Intact Numbers of Cerebellar Purkinje and Granule Cells in Sudden Infant Death Syndrome: A Stereologic Analysis and Critical Review of Neuropathologic Evidence

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Abstract

Despite much research during recent decades, the etiology and pathogenesis of sudden infant death syndrome (SIDS) remain unknown. Because of the role of the cerebellum in respiratory and cardiovascular control, it has been proposed that it plays an important role in the pathogenesis of SIDS. To date, 5 postmortem studies on the cerebellum of SIDS cases have yielded conflicting results. Using a rigorous design-based stereologic approach, we investigated postmortem cerebella from 9 SIDS patients who died between 2 and 10 months of age and from 9 age- and sex-matched control children. Neither the volumes of the cerebellar external granule cell layer, molecular layer, internal granule cell layer (including the Purkinje cell layer), and white matter nor the total numbers of Purkinje cells, granule cells in the internal granule cell layer, and the number of granule cells per Purkinje cell showed statistically significant differences between the SIDS cases and the controls. Based on these observations, we conclude that structural alterations in cerebellar development are not involved in the etiology and pathogenesis of SIDS.

Key Words: Cerebellum, Design-based stereology, Postmortem, SIDS, sudden infant death syndrome.

INTRODUCTION

Sudden infant death syndrome (SIDS) is a disease of the first year of life (1–3). It is characterized by sudden death of affected children without warning, usually after a sleep period (4–6). Although the numbers of SIDS cases are declining, it continues to be an important cause of infant death in developed countries during the first year of life (5, 7). Despite considerable research, the etiology and pathogenesis of SIDS remain unknown. Accordingly, SIDS is a postmortem diagnosis by exclusion (6, 8, 9), which has important consequences. First, there is no accepted animal model for causal research on SIDS. A PubMed search with “SIDS animal model” currently retrieved approximately 140 hits, but the actual relevance of these animal models is controversial (10, 11). Second, there are no preventive measures for SIDS. A PubMed search with “SIDS prevention” currently returned approximately 2,000 hits, including approximately 200 publications pointing to the “Back to Sleep” program that was launched by the US National Institute of Child Health and Human Development in 1992 (12–14). However, because of the current lack of knowledge about the etiology of SIDS, preventive measures must ultimately be considered empirical.

Much research has been undertaken on the cause of SIDS; these studies investigate a wide variety of organs and organ systems, including the cardiovascular system (15–17), the respiratory system with its neural control structures (18–20), the diaphragm (9, 21, 22), the lungs, including possible changes in surfactant (23–25), and other organs such as the thyroid (26), pancreas (27), and adrenal glands (28, 29). None of these studies resulted in the identification of a single or unifying cause for SIDS. The same holds true for studies of possible factors contributing to SIDS such as alterations in cytokines (30–32), stress (33), magnesium deficiency (34), and genetic factors (3, 8, 35). The hypothesis of possible adverse effects on the neural control of the cardiovascular and respiratory systems in SIDS (19, 20, 36) resulted in several postmortem studies of different parts of the brain, particularly the brainstem as the central control unit of vital functions (5, 37, 38), the hypothalamus (1, 39), and various nuclei of cranial nerves (40–42). Collectively, these studies showed at least some changes in all examined parts of the brain in SIDS (11).

Because of the role of the cerebellum in respiratory and cardiovascular control (43, 44), as well as the fact that affected children may suffer from prolonged apnea and suddenly stop breathing (18, 45), a hypothesis was formulated that the cerebellum could play an important role in the pathogenesis of SIDS. To date, 5 postmortem studies on the cerebellum of SIDS patients yielding conflicting results have been published (43, 46–49). Briefly, Oehmichen et al (46) and Riedel et al (47) reported no differences between SIDS and control cases; Cruz-Sánchez et al (43) proposed a developmental delay of the cerebellum in SIDS (43); and Lavezzi et al
(48, 49) found several changes in the cerebellar cortex in SIDS. Collectively, these studies justified the reevaluation of possible neuropathologic abnormalities of the cerebellum in SIDS using a rigorous design-based stereologic approach. Based on the recent reports by Cruz-Sánchez et al (43) and Lavezzi et al (48, 49), we hypothesized that there would be alterations in total neuron numbers and volumes of layers in the cerebellum in patients with SIDS versus age- and sex-matched controls.

