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Cerebellar Capillaries

Qualitative and Quantitative Observations in Young and Senile Rats

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Summary. Ultrastructural changes including reduced electron density, reduction in polysomes and cisternae of rough endoplasmic reticulum occur in the cytoplasm of endothelial cells and pericytes in the cerebellar cortex of senile virgin female Han: WIST-rats in comparison to 3-month old virgin rats. Processes of pericytes cover less of the capillary surface in the cerebellar cortex of senile rats; moreover, arithmetic and harmonic mean thickness of the endothelium and relative volume of mitochondria in endothelial cells and pericytes are reduced, whereas the luminal diameter of the capillaries, harmonic and arithmetic mean thickness of pericytes and their processes and of the basal laminae between endothelial cells and astrocytes (abbreviated BAL 1), pericytes and astrocytes (BAL 2) and endothelial cells and pericytes (BAL 3) increase. The increase in harmonic mean thickness of the basal laminae is statistically significant ($\alpha \leqq 0.05$) and compensates for a decrease in thickness of capillary endothelium. Consequently, the total barrier mass and thickness of cerebellar cortical capillaries in senile animals is higher than in young individuals.

Key words: Capillaries – Blood-brain barrier – Quantitative anatomy – Cerebellar cortex – Senile rats

Introduction

Numerous observations on age-related changes in the cerebellum of various species including man have been published to date. Quantitative (Hall et al. 1975; Mehraein et al. 1975; Corsellis 1976; Hinds and McNelly 1978; Glick and Bondareff 1979; Heinsen 1981), semiquantitative (Dolley 1911; Ellis

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1919, 1920; Harms 1927; Inukai 1928; Spiegel 1928; Delorenzi 1932; Ketz 1959; Wilcox 1959) and both cytological (Andrew 1936, 1939, 1941, 1955, 1956; Heinsen 1979; Nosal 1979; Fujisawa et al. 1982) and physiological (Rogers et al. 1980) changes in Purkinje cells were the subjects of these studies. Investigations on the other cerebellar neurons are rare (Cammermeyer 1963; Zs-Nagy et al. 1977; Heinsen 1978, 1979). Hassler (1965) described only few age-related vascular changes in the cerebellum, which exhibited species differences. Quantitative data on age-related vascular changes in the cerebellum are lacking. Conradi et al. (1979) only investigated the postnatal vascular growth in rats 7 to 90 days of age. Unresolved questions in geriatric research are the occurrence and etiology of nerve cell loss in aging brains. Ravens (1976) and Fang (1976) discussed the important role that age-related changes in the vascular system may play in neuronal alterations. Considerations on the role of barrier harmonic mean thickness in relation to filtration and diffusion capacity (Weibel and Knight 1964; Weibel 1970/71) and the determination of mitochondrial volume density in cerebral blood capillaries (Oldendorf et al. 1976, 1977) provide parameters that can be estimated morphometrically and relate structure with function of capillaries in young and senile animals.

Materials and Methods

Ten 3-month old virgin Hannover-Wistar rats (Han: WIST; Zentralinstitut für Versuchstierkunde, Hannover) and seven senile virgin Hannover-Wistar rats ranging in age from 34 to 40.4 months were anaesthetized with Nembutal®, fixed by transcardiac perfusion according to Palay and Chan-Palay (1974) and tissue blocks from the cerebella were embedded in Araldite®. Ultrathin sections through the vermal part of L X and the hemispheric part H VI a (Larsell 1952) were mounted onto Formvar®-coated single hole-slot grids (S2 × 1). Cross-sectioned vascular profiles were designated as capillaries when (i) their luminal diameter was smaller than 6 µm and (ii) smooth muscle cells were absent (Hammersen 1977; Lange and Halata 1979). Five perpendicularly cut capillaries from the molecular and the granular layer, respectively, of Lobule X and H VI a were photographed at an initial magnification of 9,000 × with a Philips EM 300 electron microscope. A total of 340 capillaries – 20 for each animal – was quantitatively analyzed.

A transparent sheet with a square lattice test system (Weibel 1979; $P_T = 143$, $d = 18$ mm) was laid over photographs (18 × 24 cm magnification) of capillaries (final magnification about 27000). The final magnification was regularly controlled with a grating replica, spacing of 2160 lines/mm. The test lines of the square lattice make random intersections with the capillary wall. The minimal orthogonal interception lengths (Jensen et al. 1979) of the total capillary wall and the thickness (lengths) of its components (endothelium, basal laminae including laminae rarae and processes of pericytes and perikarya of pericytes) were measured with a hand magnifying glass (10 ×) containing a millimeter scale ($d = 0.1$ mm). The original data were processed with a TI 59 pocket electronic calculator; the arithmetic and harmonic mean thickness (Weber 1980) of the capillary wall and its components were calculated with a computer program.

