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Age-dependent change of HMGB1 and DNA double-strand break accumulation in mouse brain

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ABSTRACT

HMGB1 is an evolutionarily conserved non-histone chromatin-associated protein with key roles in maintenance of nuclear homeostasis; however, the function of HMGB1 in the brain remains largely unknown. Recently, we found that the reduction of nuclear HMGB1 protein level in the nucleus associates with DNA double-strand break (DDSB)-mediated neuronal damage in Huntington's disease [M.L. Qi, K. Tagawa, Y. Enokido, N. Yoshimura, Y. Wada, K. Watase, S. Ishiura, I. Kanazawa, J. Botas, M. Saitoe, E.E. Wanker, H. Okazawa, Proteome analysis of soluble nuclear proteins reveals that HMGB1/2 suppress genotoxic stress in polyglutamine diseases, Nat. Cell Biol. 9 (2007) 402–414]. In this study, we analyze the region- and cell type-specific changes of HMGB1 and DDSB accumulation during the aging of mouse brain. HMGB1 is localized in the nuclei of neurons and astrocytes, and the protein level changes in various brain regions age-dependently. HMGB1 reduces in neurons, whereas it increases in astrocytes during aging. In contrast, DDSB remarkably accumulates in neurons, but it does not change significantly in astrocytes during aging. These results indicate that HMGB1 expression during aging is differentially regulated between neurons and astrocytes, and suggest that the reduction of nuclear HMGB1 might be causative for DDSB in neurons of the aged brain.

To identify age-related changes of gene expression and function would be one of the most effective approaches to elucidate the molecular mechanism of aging. In the nervous system, aging is not only an unavoidable process characterized by a large array of alterations in structure and function resulting in brain dysfunction and cognitive decline, but also a major risk factor for some age-related neurological diseases, such as Alzheimer's, Huntington's and Parkinson's disease [2–6]. Thus, unraveling the mechanism of brain aging would be crucially important for understanding its molecular link to neurodegeneration and for developing effective therapeutic strategies against various age-related neuropathologies.

A large body of evidence suggests that the accumulation of DNA damage is a potential candidate to cause brain aging and neurodegenerative disease [6–11]. DNA damage may affect expression of various genes involved in learning, memory and neuronal survival, to initiate a program of brain aging that starts early in adult life [12–14], especially for in post-mitotic neurons because they do not self-renew through cell proliferation. However, the region-specific and/or cell type-specific analyses of key molecules conducting DNA damage repair during aging of the brain have not been reported.

High-mobility group B1 (HMGB1) is a nuclear non-histone DNA-binding protein that belongs to the high-mobility group box family of proteins with key roles in maintenance of nuclear homeostasis [15–17]. HMGB1 binds to DNA in the minor groove without sequence specificity and unwind DNA from nucleosomes to prime transcription, recombination, genome stability and replication. The function of HMGB1 in the brain and neurodegenerative disease remain obscure, yet emerging evidence suggest that HMGB1 critically regulates DNA repair systems of base excision repair [18], DNA double-strand break (DDSB) repair [1,19,20], mismatch repair [21,22], nucleotide excision repair [23] and transcription-coupled DDSB repair [24], and we have recently found that HMGB1 associates with the DDSB-mediated neuronal damage in Huntington's disease pathology [1].

In this study, we report region-specific and cell type-specific change of HMGB1 protein expression and DDSB accumulation in the aging mouse brain. Our results suggest that HMGB1 might be a critical molecule linked to aging-dependent DNA damage accumulation in neurons and astrocytes.

Abbreviations: HMGB1, high-mobility group B1; DDSB, DNA double-strand break; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; GAPDH, glyceraldehydes-3-phosphate dehydrogenase

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Materials and methods

Animals. Experimental animals of male C57/B6 mouse (Jackson Laboratories, Bar Harbor) were subjected to four different age groups: young (0.5 month old, $n = 8$), young-adult (3 month old, $n = 6$), middle-aged (12–14 month old, $n = 6$), and aged (22–24 month old, $n = 4$). All mice were housed in adequate cages (two to five mice per cage) kept on 12-h light/dark schedule with free access to food and water, and fed with regular rodent chow (CLEA Rodent Diet CE-2; Clea Japan). For brain sample preparation, mice were deeply anesthetized and sacrificed by cervical dislocation, and brains were removed. For immunohistochemistry, mouse brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, embedded in paraffin, and sectioned into 5-μm thick slices.