MATERIALS AND METHODS

Brain Specimens

The present study was performed on postmortem cerebella (1 cerebellar hemisphere per case) obtained from 9 children with SIDS and 9 neurologically normal children who were matched to the SIDS cases with respect to age, sex, and hemisphere. Data on the control children were derived from our recent postmortem study on the development of the human cerebellum during the first postnatal year (50). Sudden infant death syndrome was diagnosed after thorough police investigations of the death scene, with review of the circumstances of death and the clinical history, and performance of a complete autopsy, including histologic, microbiologic, and toxicologic examinations according to the so-called San Diego classification (51). The mean age of the SIDS cases was $6.67 \pm 1.11$ months (mean $\pm$ SEM), ranging from 2 months of age (M2) to M10. Clinical data are shown in Table 1. The mean age of the controls was $6.39 \pm 1.17$ months, ranging from M1.5 to M10. The SIDS cases did not differ from the controls with respect to mean age (nonparametric 2-tailed Wilcoxon matched pairs signed rank test; $p = 0.500$), sex (6 males and 3 females each; Fisher exact test; $p = 1.000$), hemisphere (9 left [SIDS] vs 7 left and 2 right [controls]; Fisher exact test; $p = 0.471$), or the mean interval between death and autopsy ($30.8 \pm 6.8$ hours [SIDS] vs $29.8 \pm 6.5$ hours [controls]; Wilcoxon test; $p = 0.910$). All cerebella were collected by Andreas Büttner at the Institute of Legal Medicine, Ludwig-Maximilians-University of Munich, Munich, Germany, between 1999 and 2001. The use of these autopsy cases for scientific investigations was approved by the institutional review board of the University of Rostock, Rostock, Germany, under registration number A 2012-0053. Clinical records were available for all cases.

<p>| TABLE 1. Characteristics of the Cases |
|-----------------------------------|---------------------------------|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>SIDS Cases</th>
<th>Controls</th>
<th>No.</th>
<th>Age, months</th>
<th>Sex</th>
<th>Hemisphere</th>
<th>PMI, hours</th>
<th>No.</th>
<th>Age, months</th>
<th>Sex</th>
<th>Hemisphere</th>
<th>PMI, hours</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIDS 1</td>
<td>Control 1</td>
<td>2</td>
<td>2</td>
<td>M</td>
<td>L</td>
<td>24</td>
<td>1.5</td>
<td>M</td>
<td>L</td>
<td>Control 2</td>
<td>36</td>
<td>Heart defect</td>
</tr>
<tr>
<td>SIDS 2</td>
<td>Control 3</td>
<td>3</td>
<td>3</td>
<td>M</td>
<td>L</td>
<td>56</td>
<td>3</td>
<td>M</td>
<td>R</td>
<td>Control 4</td>
<td>27</td>
<td>Infection</td>
</tr>
<tr>
<td>SIDS 3</td>
<td>Control 5</td>
<td>3</td>
<td>18</td>
<td>M</td>
<td>L</td>
<td>39</td>
<td>4</td>
<td>M</td>
<td>R</td>
<td>Control 6</td>
<td>25</td>
<td>Waterhouse-Friderichsen syndrome</td>
</tr>
<tr>
<td>SIDS 4</td>
<td>Control 7</td>
<td>6</td>
<td>33</td>
<td>M</td>
<td>L</td>
<td>10 F L</td>
<td>7</td>
<td>M</td>
<td>L</td>
<td>Control 8</td>
<td>11</td>
<td>Unknown</td>
</tr>
<tr>
<td>SIDS 5</td>
<td>Control 9</td>
<td>7</td>
<td>9</td>
<td>F</td>
<td>L</td>
<td>64</td>
<td>9</td>
<td>F</td>
<td>L</td>
<td>Control 10</td>
<td>29</td>
<td>Suffocation (crime)</td>
</tr>
<tr>
<td>SIDS 6</td>
<td>Control 11</td>
<td>10</td>
<td>10</td>
<td>M</td>
<td>L</td>
<td>60</td>
<td>10</td>
<td>M</td>
<td>L</td>
<td>Control 12</td>
<td>29</td>
<td>MCAD deficiency/adrenogenital syndrome</td>
</tr>
<tr>
<td>SIDS 7</td>
<td>Control 13</td>
<td>10</td>
<td>32</td>
<td>F</td>
<td>L</td>
<td>60</td>
<td>10</td>
<td>F</td>
<td>L</td>
<td>Control 14</td>
<td>76</td>
<td>Sepsis</td>
</tr>
<tr>
<td>SIDS 8</td>
<td>Control 15</td>
<td>10</td>
<td>10</td>
<td>F</td>
<td>L</td>
<td>60</td>
<td>10</td>
<td>F</td>
<td>L</td>
<td>Control 16</td>
<td>7</td>
<td>Carbon monoxide intoxication</td>
</tr>
<tr>
<td>SIDS 9</td>
<td>Control 17</td>
<td>10</td>
<td>6</td>
<td>F</td>
<td>L</td>
<td>60</td>
<td>10</td>
<td>F</td>
<td>L</td>
<td>Control 18</td>
<td>7</td>
<td>Myocarditis</td>
</tr>
</tbody>
</table>

