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ORIGINAL PAPER

Age-related Differentiation in Newly Generated DCX Immunoreactive Neurons in the Subgranular Zone of the Gerbil Dentate Gyrus

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Abstract In the present study, we investigated age-related changes of newborn neurons in the gerbil dentate gyrus using doublecortin (DCX), a marker of neuronal progenitors which differentiate into neurons in the brain. In the postnatal month 1 (PM 1) group, DCX immunoreactivity was detected in the subgranular zone of the dentate gyrus, but DCX immunoreactive neurons did not have fully developed processes. Thereafter, DCX immunoreactivity and its protein levels in the dentate gyrus were found to decrease with age. Between PM 3 and PM 18, DCX immunoreactive neuronal progenitors showed well-developed processes which projected to the granular layer of the dentate gyrus, but at PM 24, a few DCX immunoreactive neuronal progenitors were detected in the subgranular zone of the dentate gyrus. DCX protein level in the dentate gyrus at PM 1 was high, thereafter levels of DCX were decreased with time. The authors suggest that a decrease of DCX immunoreactivity and its protein level with age may be associated with aging processes in the hippocampal dentate gyrus.

Keywords Aging · Dentate gyrus · Neurogenesis · Doublecortin · Gerbil

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Introduction

The Mongolian gerbil has a relatively short lifespan, but is genetically homogeneous and easily handled. In particular, this animal is an excellent model for research on aging because of its unique physiological attributes such as auditory processes, reproductive and nervous systems [1]. In addition, they are similar to humans in ways that make them particularly useful as models of stroke, epilepsy, auditory processes, and in several behavioral paradigms [2–6].

Humans and rodents have neurogenic foci and in the subgranular zone (SGZ) of the dentate gyrus and in the subventricular zone (SVZ) lining the lateral ventricle of the forebrain [7–9]. In the SVZ of the forebrain, newly generated cells migrate tangentially into the olfactory bulb, whereas in the SGZ of the dentate gyrus neurons move to the dentate gyrus and incorporate into granule cells. Neural precursor cells may provide an endogenous mechanism for brain repair and recovery from injury or disease [10]. Moreover, it has been suggested that newly generated cells might be involved in aspects of normal hippocampal function, such as, spatial learning and memory [11, 12]. Although such roles are largely conjectural, it has

generated interest in the possibility that further stimulation of neurogenesis in the brain might yield benefit in neurological diseases or in cerebral dysfunction associated with aging [13].

Doublecortin (DCX) gene encodes a 40-kDa microtubule-associated protein, which is specifically expressed in neuronal precursors in the developing and adult CNS [14]. Due to its specific expression pattern, attentions have been drawn to DCX as a marker for neuronal precursors and neurogenesis [15, 16]. In addition, DCX is frequently used as a marker of neuronal migration [15]. Although a number of reports have focused on DCX immunoreactive cells in the dentate gyrus, DCX immunoreactive neuronal precursors in the dentate gyrus in gerbils of various ages has not been conducted. Therefore, in the present study, the authors investigated age-related changes in the distribution of DCX in the dentate gyrus of the Mongolian gerbil during aging.

Experimental procedures

Experimental animals

Male Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. Postnatal month 1 (PM 1) ($n = 14$), PM 3 ($n = 14$), PM 6 ($n = 14$), PM 12 ($n = 14$), PM 18 ($n = 14$) and PM 24 ($n = 14$) gerbils were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-h light/dark cycle, and free access to food and water. The procedures for handling and caring for the animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Tissue processing for histology

For the histological analysis, seven animals at various age were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

Immunohistochemistry for DCX

Immunohistochemistry was performed under the same conditions in gerbils of different ages in order to examine whether the degree of immunohistochemical staining was accurate. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal rabbit serum in 0.05 M PBS for 30 min. They were then incubated with diluted goat anti-DCX antibody (1:50, SantaCruz Biotechnology, USA) overnight at room temperature and subsequently exposed to biotinylated rabbit anti-goat IgG and streptavidin peroxidase complex (diluted 1:200, Vector, USA). They were then visualized by staining with 3,3′-diaminobenzidine in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The sections were mounted in Canada Balsam (Kanto, Japan) following dehydration. A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures.