Immunoblotting. Brain tissues were homogenized in SDS sample buffer and boiled for 5 min. Protein concentration was quantified by using the BCA method (Micro BCA Protein Assay Reagent Kit; Pierce Chemical, Rockford). The samples were separated by SDS–PAGE, transferred onto polyvinilydene difluoride membrane Fine Traps (Nihon Eido, Tokyo, Japan) through a semidry method, blocked by 5% milk in TBS with Tween-20 (TBST) (10 mM Tris/Cl, pH 8.0, 150 mM NaCl, 0.05% Tween-20), and incubated with appropriate antibodies as described below. The filters were incubated with each primary antibody for over night at 4 °C, with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature in 5% milk/TBST. Finally, the target molecules were visualized through an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences, Hong Kong) on the X-ray film (Kodak, New York). The

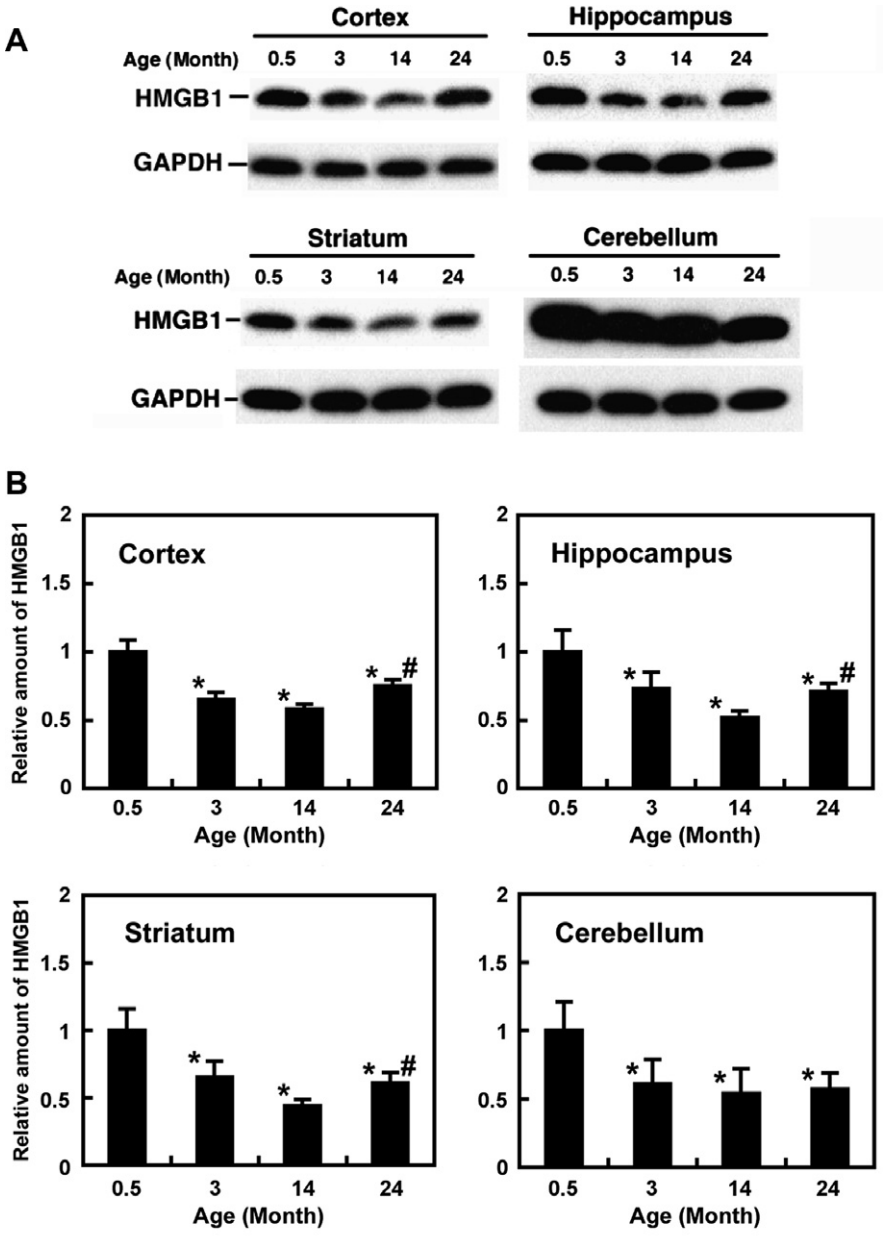

Fig. 1. Immunoblot analysis of HMGB1 expression in various brain regions of aging mouse. (A) HMGB1 expressions in cortex, hippocampus, striatum, and cerebellum of aging brain were examined by immunoblotting. One experiment representative of three is shown. (B) Densitometric analysis of HMGB1 expression in the aging brain. HMGB1 expressions in different brain regions detected by immunoblotting are expressed as a ratio relative to those of GAPDH at the same age. The value for 0.5 month of age arbitrary defined as 1.0. The bars represent means ± SD of the results from four animals. $^{*}p < 0.03$ vs. the value of 0.5 month of HMGB1. $^{\#}p < 0.05$ vs. the value of 14 month of HMGB1.

