FULL PAPER *Anatomy*

Doublecortin-Immunoreactive Neuronal Precursors in the Dentate Gyrus of Spontaneously Hypertensive Rats at Various Age Stages: Comparison with Sprague-Dawley Rats

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ABSTRACT. Spontaneously hypertensive rats (SHRs) are widely accepted in medical research because this model has been used for studies in neurodegenerative diseases such as vascular dementia and stroke. In the present study, we observed newly generated neuronal precursors using doublecortin (DCX, a marker of neural proliferation and differentiation) in the subgranular zone of the dentate gyrus in SHRs compared to Sprague-Dawley rats (SDRs) at various age stages. DCX immunoreactivity, immunoreactive cell numbers and its protein level in the dentate gyrus of the SHRs were higher than those in the SDRs at postnatal month 1 (PM 1). At PM 8, DCX immunoreactivity, immunoreactive cell numbers and protein levels in both groups were markedly decreased compared to those at PM 1; however, they were higher than those in the SDRs. They were decreased in the both groups with age: DCX immunoreactive cells in the SDRs were few at PM 12. Our results indicate that newly generated neuronal precursors are more abundant in SHRs than in SDRs during their life.

KEY WORDS: development, doublecortin, neurogenesis, spontaneously hypertensive rat.

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Spontaneously hypertensive rats (SHRs) are normotensive at birth and gradually develop stable hypertension during the first months of life. These rats are used as a genetic model of hypertension, which is widely accepted in medical research because it has features in common with idiopathic hypertension in man [20, 21]. Moreover, this animal model has been used recently in studies of neurodegenerative diseases, such as, vascular dementia and stroke, because hypertension is a primary risk factor of these conditions [9, 10].

It has been reported that the rate of granule cell generation in the dentate gyrus of the adult mouse is dependent on strain [14, 15]. In addition, significantly more newly generated cells and greater proliferation were found in the dentate gyrus of the young SHRs than in the young Sprague-Dawley rats (SDRs) [22]. However, they examined newly generated cells in the dentate gyrus using the thymidine analog bromodeoxyuridine (BrdU), which is labeled on newly generated glial cells as well as neuronal precursors [18].

DCX gene encodes a 40-kDa microtubule-associated protein, which is specifically expressed in neuronal precursors in the developing and adult CNS. It is known that during CNS development DCX expression is associated with the migration and differentiation of neuronal precursors [3, 4, 13], and as a result, DCX is frequently used as a marker of newly generated neurons, not glial cells. In the present study, we investigated differences in distribution of DCX immunoreactive neuronal precursors in the dentate gyrus of SHRs and SDRs at various age stages.

MATERIALS AND METHODS

Experimental animals: Male SHRs and SDRs were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. Postnatal month 1 (PM 1) (*n*=12), PM 8 (*n*=12) and PM 12 (*n*=12) rats in each strain were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr 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.

Immunohistochemistry for DCX: To obtain the accurate data for DCX immunoreactivity, 7 animals in each group were used same conditions. The animals were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by

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4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 hr. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

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 (diluted 1:50, Delaware, SantaCruz Biotechnology, CA) overnight at 4°C and subsequently exposed to biotinylated rabbit anti-goat IgG and streptavidin peroxidase complex (diluted 1:200, Vector, Burlingame, CA). 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, Tokyo, 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 any structures.

Western blot analysis: To confirm changes in DCX levels in the dentate gyrus of PM 1, 8 and 12 groups, 5 animals in each group were sacrificed and used for western blot analysis. After sacrificing them and removing the brain, hippocampus was serially and transversely cut into a thickness of 400 μm on a vibratome (Leica, Wetzlar, 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, Rockford, IL). 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). 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, St Louis, MO) and an ECL kit (Pierce Chemical, Rockford, IL).

Quantification of data and statistical analysis: 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 3 layers (molecular, granule cell and polymorphic layers) through a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus, Tokyo, Japan) connected to a PC monitor. The 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 immunoreactive structures was evaluated on the basis of an 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 density was subtracted, a ratio of the OD of image file was calibrated as % (relative OD, ROD) 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., Frederick, MD), which was used to count the ROD.

