Original Article

Morphometry of the Postnatal Development of Rat Hippocampal Capillaries

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Summary:

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Background: *Although the anatomy of the adult mammalian hippocampus has been studied extensively, few reports have dealt with hippocampal cytogenesis and morphogenesis. Moreover, the capillary network, which is the functional component of the vascular bed in terms of tissue metabolic requirements, has not been studied during postnatal development of the rat hippocampus.*

Aim: *To follow the postnatal development of the capillaries in the rat hippocampal formation in view of the temporal variation in the development of its regions using morphometric parameters.*

Methods: *A sample of 37 rats was used. quantitation of the capillaries in four hippocampal regions (subiculum, regio superior, regio inferior, and dentate gyrus) at different postnatal ages (P1, 3, 5, 7, 10, 14, and 21 days) was performed using computer-aided morphometry of alkaline phosphatase positive capillary profiles. Capillary diameter, length/volume density, and intercapillary distance were measured on 30μm-thick frozen coronal sections after controlled fixation.*

Results: *There was a decrease in capillary diameter and in intercapillary distance, with a concomitant increase in capillary length density in all hippocampal regions at the second postnatal week (P10). While the intercapillary distance was significantly reduced during the second postnatal week in all regions; however, its value during the first postnatal week was at its maximum in the dentate gyrus, minimum in the subiculum, and in between both in regio superior and inferior*

Conclusions: *Capillary morphometric parameters were coincidental with the metabolic activity and volumetric growth during postnatal development. It appeared that (P10) was a decisive milestone in the growth of the hippocampal capillary network, at which capillary parameters indicated a significant increase in capillary sprouting and permeation. The repertoire of the changes in intercapillary distance was a replica of the volumetric proportional expansions of the hippocampal regions. The mean intercapillary distance was considered as the most sensitive microvascular parameter.*

Key words: *morphometry, hippocampus, capillary, postnatal development, alkaline phosphatase*

Introduction:

The term hippocampal formation is usually taken to include the hippocampus proper (Ammon's horn), the subiculum, prosubiculum, parasubiculum, and the dentate gyrus[i]. The terms "hippocampus" and "hippocampal formation" may be used interchangeably to refer only to that region from the subiculum to the dentate gyrus including Ammon's horn[ii]. The latter nomenclature was adopted in this study.

In the rodent brain, the hippocampal formation is a curved structure located on the posteromedial border of the hemisphere inferior to the corpus callosum. It extends from the rostromedially located septal area to the ventrolaterally located amygdaloid area[iii]. The anterodorsal end of the hippocampal formation toward the septum is termed septal end and the most posterior portion of the hippocampus where it curves toward the occipital cortex is referred to as the occipital bend. Ventral to the occipital bend the hippocampus turns in an anteromedial direction toward the temporal cortex to terminate at the anteroventrally located temporal end[iv, v].

Phylogenetically, the hippocampus has an archicortical structure[ii]. On the basis of characteristic differences in cellular organization, Cajal (1911)[vi] divided the hippocampus into superior (regio superior), intermediate and inferior regions (regio inferior). Lorente de No' (1934)[vii] has further differentiated four CA (Cornu Ammonis) zones, referred to as fields (CA1, 2, 3, and 4)[viii]. In this study, the following four regions

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will be demarcated: subiculum, regio superior and inferior of Ammon's horn, in addition to the dentate gyrus.

Although the anatomy of the adult mammalian hippocampus has been studied extensively, fewer reports have dealt with hippocampal cytogenesis and morphogenesis. The latter have shown that the hippocampus develops basically in the same pattern in all mammals[ix,x,xi,xii,xiii,xiv,xv,iv,xvi]. In this study, the capillary bed will be assessed during the postnatal development of four hippocampal regions.

As a general rule, capillary density is correlated with the activity and nutrient demand of a particular brain region[xvii]. There is a prominent correlation between capillary length per brain volume and local cerebral blood flow, and between the number of capillaries, local blood flow and glucose utilization in a given brain area[xviii].