Additionally, the relative volume of the capillary wall components, the relative volume of mitochondria per volume of pericytes and the percent of capillary wall covered by the basal laminae between pericyte and astrocyte (BAL 2; Fig. 2 and between endothelial cell and astrocyte (BAL 1; Fig. 2) were estimated. The relative volume (V_V) is expressed as percent of volume of a component confined in a containing space, e.g., as the total volume of mitochondria ($V_{\text{mitoch.}}$) which are dispersed in the vascular endothelium ($V_{\text{endoth.}}$). It follows $V_{\text{mitoch.}} = (V_{\text{mitoch.}}/V_{\text{mitoch.}} + V_{\text{endoth.}}) \times 100$. Likewise, the surface density (S_V) is expressed as per cent

of surface of a component in a containing volume. The relative volume can be estimated by point-counting, the surface density by intersection-counting with a set of parallel lines (Weibel 1979). The total test point number and test line length required in order to achieve a relative standard error of $\leqq 10\%$ per animal were calculated by formula 3.29 and formula 4.21 (Weibel 1979). By means of V_V and S_V estimation, it is possible to calculate the thickness of the capillary wall and its components (Weibel 1979) and to control the measurements by these stereological methods.

The cross-sectional area of the capillary lumen was measured with a MOP AM 3 (Kontron), and the mean luminal capillary diameter was calculated by the circle formula:

$$2r = D = 2\sqrt{A/\pi}.$$

Postfixation of cerebellar tissue with OsO_4, dehydration and embedding in Araldit® leads in our material to a slight swelling of the tissue in the range of 2% (Heinsen, unpublished observations). The data presented here are therefore higher than in the living state.

The section thickness of ultrathin sections was measured by Small's method (Weibel 1979). Electron-microscopic sections are not infinitely thin and therefore the outlines of electron-dense objects like mitochondria are projected through translucent sections onto the plane of observation. This so-called Holme's effect results in overestimations of tissue components and is a function of object shape and section thickness. Correction of the relative volume of mitochondria due to the Holme's effect was made by formula 4.69 (Weibel 1979) (see Discussion).

Statistical tests on the harmonic mean thickness of the capillary wall and its components and the relative volume of mitochondria in endothelial cells were performed at the "Abteilung für Medizinische Dokumentation und Statistik der RWTH Aachen" with seven t-tests (Holm 1979). Age-related changes of the other parameters, such as relative volume, arithmetic mean thickness and surface of the capillary covered by different basal laminae, could not be tested statistically since the number of animals was too low to achieve reliable statistical tests. Differences were designated as significant when the error probability was $\leqq 5\%$.

