Ultrastructural and quantitative age-related changes in capillaries of the dorsal lateral geniculate nucleus

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Abstract

An ultrastructural and quantitative study of age-related changes in the capillaries of the dorsal lateral geniculate nucleus was carried out using male Wistar rats aged 3, 24, and 28 months. The most important structural changes were found in the basal lamina: thickenings either homogeneously distributed or in specific points; spurs towards the astrocyte sheath; and osmiophilic membrane-like inclusions located within the basal lamina. Endothelial cells and pericytes showed an increase in inclusions and dense bodies in the cytoplasm. The quantitative study showed that the most pronounced alteration was the thickening of the basal lamina, which existed at 24 months. Later, at 28 months, thinning of the endothelial cells was observed together with an increase in mitochondria size and the number of pinocytic vesicles. These changes could be an endothelial cell response to compensate for the increasing transport difficulties caused by the thickening of the basal lamina.

The progressive age-related changes observed in the structure of the capillaries might have an effect on the regulation of blood and brain tissue exchanges, and thus might contribute to the development of degenerative alterations in surrounding aging neurones.

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1. Introduction

During the aging process there is a progressive loss of sensorial function, which, in the visual system, manifests as the impairment of certain visual abilities. This is attributed, to a great extent, to degenerative alterations in the different components of the visual pathways [48,49]. These alterations have been reported in the retina, where loss of ganglion cells has been found [5,38] as well as thinning of the fibre layer of the optic nerve [32].

Among the components of the central visual pathways the dorsal lateral geniculate nucleus (dLGN) was chosen because it is the main nerve centre between the retina and the cortex. In previous works, we have reported an increase in dLGN volume as well as neuronal hypertrophy [12,60,61] and also found degenerative alterations in the relay neurons, which in some cases leads to apoptosis [59].

There is evidence connecting the progressive functional deterioration of the CNS due to aging to alterations in brain capillaries. The endothelium of these capillaries is the morphological substrate of the blood–brain barrier (BBB) [26,28,36]. The integrity of this barrier is considered crucial for ensuring the proper supply of nutrients to the neurons, as well as their protection against toxins and by-products [14,44,47,51].

The histological changes in brain capillaries most often described are those referring to the thickening of the basal lamina (BL) [3], the thinning and loss of endothelial cells [1,19], and glial fibrillary proliferation [42]. Other less frequent changes are reported in the mitochondria, pinocytic vesicles, and pericyte coverage, but these vary from one study to another depending on the experimental species used and the CNS area investigated [20,47,54,55].
In order to establish the relationship between CNS capillaries and aging, morphometric methods with optical microscopy were applied to the study of microvascularization in the dLGN, which showed a decrease in volume fraction and capillary diameter [62]. Following these results, it was thought that deeper knowledge of the alterations found in capillary wall components would help to establish their causal relationship with neuronal degenerative changes and secondary functional impairment. To this end, ultrastructural quantitative methods were used, which are able to detect changes in BBB component, which would remain undetected by qualitative methods, and also provide data on the functional state of BBB components.

2. Materials and methods

2.1. Animals

Fifteen male Wistar rats, 3, 24, and 28 months old (n = 5 per age group) were studied. Given that the mean mortality age for this strain of rats is 24.6 months [17], the oldest two groups were considered to be aged animals. The mean weight of the animals was 241 ± 16 g at 3 months, 508 ± 30 g at 24 months, and 371 ± 30 g at 28 months. The animals were kept in a specific pathogen-free and controlled environment with automatic temperature control (23 ± 0.5 °C), relative humidity (55 ± 5%), and 12 h light/dark cycles. They had free access to food and water consistently throughout their life and had been raised by a specialist rat breeder with particular attention to hygiene and regular veterinary inspections. All procedures were carried out in accordance with the European Community Council Directive (86/609/EEC) for the care and use of laboratory animals.