The control cases 1 to 9 (examined in Kiessling et al [50]) matched the SIDS cases 1 to 9 with respect to age, sex, and hemisphere.

F, female; L, left; M, male; MCAD, medium-chain acyl-CoA dehydrogenase; PMI, postmortem interval (time between death and autopsy); R, right; SIDS, sudden infant death syndrome.

Tissue Processing

Histologic processing was performed at the Department of Neuroanatomy, Ludwig-Maximilians-University of Munich. In all cases, the cerebella were divided medially and either the left or the right hemisphere was available for each case (Table 1). All cerebellar hemispheres were immersed fixed in 10% formalin for 10 to 12 years and rinsed in tap water for up to 1 week before histologic processing. The cerebellar hemispheres were then cryoprotected by immersion in graded sucrose solutions (10%, 20%, and 30% in Tris-buffered saline) at 4°C until the tissue sank to the bottom of the immersion jar; they were then quickly frozen and cut into complete series of 100-μm-thick sagittal sections using a cryostat (Type CM 1950; Leica Microsystems, Wetzlar, Germany) and C35 blades (Feather Safety Razor, Osaka, Japan). From the complete series of sections, every 48th section was selected with a random start to obtain a systematically and randomly sampled series (50, 52) of 7 to 10 sections per case depending on substantial interindividually differences in the size of the cerebella (Tables 2, 3). The selected sections were stained with cresyl...

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violet, mounted, and coverslipped. The volume of the cerebellar external granule cell layer was determined on another systematically and randomly sampled series of 5 sections per case, yielding an average section sampling interval of 75 (range, 54–96).

Stereology

Stereologic analysis was performed on a computerized stereology workstation, consisting of a modified light microscope (Axioskop; Zeiss, Jena, Germany) with Plan-Neofluar objectives 1.25× (numerical aperture [NA], 0.035), 20× (NA, 0.50), and 100× (oil; NA, 1.30) (Zeiss), motorized specimen stage for automatic sampling (Type MAC 6000; Ludl Electronics, Hawthorne, NY), focus encoder (Type MT 1271; Heidenhain, Traunreut, Germany), CCD color video camera (1,600 × 1,200 pixels; MBF Bioscience, Williston, VT), and stereology software (Stereo Investigator version 10; MBF Bioscience).

The boundary of the cerebellum was traced on each selected section on video images displayed on the monitor of the stereology workstation using the 1.25× objective. Total neuron numbers were then estimated with the optical fractionator method (53). All Purkinje cells (investigated with the 20× objective) and granule cells within the internal granule cell layer (investigated with the 100× objective) whose nucleus top came into focus within unbiased virtual counting spaces distributed in a systematic-random fashion throughout the delineated cerebellum were counted. Tables 2 and 3 summarize all of the details of the stereologic counting procedures. The stereologic sampling parameter “area sampling fraction” was individually adjusted because of substantial interindividual differences in the size of the cerebella. On average, 570 (range, 439–693) Purkinje cells were counted, with an average of 935 (range, 584–1,811) unbiased virtual counting spaces (Table 2), and 568 (range, 410–837) granule cells, with 921 (range, 581–1,172) unbiased virtual counting spaces.

### TABLE 2. Parameters of the Stereologic Procedure for Determining Total Numbers of Purkinje Cells Using the Optical Fractionator Method

