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Age-related alterations of Nestin-immunoreactive neurons in rat basal forebrain with aged memory deficit

Dongpei Li [a,1], Jianmin Wang [a,1], David T. Yew [b], E. Lucy Forster [b], Zhibin Yao [a,*]

[a] Department of Anatomy and Neurobiology, Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2nd. Road, Guangzhou, Guangdong 510080, PR China
[b] Department of Anatomy, Chinese University of Hong Kong, Shatin, NT, Hong Kong, PR China

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ABSTRACT

Age-related and aged memory deficit changes in Nestin-immunoreactive (Nestin-IR) neurons were studied following recent evidence of distinct Nestin-IR neurons within adult rat basal forebrain. Morris water maze task assessed spatial learning capacity of 3- and 24-month rats (aged-impaired and aged-unimpaired groups). Nestin-IR neuron distributional and morphological features were investigated by immunohistochemistry and positive neuronal number calculation. Nestin-IR neuron number declined with aging, especially aged-impaired. Significant negative correlations existed between average escape latencies and Nestin-IR neuron number in medial septum-diagonal band of Broca (MS-DBB). Correlations of rostral portion [medial septum (MS) and vertical limb diagonal band (vDB)] were higher than caudal portion [horizontal limb diagonal band (hDB)]. Aged-impaired showed reduced complexity of Nestin-IR neuron dendrite arborization and dendritic length. Nestin-IR astrocyte-like cells appeared scattered among Nestin-IR neurons on some aged-impaired slices. In conclusion, aged-impaired rats showed worse cognitive spatial performance and less Nestin-IR neuronal number compared to aged-unimpaired. Nestin-IR neuronal loss and morphological changes are some pathological characteristics of rat aged basal forebrain and may be important in neurobiological mechanisms of brain aging and aged memory deficit.

1. Introduction

Nestin is an intermediate filament protein expressed in dividing cells during the early stages of development in the central nervous system (CNS), peripheral nervous system (PNS), and in myogenic and other tissues. Upon differentiation, Nestin becomes down-regulated and is replaced by tissue-specific intermediate filament proteins. Its expression is reinduced in the reactive astrocytes of adult during pathological situations, such as the formation of the glial scar after CNS injury and during regeneration of injured muscle tissue. Although it is utilized as a marker of proliferating and migrating cells, relatively little is known about its functions (Michalczyk and Ziman, 2005). In our previous studies, we identified a distinct group of Nestin-IR neurons in the rostral part of the basal forebrain (medial septum-diagonal band of Broca, MS-DBB) in normal adult rats and human being (Gu et al., 2002; Wang et al., 2006). These Nestin-IR neurons were exclusively distributed in the MS-DBB and intermingled with Choline acetyltransferase-immunoreactive (ChAT-IR), Parvalbumin-immunoreactive (PV-IR) or Nicotinamide adenine dinucleotide phosphate-diaphorase reactive (NADPHd-reactive) neurons. However, no colocalization was found between Nestin-IR and PV-IR neurons. Only about 35% of Nestin-IR neurons were ChAT-IR and 8–12% were NADPHd-reactive. Thus, these Nestin-IR neurons are distinguishable from cholinergic and GABAergic neurons. Furthermore, retrograde tracing revealed that a significant portion of these Nestin-IR neurons projected to the hippocampus (Wang et al., 2003; Li et al., 2006). However, the significance of Nestin expression in adult neurons remains to be explored. Nestin-IR neurons within these neurons may regulate the functions of the cells through different mechanisms compared with those within astrocytes.

Basal forebrain is a heterogeneous region that has received much attention for its suggested role in learning and memory, and its involvement in the pathophysiology of Alzheimer's disease (AD) (Semba, 2000). Among the neurons identified in the basal forebrain, cholinergic neurons are the most abundant. Cholinergic hypothesis

* Corresponding author. Tel.: +86 20 87330656; fax: +86 20 87330709.
E-mail addresses: lidp@mail.sysu.edu.cn, dongpeili@hotmail.com (D. Li), yao.zb@163.com (Z. Yao).
[1] Dongpei Li and Jianmin Wang contributed to this work equally and were listed as co-first authors.