The number of DCX immunoreactive cells was calculated by using an image analyzing software system (Optimas 6.5, CyberMetrics, North Reading, MA). Cell counts were obtained by averaging the counts from 30 sections taken at the same level of the dentate gyrus.

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

Differences in DCX immunoreactivity between SDRs and SHRs: In PM 1 groups, DCX immunoreactivity was found in the subgranular zone of the polymorphic layer of the dentate gyrus in SDRs and SHRs (Figs. 1A-1D). DCX immunoreactivity in SHRs was stronger than that in SDRs (Fig. 2), and the DCX immunoreactive processes in SHRs, which projected to granule cell layer, were more abundant than those in SDRs. In the PM 8 groups, DCX immunoreactivity in both SDRs and SHRs was significantly lower than that in the PM 1 groups (Figs. 1E-1H and 2); however, DCX immunoreactive processes were well developed in the granule cell layer. In PM 12 groups, DCX immunoreactivity was very low (Figs. 1I-1L and 2): at this time, DCX immunoreactive processes in the SHRs were well stained with DCX antibody, however, those in the SDRs were very poor in development.

Differences in number of DCX immunoreactive neuronal precursors between SDRs and SHRs: In PM 1 groups, DCX immunoreactive cells in the subgranular zone in SHRs were higher in numbers than those in SDRs (Fig. 3). In the PM 8 and 12 groups, numbers of DCX immunoreactive cells in both SDRs and SHRs were significantly decreased compared to those in the PM 1 groups (Fig. 3); DCX immunoreactive neurons in the SHRs were much higher in numbers than those in the SDRs.

Differences in DCX protein levels between SDRs and SHRs: Western blot results in the dentate gyrus of SDRs and SHRs at various ages stages were similar to the immunohis-