In this study, the capillary bed will be exposed on the account of preference of their endothelium to stain for alkaline phosphatase (APase). In 1941, Gomori described staining of APase in the endothelium of capillaries of the nervous system of the rat. Arteries less than (15 μm) in luminal diameter were found to stain abruptly and intensely: the staining continued within the capillary network with a gradual fading in early venules where no APase activity was found in the walls of veins[xix]. Thus, a distinction is easily made between capillaries and terminal arterioles which stain strongly, and venules which stain very weakly or not at all[xx]. The venous absence of APase activity is not yet understood. In addition, the activity of APase in capillaries and arterioles remains unexplained[xxi]. Some workers suggested that the microvascular endothelial APase is involved in the process of active transport similar to its function in the kidney and small intestine[xix,xxii,xxiii,xxiv,xxv].

The morphometric parameters scrutinized in this study were: capillary diameter, length/volume density, and intercapillary distance. Capillary length density is used as a guide for comparison of regional vascularity within the same organ[xxvi,xxvii,xxviii]. Intercapillary distance is regarded as a sensitive parameter for assessment of age-related changes in vascualrity[xxix,xxx].

Materials and methods

Thirty-seven albino rats *(rattus rattus norvegicus albinus)* were used. The animals were sacrificed when they were (1, 3, 5, 7, 10, 14 and 21) days old; five–six animals per each age. Day one of postnatal age (P1) indicated the first 24 hours after birth. Animals were decapitated under ether anesthesia and the whole brain was taken.

A general histological study was performed on one animal for each postnatal age to provide a histological basis for the identification of different regions in the hippocampal formation at all ages studied. The brain was fixed in Bouin's fixative[xxxi] for 24 hours, then dehydrated, cleared, and embedded in paraffin. Coronal sections of (10μm) thickness were obtained. The sections were stained with cresyl violet stain[xxxii].

For the APase histochemical study that was carried out to reveal the capillary bed, the whole brain was fixed in neutral buffered formaldehyde at (4°C) for (12-24 hours). Following fixation, a brain area comprising the hippocampal formation was trimmed, frozen at (–25°C to –20°C), and sectioned (30μm thickness) in the coronal plane using a freezing microtome. An average of 40 sections from each cerebral hemisphere was collected. Since the sections of fixed blocks have a tendency to float off the cover slip during incubation, sections were picked up on cover slips coated in a gelatin-formaldehyde mixture[xxxi]. Picked sections were allowed to dry for 30-45 minutes at room temperature before incubation.

The activity of APase was demonstrated using the azo dye coupling method where 1-alpha naphthyl phosphate (disodium salt) was used as the substrate and fast blue RR salt as the diazonium salt[xxxiii]. For each animal, (10-15) sections were chosen for measurements. These sections contained all the hippocampal regions. The regions were identified depending on comparisons with cresyl violet stained sections for the same postnatal age.

The morphometric study was performed by using an image analysis computer system (Kontron) in conjunction with Reichert Jung Polyvar microscope to which a macrodual zoom was attached. The use of the latter allows computations to be performed directly on tissue sections visualized in the microscope. The basic units of the image analysis system are: control computer, digitizer tablet, and cursor. The digitizer tablet extracts the position of coordinates by means of a freely movable scanning element (cursor) which is manually moved across the digitizer tablet. A (MOP-videoplan, V. 5.41) software converts the raw data obtained through the digitizer tablet into geometrical parameters the choice of which is pre-selected by the user.

The cursor is equipped with a light emission diode which can be seen as a light spot through the microscope. Thus during measurements, cursor movement across the digitizer tablet was indicated by the light spot movement during tracing. The image seen through the microscope eyepiece is a dual (superimposed) image consisting of: first, the microscope (tissue section) image transmitted through the objective lens, and second, the digitizer tablet image transmitted through the macrodual zoom.