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Σs</th>
<th>ssf</th>
<th>sl-g, μm</th>
<th>sl-ucf, μm</th>
<th>astf (× 10³)</th>
<th>h, μm</th>
<th>t, μm</th>
<th>tsf</th>
<th>Σuvcs</th>
<th>Σn</th>
<th>CE</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>48</td>
<td>2,300</td>
<td>130</td>
<td>0.313</td>
<td>45</td>
<td>44.9</td>
<td>1.14</td>
<td>720</td>
<td>693</td>
<td>0.038</td>
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<tr>
<td>2</td>
<td>8</td>
<td>48</td>
<td>2,800</td>
<td>130</td>
<td>0.464</td>
<td>45</td>
<td>53.8</td>
<td>1.21</td>
<td>584</td>
<td>551</td>
<td>0.043</td>
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<tr>
<td>3</td>
<td>8</td>
<td>48</td>
<td>2,800</td>
<td>150</td>
<td>0.348</td>
<td>45</td>
<td>50.1</td>
<td>1.16</td>
<td>834</td>
<td>637</td>
<td>0.040</td>
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<td>4</td>
<td>8</td>
<td>48</td>
<td>2,800</td>
<td>130</td>
<td>0.464</td>
<td>45</td>
<td>53.3</td>
<td>1.19</td>
<td>821</td>
<td>550</td>
<td>0.043</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>48</td>
<td>2,800</td>
<td>150</td>
<td>0.348</td>
<td>45</td>
<td>60.8</td>
<td>1.35</td>
<td>964</td>
<td>571</td>
<td>0.042</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>48</td>
<td>2,800</td>
<td>130</td>
<td>0.348</td>
<td>45</td>
<td>56.1</td>
<td>1.25</td>
<td>1,116</td>
<td>538</td>
<td>0.043</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>48</td>
<td>2,800</td>
<td>150</td>
<td>0.348</td>
<td>45</td>
<td>58.5</td>
<td>1.30</td>
<td>1,181</td>
<td>439</td>
<td>0.048</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>48</td>
<td>3,200</td>
<td>150</td>
<td>0.455</td>
<td>45</td>
<td>49.2</td>
<td>1.14</td>
<td>1,053</td>
<td>595</td>
<td>0.041</td>
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<tr>
<td>9</td>
<td>9</td>
<td>48</td>
<td>2,800</td>
<td>150</td>
<td>0.348</td>
<td>45</td>
<td>57.4</td>
<td>1.28</td>
<td>1,142</td>
<td>560</td>
<td>0.042</td>
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</tbody>
</table>

Mean values 8.2

The sampling parameters sl-g and sl-ucf (and thus, astf) were individually adjusted because of substantial interindividual differences in the size of the cerebella.

Σs, number of analyzed sections; ssf, reciprocal value of the section sampling fraction; sl-g, side length in XY directions of the grid used to determine the systematic-random (SRS) positions of the unbiased virtual counting spaces; sl-ucf, side length in XY directions of the unbiased counting frames that served as basis for the unbiased virtual counting spaces; astf, reciprocal value of the area sampling fraction; h, height of the unbiased virtual counting spaces; t, measured actual average section thickness of the sections cut at 100 μm after histologic processing; tsf, reciprocal value of the thickness sampling fraction; Σuvcs, number of unbiased virtual counting spaces used; Σn, number of Purkinje cells counted; CE, predicted coefficient of error of the estimated total numbers of Purkinje cells using the prediction method as described in Schmitz and Hof (52) and Schmitz (54).

### TABLE 3. Details of the Stereologic Procedure for Determining Total Numbers of Granule Cells in the Internal Granule Cell Layer Using the Optical Fractionator Method

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Σs</th>
<th>ssf</th>
<th>sl-g, μm</th>
<th>sl-ucf, μm</th>
<th>astf (× 10³)</th>
<th>h, μm</th>
<th>t, μm</th>
<th>tsf</th>
<th>Σuvcs</th>
<th>Σn</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>48</td>
<td>2,300</td>
<td>10</td>
<td>52.9</td>
<td>5</td>
<td>44.9</td>
<td>9.0</td>
<td>716</td>
<td>471</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>48</td>
<td>2,800</td>
<td>10</td>
<td>78.4</td>
<td>5</td>
<td>53.8</td>
<td>10.8</td>
<td>581</td>
<td>410</td>
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<td>3</td>
<td>8</td>
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<td>2,800</td>
<td>10</td>
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<td>50.1</td>
<td>10.0</td>
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<td>586</td>
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<td>2,800</td>
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<td>53.3</td>
<td>10.7</td>
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<td>122.5</td>
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<td>60.8</td>
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<td>461</td>
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<td>1,024</td>
<td>837</td>
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<tr>
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<td>9</td>
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<td>8</td>
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<td>5</td>
<td>57.4</td>
<td>11.5</td>
<td>1,123</td>
<td>658</td>
</tr>
</tbody>
</table>

Mean values 8.2

The sampling parameters sl-g and sl-ucf (and thus, astf) were individually adjusted because of substantial interindividual differences in the size of the cerebella.