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of AD and cholinergic-boosting strategies in the treatment approaches have been well documented (Chandra et al., 2008; Pakaski and Kalman, 2008). Besides, GABAergic neurons rank the second (Walker et al., 1989; Gritti et al., 1993). The basal forebrain contains neurons also express a number of neuropeptide transmitters. However, most of them co-localized with cholinergic neurons (Semba, 2000). It is interesting to note that, during the process of aging, the total number of neurons in the basal forebrain showed a significant decrease of 30%, whereas the number of GABAergic neurons remained unchanged in this brain region. The loss of cholinergic neurons accounted for only 20% of the total age-related neuron loss (Smith and Booze, 1995). This result suggested that there must be some non-cholinergic, non-GABAergic neurons that contribute to the cell loss during aging within the basal forebrain. Therefore, we hypothesized that a decrease in the number of Nestin-IR neurons in the adult basal forebrain might be a significant part of the neuron loss during the process of aging and that Nestin-IR loss may be a contributing factor in age-related degenerative disorders. To test these possibilities, in the present study we investigated age related and aged memory deficit changes of the cell number and morphological characters of Nestin-IR neurons in rat basal forebrains.

2. Experimental procedures

2.1. Experimental animals

A total of 60 male Sprague–Dawley rats were obtained from Experimental Animal Center of Zhongshan School of Medicine, Sun Yat-sen University. All experimental procedures were performed in accordance with the guidelines of the ethical treatment of experimental animals of the Science and Technology Bureau of Guangdong Province which consistent with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80–23). Data were compared from animals in three age groups: 1 month ($N = 15$), 3 months ($N = 15$) and 24 months ($N = 30$). Animals had food and water available and were kept under standard conditions in our animal room with a constant 12 h light/12 h dark cycle.

2.2. Behavioral testing

The behavioral testing was carried out for 3- and 24-month groups in the Morris water maze (Morris, 1984). A circular tank (130 cm in diameter, 50 cm high) was filled up to 30 cm high with water that was maintained at a temperature of 22–26 °C. The pool was divided into four quadrants, with four starting locations, north (N), east (E), south (S), and west (W), at equal distances from the rim. A clear Plexiglas platform (10 cm in diameter) was submerged 1.5 cm under water at the center of the northeast quadrant of the pool, approximately 45 cm from the sidewalls. The swimming path of the rats was recorded using a video camera mounted above the center of the pool and analyzed using a video tracking and analysis system. The pool was surrounded by many cues external to the maze: these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout training.

Each rat received eight trials per day (four trials per block, two blocks per day) for five consecutive days, and each trial lasted a maximum of 120 s. Rats were placed randomly in the tank from one of four starting points and allowed to swim for 120 s or until they escaped by finding the platform. On the last day of testing, the rats received an additional trial (spatial bias test), where the platform was removed from the tank and the rats were allowed to swim for 120 s. Total swim time (escape latency) and total swim distance in the target quadrant were recorded for each rat for each trial.

2.3. The screening of aged memory deficit rats

The spatial memory of the 24-month rats was assessed by comparison with the mean escape latencies of 3-month rats based on a standardized assessment described previously (Martinez-Serrano et al., 1996). In brief, those escape latencies longer than the mean + 2 S.D. (standard deviation) of the escape latencies of 3-month rats were sorted to aged-impaired group. The aged-unimpaired rats were those with escape latencies shorter than the mean + 1 S.D. of the escape latencies of 3-month rats. Those escape latencies between the mean + 1 S.D. and the mean + 2 S.D. of the escape latencies of 3-month rats were screened out.

