Chapter 10
Acute Stress Exposure Preceding Global Brain Ischemia Accelerates Decreased Doublecortin Expression in the Rat Retrosplenial Cortex

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Abstract **Background:** Psychological distress is a risk factor of stroke in humans and worsens the behavioral and neurological outcomes. In rats, acute stress exposure preceding ischemic events attenuates learning and memory. The retrosplenial cortex (RS) plays an important role in these functions, and global brain ischemia (GBI) or acute stress exposure can induce a decrease in expression of the immature neuronal marker, doublecortin (DCX), in the RS. However, little is known about the DCX expression in the RS after stress exposure prior to GBI. **Methods:** Eighteen male Sprague–Dawley rats were used. Acute stress exposure was applied as the forced swim paradigm and GBI was induced by bilateral common carotid arterial occlusion for 10 min. The rats were divided into three groups: GBI model preconditioned by stress ($n=6$, Group P), GBI model preconditioned by non-stress ($n=6$, Group G), and controls ($n=6$, Group C). We performed immunohistochemistry to observe and analyze the DCX-expressing cells and Fluoro-Jade B (FJB) staining to detect cell death in the RS after GBI in each group. **Results:** The total number of DCX-expressing cells was 1,032, 1,219, and 1,904 in Group P, Group G, and Group C, respectively. The mean number of DCX-expressing cells per unit area was significantly lower in Group P and Group G than in Group C ($P<0.001$). Moreover, the number was significantly lower in Group P than in Group G ($P<0.05$). In each group, no FJB positive cells were observed. **Conclusion:** DCX plays an important role in various cytoskeletal changes. Preconditioning by acute stress exposure accelerated the decrease in DCX expression in the RS after GBI.

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10.1 Introduction

Psychological distress is a risk factor for stroke in humans [1], and acute stress exposure prior to brain ischemia worsens the behavioral and neurological outcomes in rats [2]. Acute stress preconditioning attenuates learning and memory function especially in ischemic rats [2]. However, little is known about the cellular mechanisms involved between acute stress and brain ischemia.

The retrosplenial cortex (RS), the dorsal cingulate cortex, plays an important role in these functions. The RS is associated with memory and visual spatial functions in rats [3, 4] and primates [5]. Memory [6, 7] and learning [8, 9] represent a complex process based on functional and structural changes at the molecular, synaptic, neuronal, and circuitry levels.

Doublecortin (DCX)-expressing cells play a key role in the plasticity of the brain. DCX is involved in various cytoskeletal changes [10, 11]. DCX-expressing cells exist within the cerebral neocortex and allocortex of the adult brain and are apparently associated with a structural plasticity [12–14].

Global brain ischemia (GBI) induces a decrease in DCX expression in the rat RS, though the mechanisms between GBI and the decreased DCX were not elucidated [15]. Moreover, acute stress exposure induces a similar decrease in the rat RS [16]. These findings suggest a decrease in plasticity potential in the RS after GBI or acute stress exposure.

We propose that preconditioning by acute stress can accelerate the decrease in DCX-expressing cells after GBI, and the resultant findings may help to elucidate cellular mechanisms involved between acute stress and brain ischemia.

10.2 Materials and Methods

10.2.1 Stress Preconditioning and GBI Model

Eighteen male Sprague–Dawley rats (body weight, 250–300 g) were used in the present study. Acute stress exposure was applied as the forced swim paradigm (day 1, 15 min; day 2, 5 min) and the rats with immobile behavior were employed [17]. Transient GBI was induced by bilateral common carotid arterial occlusion for 10 min (day 3) [18]. The rats were divided into three groups: stress exposure preceding GBI ($n=6$, Group P), non-stress exposure preceding GBI ($n=6$, Group G), and controls ($n=6$, Group C). The animals were purchased from Charles River Laboratories (Saitama, Japan) and bred at the Animal Housing Facility of Nihon University. The colony was maintained at 22–23 °C on a 12-h light/dark cycle (lights on at 08:00).

