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ORIGINAL PAPER

Comparison of the Effects of Cuprizone on Demyelination in the Corpus Callosum and Hippocampal Progenitors in Young Adult and Aged Mice

Kyu Ri Hahn[1] · Woosuk Kim[1,2] · Hyo Young Jung[1,3] · Hyun Jung Kwon[4,5] · Dae Won Kim[5] · In Koo Hwang[1] · Yeo Sung Yoon[1]

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Abstract

Cuprizone is commonly used to induce neuronal demyelination in mice. In the present study, we compared the cuprizone-induced demyelination in the corpus callosum and investigated the effects of cuprizone on proliferating cells and neuroblasts in the dentate gyrus of young adult and aged mice. 5-week- and 23-month-old mice were fed a normal diet or a 0.2% cuprizone-enriched diet for 5 weeks. Mice fed a cuprizone-supplemented diet showed a significant reduction in myelin basic protein-positive structures in the corpus callosum, with the reduction in myelinated fibers being confirmed by electron microscopic analysis. In addition, we observed a marked increase in Ki67-positive proliferating cells and doublecortin-immunoreactive neuroblasts in young adult mice in response to cuprizone treatment, although not in aged mice, as the basal levels of these cells were significantly lower in these older mice. Furthermore, Ser133-phosphorylated cAMP response element-binding protein (pCREB)-positive nuclei and brain-derived neurotrophic factor (BDNF) protein levels were significantly reduced in young adult mice following cuprizone treatment in young adult, although again not in the aged mice. However, in both young adult and aged mice, there were no significant reductions in hippocampal mature neurons in response to cuprizone treatment. These observations indicate that in the mice of both age groups a cuprizone-supplemented diet contributes to an increase in demyelination in the corpus callosum and neural progenitor cells in the dentate gyrus, although the damage is more pronounced in young adult mice. This demyelination and reduction in neural progenitor cells may be associated with changes in the levels of BDNF and pCREB in the dentate gyrus.

Keywords Cuprizone · Aging · Hippocampus · Myelination · Neurogenesis

✉ Yeo Sung Yoon
ysyoon@snu.ac.kr

1 Department of Anatomy and Cell Biology, College of Veterinary Medicine, and Research Institute for Veterinary Science, Seoul National University, Seoul 08826, South Korea

2 Department of Anatomy, College of Veterinary Medicine, and Veterinary Science Research Institute, Konkuk University, Seoul 05030, South Korea

3 Department of Veterinary Medicine & Institute of Veterinary Science, Chungnam National University, Daejeon 34134, South Korea

4 Department of Biomedical Sciences, and Research Institute for Bioscience and Biotechnology, Hallym University, Chuncheon 24252, South Korea

5 Department of Biochemistry and Molecular Biology, Research Institute of Oral Sciences, College of Dentistry, Gangneung-Wonju National University, Gangneung 25457, South Korea

Introduction

Multiple sclerosis (MS) is a chronic immune disease characterized by demyelination and axonal damage in neurons of the central nervous system. A prominent feature of post-mortem MS patients is demyelination in the hippocampus, an area of the brain that functionally processes learning and memory, in association with hippocampal atrophy [1]. Approximately 25–60% of MS patients with cognitive impairment show hippocampal dysfunction [2], which has a considerable impact on these individuals' quality of life.

The copper-chelating agent cuprizone (CPZ) has been demonstrated to induce neuronal demyelination in the brain [3], and is commonly used to promote myelin sheath damage and oligodendrocyte loss in the brain [4]. In a previous study, we and our colleagues demonstrated that CPZ affects myelin basic protein (MBP) in the hippocampus, as

well as cell proliferation and differentiated neuroblasts in the dentate gyrus [5]. Previous studies have also confirmed that the administration of dietary CPZ to adult mice results in demyelination of nerves, whereas remyelination occurs in response to the resumption of feeding on a normal diet [6]. Furthermore, CPZ-fed mice have been observed to show reductions in synaptic transmission and firing in the CA1 pyramidal cells of hippocampal slices [7]. In this regard, the findings of a recent study have indicated that the dentate gyrus and hippocampal CA4 region are most susceptible during the early stages of MS [8]. In CPZ-fed mice, adult hippocampal neurogenesis has been found to undergo a marked reduction in response to the inhibition of neural stem cell proliferation [5, 9], whereas it has been established that CPZ-induced demyelination does not induce the neurogenesis or migration of neuroblasts following ischemic damage in the mouse brain [10].