2.2. Electron microscopy

Before sacrifice, animals were anaesthetized with 8% chloral hydrate (0.1 ml/30 g body weight) administered intraperitoneally. Intracardiac perfusion was performed with heparinized phosphate buffer (PB) 0.12 M (pH 7.3, 37 °C) followed by 2% glutaraldehyde, 2% paraformaldehyde in 0.12 M PB (pH 7.3, 37 °C) containing 1% calcium chloride. After perfusion the brains were removed and stored in the same fixative overnight at 4 °C. Coronal sections including the entire dLGN were cut the following day on a Vibratome. The dLGN were dissected out and cut into small pieces of similar dimensions (<1 mm³), between 4 and 6 per animal. Tissue blocks were postfixed in buffered 1% osmium tetroxide in 0.12 M PB (pH 7.3) for 1 h. Blocks were dehydrated in a graded series of ethanol and embedded in Epon-812 at random orientations. Semithin sections of 1 μm thickness were obtained on an LKB ultramicrotome and stained with 1% toluidine blue. Ultrathin silver sections were obtained. They were contrasted with uranil acetate and lead citrate and examined on an LKB electron microscope.

2.3. Sampling procedure

An average of three blocks per animal were randomly selected (10-20 per group) to estimate morphometric parameters. Only one ultrathin section per tissue block was selected. From each section, five profiles of capillaries with a complete outline were photographed at 5000× original magnification (50 per group) for morphometric analysis of the entire vessel profile. Ten micrographs at 8300× original magnification (100 per group) were taken of the same capillaries to study capillary wall components and endothelial cell organelles. The capillaries chosen had the nucleus of the endothelial cell visible, were cut in cross-section, and were approximately 10 μm or less in diameter. Micravessels were considered to be cut in cross-section if the ratio of the largest diameter to the smallest diameter was less than 1.5 [1,54].

2.4. Morphometric analysis

The morphometric parameters were computed with an automatic image analyser Visilog Software version 5.2. The micrographs at 5000×, enlarged to give a final magnification of 12,000×, provided morphometric estimates of the following parameters: capillary and luminal area and endothelial nucleus diameter and area. The micrographs at 8300×, enlarged to give a final magnification of 166,000×, provided morphometric estimates of the following parameters:

- **Mean endothelial thickness (T Ec)** was measured orthogonally at ten equally spaced points around the entire perimeter. Basal lamina located between endothelial cells and pericytes were excluded because this location is much thinner and parts might be missing [18].
- **Mean area of mitochondria (AM) and pericyte processes (AP)**. This parameter was calculated by outlining the structure under study and then calculating the accumulated mean area per age group. Volume fraction of pericyte processes (Vv Pe). This parameter helps to determine the volume occupied by pericyte processes inside the capillary wall. This was calculated with the following formula: \( Vv Pe = \frac{\Sigma AP}{AC} \frac{AM}{AP} \). Where \( \Sigma AP \) is the sum of the areas of pericyte processes located in the capillary wall, and \( AC \) is the area of the capillary wall calculated from the total area and the lumen area. The values obtained were given in %.
- **Mitochondrial and pinocytic vesicles density (NAM and NVp)** were defined as the number of mitochondrial or vesicular profiles per square micrometer of endothelial cytoplasm. Mitochondrial and vesicular profiles were counted. The reference area was calculated by summing the areas of three or five cytoplasm zones for mitochondria and vesicles, respectively. The fields selected represented most of the endothelial cytoplasm except for the area containing...
the nucleus. The data obtained was accumulated by age group.

2.5. Statistical analysis

The morphometric values determined for each animal were used to calculate the mean and the standard error of the mean (S.E.M.) for each group. Statistical analyses were performed using the commercially available software SPSS 8.0. Comparisons between groups were done using either analyses of variance (ANOVA) or the non-parametric Kruskal–Wallis test. Where significant differences were found for a particular variable, a multiple comparison test was carried out. Statistically significant differences were considered at $P < 0.05$.

3. Results

In the 3-month-old rats, dLGN capillaries show the morphological characteristics typical of CNS continuous capillaries (Fig. 1). The endothelial cells are linked by tight junctions creating a continuous and fine layer around the lumen and numerous mitochondria, but few pinocytic vesicles are observed in the cytoplasm. The BL forms a regular thin layer around endothelial cells and surrounds pericyte processes (Fig. 2). Morphological alterations in the different components of the capillary wall were observed in 24- and 28-month-old rats (Fig. 3). The cytoplasm of endothelial cells shows inclusions and vacuoles with different morphology. The most common are dense bodies of irregular shape and variable size (Fig. 4A). There are also vacuoles apparently without content or with a

Fig. 3. Capillary from the dLGN of a 28-month-old rat. The endothelial cytoplasm is thinner than at 3 months. The basal lamina is thicker and shows alterations in its structure (arrow, enlarged in Fig. 4C). Scale bar: 1 \( \mu \)m.

