Sudden Infant Death Syndrome: Increased Number of Synapses in the Hypoglossal Nucleus

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Abstract. The medulla was sampled from nine cases of sudden infant death syndrome (SIDS) and from six age-matched control cases without neurological disease. Morphometric analyses were performed on serial Nissl sections through the hypoglossal nucleus on the left side of the medulla. The total volume of the nucleus and both the numerical density (Nv, cells per mm³) and total number of neurons were measured. Tissue from the remaining hypoglossal nucleus was prepared for electron microscopy using the ethanolic phosphotungstic acid method to stain synaptic contacts. Stereological analyses were performed to determine the Nv and total number of synapses. Total volume of the hypoglossal nucleus was significantly greater (36%) in SIDS cases than in controls. The Nv of neurons was significantly less than in controls (28%), although the total number of neurons did not differ significantly. The mean profile area of motor neuron cell bodies was significantly greater (30%) in SIDS cases, with no differences in the mean profile areas for interneurons or glia. The Nv of synapses did not differ significantly between SIDS cases and controls, although the total number of synapses was greater (61%) in SIDS. These abnormalities in growth indicate a greater volume of neuropil in a hypoglossal nucleus containing a normal complement of neurons. The greater number of synapses in SIDS cases is consistent with a failure to eliminate normally extraneous synapses during early development.

Key Words: Growth and development; Hypoglossal nucleus; Medulla; Morphometry; Motor neurons; Sudden infant death; Synaptogenesis.

INTRODUCTION

Sudden infant death syndrome (SIDS) has been defined as “the sudden death of an infant under one year of age which 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). A recent focus of research on SIDS has been the possibility that an abnormal development of neuronal circuitry, involving brainstem neurons which regulate respiratory breathing and arousal, underlies the cardiorespiratory instability characteristic of susceptible infants (2–10). Abnormalities in the early postnatal development of the central nervous system have been reported in SIDS (for reviews, see 11, 12). These include increased brain weight (13–15), increased volume of the brainstem (16) and altered tissue concentrations of catecholamines, β-endorphin, met-enkephalin and substance-P (17–20). Hypomyelination of axons in the central nervous system has been reported (14), as well as decreased numbers of small myelinated axons in the vagus nerve (21, 22). In SIDS cases the density of dendritic spines on various respiratory neurons in the brainstem has been shown to be 75–180% greater than in control cases (23, 24). In the central reticular nucleus of the medulla, which includes neurons of the ventral respiratory group, the density of synapses was found to be 38% greater in SIDS cases (25). This increased density of both dendritic spines and synapses in respiratory nuclei of the brainstem presumably reflects a persistence of normally transient synaptic contacts on brainstem neurons during early postnatal development.

In a previous morphometric study, we have demonstrated an increased volume of the hypoglossal nucleus in SIDS cases (15). Linear regression analysis revealed that the rate of increase in the volume of the hypoglossal nucleus during early postnatal development was 79% greater in SIDS cases than in age-matched control cases, suggesting accelerated growth. Although the total number of hypoglossal neurons in SIDS cases did not differ from controls, the numerical density of neurons (Nv, cells per mm³) was decreased by 30%, indicating a greater volume of neuropil. Since the Nv of glial cells was normal, the increased volume did not appear to result from gliosis. The disproportionately rapid increase in the volume of neuropil in the hypoglossal nucleus of SIDS cases most likely results from a more intricate plexus of axons and dendrites among the cell bodies of hypoglossal neurons.

The present study was conducted to compare postnatal changes in the Nv and total number of both neurons and synapses in the hypoglossal nucleus of SIDS cases and age-matched control cases without neurological disease. Although this nucleus is not generally included among classically defined respiratory centers (26), discrete populations of hypoglossal motor neurons with peak activities during inspiration, expiration or transitional phases have been identified on the basis of respiratory-related patterns of discharge (27).