Σs, number of analyzed sections; ssf, reciprocal value of the section sampling fraction; sl-g, side length in XY directions of the grid used to determine the systematic-random (SRS) positions of the unbiased virtual counting spaces; sl-ucf, side length in XY directions of the unbiased counting frames that served as basis for the unbiased virtual counting spaces; astf, reciprocal value of the area sampling fraction; h, height of the unbiased virtual counting spaces; t, measured actual average section thickness of the sections cut at 100 μm after histologic processing; tsf, reciprocal value of the thickness sampling fraction; Σuvcs, number of unbiased virtual counting spaces used; Σn, number of Purkinje cells counted; CE, predicted coefficient of error of the estimated total numbers of Purkinje cells using the prediction method described in Schmitz and Hof (52) and Schmitz (54).
Table 3). Neurons were differentiated from glial and endothelial cells by histologic criteria. Purkinje cells showed a large cytoplasm and a prominent nucleolus within a pale nucleus (Fig. 1A–C). Granule cells were much smaller than Purkinje cells and were identified by the absence of cytoplasmic staining (Fig. 1A, B, D). Glial cells within the internal granule cell layer were identified by the absence of cytoplasmic staining and intense staining of the nucleus, with dispersed chromatin and lack of a nucleolus (Fig. 1E). Granule cells outnumbered glial cells within the internal granule cell layer by a factor of approximately 10. Golgi cells in the internal granule cell layer were rare and showed a larger nucleus than the granule cells (Fig. 1F). Estimated total neuron numbers were calculated from the numbers of counted neurons and the corresponding sampling probability (52, 54).

The volumes of the cerebellar external granule cell layer, molecular layer, internal granule cell layer (including the Purkinje cell layer), and white matter were analyzed using the Cavalieri principle (52, 55). The projection areas of these layers were determined on each selected section, the data from all sections were summed up, and this value was multiplied with the interval of cresyl violet–stained sections (i.e. 48) and the average actual section thickness after tissue processing (determined with the stereology workstation) (Tables 2, 3). The projection areas of the cerebellar molecular layer, internal granule cell layer, and white matter were determined with point counting (52, 55); the upper right corner of the unbiased virtual counting spaces used for counting Purkinje cells was used as the counting criterion. On average, 259 hits (range, 95–385) of the upper right corner of the unbiased virtual counting spaces used for counting Purkinje cells were recorded for the molecular layer, 330 (range, 210–420) hits for the internal granule cell layer, and 165 (range, 117–209) hits for the white matter. The projection areas of the cerebellar external granule

![Image](https://example.com/image)

**FIGURE 1.** (A, B) Representative high-power photomicrographs of a sagittal section of the left cerebellar hemisphere from a 10-month-old child with SIDS. The same microscopic field is shown at focal planes of 12.5 μm (A) or 48 μm (B) below the upper surface (cut section thickness, 100 μm; average section thickness after histologic processing, 60.3 μm). Nucleoli of 2 Purkinje cells are evident at positions a (A) and b (B). At the corresponding positions a' (B) and b' (A), these nucleoli are out of focus. At the positions c (A) and c' (B), there is another Purkinje cell that is out of focus in both (A) and (B). Granule cells that are evident at positions d (A), e (A), and f (B) cannot be seen at the corresponding positions d' (B), e' (B), and f' (A), respectively. Accordingly, it was possible to count cerebellar Purkinje cells and granule cells in 100-μm-thick sections with unbiased virtual counting frames using the optical fractionator method. (C, D) Representative high-power photomicrographs of the same sagittal section, showing unbiased counting frames positioned on the Purkinje cell layer (C) and the internal granule cell layer (D). The unbiased counting frames represent both top and bottom of unbiased virtual counting spaces (52); they are shown at the size used for counting cerebellar Purkinje and granule cells. A cell was only counted when it was either within the unbiased counting frame or hit only the inclusion lines (dotted lines; g in [D]), but not the exclusion lines (solid lines; h in [D]) of the unbiased counting frame. (E, F) Representative high-power photomicrographs of the same sagittal section, showing a glial cell (mostly likely an astrocyte) (arrow in [E]) and a Golgi cell (arrow in [F]) within the internal granule cell layer. Note the absence of cytoplasmic staining and intense staining of the nucleus of the glial cell, and the large nucleus and cytoplasm of the Golgi cell. IGL, internal granule cell layer; PCL, Purkinje cell layer; ML, molecular layer. Scale bars = (A, B) 11 μm; (C) 115 μm; (D–F) 10 μm.
cell layer were determined with a separate (also individually adjusted) point-counting grid, with an average distance between the points in XY directions of 1,667 µm (range, 1,300–2,000 µm). From the averaged 1,416 (range, 1,328–1,497) points hitting the entire cerebellum, 131.5 (range, 75–212) points hit the cerebellar external granule cell layer. The ratio “points hitting the cerebellar external granule cell layer/points hitting the entire cerebellum (including the external granule cell layer)” served as a measure for the relative volume of the cerebellar external granule cell layer.