Fig. 4. Endothelial morphological alterations at 28 months. (A) Presence of irregularly-shaped dense bodies in the cytoplasm close to the nucleus. (B) Vacuoles containing granular-like material in the cytoplasm. (C) Junctional complex between endothelial cells, made up of a tight junction (Tj) and an adherens junction (AJs). The mitochondria do not show morphological alterations. Thicken basal lamina (Bl) splits the endothelial cell from the underlying astrocytic processes (As). Scale bar: (A) 0.5 \( \mu \)m; (B) 0.4 \( \mu \)m; (C) 0.08 \( \mu \)m.
Table 1

Quantitative analysis of endothelial cell

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>TEC (μm)</th>
<th>AM (μm²)</th>
<th>NAM (no./μm²)</th>
<th>NAPV (no./μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.10 ± 0.68 × 10⁻³</td>
<td>0.07 ± 6.47 × 10⁻³</td>
<td>4.29 ± 0.74</td>
<td>7.45 ± 0.55</td>
</tr>
<tr>
<td>3–24</td>
<td>↓10%</td>
<td>142.85%</td>
<td>74.66%</td>
<td>74.66%</td>
</tr>
<tr>
<td>24</td>
<td>0.09 ± 1.67 × 10⁻³</td>
<td>0.10 ± 1.01 × 10⁻²</td>
<td>4.49 ± 0.31</td>
<td>7.56 ± 0.44**</td>
</tr>
<tr>
<td>24–25</td>
<td>↓11.11%</td>
<td>↓10%</td>
<td>↓10%</td>
<td>4.66%</td>
</tr>
<tr>
<td>28</td>
<td>0.08 ± 1.20 × 10⁻³</td>
<td>0.09 ± 6.31 × 10⁻³</td>
<td>4.26 ± 0.26</td>
<td>7.56 ± 0.44**</td>
</tr>
<tr>
<td>28–25</td>
<td>↓11.11%</td>
<td>↓10%</td>
<td>↓10%</td>
<td>5.12%</td>
</tr>
<tr>
<td>28–28</td>
<td>↓14.44%</td>
<td>↓8.65%</td>
<td>↓8.65%</td>
<td>35.31%</td>
</tr>
</tbody>
</table>

TEC: endothelial cell thickness; AM: mitochondrial mean area. NAM: mitochondrial density; NAPV: pinocytotic vesicles density. Data are mean ± S.E.M. Significant differences: (*) 3–24; (**) 24–28; (***) 3–28. P < 0.05. Percentage of variation between groups.

Table 2

Quantitative analysis of endothelial cell nucleus

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>AEN (μm²)</th>
<th>DEN (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5.47 ± 0.35</td>
<td>5.44 ± 0.14</td>
</tr>
<tr>
<td>3–24</td>
<td>↑22.85%</td>
<td>↑6.25%</td>
</tr>
<tr>
<td>24</td>
<td>6.72 ± 0.80</td>
<td>5.78 ± 0.17</td>
</tr>
<tr>
<td>24–28</td>
<td>↓14.44%</td>
<td>↓8.65%</td>
</tr>
<tr>
<td>28</td>
<td>5.77 ± 0.34</td>
<td>5.28 ± 0.13</td>
</tr>
</tbody>
</table>

AEN: endothelial nucleus area; DEX: endothelial nucleus diameter. Data are mean ± S.E.M. Percentage of variation between groups.

granular-like material (Fig. 4B). Organelles and cellular junctions do not show visible morphological changes (Fig. 4C). Quantitative methods applied to endothelial cells showed a significant thinning of cytoplasm at the ages of 24 and 28 months (Table 1). Mitochondria showed a significant increase in mean area (AM) in the two older groups (Table 1). Their density (NAM) in relation to the endothelial area does not vary in a significant way (Table 1).

The density of pinocytic vesicles (NAPV) in endothelial cells shows an important and significant increase in the 28-month-old rats, when compared to the two earlier ages when the density remains constant (Table 1). Morphological alterations in the nucleus of endothelial cells were not observed and the parameters analysed do not show significant variations (Table 2).

