>
<td>0.71</td>
</tr>
<tr>
<td>Pontine</td>
<td>0.19 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>0.36</td>
<td>65.7 ± 4.5</td>
<td>71.9 ± 4.1</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* The values are expressed as age-adjusted mean OD ± SEM for NR1 mRNA and age-adjusted mean % positive ± SEM for NR1 protein.

DISCUSSION

This study examined the expression of NR1 mRNA and protein in the brainstem of human infants and assessed whether expression was increased in SIDS infants compared to non-SIDS infants. The 2 major findings of this study are that NR1 mRNA was increased in 6 medullary nuclei and NR1 protein expression was only altered in 2 of the same 6 medullary nuclei of SIDS infants. Taken together, these findings support the hypothesis that abnormalities of the NMDA receptor are present in the brainstem of SIDS infants.

NR1 mRNA and Protein Expression in the Infant Brainstem

In this study, we report for the first time the distribution and quantification of NR1 mRNA and NR1 protein expressions in 8 nuclei of the mid medulla and 2 nuclei of the rostral pons in human infants. The localization of NMDA receptors in the human infant brainstem has been studied by autoradiography (11). However, such ligand-binding studies are limited in that they do not discriminate between the different subtypes of receptors nor do they give detailed information regarding the cellular distribution of the receptor. Methods such as in situ hybridization and immunohistochemistry allow for specific cellular localization of receptors and their subtypes.
Our results show that NR1 mRNA and protein are widely distributed in the mid medulla and rostral pons in the human infant, which is consistent with the distribution of NR1 mRNA and protein reported in the piglet (14), cat (15), rat (13, 16), and mouse (17). Considering the wide distribution of NR1 in the infant brainstem, we suggest that the NMDA receptor has functional significance in all nuclei controlling cardiorespiratory, sleep, and/or arousal mechanisms, possibly in a quantifiable fashion.

The quantitative pattern of NR1 mRNA and protein expression among the nuclei were similar, with both NR1 mRNA and protein expressions greater in the XII nucleus and least in the NTS, a pattern similarly observed for NMDA receptor binding by autoradiography in the infant medulla (11). Interestingly, AMPA and kainate binding in the infant medulla were opposite, with higher AMPA and kainate binding in the NTS compared to the XII nucleus (8, 11). Taken together, these findings suggest that neurons of the XII may be functionally more dependent on the NMDA glutamatergic receptors compared to neurons of the NTS, which may have a tendency to be functionally dependent on the AMPA and kainate glutamatergic receptors.

Although NR1 mRNA was found in all neurons of all the nuclei studied, this does not imply all mRNA is translated into protein or functional NMDA receptors. It has been shown that the NR1 subunit that was abundantly expressed intracellularly is only transported to the plasma membrane when it is coassembled with an NR2 subunit (32). Thus, it remains possible that the expression and distribution of receptor subunits is different from that of functional receptors. This may explain some of the differences in the detection levels and expression pattern of NMDA in the human infant brainstem when comparing in situ hybridization and immunohistochemical results to the autoradiographic results of Panigrahy et al (11).

Non-SIDS vs SIDS

As hypothesized, NMDA receptor expression was different in SIDS infants compared to non-SIDS infants. However, all such changes were restricted to nuclei within the mid-medulla. The changes were more evident for NR1 mRNA than for NR1 protein where mRNA was increased in 6 nuclei. The pattern of changes in NR1 expression support our hypothesis that NMDA excitotoxic mechanisms may contribute to neuronal cell death in SIDS infants. However, given that we measured protein expression as well, we distinguished 3 patterns of change: 1) NR1 mRNA and protein were both increased in the DMNV; 2) mRNA was increased but protein decreased in the NSTT; and 3) mRNA was increased but protein unchanged in the XII, ION, vestibular and cuneate nuclei.

We did not observe any change in protein without mRNA also being affected.

At the molecular level the normal process is for NR1 mRNA to be translated to protein, which forms functional NMDA receptors by coassembling with other NMDA subunits (NR2A-D and/or NR3A,B). In this study, altered mRNA expression was translated to altered protein expression in the DMNV of SIDS infants. Although our study did not extend to the functional receptor level, abnormal heart rate and blood pressure responses have been reported in some SIDS cases (33–35) (both physiological parameters are controlled by the DMNV), suggesting some functional correlation.