The magnification was set according to "Reichert-Jung macrodual operating instructions" and was verified using a slide micrometer for the

two objective lens magnifications used (x10 and x40).

Before capillary measurements were performed, the area of the region of interest (ROI) was measured by pre-selecting the "AREA" option from the software parameters menu and tracing its perimeter at objective magnification (x10). Within this area, the following capillary morphometric parameters were determined for the APase positive profiles:

1. Capillary length: Using the "LENGTH" option from the parameters menu and at objective magnification (x10), the length of all capillary profiles was traced within the ROI. Length density (mm/mm^3) was calculated by dividing total capillary length by tissue volume. The latter was calculated by multiplying the area of ROI by its thickness (30μm).
2. Capillary diameter: In measuring the capillary diameter, the objective lens was set to (x 40) and the "DISTANCE" option was pre-selected. Capillary diameter was represented by the distance between two points facing each other at the outer edge of the capillary profile. Measurements were done at random points specified by overlying a grid of parallel lines, (1cm) apart, on the digitizer tablet. As the grid image was superimposed on the microscope section image, capillary diameter was measured at every point where a capillary crossed a grid line. In order to assess the capillary bed separately from the whole population of microvessels, diameter measurements above (10μm) were excluded from the data.
3. Intercapillary distance: With the same magnification setting and parameter option as in (2), the intercapillary distance was measured. The two points used here represented the outer (abluminal) surface of two neighboring capillaries.

The data collected were subjected to analysis of variance (ANOVA) using the "data analysis tool – Anova single factor" of Microsoft® Office Excel ME (Copyright© 1985-2001 Microsoft Corporation). A *P* value of less than (0.05) was considered as statistically significant. As Inferences from ANOVA were made[xxxiv], Tukey's honestly significant difference (HSD) test was then used to verify ANOVA significances[xxxv].

Results

General Histological Observations:

Paraffin sections stained with cresyl violet were considered as the basis for the localization of the four hippocampal regions before conducting the morphometric study. The subdivisions of the hippocampal formation were clearly distinct as early as (P3) (Fig.1). At this age, the hippocampal formation was morphologically similar to though not identical with the adult form since further development was to ensue.

APase Activity in the Hippocampal Formation:

In the rat hippocampus, APase displayed an intense activity in the form of a brown–black precipitate in the wall of microvascular tree in contrast to APase-negative parenchymal background (Fig.2). APase activity was also found in the microvasculature of the neighboring brain tissue. The level of intensity of APase activity in different regions of the hippocampal formation and at different postnatal ages was the same based on subjective evaluation.

Microvascular Parameters:

The mean capillary diameter showed a general trend of gradual increase that was interrupted by a significant decrease at (P10) in all hippocampal regions (Table-1 and Fig.3A). However, in the subiculum and regio inferior, the mean capillary diameter showed an early significant decrease at (P3) in addition to that noted at (P10) in all regions. Capillary length densities showed significant increases in all hippocampal regions by (P10) (Table-2 and Fig.3B). The mean intercapillary distance (Table-3 and Fig.3B) showed a significant increase in all the regions during the first postnatal week (P3-P5), with significant decrease in the second postnatal week particularly at (P10) that continued persistently until (P21). While the intercapillary distance was significantly reduced during the second postnatal week in all regions; however, its value during the first postnatal week was its maximum in the dentate gyrus, minimum in the subiculum, and in between both in regio superior and inferior.

Discussion:

This study confirmed that APase histochemistry provides an obvious superiority to conventionally stained histological sections in demonstrating microvessels on the account of absence of the enzymatic activity in the hippocampal parenchyma[xxvi,xxix,xvi].