MATERIALS AND METHODS

Brains from nine SIDS cases and six non-SIDS control cases were selected after autopsy for inclusion in this study. The SIDS
TABLE I
Clinical Variables for SIDS Cases and Controls

<table>
<thead>
<tr>
<th></th>
<th>SIDS</th>
<th>Control</th>
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<tbody>
<tr>
<td>Cases (n)</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Age (postconceptional weeks)*</td>
<td>49.8 ± 2.8</td>
<td>49.9 ± 7.1</td>
</tr>
<tr>
<td>Postmortem interval (hours)*</td>
<td>16.5 ± 2.3</td>
<td>13.3 ± 2.5</td>
</tr>
<tr>
<td>Male/female cases</td>
<td>3/6</td>
<td>2/4</td>
</tr>
<tr>
<td>FT/PT infants</td>
<td>7/2</td>
<td>5/1</td>
</tr>
<tr>
<td>Gestational age at birth for PT infants (weeks)</td>
<td>34, 36</td>
<td>36</td>
</tr>
<tr>
<td>Body weight (kg)*</td>
<td>5.82 ± 0.51</td>
<td>5.00 ± 0.78</td>
</tr>
<tr>
<td>Brain weight (g)*</td>
<td>663 ± 47</td>
<td>558 ± 88</td>
</tr>
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</table>

* Values are expressed as the mean ± standard error of the mean.

...thionin. Adjacent sections were stained with hematoxylin and eosin.

From the right half of each medulla 6–8 blocks of tissue (1.0 × 1.0 × 0.5 mm) were sampled for electron microscopy from the hypoglossal nucleus. These blocks were stained using a modification of the ethanolic phosphotungstic acid (EPTA) method, which reliably stains synaptic contacts in human autopsy material with postmortem intervals as long as 24–36 hours (28–30). Briefly, blocks of fixed tissue were washed in phosphate buffer and dehydrated to 95% ethanol. Blocks were stained for 90 minutes in 1% phosphotungstic acid in absolute ethanol, slightly hydrated by the addition of 2 drops of 95% ethanol per 10 ml of staining solution. The blocks were equilibrated in propylene oxide and embedded in Araldite. Ultrathin sections of silver-grey interference color (60 nm) were cut and mounted on #200 square mesh grids.

After sampling tissue from the medulla, an additional 15–18 blocks were cut from standardized regions in the remaining brain and spinal cord. Following fixation by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 10–15 days, the blocks were embedded in paraffin, sectioned at 8 μm and stained with hematoxylin and eosin. These sections were used for the pathological examination of all cases.

The serial Nissl sections were used to determine the total volume of the hypoglossal nucleus and both the Nv and total number of hypoglossal neurons. All histological sections were coded to prevent experimenter bias. For the total volume of the hypoglossal nucleus, individual sections were examined at a final magnification of ×40 and the area of the nucleus was measured in mm² (Bioquant System IV, R&M Biometrics, Nashville, TN) following the boundary criteria of Olszewski and Baxter (31) as illustrated in Figure 1. Volume was calculated from:

\[ V = \Sigma A \times T \times 4, \]

where \( \Sigma A \) is the sum of area measurements, \( T \) is section thickness and 4 is the periodicity of the section sample. The \( N_v \) of neurons was measured using the method of Abercrombie (32). Briefly, sections were examined at a final magnification of ×400. Neurons were counted when their nuclear profiles contained a distinct nucleus. The \( N_v \) was calculated from:

\[ N_v = N_s/((D + T)/2), \]

where \( N_s \) is the number of neurons per unit area of section, \( D \) is the mean diameter of the nucleus and \( T \) is section thickness.