During aging, pathological changes can lead to a reduction in hippocampal neurogenesis and diminished hippocampal function relating to learning and memory [11]. In particular, senescence-accelerated mice showed a significant reduction in MBP and 2′,3′-cyclic nucleotide 3′-phosphodiesterase immunoreactivity in the hippocampal CA1 region compared with that in control mice [12]. However, the susceptibility to CPZ varies with age. For example, in middle-aged (10-month-old) mice, CPZ-induced microglial activation in the corpus callosum is more prominent than that in young (2-month-old) mice [13].

The CPZ-induced demyelination model is typically based on the use of mice of 8 to 10 weeks of ages, as this generally guarantees reproducible oligodendrocyte apoptosis and demyelination with a high probability [14]. Mice of 8 to 10 weeks of age can be considered equivalent to humans under the age 18 years, and 6-month-old mice are considered mature adults. Doucette et al. have estimated mouse age in terms of human years, indicating that 2-, 6-, 12-, and 16-month-old mice would be chronologically equivalent to 20-, 30-. 42-, and 50-year-old humans [15]. However, few studies have been conducted on the effects of CPZ-induced demyelination on cell proliferation and differentiated neuroblasts in the dentate gyrus using older mice comparable to humans aged 65 years or older. Aging causes neurobiological alterations in the hippocampus such as increased oxidative stress and neuroinflammation as well as reduced synaptic plasticity and neurogenesis [16, 17]. In this experiment, we tried to observe the effects of CPZ-induced demyelination on hippocampus depending on age using young adult (5 weeks of age) and aged (23 months of age). Accordingly, in the present study, we investigated the differential effects of CPZ-induced demyelination on cell proliferation and neuroblast differentiation in the dentate gyrus of young adult and aged mice.

Materials and Methods

Experimental Animals

4-week- and 23-month-old male C57BL/6 mice were purchased from Central Laboratory Animal Inc. The mice were housed in animal facilities under conditions of an appropriately controlled temperature (22 ± 2 °C) and humidity (60% ± 5%) and a 12-h light/12-h dark cycle, with free access to food and tap water. The animals were maintained in cages, and the handling and care of animals was in accordance with the guidelines of current international laws and policies. [National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, Publication No. 85–23, 1985, revised 1996], and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-160929-5-2 and SNU-190314–12-2).

Experimental Groups and Treatments

Mice at each age (5 weeks and 23 months of age) were divided into one of the following two groups: those fed a normal diet (control group), and those fed a CPZ-supplemented diet (CPZ group). The CPZ-supplemented diet was prepared by adding 0.2% CPZ to chow diets as previously described [5]. Both normal and CPZ-supplemented diets were administered for 5 weeks.

Tissue Processing

The mice were anesthetized with a mixture of alfaxalone (Alfaxan, 75 mg/kg; Careside, Seongnam, South Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, South Korea), and thereafter were 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), using a flexible tube (HV-06,409-16; Masterflex, Vernon Hills, IL, USA). The brains were removed and post-fixed for 12 h in the same fixative. Brain tissue was cryoprotected by immersing overnight in 30% sucrose in 0.1 M PB, and subsequently cut into 30-µm-thick sections using a cryostat (Leica, Wetzlar, Germany). Paraformaldehyde solution was made by dissolving paraformaldehyde power in 0.1 M PB on a stirring hot plate at 60 °C, and then the solution was cooled to use 4% paraformaldehyde.

Ultrastructural Analysis of Myelinated Fiber

The corpus callosum was dissected out from perfused brains after fixation with paraformaldehyde, and the tissues were

cut into 1-mm segments on a glass slide. The tissues were post-fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in PBS for 2 h, after which the fixed tissues were incubated with 1% osmium tetroxide for 2 h and dehydrated with a serial gradient of alcohol. Finally, the tissues were embedded in Spurr's resin and polymerized at 60 °C for 2 days. Embedded tissues were cut into 70-nm-thick sections using an ultramicrotome (Leica), which were placed on 200-mesh nickel grids. Tissues were stained with lead citrate and uranyl acetate and observed under an electron microscope (JEOL, Tokyo, Japan).