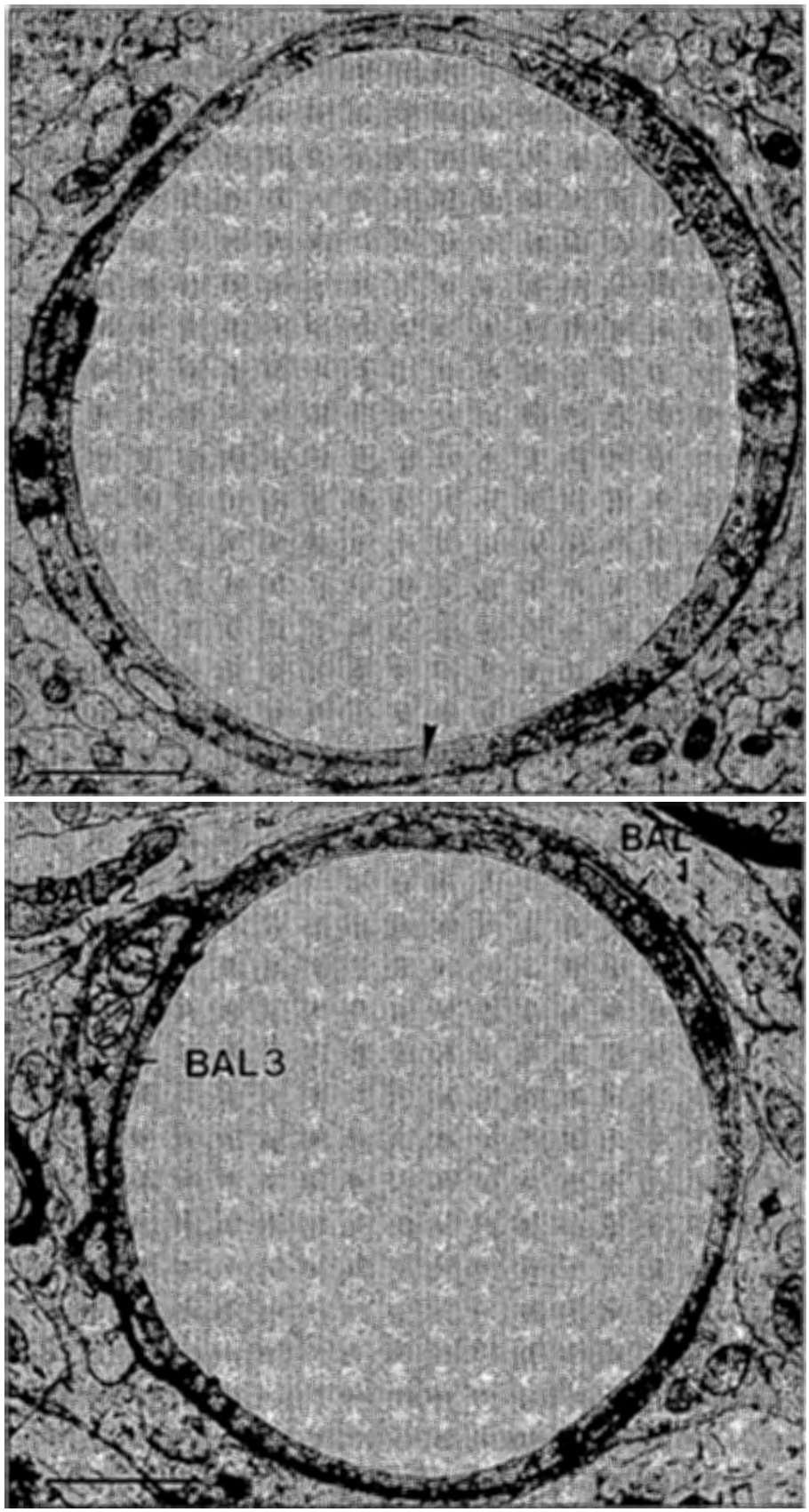
Results

Qualitative Observations

The luminal diameter of the capillaries, our main criterium for classifying cross-sectioned vascular profiles as capillaries, varied to a great extent. We observed capillaries with a large diameter up to 6 μm and others with a small diameter of 3 μm. The glial and neuronal cells surrounding these small capillaries did not show signs of swelling due to inadequate fixation or age-related astroglial hypertrophy. Capillaries with extremely large and small diameters were nearly equally distributed in the molecular and granular layer of young and senile animals.

The thickness of the capillary walls varied depending on the fact whether the nuclei of endothelial cells or pericytes were cut in the sections. At these sites the normally thin capillary wall could be 10–15 times thicker (Figs. 1–3). We felt that the nucleus of endothelial cells in young animals was somewhat larger than in the senescent ones, the heterochromatin in the older animals being condensed as a broad irregular rim at the nuclear membrane (Fig. 3). Similar changes occurred in the karyoplasm of pericytes.

The cytoplasm of the endothelial cells is more electron dense in young animals, the number of free ribosomes and that of the stacks or rough endoplasmic reticulum being considerably higher (Figs. 1, 2). The diameter of the mitochondrial profiles in young animals was obviously larger. The diameter and density of cytoplasmic vesicles did not change conspicuously.


BAL 1
BAL 2
BAL 3

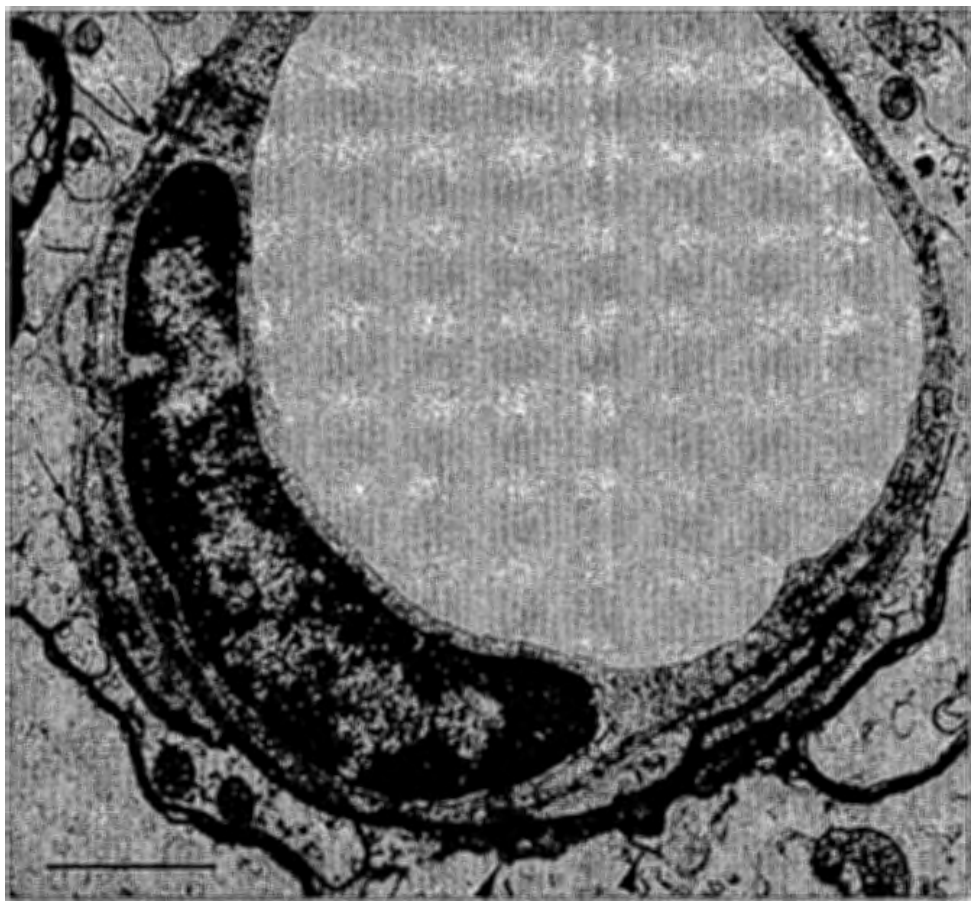


Fig. 3. Capillary from the granular layer of a 34-month old Han: WIST-rat. Splitting of the basal lamina is indicated by arrows, irregular thickening by arrowheads. These events were rather rare in our material. Bar 1 μm