Data were collected separately for hypoglossal motor neurons and interneurons. Motor neurons were identified by their larger diameters and dense accumulation of Nissl bodies in the cytoplasm (Fig. 2). Interneurons were identified as smaller profiles with round to ovoid cell bodies containing scant cytoplasm with relatively uniform staining intensity (Fig. 2). The \( N_s \) of glia was measured using the same method, although the nucleus was employed as the target object and cells were counted only when their profiles contained a distinct nucleus. The total number of neurons for each nucleus was calculated from estimates of \( N_s \) and hypoglossal volume. For individual neurons the profile areas of the cell body, nucleus and nucleolus were measured at a final magnification of ×1,000 in the plane of focus which contained the nucleolus. Calculations of mean profile area were based on approximately 300–400 motor neurons and 200–250...
interneurons in each hypoglossal nucleus. For glial cells the mean profile area of the nucleus was determined from approximately 500-600 cells per case.

The EPTA-stained ultrathin sections were used to measure the $N_v$ of synapses in the hypoglossal nucleus. Electron micrographs were randomly sampled on sections from 4–5 blocks per nucleus. Sections were photographed at a magnification of $\times 4,800$ using Kodak #4489 e.m. film (8.3 $\times$ 10.2 cm sheets) which was developed in D-19 for 4.5 minutes to obtain maximum contrast. Individual negatives were illuminated on a light table and examined with a dissecting microscope (variable magnification $\times 6–12$) for a final magnification of $\times 25,800–51,600$. For each nucleus a sample of 70–80 negatives was obtained, resulting in a sample area of approximately 36,000 $\mu$m$^2$. The $N_v$ of synapses in the hypoglossal nucleus was calculated from:

$$N_v = (2 \times N_s)/(D + T),$$

where $N_s$ is the number of synaptic contacts per unit area of micrograph, $D$ is the mean length of individual contacts, and $T$ is section thickness (25, 28–30).

RESULTS

The morphological appearance of the hypoglossal nucleus was relatively mature in the youngest cases. The boundaries of this nucleus were conspicuous by the presence of large motor neurons (Fig. 1). Throughout most of the serial sections for each case the hypoglossal nucleus was limited dorsomedially by the floor of the fourth ventricle, dorsolaterally by the nucleus intercalatus and the dorsal nucleus of the vagus, ventrolaterally by the central reticular nucleus and ventrally along the most rostral third by the nucleus of Roller. The gross morphology of the hypoglossal nucleus did not differ consistently between SIDS cases and controls. In many of the SIDS cases there appeared to be a subtle decrease in the boundary area of the nucleus with a corresponding decrease in the density of neurons (Fig. 1). In both SIDS cases and controls no signs of necrosis, inflammation or gliosis were observed in the central nervous system following examination of all histological sections stained with either thionin or hematoxylin and eosin.

The results of the morphometric analyses are presented in Table 2 and Figure 4. The total volume of the hypoglossal nucleus was significantly greater in SIDS cases when compared to controls (36%, $p < 0.05$). The $N_v$ of hypoglossal neurons was significantly less in SIDS cases.
Fig. 2. Neurons of the hypoglossal nucleus in a control case at 43 weeks of age. Motor neurons (left) were distinguished from interneurons (right) by their larger diameters and dense accumulation of Nissl bodies in the cytoplasm. The nuclei of glial cells appeared as round to ovoid profiles with moderate staining of the chromatin. Nissl stain (thionin), bar = 15 μm.

for both motor neurons (28%, p < 0.05) and interneurons (27%, p < 0.05). Using estimates of Nν and total volume to calculate the total number of neurons in the hypoglossal nucleus, no significant differences were found between SIDS cases and controls. In both groups the total number of neurons in one nucleus ranged from approximately 9,000–14,000, with 6,000–10,000 motor neurons and 2,800–3,600 interneurons. These results indicate that the hypoglossal nucleus in SIDS cases contains the normal complement of neurons in a nucleus of greater volume. This resulted in a greater separation of neuronal cell bodies with a decrease in packing density and an increase in the volume of neuropil among the neurons. The Nμ of glia did not differ significantly between SIDS cases and controls, indicating that glialosis was not a factor in the increased volume of the nucleus and the decreased Nν of neurons.

