

Expression of Glucocorticoid Receptor and Coactivators in Ependymal Cells of Male Rats

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Received March 25, 2014; accepted June 24, 2014; published online July 31, 2014

Glucocorticoid receptor (GR) is a ligand-activated nuclear receptor which is widely distributed in the brain. Many types of neurons and glial cells are known to express GR, but the expression of GR in ependymal cells has yet to be identified. The present study therefore was undertaken to determine whether ependymal cells express GR and coactivators of GR, such as steroid receptor coactivator 1 (SRC-1) and p300. GR immunoreactivity was found in cells immunopositive to vimentin, a marker of ependymal cells, around the third ventricle (3V), the lateral ventricle (LV), the cerebral aqueduct and the fourth ventricle (4V), whereas the expression of GR in vimentin-immunoreactive (ir) cells was significantly reduced by adrenalectomy (ADX) in male rats. Vimentin-ir cells also expressed both SRC-1 and p300 at around 3V, LV, the cerebral aqueduct and 4V. ADX had no effect on the expression of SRC-1 or p300 in vimentin-ir cells. These results suggest that glucocorticoid may exert effects on ependymal cells through binding to GR followed by association with SRC-1 and p300 to maintain brain environment under stressful conditions.

Key words: glucocorticoid receptor, ependymal cell, p300, steroid receptor coactivator 1 (SRC-1), adrenalectomy

I. Introduction

Glucocorticoids are secreted from the adrenal cortex, especially under stressful conditions, and are activated by binding to corticosteroid receptors. There are two known corticosteroid receptors: mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). GR has a lower affinity for corticosteroids than MR and is widely distributed in the brain [11]. Glucocorticoids are known to influence brain development, reproduction, metabolic systems, learning and memory, synaptic plasticity, neurogenesis and the central immune responses in the brain [13, 15, 16, 24, 33].

GR is a ligand-activated nuclear receptor. Ligand-bound GR is translocated into the nucleus and binds directly to glucocorticoid response elements of the target

gene. Steroid receptor coactivator-1 (SRC-1), a member of a larger family of p160 proteins, interacts with ligand-bound nuclear receptors in a ligand-dependent manner. SRC-1 can interact with p300 and its family member, CREB-binding protein (CBP), which is a transcriptional coactivator [30]. SRC-1 and p300/CBP form complexes [34] and have been immunohistochemically shown to colocalize in the rat hippocampus, which expresses GR [21]. These coactivators are required for transcriptional regulation by nuclear receptors, and for enhancing transcriptional activity.

The ependymal cell, a type of glial cell which lines the ventricle wall, provides trophic and metabolic support for progenitor cells [5]. Ependymal cells have been reported to also express glucokinase (GK) which acts like a glucose sensor, aquaporins that are channels to control water movement, and organic ion transporters including organic cation transporter 3 (OCT3) and multidrug resistance protein 5 [5, 25]. Hence, ependymal cells might play several important roles in the brain.

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GR is known to be expressed in many types of neurons and glial cells [3], but the expression of GR in ependymal cells is still unknown. In this study, the expression of GR and the coactivators in ependymal cells was examined immunohistochemically, and the effect of adrenalectomy (ADX) on their expression was determined.

II. Materials and Methods

Animals

Adult male Wistar rats (Saitama Experimental Animal Supply, Saitama, Japan) weighing 200–270 g were housed in a controlled (14 hr light/10 hr darkness, 6:00 am light on) environment with free access to food and water. The animals underwent bilateral ADX or sham operation under isoflurane anesthesia. ADX animals were given 0.9% NaCl throughout the experimental course. Three weeks after operation, rats were deeply anesthetized with sodium pentobarbital and perfused through the heart with physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). As an intact-control, non-operated normal male rats were also fixed by the same procedure. The brain was removed from the skull and kept in the same fixative overnight at 4°C. The brain was then immersed in 0.05 M PB containing 30% sucrose at 4°C for 4 days to protect it from cryodamage, and sectioned along the coronal plane at 40 µm on a cryostat (CM-3050-S, Leica, Wetzlar, Germany). All experimental procedures involving live rats were performed according to NIH guidelines (Guide for the Care and Use of Laboratory Animals, 2011) and approved by the Committee on Animal Research of Nippon Medical School.

Dual-labeling fluorescence immunohistochemistry with GR and vimentin

The sections were washed in 0.1 M PB containing 0.9% NaCl and 0.3% Triton-X 100 (Wako, Osaka, Japan) (PBST), and incubated with blocking buffer (1% bovine serum albumin (Sigma, St Louis, MO), 5% normal donkey serum, 0.02% sodium azide in PBST) for 90 min, followed by anti-GR antibody (kindly provided by Prof. M. Kawata, Kyoto Prefectural University of Medicine, rabbit polyclonal, 1:5,000 [19]) and anti-vimentin, which is a marker of ependymal cells (mouse monoclonal, 1:10,000, Millipore, Temecula, CA) diluted with 0.1 M PBST at 4°C for 5 days. After washing in 0.1 M PBS, the sections were incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:800, Invitrogen, Eugene, OR) and Alexa Fluor 568-conjugated donkey anti-rabbit IgG (1:800, Invitrogen) diluted with 0.1 M PBS for 2 hr at room temperature (RT). Then, the sections were washed in 0.1 M PBS, and incubated with 4,6-diamidino-2-phenylindole (DAPI, 1:5,000, Roche, Mannheim, Germany) diluted with 0.1 M PBS for visualizing cell nuclei. After washing, the sections were mounted on glass slides using Vectashield (H-1400, Vector Laboratories, Burlingame, CA) as mount-

ing medium. Fluorescence images were obtained using confocal laser microscopy (LSM710, Carl Zeiss, Oberkochen, Germany). Immunoreactive (ir) cells were counted in the images displayed on the monitor. The sum of the cell number in the brain sections was obtained. The number of GR +vimentin-ir or vimentin-ir cells was counted in three sections containing the third ventricle (3V) and the lateral ventricle (LV), or the cerebral aqueduct, and in five sections containing the fourth ventricle (4V).