Purely visual interpretations on this aspect must be accepted with caution. With the final magnification used we could not observe gross morphological changes in the interendothelial junctions. The endothelial cells contained few lysosomes or residual bodies in young or old animals. The size of the lysosomes was comparable to that of the endothelial mitochondria.

Fig. 1. Capillary from the molecular layer of a 3-month old Han: WIST-rat. The cytoplasm of pericytes (*asterisks*) is less electron dense than the cytoplasm of endothelial cells. It contains fewer free ribosomes; cisternae of the rough endoplasmic reticulum are frequently distended. *Arrowhead* points to close apposition of endothelial cell and pericyte (gap $\approx$ 7 nm) without intervening BAL 3. Bar 1 μ

Fig. 2. Capillary from the granular layer of a 38-month old Han: WIST-rat. An obvious thickening of the basal lamina between endothelial cells and astrocytes (*BAL 1*), pericytes and astrocytes (*BAL 2*) and endothelial cells and pericytes (*BAL 3*) has occurred. Loss of free ribosomes and cisternae of the rough endoplasmic reticulum in senile animals. Mitochondria in pericytes (*asterisks*) are larger than the endothelial mitochondria and possess less cristae. Bar 1 μm

Nuclei and surrounding cytoplasm of pericytes were always ensheathed by two different basal laminae, which we designated for the sake of simplicity as BAL 2 (Fig. 2; basal lamina between pericytes and astroglial cells) and BAL 3 (Fig. 2; basal lamina between endothelial cells and pericytes). Unlike the nuclei of endothelial cells, the nuclei of pericytes were more rounded. The side of the nucleus facing the endothelium was slightly impressed by the capillary lumen. At variance with the endothelial nuclei the pericyte nuclei covered less of the capillary surface than the flat elliptical endothelial cell nuclei clinging sometimes to three quarters of the surface of the capillary wall. We concluded from cross sections that the perikarya of pericytes emitted a few thick longitudinally running round processes (Fig. 2, asteriks), which were ensheathed by BAL 2 and BAL 3, like the parent cytoplasm. Flat circularly winding processes of pericytes were frequently encountered in cross sections covering $^1/_4$ or in rare cases $^1/_2$ of the capillary wall (Fig. 1, asterisks). The cytoplasm of longitudinal and circular processes of pericytes was less electron dense than that of the endothelial cells. Especially in young animals circular pericyte processes and the adjacent outer wall of endothelial cells were separated by an incomplete BAL 3 (Fig. 1, arrowhead). The plasmalemma of both cell types approached approximately 7 nm. In the senile animals demarcation of both cell types by BAL 3 (Fig. 3) was more complete. Mitochondria were selectively located in the thick longitudinal processes and the perikaryal cytoplasm of pericytes (Fig. 2, asterisks). They were considerably larger than the endothelial cell mitochondria but contained conspicuously fewer cristae. Mitochondria tended to be swollen in senile animals. Other organelles such as the Golgi apparatus and the cisternae of the rough endoplasmic reticulum, filled with a flocculent material, were also encountered in the circular processes of the pericytes (Fig. 1, below asterisks). Free ribosomes were less frequent than in the endothelial cytoplasm. Lysosomes and/or residual bodies were larger and their internal structure was more complicated than in endothelial cells. Their frequency was, however, rather low in the pericytes of the capillaries. Microfilaments tended to increase in number in senile animals. A few bundles ran in a longitudinal course through the perikaryal cytoplasm.

The most striking age-related change was the thickening of the basal laminae (Figs. 1–3). Besides this thickening, only few other morphological changes occurred. Sometimes BAL 2 was irregularly thickened where two processes of astrocytes were apposed (Fig. 3, arrowheads). A splitting of basal laminae was only infrequently observed (Fig. 3, arrows).