Immunohistochemistry for DCX, Ki67, pCREB, MBP, and NeuN

Tissue sections were treated with 0.3% H_2O_2 in PBS for 30 min and then incubated with 5% normal goat serum in 0.1 M PBS. Thereafter, the sections were initially incubated overnight with rabbit anti-Ki67 (1:1,000; Abcam, Cambridge, UK), rabbit anti-doublecortin (DCX, 1:2,000, Abcam), rabbit anti-phosphorylated (ser133) cAMP response element-binding protein (pCREB, 1:400; Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit anti-MBP (1:200; Merck Millipore, Temecula, CA, USA), or mouse anti-NeuN antibody (1:1000; Merck Millipore). Two days later, the sections were sequentially treated with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) and streptavidin-peroxidase complex (1:200; Vector) for 2 h at 25 °C. Sections were visualized with 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) in 0.1 M Tris–HCl buffer (pH 7.2) and mounted on gelatin-coated slides. For MBP immunofluorescence staining, the sections were incubated with Cy3-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at 25 °C. Thereafter, the sections were mounted in a water-soluble mounting medium (Fluoromount-G®; SouthernBiotech, Birmingham, AL, USA) on gelatin-coated slides.

Western Blot Analysis for Brain-Derived Neurotrophic Factor

To elucidate the mechanisms associated with changes in proliferating cells and differentiated neuroblasts in the demyelinating hippocampus of young adult and aged mice, the expression of brain-derived neurotrophic factor (BDNF), a major contributor to young adult hippocampal neurogenesis, was analyzed based on western blotting. After having been fed for 5 weeks on control or CPZ-supplemented diets, mice were sacrificed by treatment with alfaxalone and xylazine. The brains were rapidly removed from the skulls of mice and 500-µm-thick coronal sections were obtained. The hippocampal dentate gyrus was excised from the sections using a blade, and western blot analysis was conducted as previously described, [5] using BDNF (1:1,000) and β-actin (1:5,000) antibodies (Biosensis Pty Ltd., Thebarton, SA, Australia).

Quantification of Data and Statistical Analysis

The numbers of Ki67-, NeuN-, and pCREB-immunoreactive nuclei were determined using ImageJ software (version 1.53; National Institutes of Health, Bethesda, MD, USA), whereas MBP and DCX immunoreactivities were quantified as a summation of gray scale (0–255) and pixel numbers using ImageJ software (version 1.53; National Institutes of Health). In addition, areas of myelinated fibers were calculated using ImageJ software. With the exception of those used for electron microscopy, all sections were obtained from an area 1.82 to 2.32 mm caudal to the bregma, based on reference to a mouse atlas [18], separated by intervals of 150 µm, and all counts from all sections were averaged. Results are presented as the means ± standard deviation. The MBP and DCX immunoreactivity was expressed as a relative optical density (ROD) vs. vehicle-treated young adult group, respectively to compare the density easily among groups. Statistical analysis (age × CPZ treatment) was performed using two-way analysis of variance (ANOVA), and further comparisons were performed using Bonferroni post-tests. Statistical significance was set at $p < 0.05$.

Results

Effects of CPZ on Myelinated Fibers in Young Adult and Aged Mice

The changes in myelinated fibers in response to dietary administration of CPZ were assessed by immunohistochemical staining for MBP and confirmed by electron microscopic analysis of the corpus callosum (Fig. 1). In all assessed groups, MBP immunoreactivity was mainly detected in the alveus and corpus callosum, although there were significant differences among the groups. MBP immunoreactivity was found to be highest in the young adult control group, whereas in the young adult CPZ and aged-control groups, structures showing MBP immunoreactivity were less visible, and the levels of immunoreactivity were significantly reduced to 68.0% and 78.4% that in the young adult control group, respectively. Few MBP immunoreactive structures were detected in the aged CPZ group, and the level of immunoreactivity was found to be 30.3% that in the young adult control group. Two-way ANOVA revealed a significant interaction between age and CPZ treatment with respect to differences in MBP immunoreactivity ($F = 5.22$, $DFn = 1$, $DFd = 23$, $p = 0.0319$).