The differential change between NR1 mRNA and protein we observed in the NSTT of our SIDS group, i.e. increased mRNA and decreased protein, has been reported in cerebellar granule cell cultures in response to chronic depolarization (36) in some brain areas of the mouse following chronic ethanol treatment (37) and in the DMNV of our piglet model after intermittent hypercapnic-hypoxia (IH) (14). Possible explanations for this differential change include receptor downregulation and/or neuronal loss. Stereological counting would be required to confirm loss of neurons and was not part of this study. Although no other study to date has investigated for cell loss in the NSTT in SIDS infants, cell loss has been studied in the XII and DMNV of the SIDS brainstem, although results are conflicting (38–40). In the ION, a marginal decrease in neuronal numbers has been reported (41). The issue of cell loss as a specific neuropathological finding in SIDS infants appears to remain open. The association between increased apoptosis and cell loss is relevant to this discussion. Previously, we found increased apoptosis in the NSTT of the SIDS medulla (9), which may support the contention of cell loss being the reason for the decrease in NR1 protein observed in the current study. Given that increased NMDA receptor expression suggests an increased vulnerability to excitotoxic neuronal damage and cell death, our finding of increased NR1 mRNA may underlie increased neuronal apoptosis in SIDS infants.

The dissociation between mRNA and protein expression in the XII, ION, vestibular, and cuneate nuclei of SIDS infants may reveal a problem of mRNA turnover, with overabundance of mRNA that is not expressed at the protein level. This suggests a defect in translation of mRNA to protein in some nuclei of the SIDS medulla. Alternatively, the changes in mRNA may have commenced without time for translation to protein prior to death. This would also suggest that the pathological process resulting in increased NR1 expression is occurring at a faster rate in the DMNV compared to the other nuclei.
It is uncertain what functional changes would result from the dissociation between mRNA and protein, particularly since the protein expression is “normal.” If the proper level of NR1 protein is present but mRNA is not, would this have functional significance? It remains possible that although mRNA was affected in the XII, ION, vestibular and cuneate nuclei, this would not have translated to physiological compromise.

The lack of significant changes in NR1 mRNA and/or protein levels amongst other nuclei in SIDS infants does not preclude the involvement of NMDA receptors within those nuclei. We only studied for the NR1 subunit, which, although is obligatory for functional NMDA receptors, does not determine the electrophysiological properties of the receptor that are regulated by the NR2 (A-D) subunits (13). Future studies of other NMDA receptor components, for example, NR2 subunit expression (via in situ hybridization and immunohistochemistry) and pharmacological studies for NMDA receptor site binding would clarify these issues when comparing SIDS infants to non-SIDS infants. Such studies will provide a better understanding of the role of NMDA receptors in mediating responses under the pathological conditions hypothesized in SIDS infants, such as hypoxia, hypercapnia, and ischemia, alone or in combination.

Possible Mechanism(s) Involved in NMDA Receptor Expression Changes

The precise mechanism involved in the changes of NR1 mRNA and protein expression is not known. So, it is not clear whether the changes in NMDA expression could actually cause death, or instead, be the result of some event (e.g. prolonged or repeated apnea) preceding and underlying death from SIDS. If the changes in NR1 caused death in SIDS, this would imply that SIDS infants have a pre-existing defect in the NMDA receptor expression and thus, a predisposing vulnerability to an exogenous stressor. If this vulnerability overlaps with a critical developmental period and the presence of an exogenous stressor, the infant will die of SIDS as proposed by the triple-risk model (42). If the NMDA receptor changes occur as a result of the mechanism of death in SIDS, this would imply that the changes in the NMDA receptor expression are secondary to other factors and most likely induced by the exogenous stressor. An hypoxic stimulus is a strong candidate as the common exogenous stressor in the above scenarios since the NMDA receptor is highly vulnerable to hypoxic exposures. Studies of non-SIDS infants who died with known prior exposure to moderate or severe hypoxia would help to clarify these issues.