following primary and secondary antibodies were used: HMGB1 (rabbit, 1:2000, Abcam, Cambridge), GAPDH (mouse, 1:20,000, Chemicon), anti-mouse IgG (1:3000, Amersham Biosciences), and anti-rabbit IgG (1:3000, Amersham Biosciences). Immunoblotted bands were quantified using the Image J 1.32 software (National Institutes of Health, Bethesda, MD) after densitometric scanning of the films.

Immunohistochemistry. The paraffin-embedded brain sections were deparaffinized, rehydrated, and then microwaved in 10 mM of citrate buffer, pH 6.0, at 120 °C for 15 min. These sections were incubated sequentially with 3% hydrogen peroxide for 20 min at room temperature to inhibit endogenous peroxidase, followed by incubation with primary antibodies for overnight at 4 °C, and finally with Alexa Fluor 488 and 594-labeled anti-IgGs (Invitrogen) for 1 h at room temperature. The following primary antibodies were used: HMGB1 (rabbit, 1:1000, Abcam), GFAP (mouse clone GA5 Cy3-conjugated, 1:500, Invitrogen), γH2AX (mouse clone JBW301, 1:100, Millipore, Billerica), neuron-specific enolase (NSE; rabbit ready for use, Nichirei, Osaka), and NeuN (mouse clone A60, 1:500, Millipore).

Immunofluorescence signal intensity was measured as previously described [1,25] by using MetaMorph software (Universal Imaging Corporation, Downingtown). Immunostained cells were selected at random, and total mean fluorescence was calculated for each sample. More than 50 cells from at least three different slides were measured and averaged for each data point. Cortex (layers II–V), hippocampus (CA1 region), striatum, and cerebellum (granular layer) were selected for the staining and measurement.