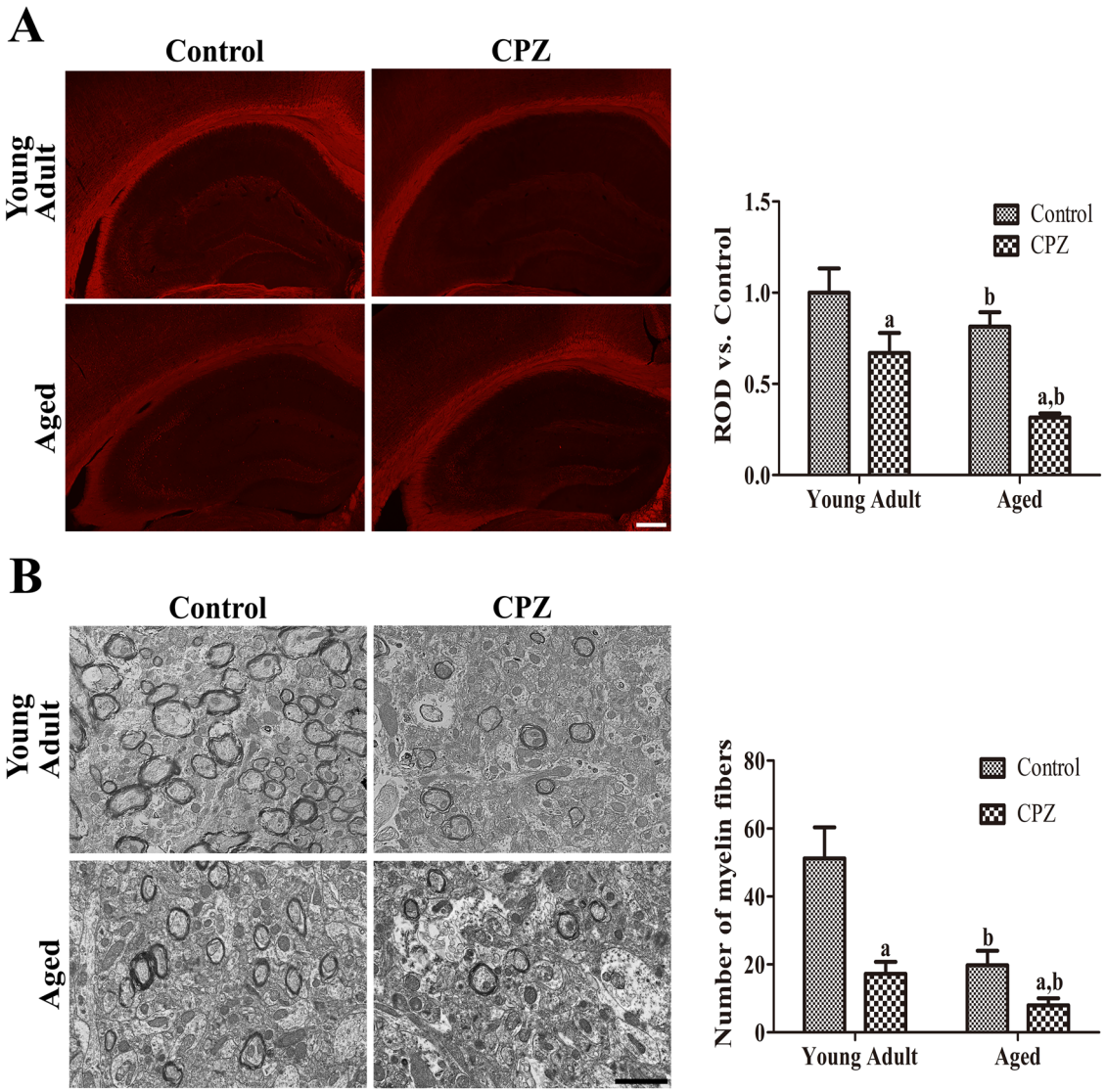

Fig. 1 Immunofluorescence staining of myelin basic protein (MBP) in the hippocampus (**A**) and ultrastructural study of the corpus callosum (**B**) of vehicle-treated young adult (young adult control), cuprizone-treated adult (young adult CPZ), vehicle-treated aged (aged control), and CPZ-treated aged (aged CPZ) mice. Scale bar = 50 µm (**A**), 2 µm (**B**). The relative optical density (ROD) corresponds to the immunoreactivity value of MBP in the dentate gyrus of the control group. The ROD of MBP fluorescent intensity and the area of myelinated fibers are also shown (n = 7 per group; $^{a}p < 0.05$, significantly different between the control and CPZ groups; $^{b}p < 0.05$, significantly different between the young adult and aged groups). All data are presented as the means ± standard deviation