Western blot analysis

To confirm changes in DCX levels in the dentate gyrus of gerbils at various age, five animals in each group were sacrificed and used for western blot analysis. After sacrificing them and removing the brain, it was serially and transversely cut into a thickness of 400 μm on a vibratome (Leica, Germany), and the dentate gyrus was then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing EGTA (pH 8.0), 0.2% NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM PMSF and 1 mM DTT. After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, USA). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, USA). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with goat anti-DCX antiserum

(1:100), peroxidase-conjugated rabbit anti-goat IgG (Sigma, USA) and an ECL kit (Pierce Chemical, Rockford, IL, USA).

Quantification of data and statistical analysis

All measurements were performed in order to ensure objectivity in blind conditions, by two observers for each experiment, carrying out the measures of experimental samples under the same conditions.

In order to quantitatively analyze DCX immunoreactivity, the corresponding areas of the dentate gyrus were measured from 25 sections per animal. Images of all DCX immunoreactive structures were taken from three layers (molecular, granule cell and polymorphic layers in the dentate gyrus) through an AxioM1 light microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss, Germany) connected to a PC monitor. Images were digitized into an array of 512×512 pixels corresponding to a tissue area of 140×140 μm (40× primary magnification). Each pixel resolution was 256 gray levels. The staining intensity of all DCX immunoreactive structures was evaluated on the basis of a optical density (OD), which was obtained after the transformation of the mean gray level using the formula: OD = log (256/mean gray level). The OD of background was taken from areas adjacent to the measured area. After the background OD was subtracted, a ratio of the OD of image file was calibrated using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software. The result of the Western blot analysis was scanned, and the quantification of the Western blotting was done using Scion Image software (Scion Corp., USA), which was used to count the OD. The relative percentage of control level was shown in the graph.

Data are expressed as the mean ± SEM. The data were elevated by a one-way ANOVA SPSS program and the means assessed using Duncan's multiple-range test. Statistical significance was considered at $P < 0.05$.

Results

Age-related change in DCX immunoreactivity

Doublecortin immunoreactivity in the PM 1 group was found in the SGZ of the dentate gyrus of the gerbil (Fig. 1a): at this age stage, DCX immunoreactive neuronal precursors with well-developed processes were not detected (Fig. 2a). In the PM 3 group, the density of DCX immunoreactivity in the SGZ was decreased compared to that in the PM 1 group (Figs. 1b and 3), however, many DCX immunoreactive neuronal precursors were found in the granule cell layer, and these cells had well-developed processes, which projected to the molecular layer (Fig. 2b). In the PM 6 group, the density of DCX immunoreactivity was lower than that in the PM 3 group, however, DCX immunoreactive neuronal precursors were well incorporated into the granule cell layer of the dentate gyrus (Figs. 1c, 2c and 3). In the PM 12 and PM 18 groups, some DCX immunoreactive neuronal precursors neurons were detected in the dentate gyrus, however, these cells had well-developed processes which stretched to the molecular layer (Figs. 1d, e, 2d, e and 3). In the PM 24 group, a small number of DCX immunoreactive neuronal precursors were found, which did not have well-developed processes (Figs. 1f, 2f and 3).

Age-related change in DCX protein levels

Western blot findings in the gerbil dentate gyrus at various age stages coincided with immunohistochemical changes (Fig. 4). DCX protein level in the dentate gyrus in the PM 1 group was high. The levels of DCX protein, thereafter, were decreased with time. At PM 24, DCX level was about 30% of that in the PM 1 group.

Discussion

Several methods can be used to detect newly generated cells (neurons and glia) in the CNS. Retroviral incorporation requires invasive intracranial injection, but causes parenchymal lesions and possible inflammatory reactions [17], whereas bromodeoxyuridine (BrdU) integrates into the DNA of dividing cells, but is diluted by multiple subsequent divisions [18]. On the other hand, DCX is a cytoskeletal protein that is transiently expressed only in newborn neurons [19], and furthermore is used as a marker of neuronal progenitors. It has been reported that DCX is expressed in differentiated neurons, suggesting a role for DCX in neuronal plasticity, axonal outgrowth, or synaptogenesis [20, 21].