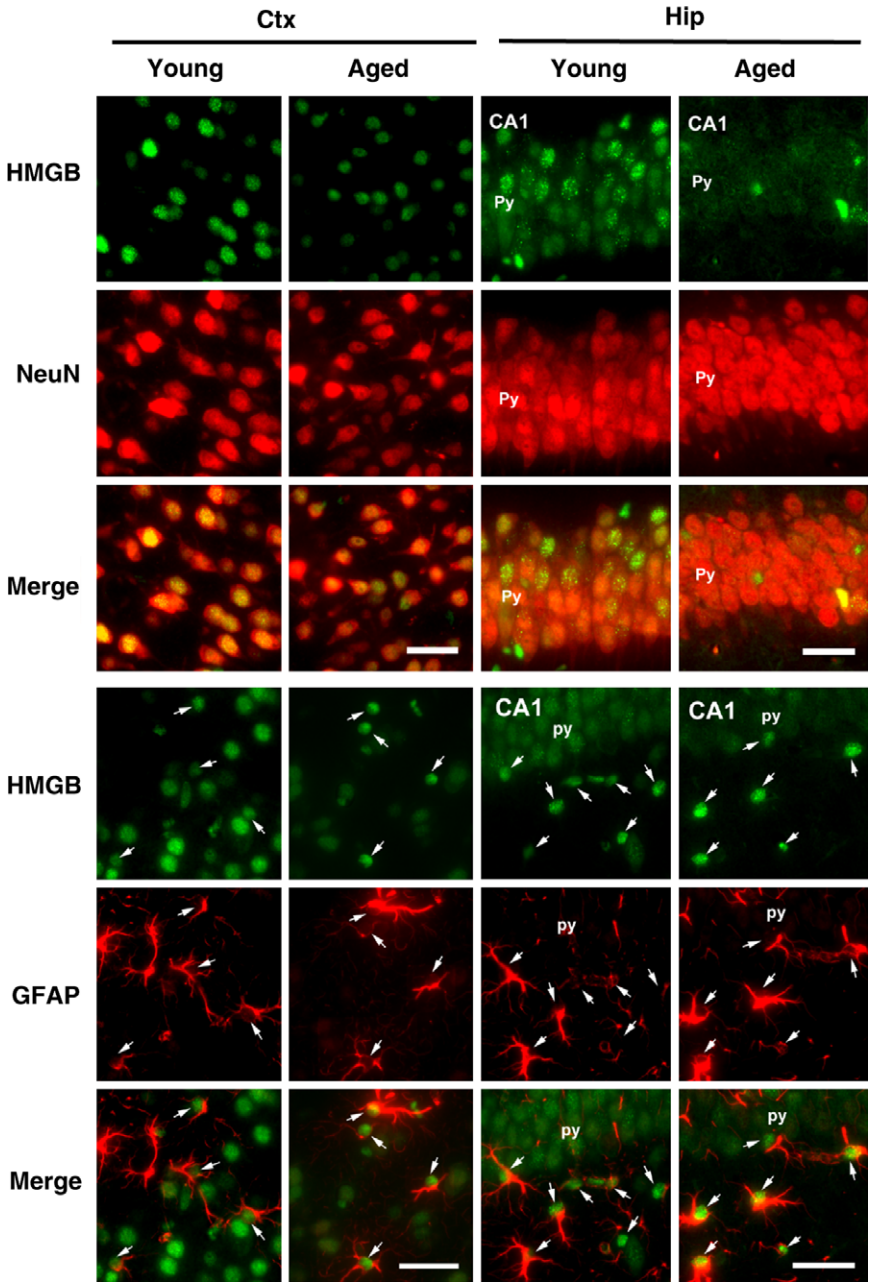

Fig. 2. Immunofluorescence detection of HMGB1 in young and aged neurons and astrocytes. (Upper) Cortex (Left) and hippocampus (Right) of young (0.5 month of age) and aged (24 month of age) brains were stained by antibodies against HMGB1 (green) and a neuron marker, NeuN (red). (Lower) Cortex (Left) and hippocampus (Right) of young (0.5 month of age) and aged (24 month of age) mouse brains were stained by antibodies against HMGB1 (green) and an astrocyte marker, GFAP (red). CA1, CA1 region of the hippocampus; py, pyramidal cell layer. Scale bar: 25 μm.