Subjectively, the intensity of APase activity within the hippocampal capillaries in comparison with APase activity in other brain regions was not different. Previous workers did not report intensity variations. However, in this study, the use of controlled fixation may be considered as an improper factor for accurate photometric quantitation of APase activity. Nevertheless, the sharp localization of the final reaction product, rather than intensity, was targeted in this study. Controlled fixation with neutral buffered formaldehyde was useful for preventing diffusion and thus for the sharper localization of the enzymatic activity, with an inherent superiority in increasing the accuracy of morphometric quantitation, particularly regarding the diameters. In practice, fixation also helped better trimming and handling of a tissue difficult to manipulate such as the fresh brain.

The differential staining of microvessels provided a more accurate tool for screening capillaries (the blood exchange compartments). It excluded the larger arterioles and venules, such exclusion cannot be provided as easily on structural basis[xxxvi]. The terminal arterioles which stained strongly for APase cannot be differentiated from the capillary bed on histochemical basis. The difficulty in differentiating terminal arterioles from capillaries on histochemical and structural basis has necessitated resorting to vascular diameters in differentiating capillaries from arterioles. The 10μm-diameter cut-value that was used in this study encompassed all the capillary compartment though not exactly excluding terminal arterioles whose diameter might overlap that of capillaries[xxxvii].

Evaluation of capillary morphometric parameters:

The capillary morphometric parameters which were used in this study are considered to be indicators of the exchange potential of the capillary bed[xxxvii], and the degree of growth of each region at different ages[xxxviii]. In addition, if the capillary bed is regarded as a continuous cylinder, estimates regarding capillary surface area and volume densities can be derived[xxxix] for comparative evaluation of the degree of vascularity[xl,xxxviii].

The mean capillary diameter in CA1 region of adult Fischer-334 rats is reported to be (6.1±0.13μm), with mean intercapillary distance of (68.5±3.9μm)[xxix]. In Wistar rats, the mean capillary diameter in the hippocampal formation, in general, ranged between (4.5-6.0μm)[xxxviii]. In other regions of the central nervous system, a capillary density of (251±103 mm/mm^3) and diameter of (5.04±1.34μm) has been reported in the human visual cortex[xxvi]. In the rat pineal gland, which is considered as a neuroendocrine organ, the mean capillary diameter is (5.7±0.41μm), and the length density is (341±58 mm/mm^3)[xxviii]. The mean intercapillary distance has been shown in different sites of the brain to be (123.0±4.2 μm)[xli].

Capillary parameters and growth:

The general trend of increase in capillary diameters is consistent with age increase[xxxvii,xlii,xxx]. On the other hand, the decrease in the mean capillary diameter at certain ages is mainly correlated with vascular sprouting at those ages[xliii]. In this study, and especially during (P10), it seems that a spell of newly formed capillaries possessing smaller diameters resulted in the shortly maintained reduction in the mean capillary diameter.

The general trend of increase in the capillary length density is concomitant with the permeation of the growing tissue with newly formed capillaries. The bout of significant increase in length density at (P10) may be considered as an abrupt acceleration in the growth process. Apart from the general trend of increase, local and temporal fluctuations could be attributed to uneven relationship between the degree of microvascular and parenchymal postnatal growth.

The intercapillary distance, which may be considered as the most sensitive parameter for the degree of vascularity in a region[xxx], had went through a period of increase during the first postnatal week. The increase in the mean intercapillary distance suggested an increase in tissue volume in a region that is not paralleled by an equivalent increase in capillarity. The decrease in the mean intercapillary distance that followed in the second postnatal week onwards could be considered as a reflection to the subsequent increase in vascularity.

The fact that the capillary length density does not increase significantly during the first postnatal week while there is an increase in the intercapillary distance denotes disproportional increase in tissue volume with a minimal sprouting of vessels. During the second postnatal week (particularly at P10), the decrease in both the intercapillary distance and mean capillary diameter together with increase in length density could be explained, all together, as a manifestation of an accelerated process capillary sprouting at a time when there is a relatively slower increase in volumetric expansion.