The mean profile area of motor neuron cell bodies was significantly greater in SIDS cases than in controls (30%, p < 0.02). Similarly, the mean profile area for the nucleolus of motor neurons was found to be 19.46 ± 0.67 μm² in SIDS cases and 16.47 ± 0.65 μm² in controls, demonstrating a significant increase in SIDS cases (18%, p < 0.01). However, the mean profile area for the nucleus of motor neurons did not differ significantly between the groups (236 ± 15 μm² in SIDS and 210 ± 21 μm² in controls). An examination of the size-frequency histograms for neuronal profile area in individual cases revealed no consistent differences in the overall range of measurements between the groups. These results suggest that the increase in mean profile area for hypoglossal motor neurons in SIDS cases resulted from a shift in the entire distribution toward profiles of greater area, rather than from a loss of the smallest neurons. No significant differences were found between the groups in the mean profile areas of interneurons or glia (Table 2).

Examination of the EPTA-stained sections by electron microscopy revealed numerous synaptic contacts in the neuropil of the hypoglossal nucleus (Fig. 3). Synapses were identified by a prominent synaptic cleft and high-contrast staining of the presynaptic projections and post-synaptic density. In many profiles an intracellular line could be distinguished (Fig. 3A). This modification of the EPTA staining method produced a low-contrast staining of certain background structures, including neurofilaments, microtubules, ribosomes and intranuclear proteins. The staining of neurofilaments in the presynaptic terminals assisted in the identification of the smaller synaptic profiles.

TABLE 2
Morphometric Variables for the Hypoglossal Nucleus in SIDS Cases and Controls

<table>
<thead>
<tr>
<th></th>
<th>SIDS</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>Volume (mm³)</td>
<td>5.067 ± 0.363</td>
<td>3.732 ± 0.432*</td>
</tr>
<tr>
<td>Nν (cells per mm³)</td>
<td>1,681 ± 81</td>
<td>2,335 ± 277*</td>
</tr>
<tr>
<td>Motor neurons</td>
<td>600 ± 35</td>
<td>836 ± 89*</td>
</tr>
<tr>
<td>Interneurons</td>
<td>71,090 ± 2,414</td>
<td>72,828 ± 3,431</td>
</tr>
<tr>
<td>Total number of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>neurons</td>
<td>11,354 ± 481</td>
<td>11,210 ± 637</td>
</tr>
<tr>
<td>Motor neurons</td>
<td>8,355 ± 430</td>
<td>8,236 ± 551</td>
</tr>
<tr>
<td>Interneurons</td>
<td>2,998 ± 111</td>
<td>2,935 ± 126</td>
</tr>
<tr>
<td>Mean cell profile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>areas (μm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motor neurons</td>
<td>844.4 ± 42.8</td>
<td>649.5 ± 52.4**</td>
</tr>
<tr>
<td>Interneurons</td>
<td>173.6 ± 12.3</td>
<td>156.2 ± 7.8</td>
</tr>
<tr>
<td>Glia</td>
<td>27.0 ± 0.9</td>
<td>27.2 ± 2.1</td>
</tr>
<tr>
<td>Hypoglossal synapses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nν (×10⁶ per mm³)</td>
<td>2.339 ± 0.136</td>
<td>2.115 ± 0.218</td>
</tr>
<tr>
<td>Nμ (×10⁶ per mm³)</td>
<td>43.266 ± 3.069</td>
<td>38.743 ± 3.289</td>
</tr>
<tr>
<td>Mean contact length (μm)</td>
<td>0.308 ± 0.007</td>
<td>0.312 ± 0.015</td>
</tr>
<tr>
<td>Total number (×10⁶)</td>
<td>12.041 ± 1.268</td>
<td>7.493 ± 0.400***</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard error of the mean. The statistical significance of differences between groups was determined using Student's t-test: * p < 0.05, ** p < 0.02 and *** p < 0.01.
Fig. 3. Electron micrographs of EPTA-stained synapses in the hypoglossal nucleus of SIDS cases at (A) 40 and (B) 62 postconceptional weeks of age. Synaptic contacts were identified by dense staining of the presynaptic projections and postsynaptic density. In cases less than 50 weeks of age (A) the presynaptic projections appeared as a relatively continuous serrated band along the presynaptic side of the synaptic cleft. In older cases (B) the presynaptic projections were organized into discrete masses along the presynaptic membrane. EPTA stain, (A) $\times 25,080$; (B) $\times 39,900$.