2.4. Immunohistochemistry procedures

All animals tested in the study, including 1-, 3-, and 24-month rats were terminally anesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg), then perfused intracardially with heparin saline (0.1% heparin in 0.9% saline) followed by paraformaldehyde [4% in phosphate- buffered saline (PBS); 250 ml/rat]. After fixing the animals on a stereotaxic apparatus, the forebrains (Bregma 1.70 mm∼Bregma −1.30 mm) were removed according to the stereotaxic coordinates of the rat brain (Paxinos and Watson, 1997). Following post-fixation in the same 4% paraformaldehyde solution for 24 h, the forebrains were equilibrated in sucrose (30% in PBS), sectioned (40 μm) on a freezing microtome, and collected in PBS.

Sections were treated for 10 min in 0.3% hydrogen peroxide, washed three times in PBS, and incubated in 1% bovine serum albumin (containing 0.3% Triton X-100 in PBS) for 30 min prior to overnight incubation at 4 °C with the primary antibody (Rat-401, mouse monoclone; Pharmingen; 1:1000). After a 3 × 10 min rinse in PBS, the sections were incubated in biotinylated secondary antibody (goat anti-mouse; Sigma; 1:200) at room temperature for 2 h. The sections were rinsed 3 × 10 min in PBS and incubated in streptavidin–biotin complex (Sigma; 1:200) for 2 h at room temperature. Following thorough rinsing with PBS, staining was visualized by incubation in 3,3′-diaminobenzidine solution. After immunohistochemistry staining, floating tissue sections were mounted on superfrost plus glass slides, dehydrated, cleared, and coverslipped.

2.5. Quantification

Photo documentation and image digitizing from the microscope were performed with the Leica DMR microscope (Wetzlar, Germany) with a digital firewire camera Leica MPS 60 and with image analysis software Leica Qwin version 2.8. The estimation was performed in a double-blind manner to avoid investigator bias.

Two horizontal lines through the superior border of bilateral anterior commissure and the sulcus above the optic nerve were utilized to define the boundary of MS, vDB and hDB. The Nestin-IR neurons were calculated to MS if they touched the horizontal line through the superior border of bilateral anterior commissure. Those that touched the horizontal line through the sulcus above the optic nerve were calculated to vDB. One-in-four series of sections (160 μm apart) through a 10× objective were used to measure the reference volume (Vref) of the MS-DBB which was determined by summing the traced areas of the MS, vDB and hDB for each section multiplied by the distance between sections sampled. The numerical density (Nv) was obtained from the optical dissector. The total number of Nestin-IR neurons within the MS, vDB and hDB were then estimated by combining the Nv with the Vref to give absolute numbers as described previously (West and Gundersen, 1990; Peterson et al., 1999). In addition, the mean somatic area and the grey value of the Nestin-IR neurons were also calculated.

2.6. Statistical analysis

Statistical calculations were carried out using SPSS 11.0. Statistical significance in the differences between groups was assessed by multivariate ANOVA. When appropriate, 2 × 2 comparisons were made using a least significant difference (L.S.D.) test. P-values of 0.05 or less were considered statistically significant.

The correlation coefficient between mean escape latencies and the number of Nestin-IR neurons in MS, vDB and hDB were derived by linear-regression analysis using the least-squares method.

3. Results

3.1. Water-maze performance

Escape latencies (mean in second ± S.D.) to a submerged platform were compared between 3- and 24-month groups. The average escape latency of the 3-month group is 21.39 ± 11.03. According to the standard mentioned above, rats of the 24-month group were subdivided into aged-impaired group ($N = 15$) and aged-unimpaired group ($N = 7$). Those escape latencies between the mean + 1 S.D. and the mean + 2 S.D. of the escape latencies of 3-month rats were screened out ($N = 8$) (Fig. 1).

Average escape latency to the submerged platform was plotted across blocks for each group (Fig. 2). Both the 3-month group and aged-unimpaired group showed an enhancement in escape acquisition indicated by sharply decreased escape latencies at the first four blocks. Shorter escape latencies were retained to the last trial. Aged-unimpaired group showed longer escape latencies than the 3-month group at the first block, but there were no significant differences between 3-month group and aged-unimpaired group in this measure at the other time points. Significant differences were detected in the aged-impaired group showing longer escape latencies in finding the submerged platform and