Ultrastructural observations revealed abundant myelinated fibers in the corpus callosum, whereas myelin fibers tended to be less abundant in the corpus callosum of mice in the young adult CPZ group, with the areas of myelinated fibers being significantly reduced to 33.6% of that observed for the young adult control group. A similar reduction in myelinated fibers was observed in the aged control group to a level 38.7% of that in the young adult control group. Relatively few myelinated fibers were observed in the aged CPZ group, the areas of which were significantly reduced compared with the young adult CPZ and aged control groups, to a level 15.5% of that seen in the young adult control group. Two-way ANOVA revealed a significant interaction between age and CPZ treatment with respect the areas of myelinated fibers ($F = 28.74$, $DFn = 1$, $DFd = 24$, $p < 0.0001$).

Effects of CPZ on Proliferating Cells in the Dentate Gyrus of Young Adult and Aged Mice

In the young adult control group, Ki67-positive nuclei were observed to be located in the subgranular zone of the dentate gyrus, the mean number of which (21.9/section) was highest

among the four treatment groups (Fig. 2A). The number of Ki67-positive nuclei in the subgranular zone of young adult CPZ mice had been reduced to 41.8% of that observed in the young adult control mice. Similarly, in the aged control group, there were fewer Ki67-positive nuclei in the dentate gyrus, with the number being 48.4% that observed in the young adult control group. The aged CPZ group was characterized by the lowest number of Ki67-positive cells (2.4 per section) in the dentate gyrus. Results of the two-way ANOVA test revealed a significant interaction between age and CPZ with respect to the number of Ki67-positive cells in the dentate gyrus ($F = 4.54$, $DFn = 1$, $DFd = 24$, $p = 0.0436$).

Effects of CPZ on Neuroblasts in the Dentate Gyrus of Young Adult and Aged Mice

In the young adult control group, the cytoplasm of DCX-positive neuroblasts was observed in the subgranular zone of the dentate gyrus and extended into the molecular layer (Fig. 2B). Compared with the young adult control mice, those in the young adult CPZ group were characterized by fewer DCX-positive neuroblasts and their dendrites in the dentate gyrus, and, with the exception of the polymorphic layer, DCX immunoreactivity was significantly reduced to 39.8% that observed in the dentate gyrus of young adult control mice. Similarly, in