Dual-labeling fluorescence immunohistochemistry with p300 and vimentin

Details of immunohistochemistry were described above. Briefly, the sections were incubated with anti-CBP/p300 antibody (mouse monoclonal, 1:500, Acris Antibodies GmbH, Herford, Germany) at 4°C for 2 days and then with anti-vimentin antibody (goat, 1:1,000). After 6 days in primary antibodies, sections were washed and placed in Alexa Fluor 488-conjugated donkey anti-goat IgG (1:800, Invitrogen) and Alexa Fluor 568-conjugated donkey anti-mouse IgG (1:800, Invitrogen). Cell nuclei were visualized using DAPI. Fluorescence images were obtained using confocal laser microscopy. The number of p300+vimentin-ir or vimentin-ir cells was counted in the three sections containing 3V and LV and in the two sections containing the cerebral aqueduct or 4V. The total number of the cells in the brain sections was thus obtained.

Dual-labeling fluorescence immunohistochemistry with SRC-1 and vimentin

Details of immunohistochemistry were described above. Briefly, the sections were incubated with anti-SRC-1 antibody (mouse monoclonal, 1:250, Thermo Scientific, Rockford, IL) at 4°C for 24 hr and then with anti-vimentin antibody (goat polyclonal, 1:1,000, Millipore). After 4 days in primary antibodies, sections were washed and placed in Alexa Fluor 488 anti-goat IgG and Alexa Fluor 568 anti-mouse IgG. Cell nuclei were visualized using DAPI. The number of SRC-1+vimentin-ir or vimentin-ir cells was counted in the three sections containing 3V and LV and in the two sections containing the cerebral aqueduct or 4V. The total number of the cells in the brain sections was thus obtained.

Dual-labeling fluorescence immunohistochemistry with OCT3 and vimentin

The details of immunohistochemistry were as described above. Briefly, the sections containing 3V and LV were incubated with anti-OCT3 antibody (rabbit polyclonal, 1:1,000, Alpha Diagnostic International, San Antonio, TX) [9, 27] and mouse anti-vimentin antibody at 4°C for 2 days, followed by Alexa Fluor 488 anti-mouse IgG and Alexa Fluor 568 anti-rabbit IgG. Cell nuclei were visualized using DAPI.