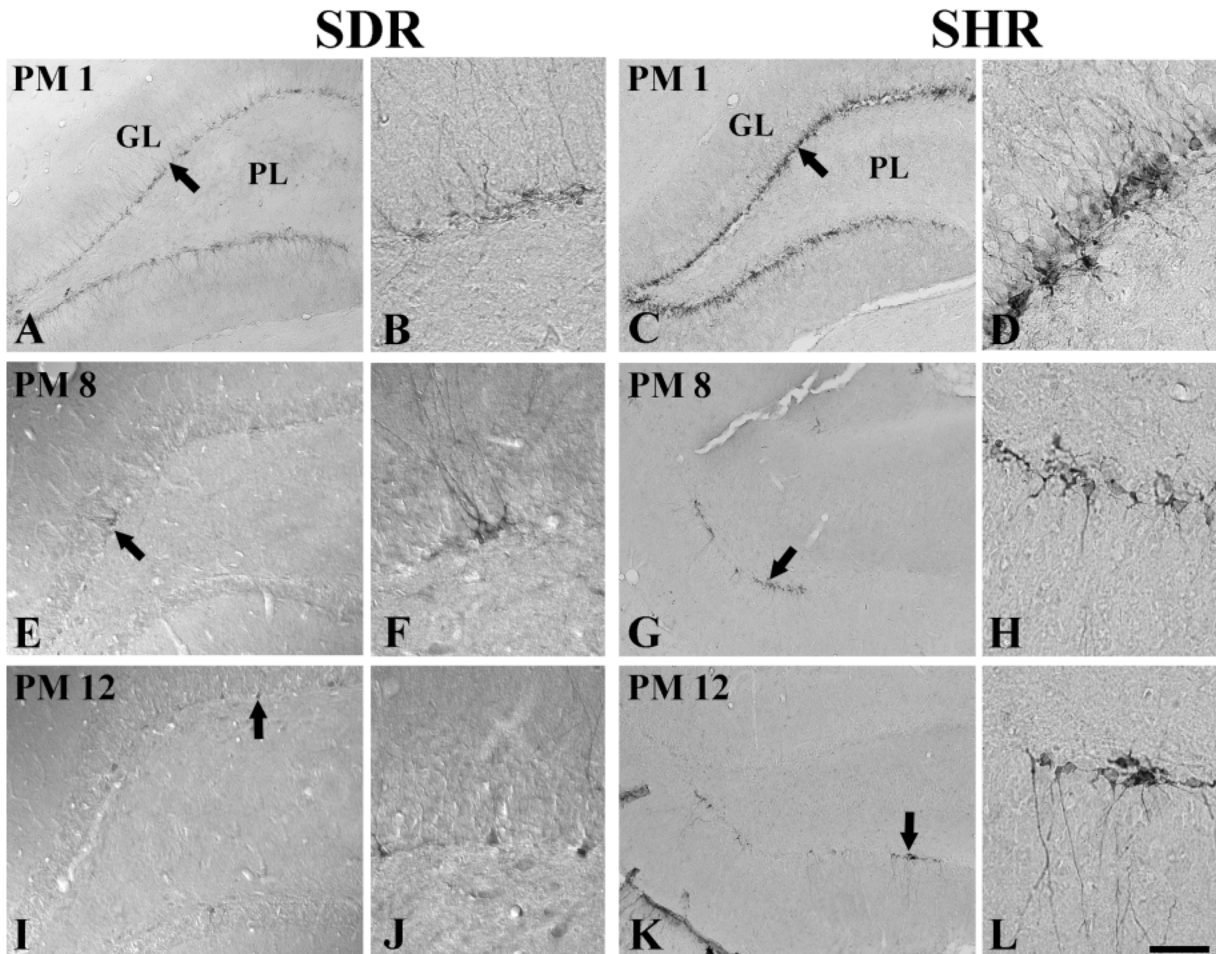

Fig. 1. Microphotographs of DCX immunoreactivity in the dentate gyrus in the SHRs and SDRs at PM 1 (A-D), PM 8 (E-H) and PM 12 (I-L). DCX immunoreactivity is detected in the subgranular zone (arrows) of the dentate gyrus. DCX immunoreactive cell numbers at PM 1 are greater in SHRs than SDRs. Their numbers are significantly decreased at PM 8. DCX immunoreactive cell numbers markedly decrease (arrows); numbers in SHRs are greater than SDRs. GL, granule cell layer; PL, polymorphic layer. Bar = 200 μm (A, C, E, G, I and K), 50 μm (B, D, F, H, J and L).

tochemical changes (Fig. 4). In PM 1 groups, DCX protein level in the SHRs was higher than that in the SDRs. In PM 8 and 12 groups, DCX protein levels in SDRs and SHRs was significantly decreased with time compared to those in the PM 1 groups; DCX protein level in each SHR group was higher than that in the corresponding SDR group.

DISCUSSION

In the present study, we observed DCX immunoreactive cell numbers in the dentate gyrus of SHRs and SDRs at various age stages. DCX immunoreactive cells were found to decrease with age in both groups. This result concurs with previous studies which found that neurogenesis dramatically declined in the dentate gyrus during aging [5, 7, 11, 12, 17, 23, 25, 28]. In addition, Kronenberg *et al.* [16] compared young adult SHR and stroke-prone SHR (SHRSP) with the genetic control WKY strain: in both SHR and SHRSP, newly generated DCX immunoreactive neurons were significantly high compared to WKY strain. However, in the present study, we found that the number of DCX immunoreactive cells, the intensity of DCX immunoreactivity and the expression level of DCX protein in the SHRs were higher than those in the SDRs at corresponding age stages. These results suggest that newly generated DCX immunoreactive cells in hippocampal dentate gyrus are more in SHRs than in SDRs at same age stage.