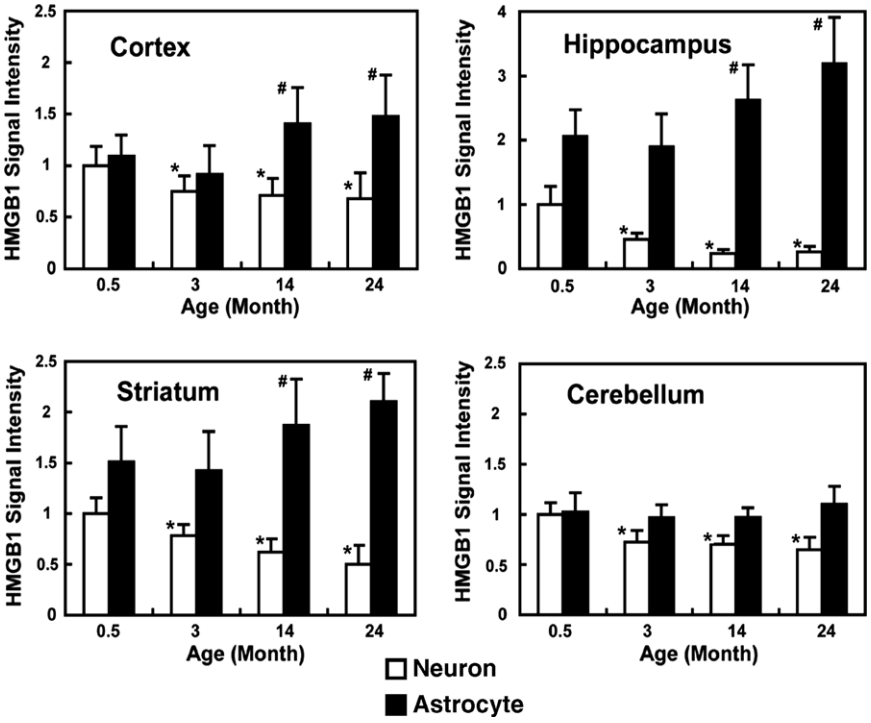

Fig. 3. Quantitative changes of nuclear expression of HMGB1 in neurons and astrocytes during aging. Immunofluorescent signal intensities of nuclear HMGB1 in neurons and astrocytes in cortex, hippocampus, striatum, and cerebellum were measured by using MetaMorph Imaging software as described in Materials and methods. The signal intensities are expressed as ratio relative to the value for 0.5 month of age defined as 1.0. The bars represent means ± SD $^{*}p < 0.001$ vs. the value of 0.5 month of neuronal HMGB1. $^{\#}p < 0.01$ vs. the value of 0.5 month of astrocyte HMGB1.

Statistical analysis. Statistical analyses were performed by the Student's *t*-test. The data presented are means ± standard deviation (SD). The *p* values <0.05 were considered to be statistically significant.

Results

HMGB1 expression is age-dependently changed in various brain regions

We analyzed protein expression levels of HMGB1 in various brain regions during aging from a half to 24 month by immunoblotting (Fig. 1). In all brain regions examined (cortex, hippocampus, striatum, and cerebellum), HMGB1 expression was highest in the young brain (0.5 month of age), and it gradually decreased during aging (from 0.5 to 14 month of age). However, interestingly, in the most aged brain (24 month of age), a slight increase of HMGB1 expression from that of middle-aged brain (14 month of age) was observed in cortex, hippocampus, and striatum. Although the expression patterns of HMGB1 were almost similar in different brain regions, the rebound increase at 24 month was not clear in cerebellum where relative HMGB1 expression was highest. Taken together, these results indicate that HMGB1 expression changes in an age-dependent manner in various brain regions.

HMGB1 expression is changed differently between neuron and astrocyte during aging of the brain

To examine the cell-type specific change of HMGB1 expression in brain aging, we next performed immunofluorescent staining of HMGB1 in neurons and astrocytes in young and aged mouse brains. HMGB1 was localized in the nuclei of neurons and astrocytes at all ages examined. Interestingly, HMGB1 expression was clearly lower in aged neurons than in young ones, whereas it was higher in aged astrocytes than in young ones (Fig. 2). We also performed quantitative analysis of HMGB1 signals in neurons and astrocytes in various regions of aging brain (Fig. 3). In neurons, HMGB1 expression was age-dependently reduced in all regions examined, and most remarkable reduction was observed in hippocampus and striatum (Fig. 3). In contrast, HMGB1 expression in astrocytes was increased during aging. Collectively, these results indicate that HMGB1 expression is changed in a cell-type specific manner during aging and suggest that the increase of HMGB1 at 24 month detected by immunoblotting (Fig. 1) reflects the increase in astrocytes (Fig. 3).

HMGB1 reduction and DNA double-strand break accumulation are inversely changed in neurons, but not in astrocytes during aging