Quantitative Results

Capillary Diameter, Relative Volume and Arithmetic Mean Thickness of Capillary Wall Components. The average luminal diameter of capillaries in the cerebellar cortex of young female Han: WIST-rats was 4.10 µm. The processes of pericytes surrounded 27.83% of the total capillary wall, or, in other words, 72.17% (BAL 1, Figs. 1, 2) of the capillary surface was exclusively established by the endothelial cell layer and the adjacent basal lamina

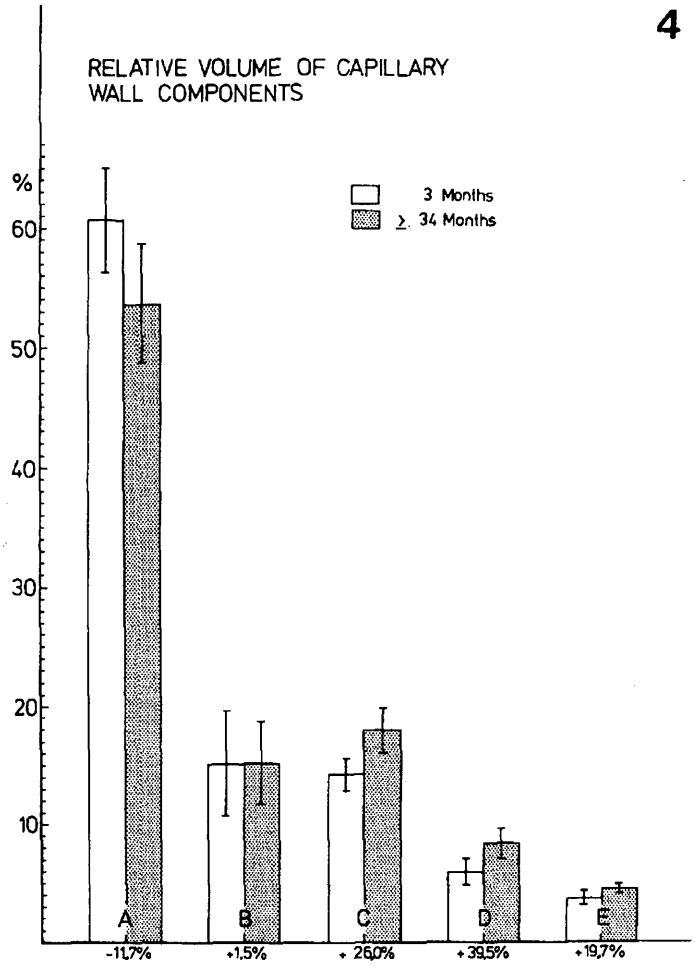
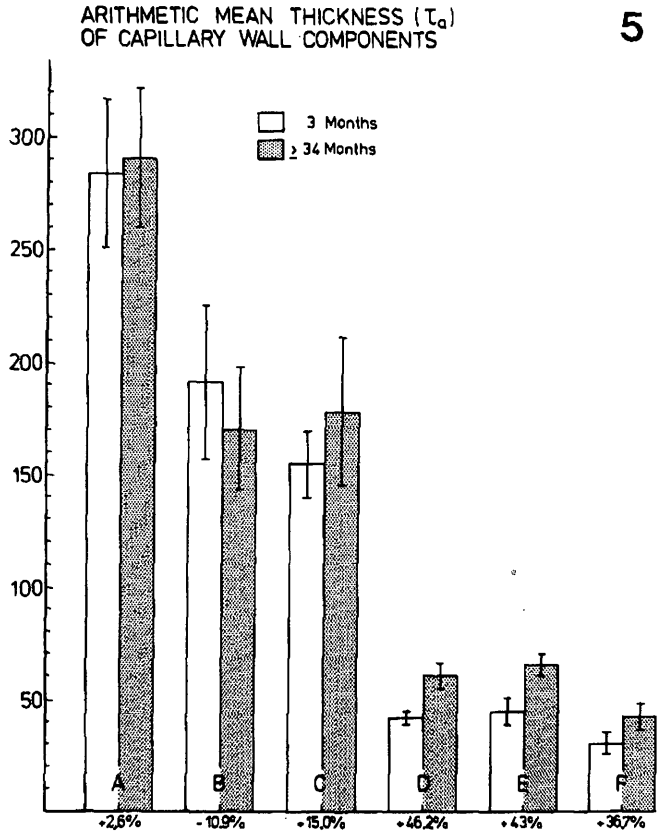


Fig. 4. Relative volume of capillary wall components determined by point counting. (*A*) endothelial cell layer, (*B*) pericytes, (*C*) BAL 1, (*D*) BAL 2, and (*E*) BAL 3. Means and standard deviations of the means. Numbers under the abscissa show age-related increase (positive sign) or decrease (negative sign) from young to senile animals

(Fig. 7a). The tissue mass of the endothelial cells was with 60.83% quantitatively the most important contributor to the entire capillary wall followed by the pericytes with 15.06%. The volume of the thin BAL 1 was nevertheless 14.29%, i.e., only 0.77% less than the mass of the entire pericytes. The combined BAL 2 and 3 contributed less than 10% to the entire capillary wall (Fig. 4).

The arithmetic mean thickness of the total capillary wall was 283.89 nm in young animals, that of the endothelium 192.37 nm and the average thickness of the processes of pericytes 156.01 nm. The three basal laminae were considerably thinner in young animals (Fig. 5).