Statistical Analysis

Except for the variables “volume of the cerebellar external granule cell layer” and “relative volume of the cerebellar external granule cell layer,” dependence of all quantitatively investigated variables from the SIDS cases and the controls on the children’s ages was tested with linear regression analysis, with age as the independent variable. The dependent variables were body length, body weight, brain weight, ratio between brain weight and body weight, volume of the cerebellar molecular layer, volume of the cerebellar internal granule cell layer, volume of the cerebellar white matter, total number of cerebellar Purkinje cells, total number of cerebellar granule cells, and the number of granule cells per Purkinje cell. Dependence of the variables “volume of the cerebellar external granule cell layer” and “relative volume of the cerebellar external granule cell layer” from the SIDS cases and the controls on the ages was tested with second-order polynomial (quadratic) nonlinear regression analysis, also with age as the independent variable.

For both SIDS and control cases, mean and SEM were calculated for all quantitatively investigated variables. Comparisons between groups were then performed using the non-parametric 2-tailed Wilcoxon matched pairs signed rank test.

Because each data set from the SIDS cases and the controls was used in 2 different tests, an effect was considered significant if its associated p value was smaller than 0.025 (0.05/2) according to the Bonferroni correction in all analyses (56). Calculations were performed using GraphPad Prism (Version 5.0 for Windows; GraphPad Software, San Diego, CA).

Photography

The photomicrographs shown in Figures 1 and 5 were produced by digital photography using a Zeiss AxioCam HRc digital camera (4,164 × 3,120 pixels; Carl Zeiss MicroImaging, Jena, Germany) attached to a Zeiss Axiopt microscope (Zeiss) and AxioVision software (version 4.8.2; Zeiss), using a 100× oil objective (NA, 1.30) (Fig. 1A, B, D–F), a 10× objective (NA, 0.30) (Fig. 1C), or a 5× objective (NA, 0.15) (Fig. 5), respectively. The final figures were constructed using Corel Photo-Paint X5 and Corel Draw X5 (both versions 15.2.0.686; Corel, Ottawa, CA). Only minor adjustments of contrast and brightness were made, without altering the appearance of the original materials.

RESULTS

Except for the variables of body weight (SIDS cases) (Fig. 2C), ratio between brain weight and body weight (both SIDS cases and controls) (Fig. 2G), and estimated total number of cerebellar Purkinje cells (both SIDS cases and controls) (Fig. 4A), linear regression analysis showed for all investigated variables statistically significant increases as a function of the children’s age, with slopes of the regression lines significantly different from zero (all p < 0.05) (Figs. 2A, C, E; 3E, G, I; 4C, E). The slopes and intercepts of the regression lines for the matched controls did not differ significantly from the corresponding slopes and intercepts of the regression lines for the matched controls (p > 0.05).
The volume of the cerebellar external granule cell layer of the SIDS cases showed no clear age-related trend, which was mainly caused by considerable interindividual differences at M9/10 (Fig. 3A). In contrast, the volume of the cerebellar external granule cell layer of the controls slightly increased between M1.5 and M5, followed by a slight age-related decrease until M10 (Fig. 3A). Nevertheless, both the SIDS cases and the controls showed clear age-related reductions in the relative volume of the external granule cell layer between M1.5/2 and M10, the oldest age investigated in this study (Fig. 3A).

No significant differences were found between the SIDS cases and the controls with respect to body length (Fig. 2B), body weight (Fig. 2D), brain weight (Fig. 2F), ratio between brain weight and body weight (Fig. 2H), estimated volume of the cerebellar external granule cell layer (Fig. 3B), estimated relative volume of the cerebellar external granule cell layer (Fig. 3D), estimated volume of the cerebellar molecular layer (Fig. 3F), estimated volume of the cerebellar internal granule cell layer (Fig. 3H), estimated volume of the cerebellar white matter (Fig. 3J), estimated total number of cerebellar Purkinje cells (Fig. 4B), estimated total number of cerebellar granule cells (Fig. 4D), or estimated number of granule cells per Purkinje cell (Figs. 4F, 5) (all \( p > 0.05 \)).