At 7 days after GBI, the rats were transcardially perfusion fixed with lactated Ringer's solution, followed by perfusion of 4 % paraformaldehyde. Coronal serial brain sections were cut in the frontal plane on a vibratome (50 μm). We identified the RS by referring to the rat atlas of Paxinos and Watson [19].

All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, National Academy Press, Washington, DC, 2003) and approved by the Animal Care and Use Committee of Nihon University.

10.2.2 Immunohistochemistry and FJB Staining

We performed immunostaining and fluorescence immunostaining with DCX to observe the cells with a plasticity potential. The primary antibodies used in this study were polyclonal goat anti-DCX antibodies (dilution 1:1000, Santa Cruz Biotechnology (California, USA)). We counted the right and left hemispheres separately on two serial sections, for a total of four regions per animal, to examine the differences in expression related to acute stress preconditioning.

We performed Fluoro-Jade B (FJB) staining to investigate the cause of any decrease in DCX-expressing cells. The FJB staining procedure was as reported previously [20].

10.2.3 Measurement and Analysis

A Biozero (BZ-8000; Keyence, Osaka, Japan) and a BZ Analyzer (Keyence) were used to prepare the microphotographs, and a VH Analyzer (Keyence) was used to count the numbers of immunostain-positive cells. We used Neurolucida (Version 3; MicroBrightField, USA) to analyze the area and cell counts without double counting of positive cells and then estimated the cell counts per unit area in the control and GBI model with/without acute stress preconditioning. The statistics software SPSS Statistics 17.0 (Japan IBM, Tokyo, Japan) was used for data analysis. We performed a one-way ANOVA followed by Tukey's tests, and $P < 0.05$ was considered as statistically significant.

10.3 Results

10.3.1 Number of DCX-Expressing Cells

Immunohistochemically DCX-expressing cells in coronal sections were observed in the RS of each case (Fig. 10.1a). Their shape gave the appearance of non-pyramidal cells, they had multiple neurites, and their diameter was 10–25 μm, indicating that they were interneurons. DCX-expressing cells were observed in the subgranular zone (SGZ) within the hippocampal dentate gyrus of each case, indicating the occurrence of neurogenesis in the SGZ (Fig. 10.1b).

The total number of DCX-expressing cells was 1,032, 1,219, and 1,904 cells in Group P, Group G, and Group C, respectively. The mean number of DCX-expressing

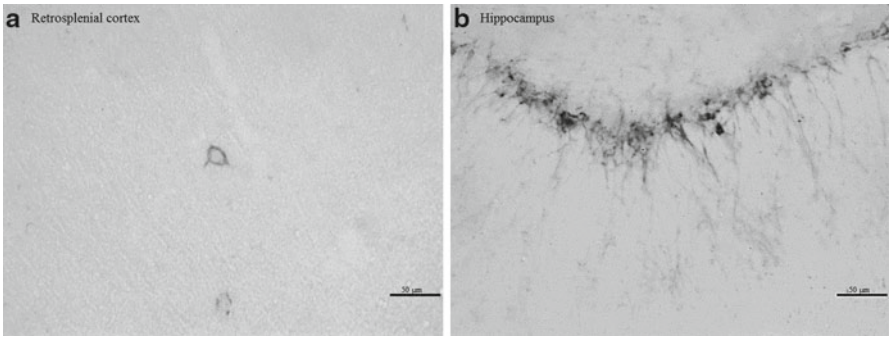

Fig. 10.1 (**a**) Doublecortin (DCX)-expressing cells in the retrosplenial cortex (*RS*) (diaminobenzidine reaction; scale bar, 50 μm; bregma posterior, 3.30 mm). This image was obtained from the RS of a global brain ischemia (*GBI*) model rat after acute stress exposure (Group P; layer, III). Similarly, DCX-expressing cells were present in the RS of the GBI model without acute stress exposure and controls, respectively. (**b**) DCX-expressing cells in the subgranular zone (*SGZ*) of the hippocampal dentate gyrus (diaminobenzidine reaction; scale bar, 50 μm). This image was obtained from the hippocampus of Group P. The cells demonstrate neurogenesis in the SGZ of the hippocampus