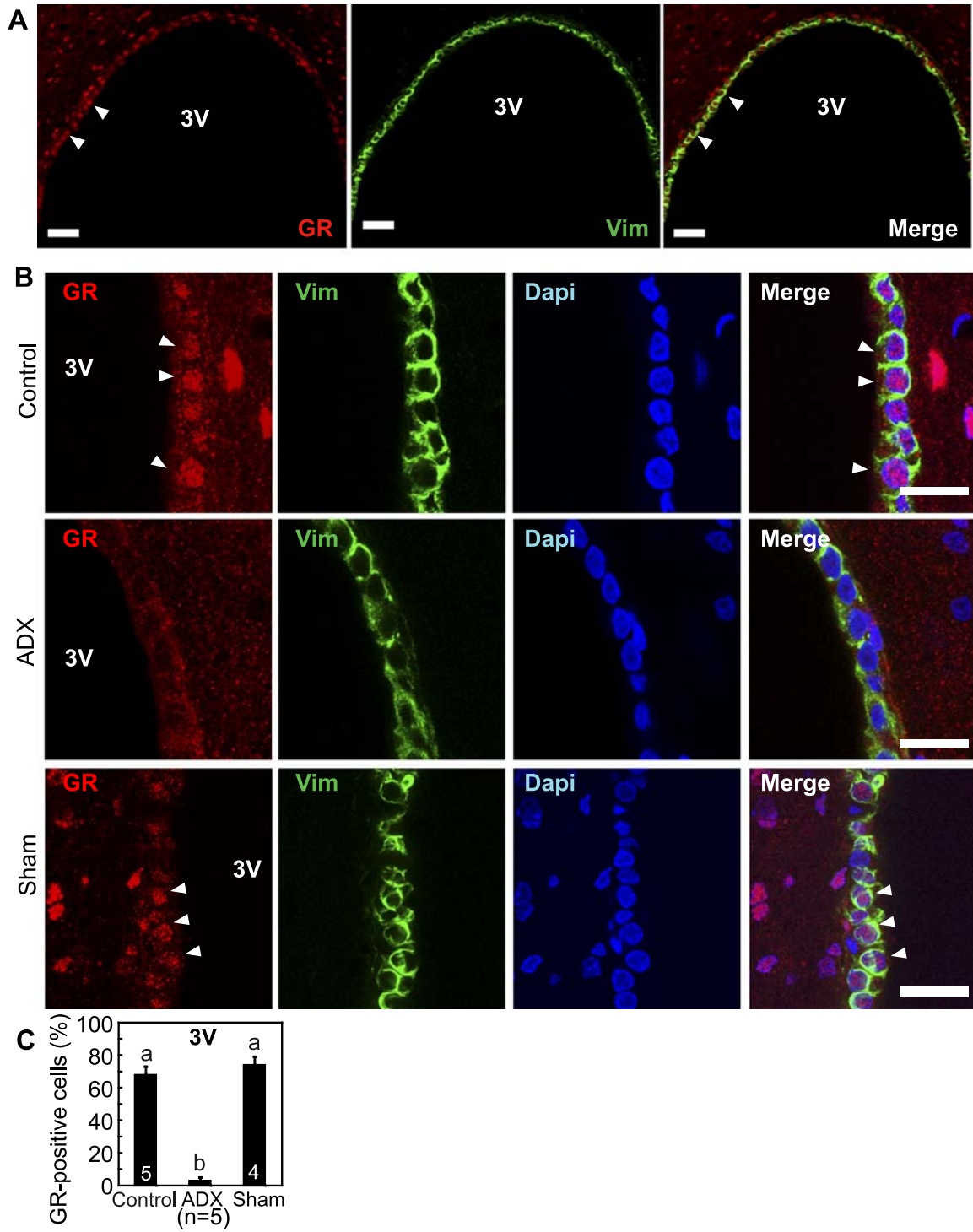


Fig. 1. Expression of glucocorticoid receptor (GR) on ependymal cells around the third ventricle (3V). **(A)** Immunofluorescent localization of GR (red), vimentin (a marker of ependymal cells, green) around 3V in intact controls. Bars=50 μ m. **(B)** Expression of GR, vimentin and DAPI (cell nuclei, blue) around 3V in controls, adrenalectomized (ADX) and sham-operated rats. Bars=20 μ m. Arrowheads indicate GR expression in ependymal cells. **(C)** Percentage of GR-ir cells to vimentin-ir cells around 3V in control, ADX and sham-operated rats. Values with a different letter are significantly different from each other ($P<0.05$, one-way ANOVA followed by Bonferroni test). Values are means \pm SEM. The number in each column represents the number of animals used.