In this study, we used the DCX to find a neuronal differentiation in the subgranular zone of the dentate gyrus at various age stages, although the direct evidence was not demonstrated in the age-related newly generated neurons of the dentate gyrus. BrdU labeling has some drawbacks to labeling the newly generated neurons in aging study, and BrdU entry into the brain depends upon several factors including nucleoside transport mechanisms, blood flow to the brain or other pharmacokinetic variables, which could be affected by age [22]. However, DCX has no effects with these exogenous values, and DCX detects only newly

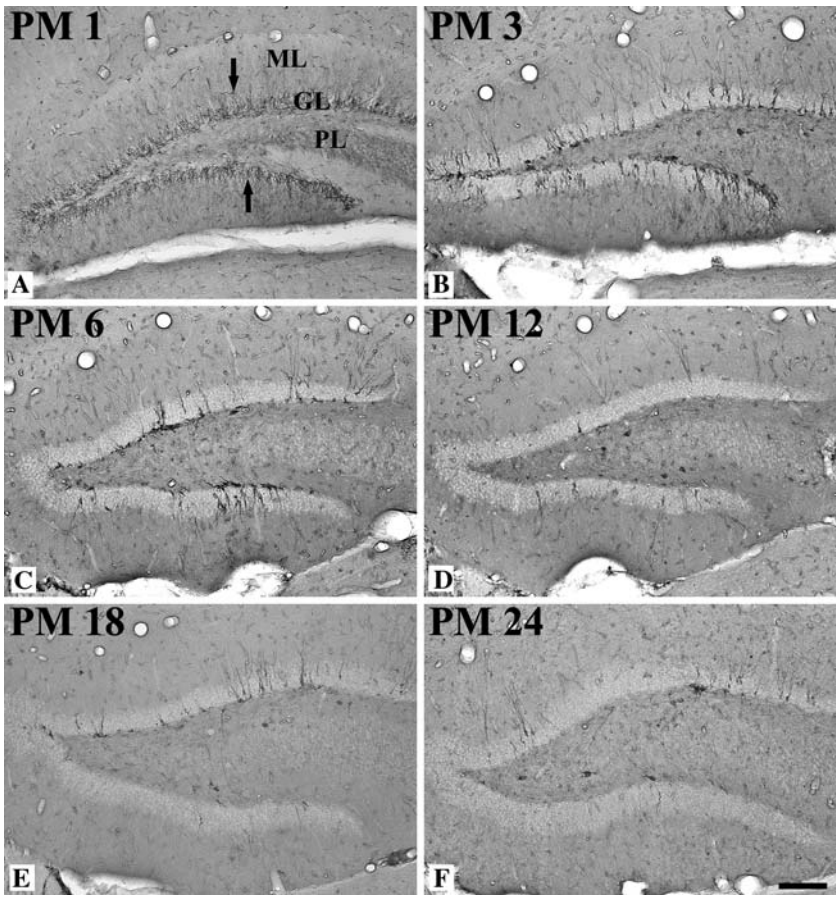

Fig. 1 Low magnification of doublecortin (DCX) immunoreactivity in the gerbil dentate gyrus at postnatal month (PM) 1 (**a**), 3 (**b**), 6 (**c**), 12 (**d**), 18 (**e**) and 24 group (**f**). DCX immunoreactivity is detected in the subgranular zone (arrows) of the polymorphic layer (PL). DCX immunoreactive cells are significantly decreased age-dependently in the dentate gyrus. GL, granule cell layer; ML, molecular layer. Bar = 100 μm

generated neurons, not glial cells (astrocytes and oligodendrocytes).