These morphometric deductions are consistent with ealier observations on the morphogenesis of the hippocampal region in which that most of the volumetric increase occurs in the first postnatal week; whereas between (P1-P7) the volume increases by 35%, it only increases about 14% between (P7-P21)[xiv,xv]. However, proportional changes are regionally variable; whereas the maximal proportional increase takes place in the dentate gyrus (50%), the least increase occurs in regio superior and inferior (11%). The increase in the subiculum is intermediate between the two extremes (23%). The proportional increase at all regions is set to about 10% during (P7-P21)[xiv,xv]. The repertoire of the intercapillary distance was a replica of the volumetric proportional changes on regional and temporal basis.

Capillary parameters as indicators of the metabolic demands:

There is now ample evidence that the level of neuronal metabolism which is in turn correlated with neuronal activity is involved directly or indirectly in the regulation of the microcirculation of the central nervous system in general[xxix]. During postnatal development of the rat brain, synapse production and dendrite extension reflect an overall increase and spread of the preexisting vessels[xviii].

The fact that the increased metabolic demand and blood glucose utilization in a region is accompanied by increased capillarization, may be connected with another phenomenon which is the appearance of high activity of enzymes involved in glycolytic and oxidative glucose metabolism in the

rat: lactate dehydrogenase and cytochrome oxidase[xliv]. Both enzymes had a lower activity in the hippocampal formation between (P1-P10) to increase between (P10-P14)[xlv,xlvi]. This is in harmony with increased capillarization observed in this study at the second postnatal week in all the hippocampal regions.

In conclusion, it seems that P10 is a decisive milestone in the growth of the hippocampal capillary network, at which capillary parameters indicated a significant increase in capillary sprouting and permeation.

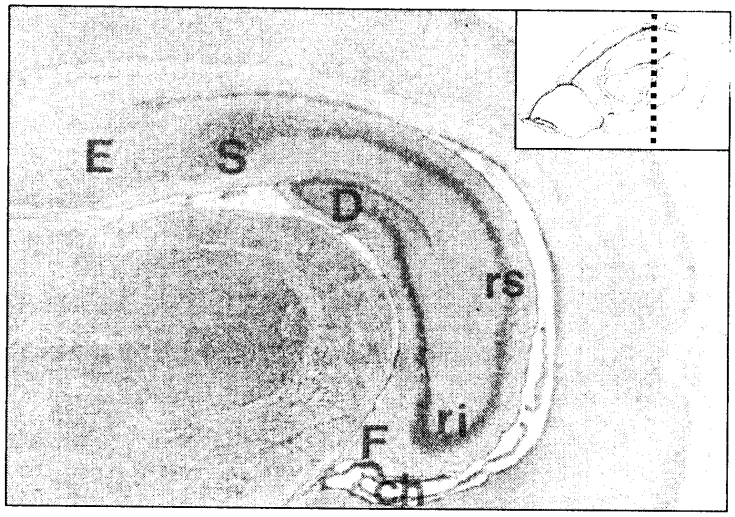

Fig.1:Rat brain hippocampus at (P3), coronal section (level shown in the inset [dotted line]). Note the hippocampal regions as adopted in this study. Dentate gyrus (D), fimbria (F), choroid plexus of the lateral ventricle (ch), regio inferior (ri), regio superior (rs), subiculum (S), and entorhinal cortex (E). Cresyl fast violet stain (x12).