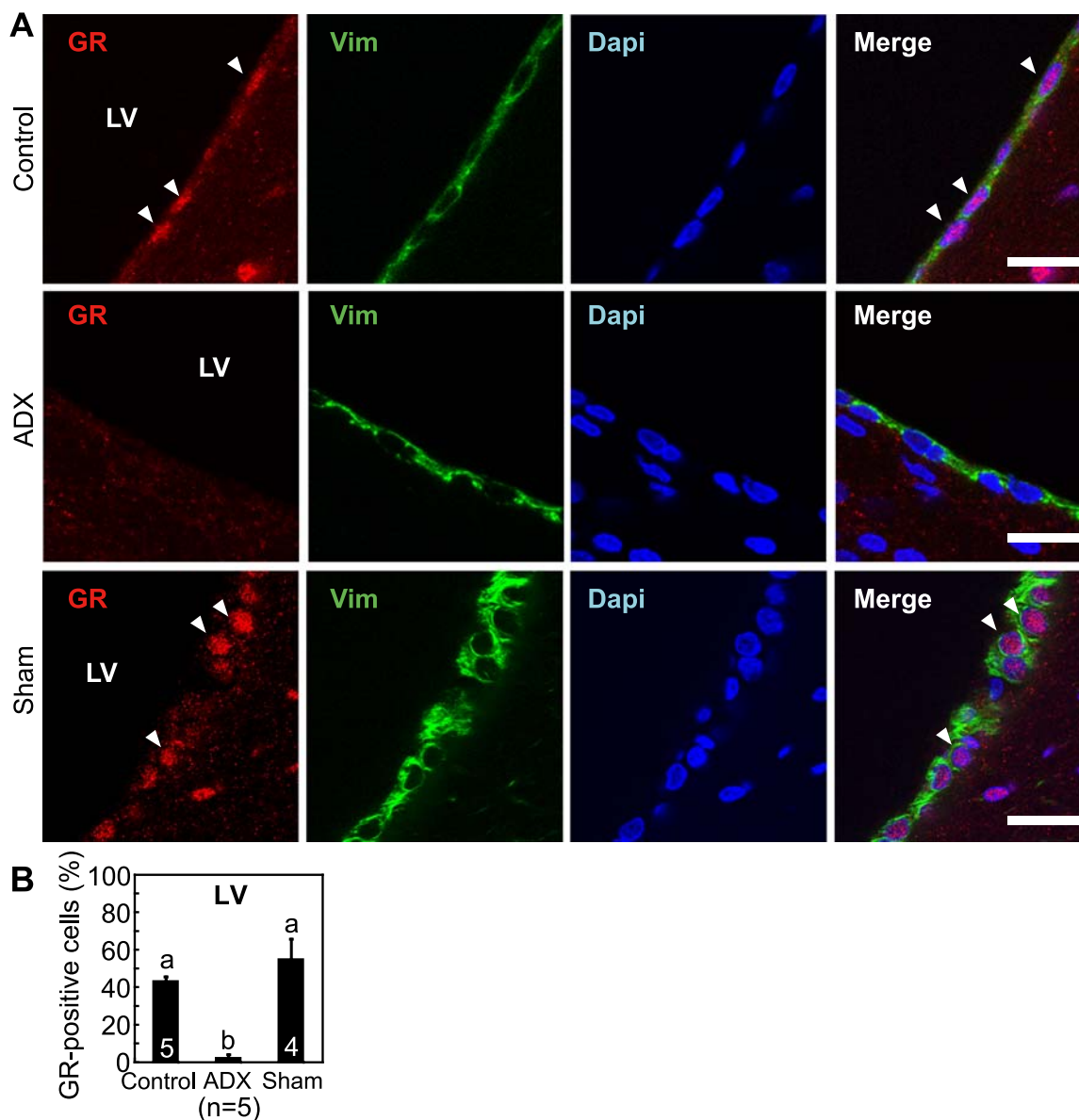


Fig. 2. Expression of glucocorticoid receptor (GR) on ependymal cells around the lateral ventricle (LV). (A) Expression of GR (red), vimentin (green) and DAPI (blue) around the LV in controls, adrenalectomized (ADX) and sham-operated rats. Bars=20 μ m. Arrowheads indicate GR expression in ependymal cells. (B) Percentage of GR-ir cells to vimentin-ir cells around LV in control, ADX and sham-operated rats. Values are means \pm SEM. Values with a different letter are significantly different from each other ($P<0.05$, one-way ANOVA followed by Bonferroni test). The number in each column represents the number of animals used.

Statistical analysis

All data are expressed as mean \pm SEM. Statistical differences in cell numbers were determined by one-way ANOVA followed by Bonferroni test (Figs. 1C, 2B, 3B and 4B). Statistical differences in cell numbers between groups were determined by unpaired Student's *t*-test (Fig. 5B and 5D).

III. Results

GR immunoreactivity was detected in cells immunopositive to vimentin, a marker of ependymal cells, around

3V, LV, the cerebral aqueduct and 4V (Figs. 1A, 2A, 3A and 4A, respectively). About 67% (1,698/2,514 cells) of vimentin-ir cells around 3V expressed GR in intact-controls, whereas the percentage of GR-ir to vimentin-ir cells was significantly lower in ADX rats (2.8 \pm 1.9%, 54/1,951 cells) (Fig. 1B and 1C). About 43% (408/957 cells) of vimentin-ir cells around LV expressed GR in controls, whereas the percentage of GR-ir to vimentin-ir cells was significantly reduced in ADX rats (2.2 \pm 1.8%, 16/783 cells) (Fig. 2). About 65% (1,885/3,155 cells) of vimentin-ir cells around the cerebral aqueduct expressed GR in controls, whereas the percentage of GR-ir to vimentin-ir cells