However, the hippocampus of the 6-month SHR is smaller than that of same-aged normotensive animals [2, 24]. It is well known that the hippocampal complex is related to learning and memory. In the majority of studies, enhanced neurogenesis has been suggested to contribute to the spatial learning test [15, 19]. In addition, it has been reported that persistent hilar basal dendrites contribute to a functional recurrent excitatory circuitry [1, 27]. However, SHRs have impaired learning-memory function and abnor-

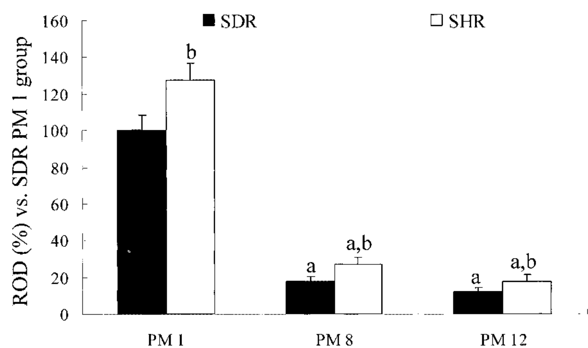

Fig. 2. Relative optical density (ROD) as % of DCX immunoreactivity in the dentate gyrus of SHRs and SDRs at PM 1, PM 8 and PM 12 (n=7 per group; [a] P<0.05, significantly different from the respective PM 1 group, [b] P<0.05, significantly different from the corresponding SDR group). Data are expressed as the means ± SEM.

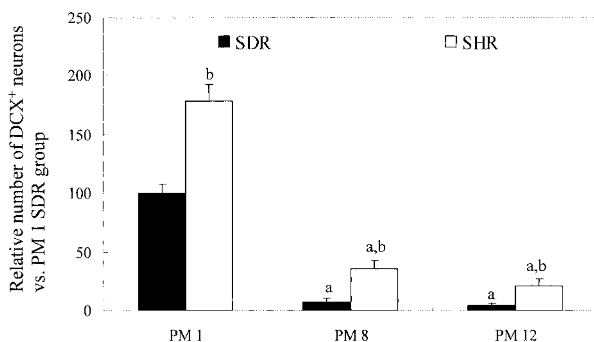

Fig. 3. Relative analysis in the cell number of DCX immunoreactive cells in the dentate gyrus (n=7 per group; [a] P<0.05, significantly different from the respective PM 1 group, [b] P<0.05, significantly different from the corresponding SDR group). Data are expressed as the means ± SEM.

mal behavior [6, 8]. These discrepancies may be associated with compensatory mechanisms designed to overcome hippocampal neurodegeneration in SHRs: *e.g.*, because neurons in the SHR hippocampus are susceptible to hypertensive damage, DCX immunoreactive neuron numbers may be elevated in the hippocampal dentate gyrus. In conclusion, DCX immunoreactivity, immunoreactive cell numbers and protein levels are significantly higher in the dentate gyrus of SHRs than in that of SDRs.

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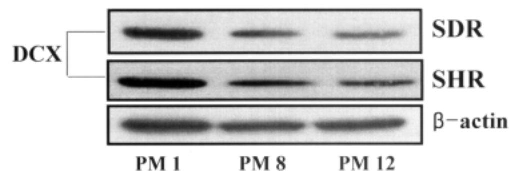

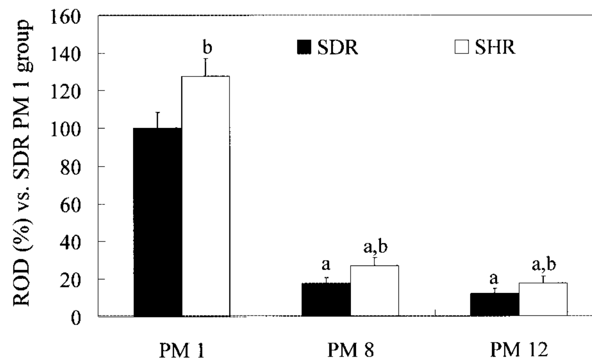

Fig. 4. The top panel shows western blot analysis for DCX in the dentate gyrus of SDRs and SHRs at PM 1, PM 8 and PM 12. The bottom panel shows relative optical density (ROD) of immunoblot bands (n=5 per group; [a] P<0.05, significantly different from the respective PM 1 group, [b] P<0.05, significantly different from the corresponding SDR group). Data are expressed as the means ± SEM.

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