Neuroscience Letters 505 (2011) 186–190

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

The effects of short-term enriched environment on capillaries of the middle-aged rat cortex

Xuan Qiu[a,b,c,1], Chen Li[b,c,1], Rong Jiang[b,c], Lin Chen[b,c], Chunxia Huang[d], Shu Yang[b,c], Wei Lu[b,c], Xiaoyan Shi[b,c], Yuanyu Zhao[b,c], Yuan Gao[e], Guohua Cheng[b,c], Yong Tang[b,c,*]

[a] Department of Laboratory Medicine, Key Laboratory of Diagnostic Medicine, Ministry of Education, Chongqing Medical University, Chongqing 400016, PR China
[b] Department of Histology and Embryology, Chongqing Medical University, Chongqing 400016, PR China
[c] Laboratory of Stem Cells and Tissue Engineering, Chongqing Medical University, Chongqing 400016, PR China
[d] Department of Physiology, Chongqing Medical University, Chongqing 400016, PR China
[e] Department of Geriatrics, The First Affiliated Hospital, Chongqing Medical University, Chongqing 400016, PR China

ARTICLE INFO

Article history:
Received 5 May 2011
Received in revised form
29 September 2011
Accepted 6 October 2011

Keywords:
Capillaries
Cortex
Middle-aged rats
Enriched environment
Stereology

ABSTRACT

There has been no study investigating the effects of enriched environment on the capillaries of cortex with new stereological methods. In the present study, both 14 month female and male Sprague-Dawley rats were randomly divided into enriched environment (EE) rats and standard environment (SE) rats. EE rats were reared in enriched environment and SE rats were reared in standard environment for 4 months. The effects of short-term enriched environment on the cortex volume and on the total volume, total length, total surface area and mean diameter of the capillaries in the cortex of mid-aged Sprague-Dawley rats were quantitatively investigated with immunohistochemistry technique and unbiased stereological methods. There were no significant differences in the cortex volume, the total length and total surface area of the capillaries in the cortex between EE rats and SE rats. The total volume of the capillaries in the cortex of female EE rats and male EE rats was significantly increased when compared to female SE rats and male SE rats. The mean diameter of the capillaries in the cortex of female EE rats was significantly decreased when compared to that in female SE rats, but there was no significant difference in the mean diameter of the capillaries in the cortex between male EE rats and male SE rats. The present results indicate that enriched environment had a positive effect on the capillaries in the cortex of middle-aged rats. The present study might provide an important morphological basis for searching the ethology strategy to delay the progress of brain aging in the future.

1. Introduction

Brain function is highly dependent on sufficient nutrients and oxygen which are provided by an important portion of the circulatory system in the target tissues. In previous studies, the age-related changes of the capillaries in brain have been reported. Qualitatively, the studies of the effects of aging on the capillary ultrastructures have shown that there were aged-related alterations in the endothelial cells, basement membranes and pericytes of capillaries [17,7,10]. Quantitatively, Kalaria [10] summarized that there was a limited but significant decrease of the capillary density in some regions of the aged brain, including the hippocampus and cerebral cortex. Meanwhile, Farkas and Luiten [7] concluded that there was compelling evidence for an age-related decline of the capillary density in the cerebrum of humans and experimental animals.

Enriched environment is an experimental paradigm which can reduce age-related structural and functional degeneration. Enriched environment can enhance learning and memory [16,11,12,31,29,19,22], neurogenesis in the dentate gyrus [11,12,19], gliogenesis in the cortex and hippocampus [22], neurite branching in the cortex and hippocampus [20,13], synapse formation in the cortex and hippocampus, growth factor increase in the cortex and hippocampus [15,28] and neurotransmitter increase in the cortex and hippocampus [18]. Previous studies showed that the vasculature of brain was also responsive to enriched environment. Greenough and colleagues demonstrated that angiogenesis in response to experience was greatest during development, was maintained during adulthood, and remained present, although diminished, during aging [2–5]. To the best of our knowledge,

* Corresponding author at: Department of Histology and Embryology, Faculty of Basic Medical Sciences, Chongqing Medical University, Chongqing 400016, PR China. Tel.: +86 23 68485635; fax: +86 23 68485868.
E-mail address: ytang062@yahoo.com.cn (Y. Tang).
[1] Co-first authors.

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doi:10.1016/j.neulet.2011.10.019

however, there has been no study investigating the effects of enriched environment on the capillaries of cortex with the new stereological methods. Therefore, the present study was designed to investigate the effects of enriched environment on the capillaries of the cortex in middle-aged male rats and female rats with immunohistochemistry techniques and the new stereological methods.

2. Materials and methods

2.1. Animals

Twenty-four female Sprague-Dawley rats (14 months old) and twenty-four male Sprague-Dawley rats (14 months old) (all provided by The Third Military Medical University, PR China) were used. Both female rats and male rats were randomly divided into EE group (12 female rats and 12 male rats) and SE group (12 female rats and 12 male rats). EE rats were reared in an enriched environment and SE rats were reared in a standard environment for 4 months. Male rats and female rats were kept in different cages. Animal care and treatment followed the National Institute of Health Guide for the care and use of Laboratory Animals (NIH Publication 85-23).