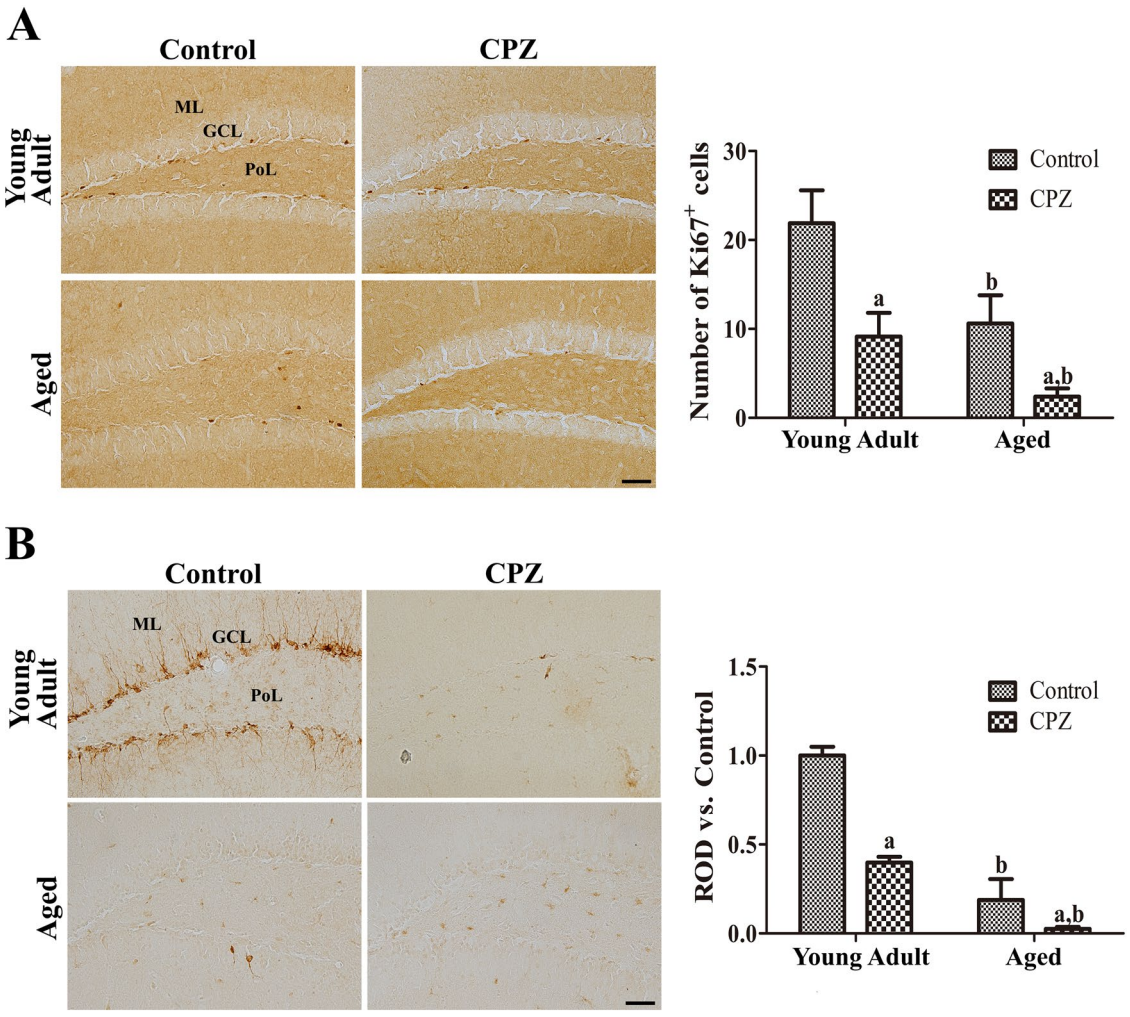

Fig. 2 Immunohistochemistry for Ki67 (**A**) and doublecortin (DCX, **B**) in the hippocampal dentate gyrus of vehicle-treated young adult (young adult control), cuprizone-treated young adult (young adult CPZ), vehicle-treated aged (aged control), and CPZ-treated aged (aged CPZ) mice. Some Ki67- and DCX-positive cells are observed in mice in the young adult control group. Note that there are few Ki67- and DCX-positive structures in the young adult CPZ and aged control groups. Fewer Ki67- and DCX-positive structures are found in the dentate gyrus of aged CPZ mice. GCL, granule cell layer; ML, molecular layer; PoL, polymorphic layer. Scale bar = 50 μm. The number of Ki67-positive cells per section and relative optical densities (ROD) of DCX-immunoreactive structures are also shown for all groups (n = 7 per group; $^{a}p < 0.05$, significantly different between control and CPZ group; $^{b}p < 0.05$, significantly different between young adult and aged group). The relative optical density corresponds to DCX immunoreactivity in the control group. All data are presented as the means ± standard deviation

the aged control group, few DCX-positive neuroblasts were observed in the subgranular zone of the dentate gyrus, and although some DCX-positive cells were detected in the polymorphic layer, their morphologies were somewhat different from those of the neuroblasts. Notably, in the aged CPZ group, we detected no DCX-positive neuroblasts in the subgranular zone of the dentate gyrus, although DCX immunoreactive structures were observed in the polymorphic layer. With the exception of the polymorphic layer, the DCX immunoreactivity in these mice was the lowest in the dentate gyri of mice in the four group (only 2.6% that in the young adult control group). Two-way ANOVA revealed a significant interaction between age and CPZ with regards to a reduction in DCX immunoreactivity ($F = 44.23$, $DFn = 1$, $DFd = 13$, $p < 0.0001$).