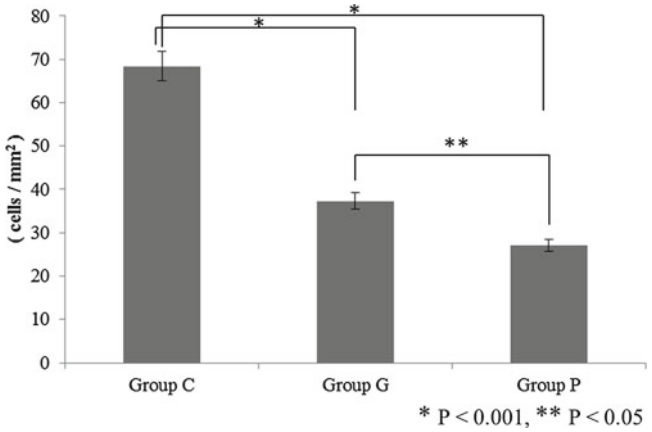

Fig. 10.2 In the global brain ischemia (*GBI*) model, regardless of the preceding acute stress exposure, significantly less doublecortin (*DCX*) expression was observed in the retrosplenial cortex (*RS*) than in the controls (Group C) per unit area ($n = 24$ samples/subregion, right and left hemispheres of two sections from each rat). Acute stress exposure preceding GBI (Group P) revealed a significantly greater decrease in DCX expression in the RS as compared to the normal condition preceding GBI (Group G)

cells per unit area (mean ± SD) was 27 ± 7.57 cells/mm^2 in Group P, 37 ± 9.68 cells/mm^2 in Group G, and 68 ± 18.16 cells/mm^2 in Group C. The mean number of DCX-expressing neurons was significantly lower in Group P and Group G than in Group C (ANOVA followed by Tukey-type test, $P < 0.001$). Moreover, the mean number was significantly lower in Group P than in Group G (ANOVA followed by Tukey-type test, $P < 0.05$) (Fig. 10.2).

10.3.2 FJB Staining

FJB staining is employed for the identification of neuronal cell death to elucidate the mechanism of decreased DCX. As regards the RS, there were no positive cells in Group P, Group G, or Group C, while FJB positive neurons were present in the hippocampal CA1 in Group P and Group G. This result for the RS did not appear to be appropriate for the observed decreased number of DCX-expressing cells.

10.4 Discussion and Conclusion

Preconditioning by acute stress exposure could accelerate the decreased levels of DCX expression in the RS after GBI. The present data showed that the DCX-expressing cells in the RS were significantly decreased after GBI regardless of preconditioning by acute stress exposure in comparison with the controls, as reported previously [15]. Furthermore, acute stress preconditioning through forced swim accelerated the decrease in DCX expression in the RS after GBI as compared to normal preconditioning.

DCX is a microtubule-binding protein and contributes to various cytoskeletal changes involved in the extension of axons and dendrites and new synapse formation in mature neurons [10, 11]. The present results indicate that acute stress preconditioning before GBI accelerated the decrease in plasticity potential after GBI in the RS, though it was not clear whether the actual plasticity decreased in the present study.

DCX-expressing cells in the cortex of adult animals are GABAergic interneurons [13]. The DCX-expressing cells in the present study appeared to be GABAergic interneurons based on their diameter, configuration with bipolar or multipolar formation, and distribution as reported for the RS [16]. It is inferred therefore that interneurons with a plastic potential in the RS may be influenced by acute stress preconditioning.

No FJB positive cells after GBI were observed regardless of acute stress preconditioning, indicating that cells did not fall into cell death after GBI in the present study. Taken together, the decreased DCX expression in the interneurons and findings of FJB staining imply that GABAergic interneurons with a plastic potential may undergo a decrease in their plastic potential without neuronal cell death [21].

Preconditioning by acute stress exposure accelerated the decrease in DCX expression in the RS after GBI. However, the molecular mechanisms between GBI, stress exposure, and the decreased DCX were not elucidated. As described above, the RS plays an important role in memory and space learning which requires complex plastic processes. Alterations in the RS may be associated with more attenuated learning and memory due to acute stress exposure preceding GBI and indicate the involvement of a cellular mechanism between stress exposure and GBI.

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