Although age-dependent accumulation of oxidative DNA damage in the brain has been implicated [6–9], little is known about the region- and cell type-specific accumulation of DNA double-strand breaks (DDSBs) in the brain aging. Recent studies have shown that HMGB1 closely relates to various DNA repair systems including DDSB repair [1,19,20]. Therefore, we analyzed the relationship between HMGB1 expression and DDSB accumulation in the brain aging, we performed immunofluorescent staining of young and aged brains by using anti-γH2AX antibody, a specific marker for DDSB. In contrast to HMGB1 expression pattern, DDSB remarkably increased in neurons in all brain regions examined during aging, but no remarkable change was observed in astrocytes (Fig. 4A). We also performed quantitative analysis of DDSB in the nucleus of neurons and astrocytes in various regions of aging brain (Fig. 4B). DDSB accumulated in neurons age-dependently, especially in hippocampus and striatum. On the other hand, no significant increase of DDSB was detected in astrocytes during aging. Taken together, these results suggest that HMGB1 reduction and DDSB accumulation are inversely correlated in neurons, while no

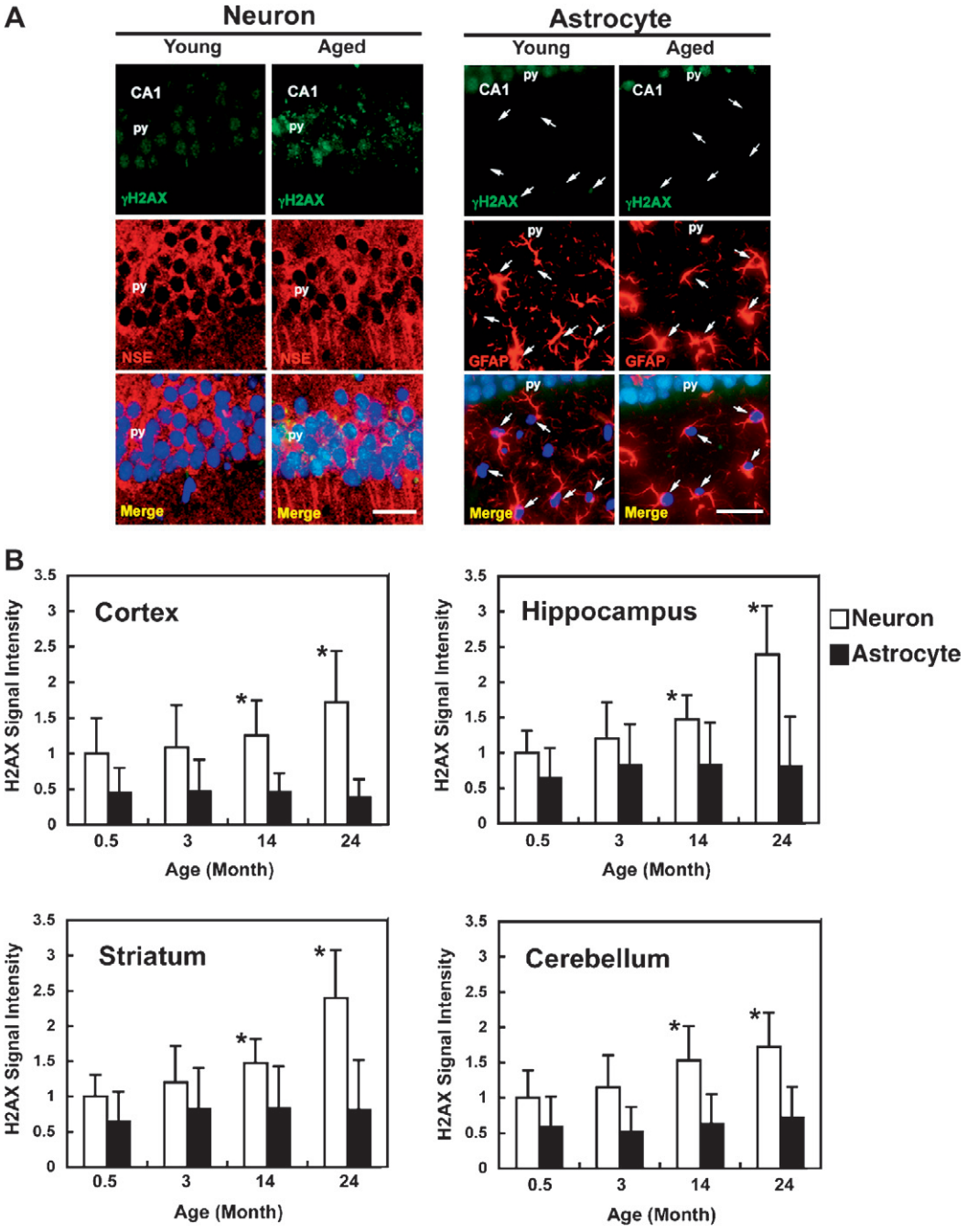

Fig. 4. Accumulation of DNA double-strand breaks (DDSBs) in the aging mouse brain. (A) Hippocampus of young (0.5 month of age) and aged (24 month of age) mouse brains were stained by antibody against a DDSB marker, γH2AX (Upper, green), and a neuron marker, neuron-specific enolase (NSE; Left middle, red) or a astrocyte marker, GFAP (Right middle, red). Arrows indicate the nuclei of astrocytes. To clarify the cell nuclear location, DAPI staining images were inserted in merged images (Lower, blue). CA1, CA1 region of the hippocampus; py, pyramidal cell layer. Scale bar: 25 μm. (B) Quantitative changes of DDSBs in neurons and astrocytes during aging. Immunofluorescent signal intensities of γH2AX in neurons and astrocytes in cortex, hippocampus, striatum, and cerebellum were measured and described by using MetaMorph Imaging software as described in Materials and methods. The signal intensities are expressed as ratio relative to the value for 0.5 month of age defined as 1.0. The bars represent means ± SD
$^{*}p < 0.001$ vs. the value of 0.5 month of neuronal γH2AX.

significant change of DDSB was observed in astrocytes (Fig. 4) that upregulates HMGB1 during aging (Fig. 3).

Discussion

In this study, we have examined the region- and cell type-specific changes of HMGB1, a key component of the DNA repair machinery, and of DDSB during the aging of mouse brain. Basically, HMGB1 reduction and DDSB accumulation are inversely correlated in neurons, while astrocytes did not show the increase of DDSB but showed an increase of HMGB1 in aged brains. In addition to the discrepancy between neurons and astrocytes, we found that, although the basic tendency was similar, such expression patterns of HMGB1 in neurons and astrocytes vary slightly among different brain regions.