Effects of CPZ on Mature Neurons in the Dentate Gyrus of Young Adult and Aged Mice

In all four experimental groups, mature NeuN-positive cells were detected in all hippocampal subregions, including the dentate gyrus (Fig. 3), and although there were no significant differences in NeuN-positive mature neurons among the groups, numbers were highest in the in the dentate gyrus of young adult control mice and lowest in that of the aged CPZ mice. Two-way ANOVA revealed no significant interaction between age and CPZ treatment with respect to the number of mature neurons ($F = 0.07$, $DFn = 1$, $DFd = 24$, $p = 0.7918$).

Effects of CPZ on the Phosphorylation at Ser133 of CREB in the Dentate Gyrus of Young Adult and Aged Mice

In the young adult control group, we detected pCREB-positive nuclei in the subgranular zone (Fig. 4A), and the average number of pCREB-positive cells in the mice in this group (65.56) was higher than that in the young adult CPZ mice (32.11), in which the numbers had decreased to 48.98% of those the young adult control mice. Comparatively, very few pCREB-positive nuclei were observed in the dentate gyrus of aged control and aged CPZ mice, with numbers being reduced to 6.86% and 1.91% of those in the aged CPZ mice, respectively. There were, however, no significant differences between the aged control and aged CPZ groups with respect to the number of pCREB-positive nuclei. However, two-way ANOVA revealed a significant interactive effect between age and CPZ treatment on the reduction of pCREB-positive nuclei ($F = 19.19$, $DFn = 1$, $DFd = 22$, $p = 0.0002$).

Effects of CPZ on Brain-Derived Neurotrophic Factor Protein Levels in the Dentate Gyrus of Young Adult and Aged Mice

Western blot analysis for brain-derived neurotrophic factor (BDNF) and β-actin was performed to examine the changes in BDNF, a major neurotrophic factor in the brain, in response to CPZ treatment and/or aging in the hippocampus (Fig. 4B). We accordingly found that levels BDNF protein in the hippocampus of young adult CPZ and aged control mice were significantly reduced to 50.60% and 24.81% of levels detected in young adult control mice, respectively. BDNF levels were found to be lowest in the aged CPZ mice, in which they were significantly reduced to 6.26% those in young adult control mice ($F = 7.60$, $DFn = 1$, $DFd = 16$, $p = 0.0140$).

Discussion

The CPZ model is believed to be a suitable animal model for studying hippocampal demyelination in human patients with multiple sclerosis [19, 20]. This well-established CPZ model is typically based on the use of young mice (8 to 10 weeks old), which display a wide range of remyelination in response to a cessation of CPZ treatment [21]. Also, one of the most serious factors affecting remyelination is aging, which becomes less effective with age [22]. We used young and old mice, because MS patients occur at various ages. In adult mice, CPZ typically causes demyelination in the hippocampus and reduces the number of neural progenitors in the subgranular zone of the dentate gyrus [5]. In addition, the findings of a more recent study have revealed a reduction in neurogenesis in mice fed a CPZ-supplemented diet [9]. In the present study, we compared the effects of CPZ on demyelination in the corpus callosum of young adult and aged mice, and accordingly observed significant reductions in MBP immunoreactivity and the number of myelinated fibers in aged mice compared with those in young adult mice. These observations are consistent with those of a previous study showing that myelin is a sensitive indicator of aging in humans [23]. In addition, we found that CPZ treatment significantly reduces MBP in the corpus callosum, which is consistent with our ultrastructural observations indicating a reduction in myelinated fibers following CPZ treatment. Previous analysis of MBP has revealed CPZ-induced demyelination in the hilar region of the dentate gyrus and splenium [13]; however, these authors failed to observe any significant reduction in MBP immunoreactivity in these regions between 2-month-old (adult) and 10-month-old (middle-aged) mice [13]. A further study has, nevertheless, presented conflicting evidence, indicating that CPZ treatment promotes a more severe reduction in MBP and demyelination in the