In the senile animals only three of the afore mentioned parameters decreased: the relative volume of the endothelial cell layer (−11.7%) (Fig. 4) and consequently the arithmetic mean thickness of this layer (−10.9%) (Fig. 5) and the relative capillary surface covered by the processes of pericytes (−4.1%) (Fig. 7a). The average diameter of the capillaries slightly increased (+2%) as did the total tissue thickness (+2.6%) (Fig. 5) and



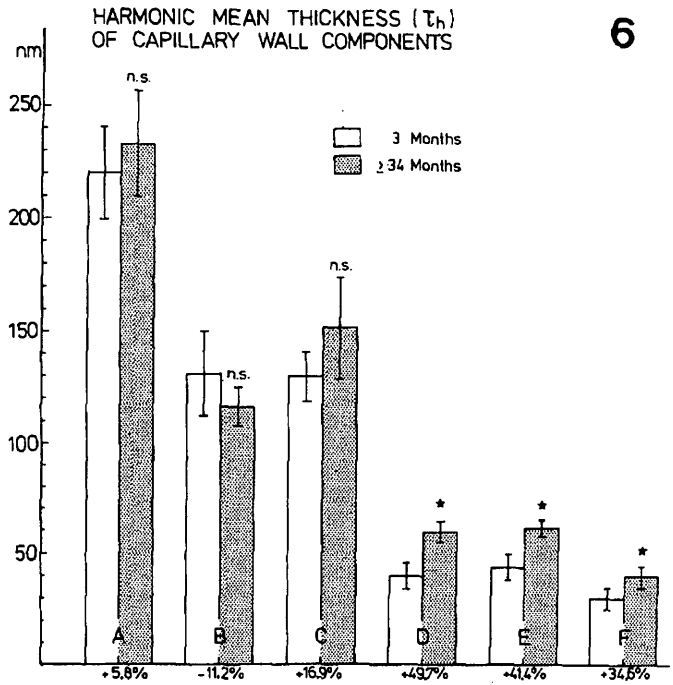


Fig. 5. Arithmetic mean thickness of the capillary wall and its components in nm. (*A*) total capillary wall, (*B*) endothelial cell layer, (*C*) pericytes and processes, (*D*) BAL 1, (*E*) BAL 2, and (*F*) BAL 3. Means and standard deviations of the means. Numbers under the abscissa show age-related increase (positive sign) or decrease (negative sign) from young to senile animals

Fig. 6. Harmonic mean thickness of the capillary wall and its components in nm. (*A*) total capillary wall, (*B*) endothelial cell layer, (*C*) pericytes and processes, (*D*) BAL 1, (*E*) BAL 2, and (*F*) BAL 3. Means and standard deviations of the means. (n.s.) age-related differences not statistically significant (*) statistically significant differences with $\alpha \leqq 5\%$. Numbers under the abscissa show age-related increase (positive sign) or decrease (negative sign) from young to senile animals

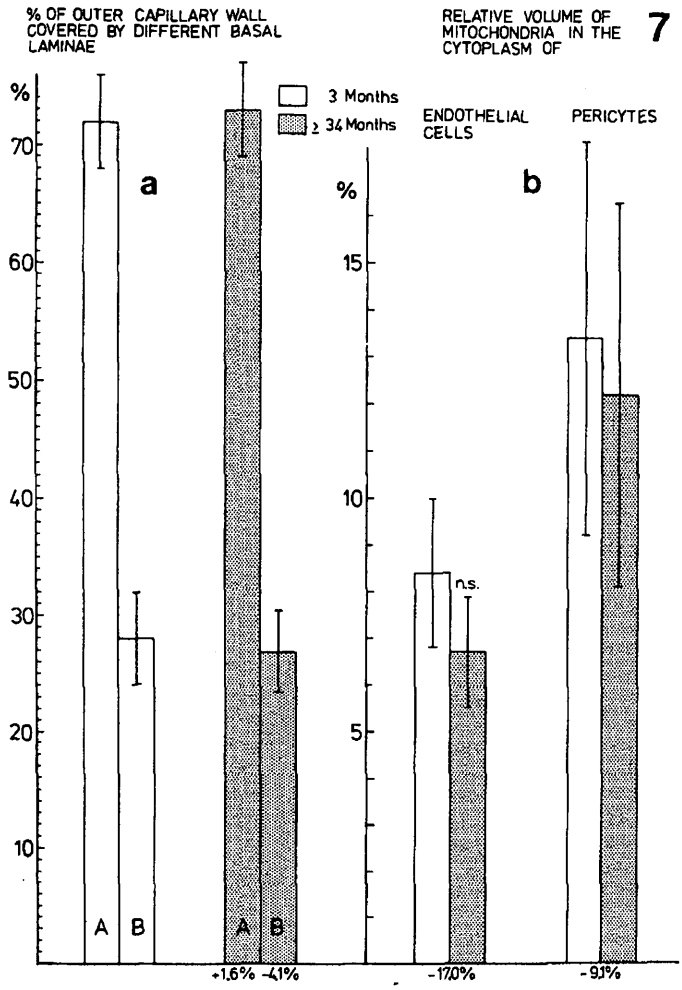


Fig. 7a. Percent of capillary surface covered by combined endothelial cells and BAL 1 (*A*) and by three and/or more layers (endothelial cells, BAL 3, pericytes and BAL 2) (*B*). Means and standard deviations of the means. Numbers under the abscissa show age-related increase (positive sign) or decrease (negative sign) from young to senile animals. **b** Relative volume of mitochondria in the cytoplasm of endothelial cells and pericytes. Means and standard deviations of the means. The decrease in volume density of endothelial mitochondria is statistically not significant (n.s.). Numbers under the abscissa show age-related increase (positive sign) or decrease (negative sign) from young to senile animals