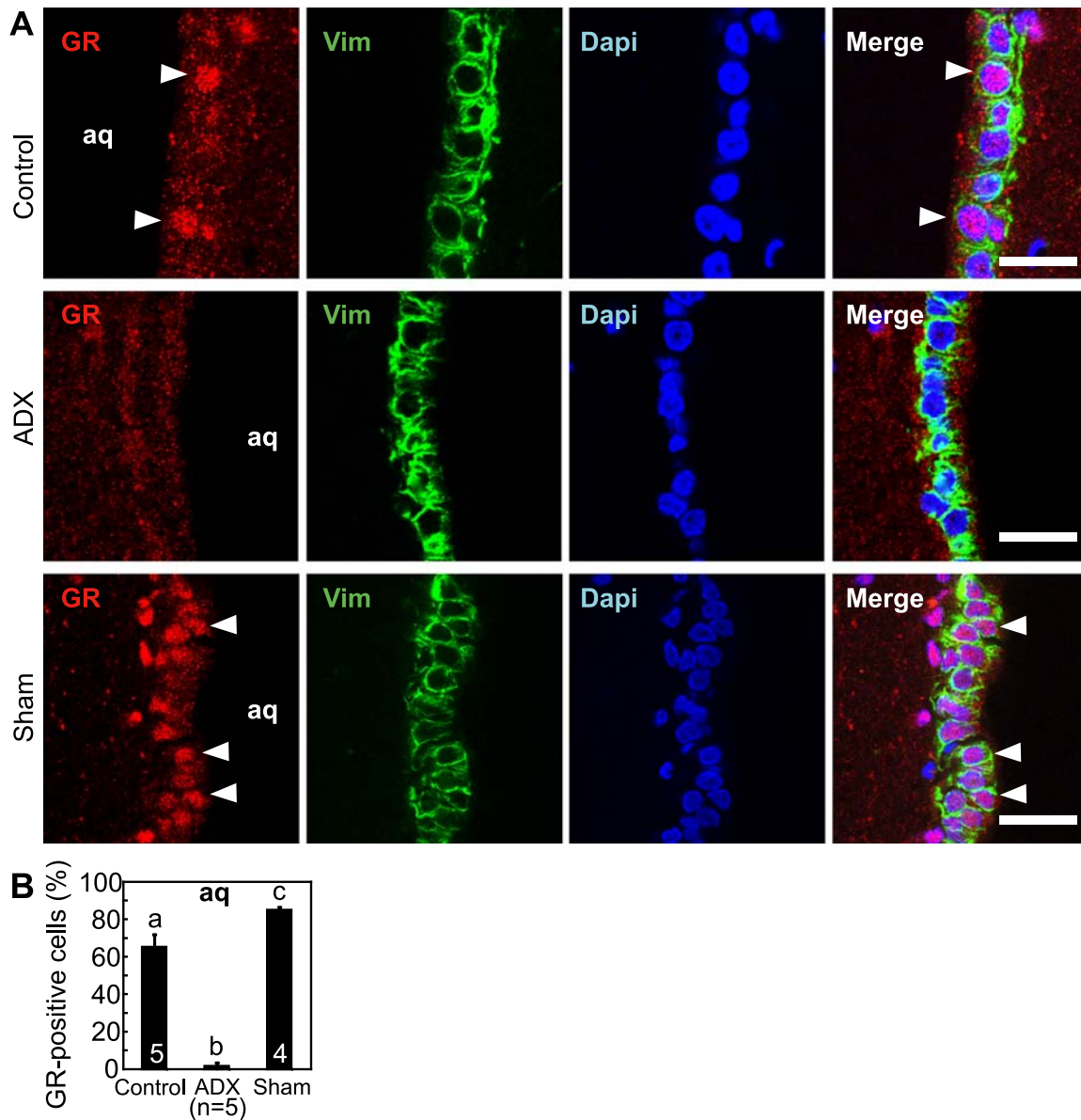


Fig. 3. Expression of glucocorticoid receptor (GR) on ependymal cells around the cerebral aqueduct (aq). (A) Expression of GR (red), vimentin (green) and DAPI (blue) around the cerebral aqueduct in controls, adrenalectomized (ADX) and sham-operated rats. Bars=20 μ m. Arrowheads indicate GR expression in ependymal cells. (B) Percentage of GR-ir cells to vimentin-ir cells around the cerebral aqueduct in control, ADX and sham-operated rats. Values are means \pm SEM. Values with a different letter are significantly different from each other ($P<0.05$, one-way ANOVA followed by Bonferroni test). The number in each column represents the number of animals used.

was significantly lower in ADX rats ($1.7\pm 1.6\%$, 37/2,181 cells) (Fig. 3). About 65% (1,623/2,558 cells) of vimentin-ir cells around 4V expressed GR in controls, whereas the percentage of GR-ir to vimentin-ir cells was significantly reduced in ADX rats ($1.9\pm 1.2\%$, 16/1,513 cells) (Fig. 4). Sham operation had no effect on the expression of GR in ependymal cells around 3V ($73.9\pm 5.0\%$, 1,336/1,756 cells), LV ($54.8\pm 10.6\%$, 1,004/1,946 cells) and 4V ($49.7\pm 5.6\%$, 805/1,642 cells), while the percentage of GR-ir cells to vimentin-ir cells around the cerebral aqueduct in sham-operated animals was significantly higher than in intact-

control animals (Fig. 3B, $85.0\pm 1.1\%$, 1,333/1,573 cells).

Vimentin-ir cells expressed both SRC-1 and p300 around 3V, LV, the cerebral aqueduct and 4V (Fig. 5). While the expression of GR in the ependymal cells was reduced by ADX, ADX had no effect on the expression of either SRC-1 or p300 (Fig. 5).

The expression of OCT3, which is a corticosterone-sensitive monoamine transporter, was analyzed by immunohistochemistry. OCT3 was expressed in vimentin-positive cells in controls (Fig. 6). ADX reduced GR expression, but ADX had no effect on the expression of OCT3 in