HMGB1 possesses two discrepant functions. In ischemic necrosis HMGB1 translocates from nucleus to cytoplasm, and it is released into extracellular space. When HMGB1 is secreted from neural cells, it functions as a pro-inflammatory cytokine-like factor

[26,27]. On the other hand, HMGB1 is essential for keeping homeostasis of cells as aforementioned. However, the physiological function of HMGB1 in the normal brain and the pathology caused by its deficiency remain to be elucidated.

In the present study, we found that HMGB1 is predominantly localized in the nuclei of neurons and astrocytes, and its expression level was markedly reduced during aging specifically in neurons. In this case, we could hardly detect cytoplasmic localization of HMGB1 both in neurons and astrocytes, at any age examined (data not shown), denying the possibility that HMGB1 is released from neurons or astrocytes to cause tissue damage. Together with the results that HMGB1 critically regulates cell vulnerability to DNA damage [1,18–24,28], our findings suggest that the reduction of nuclear HMGB1 level in neurons might be relevant to the accumulation of naturally occurring DNA damage leading to neuronal dysfunction in the aged brain.

Accumulating evidence suggests that non-repaired or mis-repaired DNA damage leads to deleterious consequences during the long life span of neurons, such as impaired transcription, genomic instability, abnormal neural functions and ultimately cell death [6–9]. Recently, chromosomal or mitochondrial DNA damage has been also implicated in Alzheimer's, Huntington's, and Parkinson's diseases [1,9–11]. On the other hand, the role of DNA damage in normal brain aging process remains largely unknown [14]. Our present study showed that HMGB1 is changed in normal aging brains in an age- and cell type-dependent manner. The negative correlation between DNA damage and HMGB1 suggests that HMGB1 might be a new parameter to assess DNA damage in neurons during aging.

At present, regulatory mechanisms underlying the changes of the HMGB1 expression are unknown. HMGB1 expression is reported to increase in reactive astrocytes [29]. However, because GFAP and HSP27 proteins, markers for reactive astrocyte, were not changed, and because morphological change of GFAP-positive astrocytes could not be detected in aged brain (Figs. 2 and 4A, data not shown), it is unlikely that the increase of HMGB1 in astrocytes during aging corresponds to reactive gliosis.

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