mass (+4.9%) of the capillary wall. The arithmetic mean thickness of the basal lamina increased considerably, that of BAL 1 +46.2%, BAL 2+43% and BAL 3+36.7%. The relative volume of BAL 1 in senescent animals was higher than the volume of pericytes (Fig. 4).

Harmonic Mean Thickness of Capillary Wall Components and Relative Volume of Mitochondria in Endothelial Cells and Pericytes. The harmonic mean thickness of capillary wall components was always lower than the arithmetic mean thickness (Figs. 5, 6), but the percent increase from young to senescent animals was higher. The increase in the harmonic mean thickness of the total barrier from 219.70 to 232.53 nm was 5.8%. The 11.2% decrease in the harmonic mean thickness of the endothelium is more than compensated

by a 16.9% increase in thickness of the pericyte processes and by an extraordinary increase in harmonic mean thickness of BAL 1 (+49.7%), BAL 2 (+41.4%) and a slighter increase of BAL 3 (+34.6%) (Fig. 6). We tested these quantitative changes by a *t*-test. Only the changes in thickness of the basal laminae were significant at an error probability $\leqq 5\%$. Neither the increase in harmonic mean thickness of the pericyte processes nor the decrease of the endothelial cell layer attained statistical significance.

The relative volume of mitochondria per volume of endothelial cell cytoplasm was 8.39% in the young and 6.96% in the senile animals (Fig. 7b). The distribution of mitochondria in the cytoplasm of endothelial cells was not at random but they appeared clustered in certain regions, not necessarily in the perikaryal cytoplasm. High standard deviations of the mean values reflect the non-random mitochondrial distribution. The 17% decrease in relative mitochondrial volume from young to senile animals was statistically not significant in our material. The relative volume of mitochondria per pericyte was always higher than that of endothelial cell mitochondria. The density of mitochondria decreased from 13.41 to 12.19% (−9.1%) (Fig. 7).

Discussion

Classification of preterminal and postterminal vessels and capillaries in the central nervous system of smaller animals is not unequivocal. For example, Lange and Halata (1979) did not find smooth muscle cells in preterminal arterial vessels in the cerebellum of the rat. In agreement with Hammersen (1977) and Lange and Halata (1979), we therefore classified all vascular profiles as capillaries when the luminal diameter was $\leqq 6$ µm. The average diameter of the capillaries in our young animals was 4.10 µm. It is comparable to the capillary diameter in the middle laminae of the visual cortex of Wistar-Kyoto rats (Knox and Oliveira 1980). Sampling procedure (Bär 1978; Weiss et al. 1982), methodological factors (Werner 1967; Hunziker et al. 1979), regional (Sosula et al. 1972; Hunziker et al. 1974; Knox and Oliveira 1980) and species differences (Hunziker et al. 1979; Burns et al. 1981) contribute to the reported differences in mean capillary diameter. Differences in the quantitative investment of the capillary wall by pericytes (Sosula et al. 1971) are further evidence for a morphological and possible functional heterogeneity in the capillary bed.

The afore mentioned factors may contribute to quantitative differences reported by various authors. The mean capillary diameter in the visual cortex of Sprague-Dawley rats decreases (Bär 1978), whereas other authors described an age-related increase of the mean capillary diameter (Werner 1967; Hunziker 1978; Knox and Oliveira 1980; Bell and Ball 1981), which is in agreement with our results.

The capillaries of the cerebellar cortex belong to the morphological type A1β (Bennett al. 1959) with complete continuous basement membrane, lacking fenestrations or pores and with a complete pericapillary cellular investment interposed between parenchymal cells and capillaries. Reese and

Karnovsky (1967) described morphological evidence that the phenomenon of the blood-brain barrier is due to tight junctions between endothelial cells and the paucity of vesicles in the cytoplasm of endothelial cells. The higher volume density of mitochondria in the endothelial cells reflects specific transport capacities of capillaries (Oldendorf et al. 1976; 1977). Weibel and Knight (1964) and Weibel (1970/71) showed that the diffusion capacity of barriers is dependent on the harmonic mean thickness of this barrier.