The estimated total numbers of Purkinje cells of both the SIDS cases and the controls did not depend on the interval between death and autopsy (Fig. 6).

**DISCUSSION**

**Validity of the Results**

The estimated mean total numbers of cerebellar Purkinje cells obtained in the present study are in agreement with stereologic estimates of mean total numbers of Purkinje cells reported elsewhere (50). Furthermore, the estimated total numbers of Purkinje cells did not depend on the interval between death and autopsy. The estimated mean volumes of the molecular layer, internal granule cell layer, and white matter found in the present study were somewhat smaller than corresponding data reported elsewhere (50). This was because of the fact that only SIDS cases aged less than 1 year were investigated in the present study, whereas the data reported elsewhere are from adults (50). Nevertheless, the age-related increase in the volumes of the molecular layer, internal granule cell layer, and white matter between M1.5 and M10 found in the present study was consistent with earlier reports (47, 57).

**Cerebellar Cell Numbers in SIDS**

None of the investigated variables showed a statistically significant difference between the SIDS and the control cases. This is in line with earlier reports by Oehmichen et al.

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**FIGURE 3.** (A-H) Volume of the external granule cell layer (EGL) (A, B), relative volume of the external granule cell layer (C, D), and volumes of the molecular layer (ML) (E, F), internal granule cell layer (IGL) (G, H), and white matter (WM) (I, J) in cerebellar hemispheres (left or right) of 9 SIDS cases (open dots in [A, C, E, G, I]; open bars in [B, D, F, H, J]) and 9 matched controls (black dots in [A, C, E, G, I]; black bars in [B, D, F, H, J]). In [A, C, E, G, and I], data are shown as a function of the children’s age. In [B, D, F, H, and J], data are shown as mean and SEM. In [A and C], solid curves (SIDS cases) and dotted curves (controls) represent the results of second-order polynomial (quadratic) nonlinear regression analysis of the corresponding variables as a function of age; the corresponding \( r^2 \) values are provided at the bottom of each panel. In [E, G, and I], solid lines (SIDS cases) and dotted lines (controls) represent linear regression lines of the corresponding variables as a function of age, with slopes significantly different from zero (\( p < 0.05 \)); the corresponding \( F \) and \( p \) values of the linear regression analysis are provided at the lower right of each panel (SIDS, SIDS cases; C, controls). In [B, D, F, H, and J], the \( p \) values of the comparison between the SIDS cases and the controls using the Wilcoxon matched pairs signed rank test are provided. Data of the controls were derived from Kiessling et al (50).
controls (black dots in [A, C, E]). In (A, C, and E), data from SIDS cases and controls are shown as a function of the children’s age. In (B, D, and F), data from SIDS cases and controls are shown as mean and SEM. In (C and E), solid lines (SIDS cases) and dotted lines (controls) represent linear regression lines of the corresponding variables as a function of age, with slopes significantly different from zero (p < 0.05); the corresponding F and p values of the linear regression lines of the corresponding variables as a function of the children’s age. In (B, D, and F), the p values of the comparison between SIDS cases and controls using the Wilcoxon matched pairs signed rank test are provided. Data of the controls were derived from Kiessling et al (50).

Thus, the variables investigated in the present study do not support the hypothesis that alterations in cerebellar development are involved in the etiology and pathogenesis of SIDS. On the other hand, studies by Cruz-Sánchez et al (43) and Lavezzi et al (48, 49) came to the conclusion that the cerebellum could play an important role in the pathogenesis of SIDS.

Oehmichen et al (46) examined the cerebellum from 12 SIDS cases and 12 age- and sex-matched controls for morphologic changes of the cerebellar cortex caused by hypoxia and, in particular, for changes in the density of Purkinje cells. They found no significant differences between the SIDS cases and the controls. These authors based their investigations on photographs of 3-μm-thick cerebellar sections; they estimated the density of Purkinje cells but not their total number. In this regard, it is important to note that changes in cell densities can reveal changes in the total numbers of the corresponding cells, and lack of changes in cell densities can indicate the absence of changes in the total numbers of the corresponding cells. However, this is not mandatory, and changes in total numbers of cells can occur without changes in cell densities (58). Moreover, the analysis of 3-μm-thick sections of the human brain does not allow performing “unbiased” estimates of total numbers of cells with modern design-based stereologic methods (52). The term “unbiased” means that the results of a design-based stereologic study are not subject to systematic errors (52, 59).