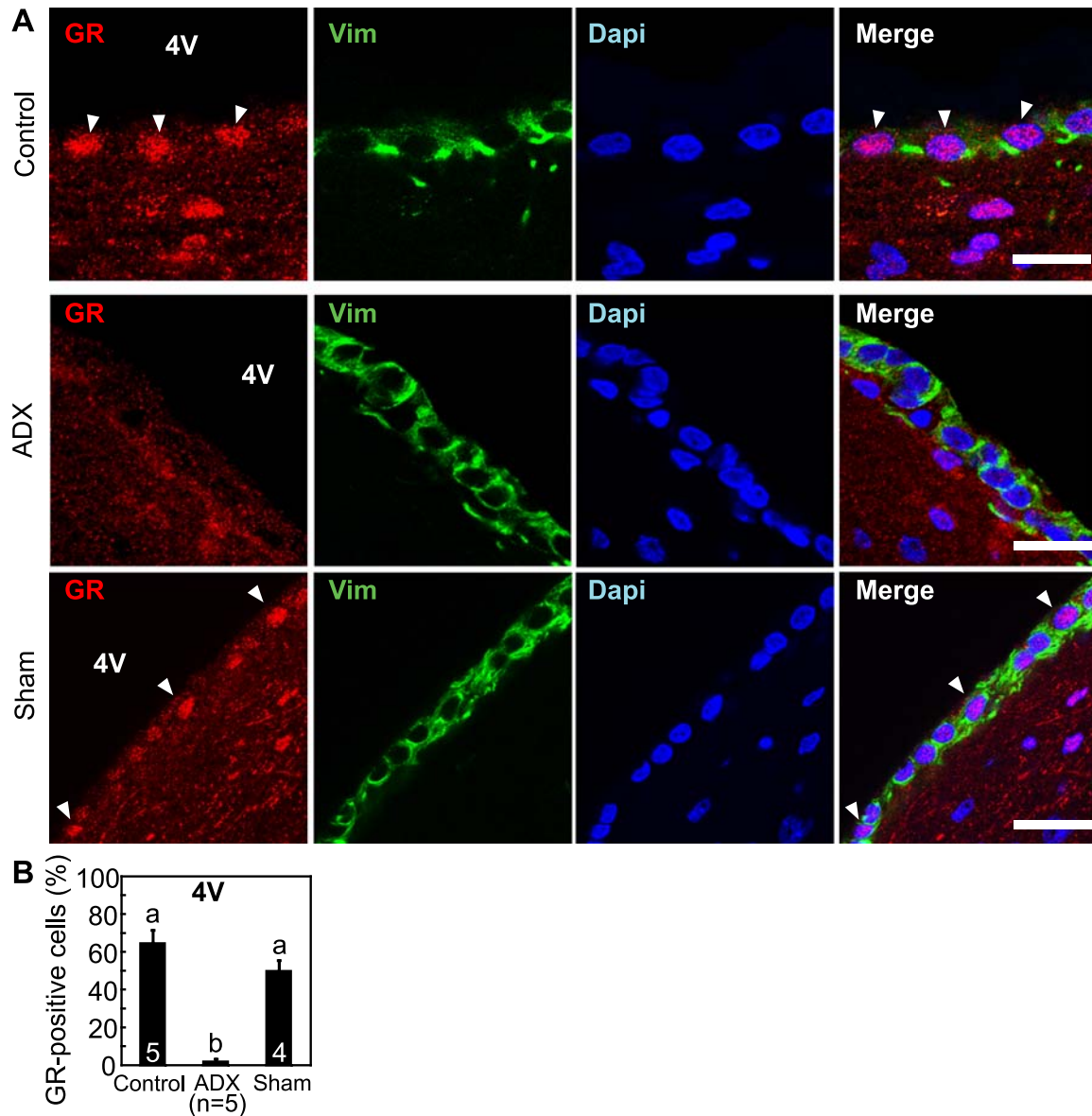


Fig. 4. Expression of glucocorticoid receptor (GR) in ependymal cells around the fourth ventricle (4V). (A) Expression of GR (red), vimentin (green) and DAPI (blue) around 4V in controls, adrenalectomized (ADX) and sham-operated rats. Bars=20 μ m. Arrowheads indicate GR expression in ependymal cells. (B) Percentage of GR-ir cells to vimentin-ir cells around 4V in control, ADX and sham-operated rats. Values are means \pm SEM. Values with a different letter are significantly different from each other ($P<0.05$, one-way ANOVA followed by Bonferroni test). The number in each column represents the number of animals used.

ependymal cells (Fig. 6). OCT3-ir cells could not be detected in the absence of anti-OCT3 antibody.

IV. Discussion

This study is the first to reveal that GR is expressed in ependymal cells lining all ventricles in the rat brain, and that the expression was diminished by ADX. This result concurred with previous studies, which demonstrated that GR is expressed in the hippocampus, and that the expression is suppressed by ADX [8, 12, 23]. Because GR is known to be localized predominantly in the cytoplasm in

the absence of ligands [20], ADX-induced suppression of GR in ependymal cells may be induced by diffusion of GR into the cytoplasm. Moreover, SRC-1 and p300, which are coactivators associated with classical steroid hormone action mediated through steroid receptors functioning as ligand-dependent transcription factors, were also expressed in ependymal cells, while the expression was unaffected by ADX. Although the reason for the discrepancy is not clear, there is the possibility that the expressions of the coactivators in ADX animals are induced by a different pathway of GR expression. It is well known that SRC-1 and p300 interact with many different steroid hormone receptors such as