The harmonic mean thickness of the total capillary barrier is about 220 nm. This is only one third of the harmonic mean thickness of the pulmonary blood-air barrier (Weibel and Knight 1964). About 72% of the barrier is invested by the endothelial cell layer and the adjacent BAL 1 only; about 28% is covered by three or more layers: endothelium, intervening processes of pericytes, BAL 2 and BAL 3. The combined endothelial cell layer and surrounding BAL 1 may be as thin as 100 nm, but in regions where endothelial cell nuclei are present the thickness may reach 1.5 µm. The minimal thickness of the cerebellar blood-brain barrier is comparable to the alveolar-capillary barrier: the maximal thickness of the pulmonary air-blood barrier is about twice as thick as the cerebellar capillary blood-brain barrier (Weibel and Knight 1964). Harmonic and arithmetic mean thicknesses of the capillary wall in the cerebellar cortex slightly increase in senile animals. The net increase in wall thickness of cerebellar capillaries in rats is at variance with the observations of Burns et al. (1979; 1981) in the aging monkey and similar to changes described by Bär and Strauch (1979) and Hinds and McNelly (1982) in rats. The harmonic mean thickness of basal laminae in the striatum (Laursen 1980) and kidney (Gundersen et al. 1978) is higher than in the present material. Basal lamina harmonic mean thickness in the olfactory bulb of senile male Charles-River rats (Hinds and McNelly 1982) is nearly identical with our reported data.

The relative volume of mitochondria per endothelial cells in cerebellar cortex is in good agreement with the results of Oldendorf et al. (1976, 1977) and Laursen (1980). The decrease in volume density of mitochondria in senile animals is statistically not significant. In this respect our results differ from the contentions of Burns et al. (1979). These authors admitted shortcommings in their quantitative procedures. None of the cited authors corrected overestimation of the volume density of mitochondria due to the Holme's effect. Weibel (1979) gives a formula (4.69) for correction. Applying this formula we have obtained correction factors of 0.77 for the young and 0.72 for the senile animals. In other words, our data are overestimations in the range of nearly 30%. We resigned to test the statistical significance with the corrected volume densities of mitochondria since the reconstruction of the mitochondrial diameter from profiles is rather complicated (for a review see Cruz-Orive 1983) and since the shape of mitochondria in young and senile animals may change.

Our quantitative data leave many questions open pertaining to the functional significance. The transport of lipid-soluble non-electrolytes, such as oxygen and CO_2 (Lübbers 1977), across the endothelium is rapid, i.e., is

limited by blood flow rather than by a slight thinning of this layer (Bradbury 1979). Specific transport systems for metabolic substrates like sugars, amino acids and monocarboxylic acids (Bradbury 1979) have been identified. At least some of these carrier systems are regulated by metabolic changes (Gjedde 1981; Oldendorf 1981) and not by barrier thickness alone. The statistically non-significant decrease in mitochondrial relative volume supplies no morphometric evidence for a decrease in energy-dependent processes. The limiting step for vesicle-mediated transport of large molecules (Renkin 1977) is vesicle formation time (for a review see Wagner and Casley-Smith 1981) rather than barrier thickness.

Physiologists have paid attention mostly to the endothelium and its role in blood-brain barrier function and neglected the basal laminae and also the pericytes. The observations of Robert et al. (1978) and Godeau and Robert (1979) elucidate endothelial – basal lamina interactions. Basal-lamina collagen controls pinocytotic activity of the endothelial cells. Age-related thickening of the basal laminae may have some effect on this mechanism.

The basal lamina serves as an extracellular scaffold (Poliwoda et al. 1965; Vracko 1970) and contributes to the structural rigidy of blood capillaries. Thickening of the basal laminae may have repercussations on capillary blood flow in senile animals and consequently on gas exchange (see above). In the renal glomerulum the basal lamina functions as a charge and size filter (Farquhar 1978; Martinez-Hernandez 1978) for larger molecules, but kidney and brain capillary basal laminae do not behave completely identical in this respect (Brendel and Meezan 1980). The basal lamina impedes larger serum molecules from diffusion into the surrounding neuropil after the acute opening of the blood-brain barrier (van Deurs 1976; Westergaard et al. 1977). The significant increase in harmonic mean thickness of the basal laminae in senile animals may delay or impede diffusion of large molecules and deleterious auto-antibodies against nerve cells (Blumenthal 1976; Nandy 1982). Enzymes have been cytochemically identified in the basal lamina of brain capillaries (Firth 1977; Karscú et al. 1977), and at least the Na^+-K^+-ATPase at the interface between blood and brain may play an additional role in the control of ionic concentrations of the extracellular fluid (Eisenberg et al. 1980).

We conclude that the structural integrity of the capillary wall in the cerebellar cortex remains remarkably undisturbed in comparison with neuronal changes (Hall et al. 1975; Corsellis 1976; Hinds and McNelly 1978; Glick and Bondareff 1979; Heinsen 1979, 1981). Unchanged physiological parameters for smaller molecules, such as metabolic rate for O_2 (Rapoport et al. 1982) and glucose utilization (Smith and Sokoloff 1982), in the cerebellum support our morphological results. Senescence results in a quantitative redistribution of capillary wall components with most pronounced changes in basal laminae thickness. Intercellular substance is expecially prone to age-related changes (for a review see Verzar 1965; Hall 1976).

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