Given that the NMDA receptor is responsible for neuronal maturation and plasticity (11, 12), changes in its expression may be associated with synaptic plasticity after injury (43). It has been suggested that NR1 mRNA upregulation may facilitate reactive synaptogenesis; a reorganization of neurons to alter their connections or form new ones in response to lesions (44). Thus, upregulation of NR1 mRNA in the SIDS medulla may be associated with transient, albeit unsuccessful, attempts at regeneration of neurons after hypoxic injury.

There is strong evidence that changes in the NMDA system are inducible during early development. We recently showed that a stimulus of intermittent hypercapnic-hypoxia (IHH), which was designed to mimic SIDS risk factors such as rebreathing in the prone sleeping position, increased NR1 mRNA levels in the XII, DMNV, and gracile nuclei of the piglet caudal medulla, and simultaneously decreased NR1 protein levels in the DMNV (14). This finding is significant for 2 reasons. First, it shows that the NMDA receptor is sensitive to the more common clinical stimulus of hypercapnic hypoxia. Second, it supports the notion that the changes in NR1 mRNA and protein expressions may be induced by postnatal environmental insults with no pre-existing defect. The changes observed in the piglets were not as dramatic nor were they present in as many nuclei as seen in the SIDS infants. It therefore remains possible that SIDS infants have a specific and innate abnormality in NMDA receptors.

Limitations of the Study

We have previously reported the use of a non-RISH method to study the cellular distribution and pattern of staining of NR1 mRNA in formalin-fixed and paraffin-embedded human brain tissue (28). One of the advantages of this non-RISH method is that it can be quantified using computer-based densitometry with measurements reported in optical density (OD) units (29, 30). However, there are 2 major issues concerning such immunodensitometric measurements: the microscopic setting and the quantitative association between mRNA and OD, both of which were accounted for in our study. Methodologically, the stability of the light source and the calibration of the measurements must be strictly controlled (29). In this study, this was achieved prior to image capturing, by standardizing the microscope settings, white balancing the camera for each tissue section, and by standardizing the exposure time.

To account for a possible nonlinear association between mRNA expression and OD values, given that results are dependent on an antigen-antibody reaction, we were rigorous in our standardization of the non-RISH method. We achieved this by including sections from all cases in the same run and by staining all cases in a duplicate run, confirming that there was <10% difference between the 2 runs for all nuclei for all cases. While this has been accepted as adequate for analyzing differences...
induced in pathological conditions (14, 30), the ideal solution would be to determine the precise relationship between mRNA levels and OD values and, therefore, have an external assay reference.

As with all SIDS pathology research, it is very hard to obtain good control groups. The 5 control cases with a postmortem diagnosis of bronchopneumonia may have experienced hypoxia prior to death. However, all 5 died suddenly and unexpectedly, so we have assumed that no chronic or repeated hypoxia occurred. Nevertheless, further verification of our findings in a larger dataset will be required.

Conclusion

This study contributes to the increasing evidence that neurotransmitter abnormalities exist in SIDS infants. We have demonstrated that NR1 mRNA and protein are widely expressed in nuclei of homeostatic function in the human infant mid medulla and rostral pons, and that the overall pattern of expression among nuclei was not different between SIDS and non-SIDS infants. Nonetheless, expression of NR1 mRNA was greater in SIDS than non-SIDS infants in 6 of 8 nuclei associated with cardiorespiratory control or facial sensation, while NR1 protein was altered in 2 of these 6 nuclei. Taken together, we suggest that NMDA receptor expression is altered in brainstem medullary nuclei that regulate cardiorespiratory functions in some SIDS infants. This abnormality could also underlie our previous report of increased neuronal cell death in the same regions in SIDS infants.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Bronwyn Relf for her technical support in establishing the nonradioactive in situ hybridization method and Mr. Dennis Dwarte (Electron Microscopy Unit, University of Sydney) for assistance in setting up the image analysis software for quantitative measurements.

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Received March 14, 2003
Revision received July 7, 2003
Accepted July 15, 2003