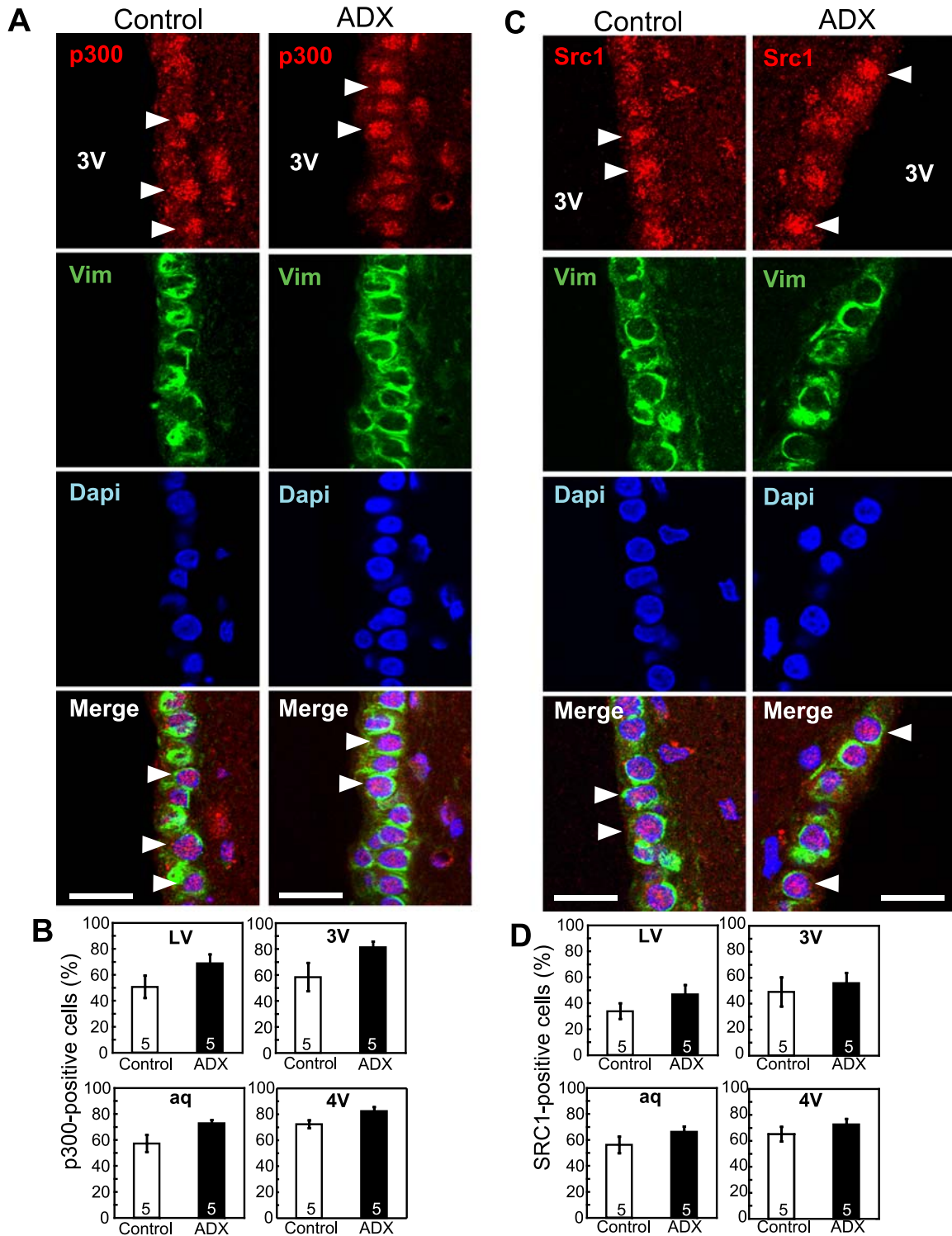


Fig. 5. Expression of coactivators on ependymal cells. (A) Expression of p300 (red), which is a glucocorticoid receptor (GR) coactivator, vimentin (green) and DAPI (blue) around the third ventricle (3V) in controls and adrenalectomized (ADX) rats. Bars=20 μ m. Arrowheads indicate p300 expression in ependymal cells. (B) Percentage of p300-ir cells to vimentin-ir cells around the lateral ventricle (LV), 3V, the cerebral aqueduct (aq) and the fourth ventricle (4V) in control and ADX rats. Statistical differences were determined by unpaired Student's *t*-test. Number in each column represents the number of animals used. Values are means \pm SEM. (C) Expression of steroid receptor coactivator 1 (SRC-1, red), which is also a GR coactivator, vimentin (green) and DAPI (blue) around 3V in controls and ADX rats. Bars=20 μ m. Arrowheads indicate SRC-1 expression in ependymal cells. (D) Percentage of SRC-1-ir cells to vimentin-ir cells. Statistical differences were determined by unpaired Student's *t*-test. Values are expressed as means \pm SEM.