In the present study, DCX immunoreactivity and its protein levels in the dentate gyrus at PM 1 were very high, and they were decreased with age: they were very low at PM 24. This result concurs with previous studies that neurogenesis dramatically declined in the dentate gyrus in the aged brain [15, 23–29]. However, these previous results did not examine the morphology of DCX immunoreactive neuronal precursors throughout the life span. In the present study, we found that DCX immunoreactive neuronal precursors at PM 1 did not show well-developed processes, however, the processes of these neurons in the SGZ of the dentate gyrus between PM 3 and PM 18 were well developed and stretched to the molecular layer.

Interestingly, the rate of new cell production declines steadily with age, e.g., it is at only 20% of the young rat level in one-year-old rats [24–28]. Moreover, it was reported that DCX immunoreactivity was about 4 times greater in the adolescent rat dentate gyrus (1 month) than in the adult dentate gyrus (4 months) [30]. In addition, Rao et al. [15] reported that numbers of DCX neurons remained unchanged between 4 and 7.5 months but progressively declined from 7.5 to 12 months in the rat dentate gyrus.

In our present study, many DCX immunoreactive neuronal precursors between PM 3 and PM 18 were found in the granule cell layer of the gerbil dentate gyrus. It has been reported that newly generated cells migrate into the granule cell layer, and form dendritic and axonal processes that have the electrophysiological characteristics of new born neurons, i.e., they make appropriate synaptic connections to carry information to and from other parts of the brain within approximately 1 month [31]. Moreover, some factors are likely to be involved in age-related cognitive decline, because the hippocampus and dentate gyrus are involved in various types of learning and memory [32]. Snyder et al. [11] suggested that newly generated cells in the adult hippocampus are involved in normal hippocampal functions, such as, spatial learning and memory. It was reported that the integration of newly generated neurons into hippocampal circuitry was decreased with aging and this phenomenon might, in part, explain the decline in learning and memory in aged rats [33]. In addition, we previously observed that the mean number of reference and working memory task errors in the aged gerbils were greater than adult gerbils [4].

Hattiangady and Shetty [34] reported that a dramatic decline in dentate neurogenesis during aging was not