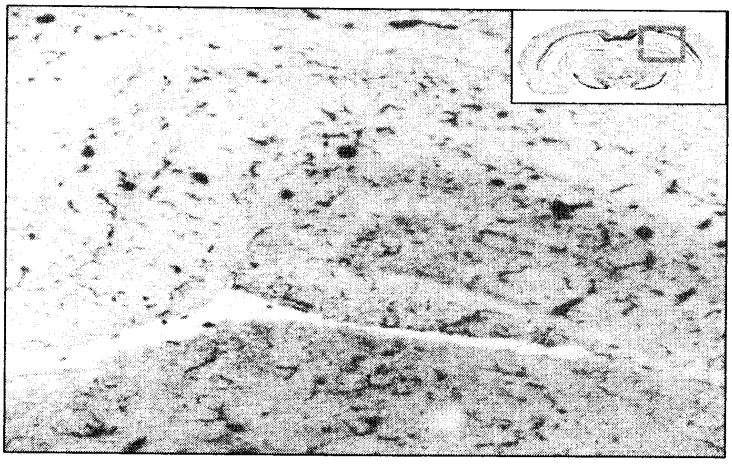

Fig.2:Rat brain hippocampus at (P3), Frozen coronal section. Hippocampal region outlined in the inset [red rectangle]. Note the brown-black APase reaction in the microvascular endothelium in contrast to brain parenchyma. Also note the comparable intensity of reaction in the adjacent brain regions (x40).