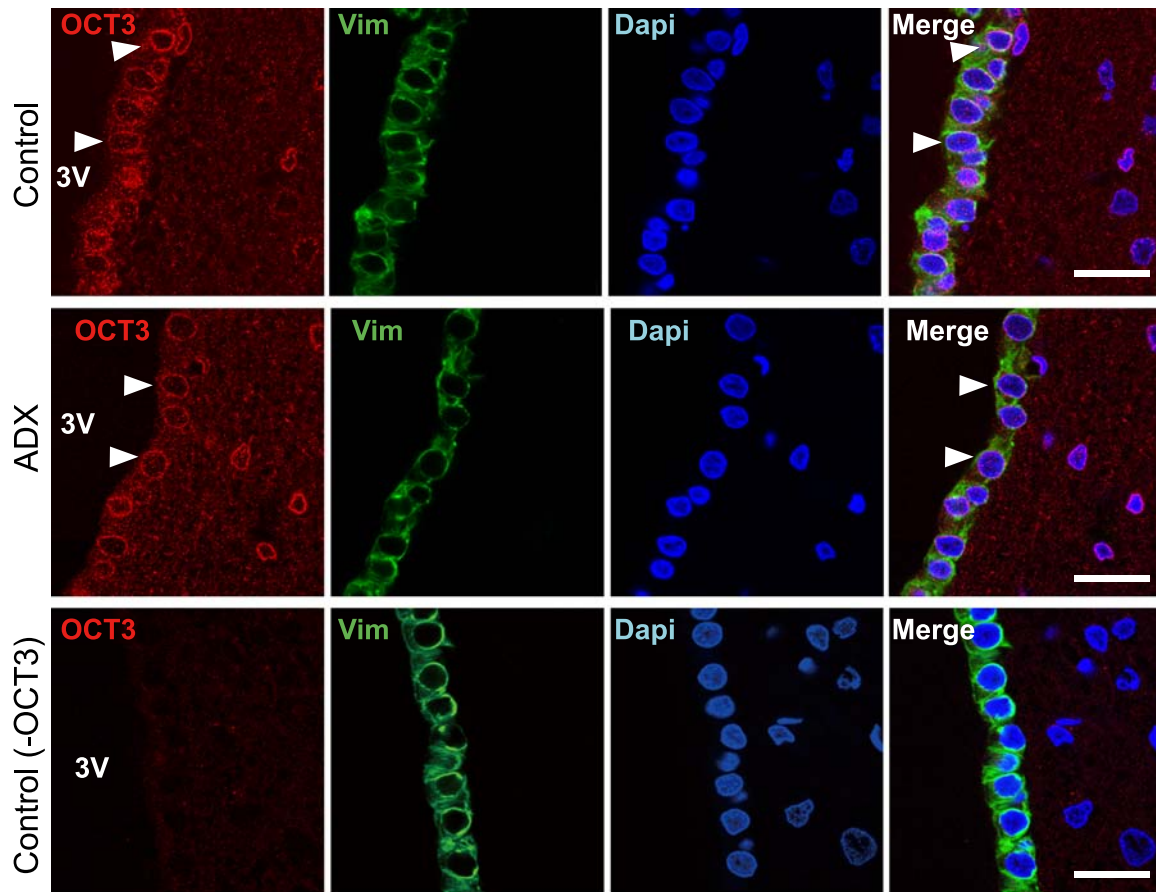


Fig. 6. Expression of organic cation transporter 3 (OCT3) in ependymal cells around the third ventricle (3V). Localization of OCT3 (red), which is a monoamine transporter, vimentin (green) and DAPI (blue) around 3V in controls and adrenalectomized (ADX) rats. Bars=20 μ m. Arrowheads indicate OCT3 expression in ependymal cells. OCT3-ir cells were not found in sections incubated in the absence of anti-OCT3 antibody (lower panels).

GR, MR, estrogen receptors (ER), androgen receptors (AR), and progesterone receptors (PR) [2, 14, 18, 22]. It has been reported that ependymal cells express MR [1]. Co-expression of GR and MR has also been reported in CA1 and CA2 hippocampal pyramidal neurons, and MR expression in CA1 and CA2 neurons was also unaffected by ADX [23]. SRC-1 is activated by Src kinase, the mitogen-activated protein kinases, growth factors and cAMP [32]. 8-Bromo-cAMP-induced activation of SRC-1 regulates the ligand-independent activation of chicken PR [26]. On the other hand, p300 shows a broad range of interaction with transcriptional factors, including GR and MR. Thus, although SRC-1 and p300 co-express and form complexes with GR in a ligand-dependent manner, another signaling pathway may be involved in the expression of SRC-1 and p300 in ependymal cells, regardless of the GR expression.

We showed that there are GR-immunopositive and GR-immunonegative ependymal cells in the rat brain. It is very difficult to clarify whether there is any difference in cellular function and/or property between these cells. One possibility is that each of the two kinds of ependymal cells plays a different functional role. The other possibility is that each ependymal cell has different levels of protein

expression, although all ependymal cells express GR. In any case, it is necessary to conduct further investigations.

To date, the function of GR in ependymal cells has yet to be elucidated. To obtain clues to determine the mechanism of the expression of GR in ependymal cells, in this study we investigated whether the expression of OCT3 is affected by ADX. OCT3 is a corticosterone-sensitive monoamine transporter, and corticosterone inhibits the transport of histamine in rat medial hypothalamic tissues [6]. Moreover, OCT3 is also expressed in ependymal cells [7]. The present study also showed that ependymal cells expressed OCT3. However, the OCT3 expression was unaffected by ADX indicating that the expression of OCT3 in ependymal cells may not be associated with corticosteroid-GR actions. On the other hand, OCT3-immunoreactivity was observed around the perikarya of ependymal cells, and was located very close to the cell nucleus similar to that observed in neurons of the amygdala [9]. Further studies are needed to clarify the details of this result.

Ependymal cells have been reported to express glucose transporter (GLUT) 1, 2, 4, GK, and monocarboxylate transporter 1, which is a transporter of ketone body, lactate and pyruvate, [10, 17, 31], suggesting that ependymal cells

might integrate the information to maintain energy homeostasis. Insulin-like growth factor (IGF-1) regulates GLUT2 and GLUT3 [29], and increases glucose transport in primary ependymal cell cultures [31]. Dexamethasone, a synthetic glucocorticoid, downregulates IGF-1 in glial cells [4]. Dexamethasone also has an inhibitory effect on GK activity in MIN6 β -cells [28]. Since GK and GLUT2 have been considered to play a key role in glucose sensing [17], GR in ependymal cells might be involved in glucose transport and glucose sensing mechanisms. Further studies are needed to elucidate these interactions.

In conclusion, this is the first study to demonstrate that ependymal cells lining the ventricles express GR and the coactivators, SRC-1 and p300. The expression of GR is thought to be ligand dependent. Although the function of corticosteroids via GR in ependymal cells is still unclear, ependymal cells are considered to be related to the maintenance of certain brain functions through GR under stressful conditions.

V. Acknowledgments

We are grateful to Professor Mitsuhiro Kawata, Department of Anatomy and Neurobiology, Kyoto Prefectural University of Medicine, for kindly providing rabbit anti-GR antibody. This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan (#S0801035, 22590230 to H.O.), and the Ministry of Education, Sports, Science and Technology (MEXT)-supported Program for the Strategic Research Foundation at Private Universities.

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