The morphology of EPTA-stained synapses in the hypoglossal nucleus did not differ remarkably between SIDS cases and controls. However, subtle morphological differences could be distinguished as a function of age. In cases less than 50 postconceptional weeks of age, the presynaptic projections were relatively continuous, having the appearance of a serrated band of irregular width running parallel to the synaptic cleft (Fig. 3A). In older cases the presynaptic projections were separated into discrete masses along the presynaptic membrane, having the morphological appearance characteristic of adult synapses (Fig. 3B).

The results of the stereological analyses are presented in Table 2 and Figure 4. Synaptogenesis in the hypoglossal nucleus of control cases was characterized by a gradual decrease in the $N_v$ of synapses from approximately 240 million synapses/mm$^3$ at 40 weeks to 130 million synapses/mm$^3$ at 84 weeks. The mean length of synaptic contacts increased from approximately 0.29 $\mu$m at 40 weeks to 0.38 $\mu$m at 84 weeks. The total number of synapses in one hypoglossal nucleus did not increase appreciably from 40 weeks to 84 weeks of age, holding at approximately 750 million synapses. Comparing SIDS cases and controls, there were no significant differences in the $N_m$, $N_n$ or mean contact length of synapses in the hypoglossal nucleus. However, the mean for total number
of hypoglossal synapses was significantly greater in SIDS cases (61%, p < 0.01).

Further analysis of these data revealed no significant differences between male and female cases and no consistent differences between full term and preterm infants. No consistent differences were observed in any of the morphometric variables as a function of postmortem interval. Correlation coefficients between postmortem interval and hypoglossal volume were not significant for SIDS cases (r = 0.392, p > 0.05) or controls (r = 0.086, p > 0.05), indicating no systematic change in volume as a function of postmortem interval. Similarly, correlations between postmortem interval and mean profile area of motor neuron cell bodies were not significant for SIDS cases (r = 0.111, p > 0.05) or controls (r = 0.314, p > 0.05). No consistent differences in any of the morphometric variables were observed as a function of attempted resuscitation.

DISCUSSION

The morphometric analyses in the present study have demonstrated developmental abnormalities in the hypoglossal nucleus in SIDS. The increased volume of the nucleus, the decreased Nv of neurons and the increased profile area of motor neuron cell bodies agree with the findings of our previous study (15), suggesting a subtle overgrowth of the hypoglossal nucleus. The fact that the Nv of glia did not differ significantly between SIDS cases and controls indicates that the increase in volume was not the result of gliosis. If the increased volume of the hypoglossal nucleus and the increased size of motor neuron cell bodies were due entirely to subtle swelling associated with cerebral edema, then one would expect to observe a corresponding decrease in the Nv of synapses. This was not the case. Although the mean profile areas of motor neuron cell bodies and nucleioli were greater in SIDS, the mean profile area of the nucleus did not differ significantly. Furthermore, profile areas for interneurons (i.e. cell body, nucleus and nucleolus) and glia did not differ between SIDS cases and controls. These results demonstrate a relatively selective hypertrophy of motor neurons in the hypoglossal nucleus. The 61% increase in the total number of hypoglossal synapses provides evidence that the pathogenesis of SIDS involves a disorder of synaptogenesis during early postnatal development. In a previous study we reported a 38% increase in the Nv of synapses in the central reticular nucleus of SIDS cases (25). Since the relatively indistinct boundaries of this region in the human medulla did not permit a reliable estimate of its volume, the total number of reticular synapses could not be determined. However, a 30% decrease in the Nv of reticular neurons and a 39% increase in the mean profile area of neuronal cell bodies were observed. The similarity of these findings to those of the hypoglossal nucleus suggest that an increase in the volume of the central reticular nucleus in SIDS is not unlikely. Thus, an increase in the total number of reticular synapses in SIDS cases would be greater than the 38% reported for Nv. The increased number of synapses in both the hypoglossal nucleus and the central reticular nucleus of SIDS cases suggests that disordered synaptogenesis during early postnatal development may be widespread in the brainstem.