2.2. Enriched environment

Enriched environment rats were kept 12 per cage in large cages (120 cm × 60 cm × 50 cm) containing a variety of stimulating objects such as tunnels, platform, running wheels, ropes, boxes, toys, balls, figurines, building blocks. Tunnels, toys and food location were changed frequently [16,11,29]. Standard environment rats were kept in regular size cages in groups of three without any stimulating objects. They were kept under a constant 12 h light-12 h dark cycle. Food and water were available ad libitum.

2.3. Tissue processing

The rats were deeply anaesthetized with 4% chloral hydrate (10 mL/kg) intraperitoneally and perfusion fixed with 4% paraformaldehyde in 0.6 M phosphate buffered saline (pH 7.4). The good staining of the capillaries in the cortex indicated the high quality of the perfusion-fixation throughout the brain. After perfusion, the cerebellum, brain stem, and cranial nerves under the pavimentum cerebri were cut and the cerebral hemispheres were taken out. Each hemisphere was coronally cut into 2-mm thick slabs, starting randomly from the rostral pole. Eight to nine slabs were obtained from each hemisphere. The left or right hemisphere was sampled at random. The cortex volume was estimated according to Cavalieri's principle [9,25,26].

The slabs of the randomly selected hemisphere were post-fixed in 4% paraformaldehyde for at least 2 h. Then, they were embedded in paraffin with the caudal surface being faced down. To get isotropic, uniform random (IUR) sections, the embedded slabs were treated with the orientator technique [9,14,23]. After the IUR surface was obtained, the tissue blocks were sectioned at 4-μm along the direction parallel to the IUR surface. We called these sections "IUR sections". The orientator technique ensures that the capillaries, in each direction of three-dimensional space, have the same probability of being sampled.

2.4. Immunohistochemistry

Immunohistochemistry was performed using the Histostain TM-Plus SP/9001 kit from ZYMED (ZSGB; Beijing, China). Briefly, the 4-μm paraffin sections were deparaffinized in xylol and rehydrated in a graded alcohol series. The sections were then immersed in citrate buffer (0.01 M, pH 6.0) and heated in a microwave oven for 15 min for antigen retrieval. After being cooled, sections were washed twice in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Endogenous peroxidase was inhibited by incubation with 3% H_2O_2 for 10 min and then washed in PBS three times for 5 min. Nonspecific binding sites were blocked with normal goat serum for 20 min at room temperature. Sections were incubated at 4 °C overnight and then 37 °C for 1 h with rabbit polyclonal anti-collagen IV primary antibody (ab6586; Abcam, Cambridge, UK) at a dilution of 1:200 in PBS. Since collagen IV is a major constituent of the basement membranes of capillaries, collagen IV was used to identify capillaries in the current study. After three 5-min washes in PBS, sections were incubated with biotinylated goat anti-rabbit IgG for 20 min at 37 °C, which was followed by three additional 5-min washes in PBS. Then, the specimens were incubated with S-A/HRP for 20 min at 37 °C, which was followed by repeated washes as described previously. Diaminobenzidine (DAB, ZLI-9032, ZSGB; Beijing, China) was used as a chromogen. Then, sections were dehydrated by sequential immersion in gradient ethanol and xylene and coverslipped.

The sections were viewed using a modified Olympus BX51 microscope (Olympus, Tokyo, Japan). A DP-70 video camera mounted on the top of the microscope was connected to a computer system. Under an oil objective lens (100×), the entire cortex region on each section was photographed. Three to five fields of vision were captured from each section. When the photographs were made under microscope, a scale marking was automatically made on the photographs to validate the magnification of images. For each section, vessels with luminal diameter of <10 μm were defined as components of the capillary net [30,1].

2.5. Stereology analysis

2.5.1. Estimation of cortex volume

On each slab of the randomly selected hemisphere, a transparent counting grid with an area of 0.4 mm^2 associated with each point was placed at random on the caudal surface. The points hitting the cortex were counted under an optical microscope. The cortex volume, V(cortex), was calculated according to the Cavalieri's principle [9,25,26]:

$$V(\text{cortex}) = 2 \times t \times \alpha(p) \times \sum P(\text{cortex}) \quad (1)$$

where t equals the slab thickness, 2 mm, $\alpha(p)$ equals the area associated with each grid point, and $\sum P$(cortex) is the total number of grid points hitting the cortex per rat hemisphere.

2.5.2. Tissue shrinkage

To estimate the volume shrinkage of cortex induced by tissue processing, two tissue blocks were randomly taken from each animal. The cortex volume of each tissue block before the tissue processing, V_{before}, was estimated according to the Cavalieri's principle [9,25,26] as described above. After tissue processing (dehydrating, embedding, and sectioning), the cortex volume of each tissue block was estimated again using described methods [21]. First, a 4 μm section was cut along the z-axis direction (or the direction of the slab's thickness) from the embedded tissue block and stained with hematoxylin. A sliding caliper was used to measure the slab's thickness after tissue processing, t'. Then, another 4 μm section was cut along the slab's coronal surface direction and was stained with hematoxylin and eosin. The point grid was randomly put on the stained coronal section. The points hitting cortex were counted (P'_{cortex}) under microscope using a 4× objective lens. The cortex volume of tissue block after the tissue processing, V_{after}, was calculated, again, according to Cavalieri's principle [9,25,26]:

$$V_{\text{after}} = t' \times \alpha(p) \times P'_{\text{cortex}} \quad (2)$$

where $\alpha(p)$ equals the area associated with each grid point.