The basal lamina shows a visible increase in thickness that is generally regular throughout the perimeter of the capillary (Figs. 4C and 5B, arrowheads). In some cases the thickening is irregular but located in specific points, with the material...
The thickening mechanism and the nature of the amorphous material accumulated in the BL are unknown. It has been suggested that they might be the result of alterations in the replacement of their components [15]. Robert et al. [43] among the mechanisms involved in the functional decline of the CNS, a key role is played by morphological alterations in capillaries, which maintain local brain perfusion and thus are essential for fulfilling the metabolic needs of normal brain function [8,25,28,52]. Their relevance has led to many studies of CNS capillaries. These studies have revealed several and different morphological changes, all of which are associated with aging but whose variability depends on the areas and species investigated [15,26,36]. Some alterations have been detected via ultrastructural quantitative studies of specific nervous areas [21,28,34,52,57].

The use of quantitative methods is of great interest because they reveal initial alterations which can be monitored throughout the whole aging process. This study, which focuses on the aging of the visual pathway, makes use of quantitative methods because the kind of degenerative lesions already reported in the dLGN [12,59–62] could be due to structural alterations in capillary walls that would eventually modify BBB functional conditions [65].

The ultrastructural study of endothelial cells revealed dense bodies of lipofuscin, which are usually associated with aging, especially in neurons and astrocytes [34,39,50,59,63]. The quantitative study of endothelial cells showed a significant thinning of the cytoplasm between the 24th and the 28th month. It has been suggested that endothelial thinning is a morphological adaptation to facilitate nutrients reaching the brain [6]. This thinning, reported both during development [27] and aging [3,4,19,24,26,52], has been interpreted as a consequence of cell elongation during vascular growth [27] while others suggest it is due to a loss of endothelial cells together with a lengthening of the remaining ones [1].

The thickening of BL is the most frequently reported change in CNS capillaries during aging. Our study showed a homogeneous distribution of thickening throughout the capillary perimeter, in dLGN capillaries, as well some local thickenings with a more irregular distribution. Increases in thickness have been reported in aged rat capillaries by Burns et al. [3], Heinsen and Heinsen [19], Hicks et al. [20], Hinds and McNelly [21], Knox and Oliveira [30], Schellini et al. [46], Topple et al. [57]. On the other hand, quantitative studies performed in human [55] and primate cortex [28] have not confirmed this thickening of the BL with aging. Local thickening has been reported in both human [9,33] and rat capillaries [8,9].

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mitochondria or in the endothelial area taken up by mitochondria [2,16,20,35,59]. In addition, an increase in the number of mitochondria and pinocytic vesicles in the dLGN capillaries is the thickening of the basal lamina and aminoacids transport systems—reliant on mitochondrial energy—suffer functional deterioration in aged brains [28,37,45]. Oxidative stress, partly caused by mitochondrial dysfunction, may be a cause of neuronal cell degeneration [23].

The quantitative data reported in the literature concerning pinocytic vesicles is very varied. Stewart [51] attributes these differences to possible errors in methodology, especially related to the thickness and orientation of sections cut and the fixation methods. In order to avoid possible sources of error, only orthogonal sections of the capillary wall were used. In addition, the cutting and fixation conditions were kept constant in the three animal group investigated.

According to our results, the density of pinocytic vesicles show a significant rise in the 28-month-old animals. It is thought that this increase suggests the harmful effect of BL alterations on the BBB’s transport functions. A similar result was obtained by De Jong et al. [8,9] in hippocampus capillaries from rats. On the other hand, in human brain capillaries, where BL does not become thicker, no pinocytic vesicle increase has been reported [55].

Several authors [8,9,22,53,55,64] have suggested a relationship between the increase of pinocytic vesicles in the endothelium of CNS capillaries and the increase of BBB permeability. However, given that the relationship between these two factors is not direct, the number of vesicles should not be the only data used to predict the BBB permeability. Rather, the data should be completed with determinations of the level of expression of transport proteins and their corresponding receptors in endothelial cell membranes [51].

Summing up the results obtained in this study of the components of dLGN capillary walls, it should be emphasized that the age-related changes do not seem to be as intense as those found in other areas of the CNS, such as the hippocampus or the cortex. The most relevant alteration found is the thickening of BL. The other alterations, such as thinning of the endothelium, an increase in mitochondria size, and a greater number of pinocytic vesicles, could be a response mechanism of the endothelial cell to compensate for the thickening of the BL.

References