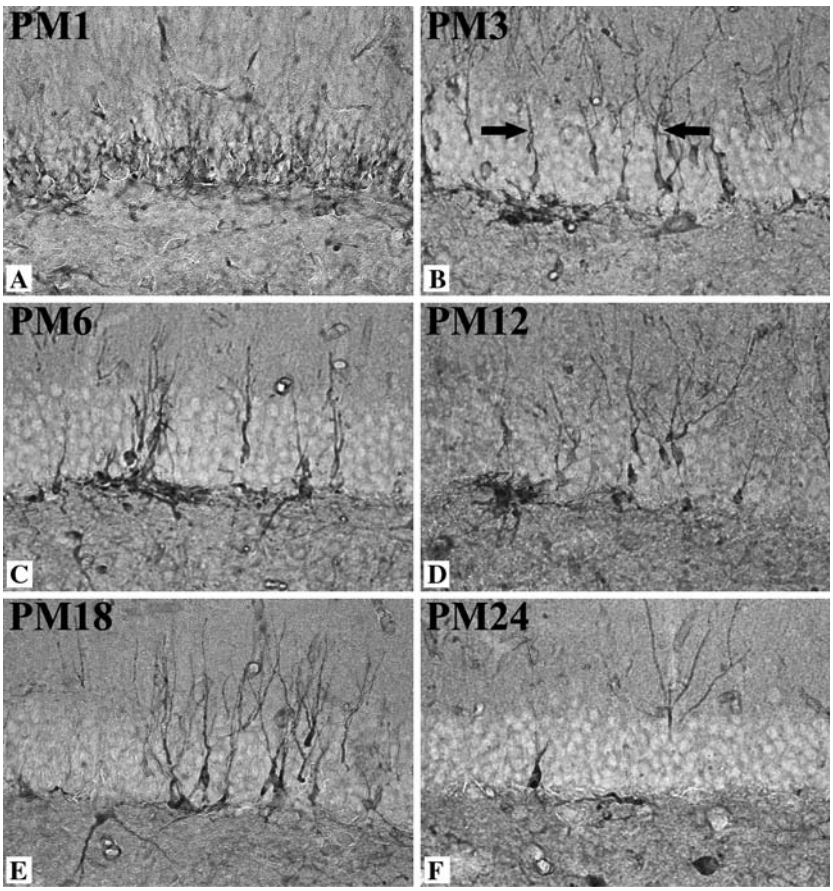

Fig. 2 High magnification of DCX immunoreactivity in the upper blade of the dentate gyrus at postnatal month (PM) 1 (**a**), 3 (**b**), 6 (**c**), 12 (**d**), 18 (**e**) and 24 group (**f**). DCX immunoreactivity at PM 1 is dispersed (**a**). DCX immunoreactive cells in the PM 3–18 groups have well-developed processes (arrows) (B–E). Bar = 25 μm

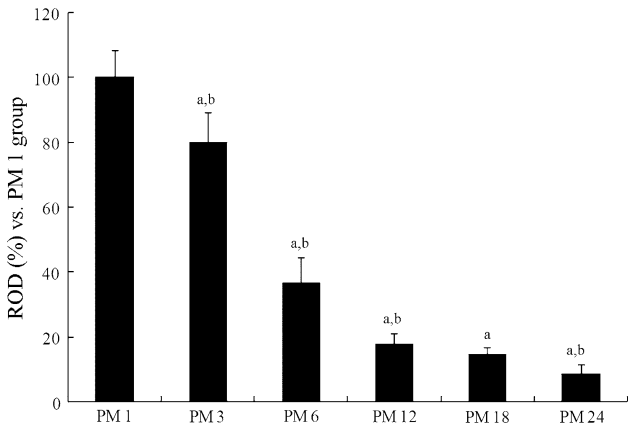

Fig. 3 Relative optical density (ROD) as % of DCX immunoreactivity in the dentate gyrus at various aged gerbils ($n = 7$ per group; $^{a}P < 0.05$, significantly different from the PM 1 group, $^{b}P < 0.05$, significantly different from the pre-adjacent group). The bars indicate the means ± SEM

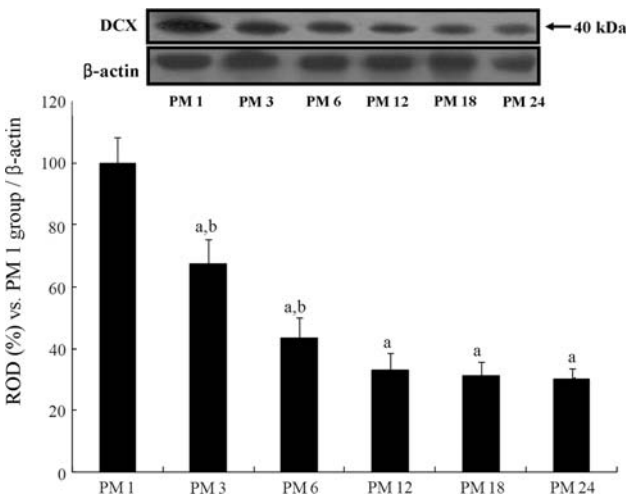

Fig. 4 Western blot analysis of DCX in the dentate gyrus derived from various aged gerbils. The relative optical density (ROD) as % of immunoblot band is also represented ($n = 7$ per group; $^{a}P < 0.05$, significantly different from the PM 1 group, $^{b}P < 0.05$, significantly different from the pre-adjacent group). The bars indicate the means ± SEM

reflected by a reduction in the number of neural stem cells in the SGZ, and suggested that it rather reflects increased quiescence and a lengthening of cell cycle times with aging, which are probably due to multiple changes in the NSC milieu. In the present study, we observed that DCX immunoreactive neuronal precursors did not show a fully-developed morphology at PM 24. This result suggests that neurogenic ability in the dentate gyrus reduced with age.

In conclusion, DCX immunoreactivity and its protein levels were high at PM 1 in the gerbil dentate gyrus, however, DCX immunoreactive neuronal precursors at PM 3 and 6 had well-developed processes. In addition, the reduction of DCX immunoreactive neuronal precursors in number and morphology at PM 24 may be associated with memory deficits in the aged hippocampus.

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