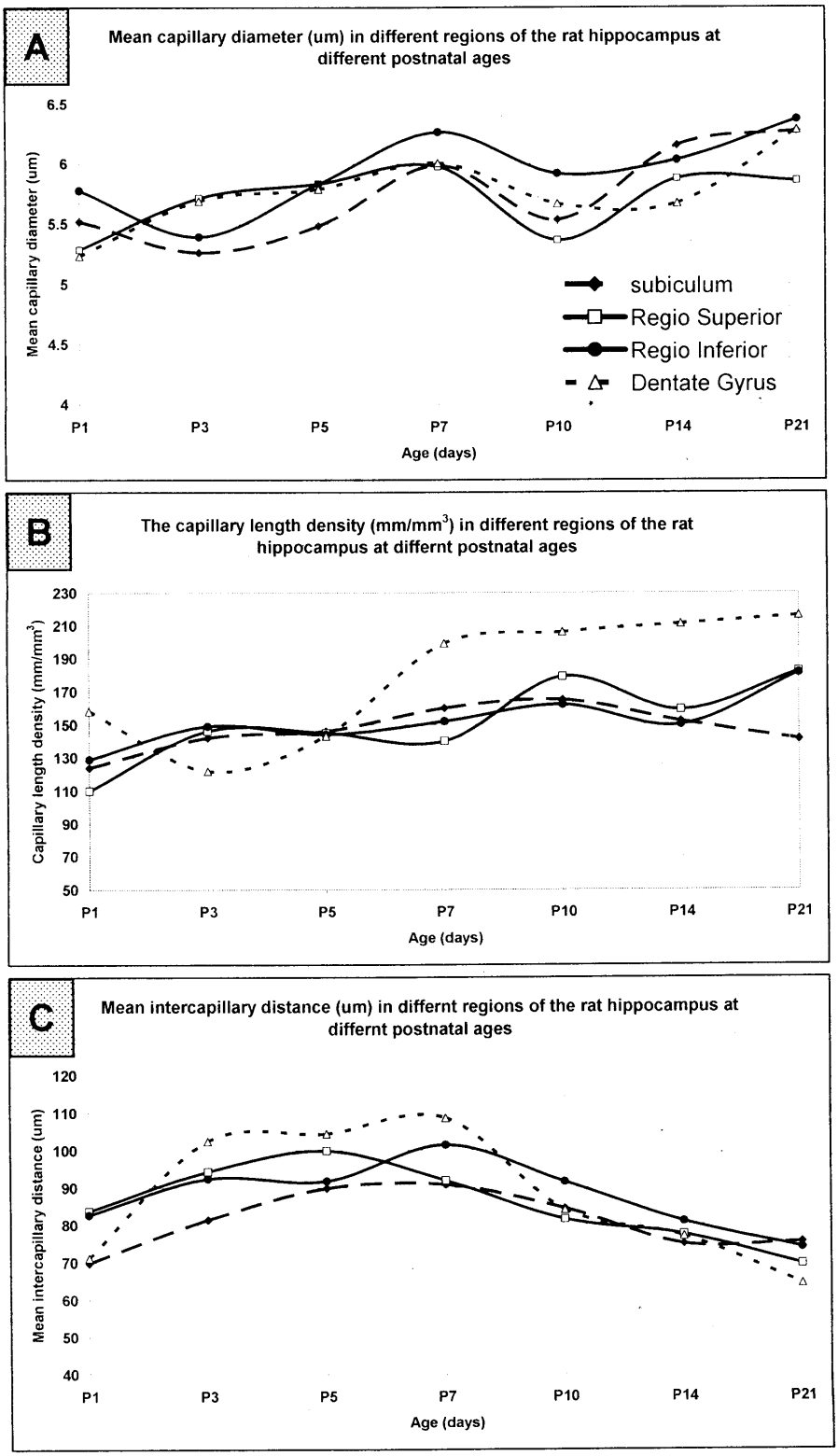

Fig.3:Mean capillary morphometric parameters in different regions of the rat hippocampus during postnatal ages (P1-P21). [A]: mean capillary diameter (μm), [B]: capillary length density (mm/mm3), [C]: intercapillary distance (μm).

Table-1: Mean capillary diameter (μm ± S.D.) in four regions of the rat hippocampus at different postnatal ages (days).

Age / Region	P 1	P 3	P 5	P 7	P 10	P 14	P 21
Subiculum	5.5 ±1.2	5.3 ±1.2	5.48 ±1.5	6.0 ±1.4	5.5 ±1.6	6.2 ±1.7	6.3 ±1.7
Regio superior	5.3 ±1.0	5.7 ±1.6	5.83 ±1.2	5.98 ±1.5	5.4 ±1.8	5.9 ±1.7	5.9 ±1.1
Regio inferior	5.8 ±1.1	5.4 ±1.4	5.83 ±1.3	6.3 ±1.5	5.9 ±1.8	6.0 ±1.6	6.4 ±1.3
Dentate gyrus	5.2 ±1.2	5.7 ±1.1	5.8 ±1.3	6.0 ±1.4	5.7 ±1.2	5.7 ±1.5	6.3 ±1.1

Table- 2: Capillary length density [length/tissue volume] (mm/mm3 ± S.D) in four regions of the rat hippocampus at different postnatal ages (days).

Age / Region	P 1	P 3	P 5	P 7	P 10	P 14	P 21
Subiculum	124 ±26	142 ±51	146 ±37	160 ±49	165 ±48	152 ±27	141 ±35
Regio superior	110 ±19	146 ±38	145 ±29	140 ±47	179 ±44	159 ±31	182 ±40
Regio inferior	129 ±28	149 ±36	144 ±28	152 ±42	162 ±31	150 ±38	181 ±29
Dentate gyrus	158 ±19	122 ±49	143 ±31	199 ±43	206 ±37	211 ±52	216 ±33

Table-3: Mean intercapillary distance (μm ± S.D) in four regions of the rat hippocampus at different postnatal ages (days).

Age / Region	P1	P3	P5	P7	P10	P14	P21
Subiculum	69.8 ±23.4	81.4 ±17.0	89.9 ±27.8	90.8 ±31.3	84.4 ±24.1	74.9 ±30.7	75.2 ±23.6
Regio superior	83.7 ±24.3	94.3 ±28.9	99.7 ±29.8	91.9 ±31.8	81.5 ±24.0	77.4 ±27.0	69.5 ±20.3
Regio inferior	82.7 ±27.7	92.4 ±23.9	91.8 ±29.6	101.4 ±29.4	91.7 ±24.1	80.9 ±33.4	73.8 ±19.4
Dentate gyrus	71.2 ±23.1	102.4 ±26.2	104.4 ±36.4	108.6 ±59.4	84.5 ±23.2	76.8 ±29.3	64.2 ±20.1

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