Moreover, the evaluation of photographs (as well as the investigation of 3-dimensional structures, such as cells in thin tissue sections) has been proven unsatisfactory in determinations of total cell numbers (52, 60, 61). Finally, isolated studies of Purkinje cells during the development of the cerebellum must be considered incomplete. Rather, combined examinations of the total numbers of Purkinje and granule cells may provide clues about possible developmental disorders of the cerebellum based on the so-called numerical matching hypothesis (62-64). The numerical matching hypothesis is based on the finding that, during cerebellar development, survival of the granule cells depends on the presence of intact Purkinje cells (65). Therefore, the number of granule cells per Purkinje cell arises in a fixed ratio and additionally formed granule cells die physiologically (62, 63, 65).

Cruz-Sánchez et al (43) investigated 19 SIDS cases and 12 age-related controls and found an increased cell density in the external granule cell layer in the cerebella from the SIDS cases versus the controls. These authors argued for the existence of a developmental delay in the cerebellum in SIDS patients either because of delayed differentiation of the precursors of the cerebellar granule cells or their delayed migration from the external to the internal granule cell layer. However,
these authors investigated only tissue samples from the culmen (as did Riedel et al [47]). The tissue samples were processed into 5- and 25-μm-thick sections to determine the cell density in the external granule cell layer. No methodologic details were provided about measurements, except for citing an editorial on stereology (60).

Finally, Lavezzi et al examined the cerebella of SIDS cases and controls using immunohistochemical methods. Specifically, a first study (48) investigated the cerebella from 20 SIDS cases and 18 controls; in a subsequent study, they investigated the cerebella from 20 SIDS cases and 11 controls (49). In both studies, the authors found several changes in the cerebellar cortex in SIDS that were, however, inconsistent. In a 1-month-old SIDS case, they reported (in both studies) apoptosis of almost all cells in the internal granule cell layer; and in a 7-month-old SIDS child, they reported evidence of apoptosis of all Purkinje cells. In both studies, case information is either missing or scarce and the cases seem to be unmatched. Furthermore, Lavezzi et al used the TUNEL method for the detection of apoptosis, which is not specific for the detection of apoptosis because it also reveals DNA repair processes in TUNEL-positive cells (67). In our assessment, the findings by Lavezzi et al seem unconvincing for a significant role of the cerebellum in the pathogenesis of SIDS, and it is possible that the individual findings in these studies were not related to SIDS.

In summary, the results of the present study (together with data from Oehmichen et al [46] and Riedel et al [47]) imply that microscopic investigation of cerebellar sections from SIDS cases using routine stains fail to reveal quantifiable pathologic abnormalities. This does not indicate, however, that other developmental abnormalities of the cerebellum should be ignored in SIDS. It is indeed possible that such alterations occur at the subcellular level and involve potential deficits in synaptic connectivity and function. For example, SIDS was shown to be associated with decreased α7 acetylcholine receptor (nAChR) subunit expression in the caudal nucleus of the solitary tract and the gracile and cuneate nuclei, as well as with decreased β2 nAChR subunit expression in the caudal nucleus of the solitary tract and increased β2 nAChR subunit expression in the facial nucleus (68). Similar alterations in the expression of nAChR subunits could also occur in the cerebellum. It is known from animal studies that heteromeric nAChRs enhance synaptic glutamate and GABA transmission.

FIGURE 5. Representative photomicrographs of sagittal sections stained with cresyl violet (section thickness, 100 μm) of left cerebellar hemispheres from control and SIDS cases at postnatal day 1 (P1) and 3 (M3), 7 (M7), and 10 (M10) months of age, showing the external granule cell layer (EGL), molecular layer (ML), Purkinje cell layer (PCL), internal granule cell layer (IGL), and white matter (WM). Note the age-related increase in the thickness of the molecular layer and the internal granule cell layer and the age-related decrease in the thickness of the external granule cell layer. It was not possible to assess the actual amount of age-related alterations in the volumes of these layers or potential differences between SIDS cases and controls from these photomicrographs. Scale bar = 100 μm.
onto Purkinje cells, pointing to a possible functional role of nAChRs in regulating cerebellar ontogeny (69–71). Accordingly, unraveling the potential role of nAChRs subunit expression in the cerebellum in SIDS, in the context of possible alterations of its synaptic connectivity, may represent an attractive novel target for further research on the neuropathology of SIDS.

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