Given the absence of necrosis and inflammation in the medulla of these SIDS cases, the significant increase in the total number of synapses in the hypoglossal nucleus would not reflect axonal sprouting in response to injury. It remains uncertain as to whether the increased number of synapses results from an initial overproduction of synapses during the progressive phase of synaptogenesis or from a failure to eliminate normally transient synaptic contacts during the regressive phase. Previous studies of developmental changes in the density of dendritic spines on hypoglossal neurons in SIDS cases are equivocal in this regard. Quattrochi and collaborators (23) reported that normal development of the hypoglossal nucleus during the first postnatal year is characterized by a decrease in spine density. In SIDS cases the density of dendritic spines exceeded normal values by 113% (23), suggesting a persistence of normally transient dendritic spines and their associated synaptic contacts. In a subsequent study, Takashima and Becker (24) found no difference between SIDS cases and controls in the density of dendritic spines on hypoglossal neurons. The results of the present study suggest that normal development of the hypoglossal nucleus is characterized by a decrease in the Nv of synapses, while the total number of synapses did not change appreciably during the first postnatal year. Given the relatively small number of control cases employed in this study, an accurate determination of normal postnatal changes in these variables would require a larger sample. Although the Nv of synapses did not differ between SIDS cases and controls, the significant increase in total number indicates the presence of anomalous axonal projections within the hypoglossal nucleus of SIDS cases.

Stereological analyses of synaptogenesis by electron microscopy do not permit the identification of parent neurons giving rise to these extraneous projections. The identification of anomalous axonal projections to the hypoglossal nucleus from various afferent sources remains an important objective for research on the pathogenesis of SIDS. There are two distinct populations of neurons in the hypoglossal nucleus (33, 34). The large multipolar motor neurons, which are thought to be cholinergic (35), innervate the extrinsic muscles of the tongue. There is no evidence that these motor neurons give rise to recurrent collaterals (36), which could contribute to the increased number of synapses. The second population of small interneurons, which are thought to be GABAnergic (37), have locally arborizing axons. Major sources of afferent
input to the hypoglossal nucleus in the adult include neurons of the parvocellular reticular nucleus, the spinal trigeminal nucleus, the dorsal nucleus of the vagus, the nucleus of the solitary tract, the medial parabrachial nucleus, the nucleus subcoeruleus and the nucleus raphe obscurus (36, 38, 39). Axon remodeling and synapse elimination involving afferents to the hypoglossal nucleus from these regions have not been studied systematically during normal development. Respiratory nuclei of the brainstem which project to the hypoglossal nucleus include the dorsal nucleus of the vagus, the nucleus of the solitary tract and the medial parabrachial nucleus (26). The failure to eliminate normally transient projections from these nuclei or from other anomalous nuclei during early postnatal development would likely contribute to the respiratory instability characteristic of infants susceptible to SIDS. Experimental studies in newborn kittens have shown that oral stimulation, which involves predominantly trigeminal afferents, can provoke episodes of apnea (40). The duration of the apnea was greatest in neonates and decreased with age until the third postnatal week. The abnormal development of afferents to the brainstem from the spinal trigeminal nucleus could conceivably prolong this period of reduced threshold to apnea. The relationship between an increase in the number of hypoglossal synapses and the reorganization of afferent input to the nucleus from various sources during postnatal development remains to be investigated.

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