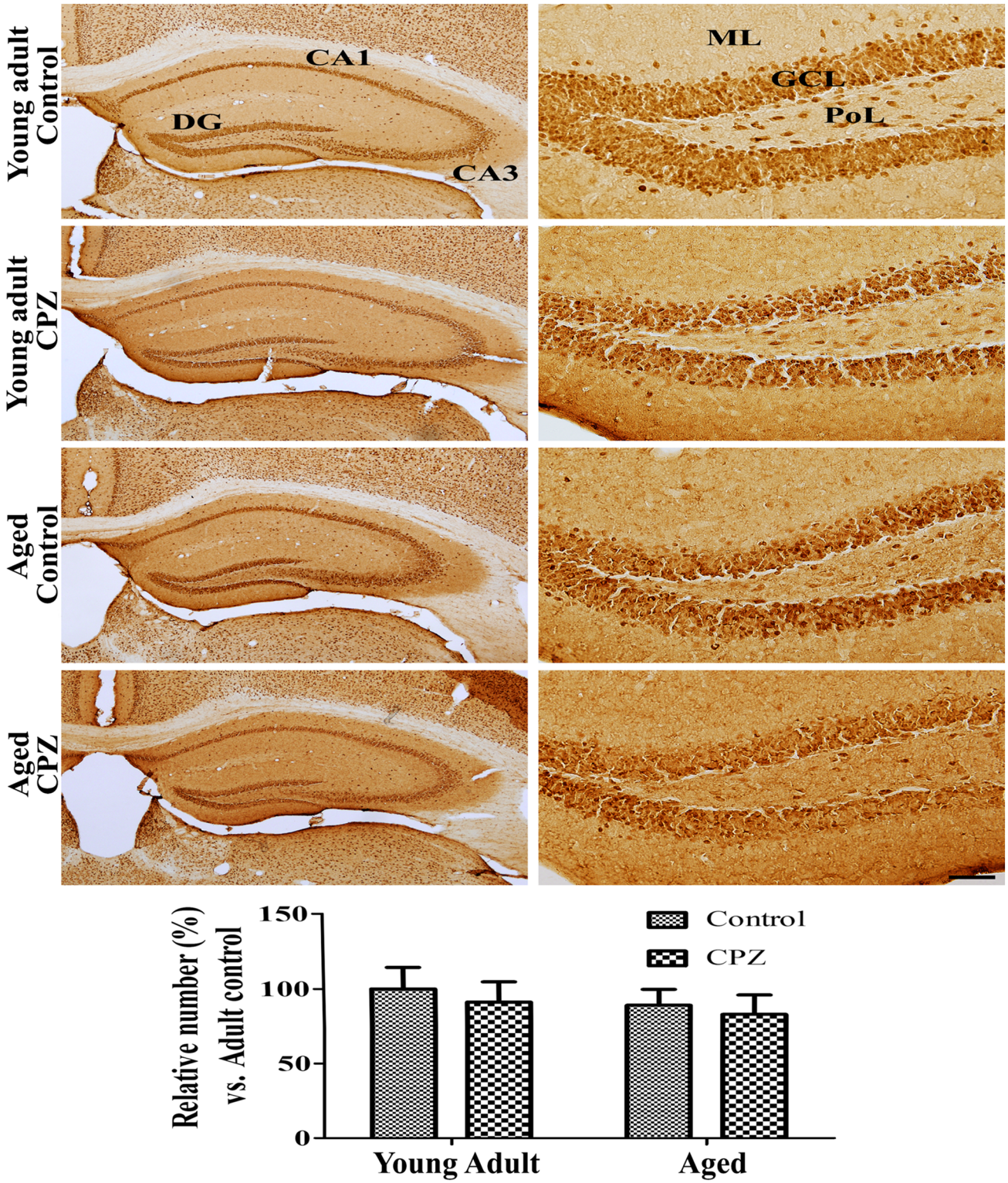

Fig. 3 Immunohistochemistry for neuronal nuclei (NeuN) in the whole hippocampus and hippocampal dentate gyrus of vehicle-treated young adult (young adult control), cuprizone-treated young adult (young adult CPZ), vehicle-treated aged (aged control), and CPZ-treated aged (aged CPZ) mice. Note that there are slight non-significant reductions in the number of NeuN-positive cells in the dentate gyrus of young adult CPZ, aged control, and aged CPZ compared with the young adult CPZ group. *GCL* granule cell layer, *ML* molecular layer, *PoL* polymorphic layer. Scale bar = 50 μm. Data are presented as the means ± standard deviation

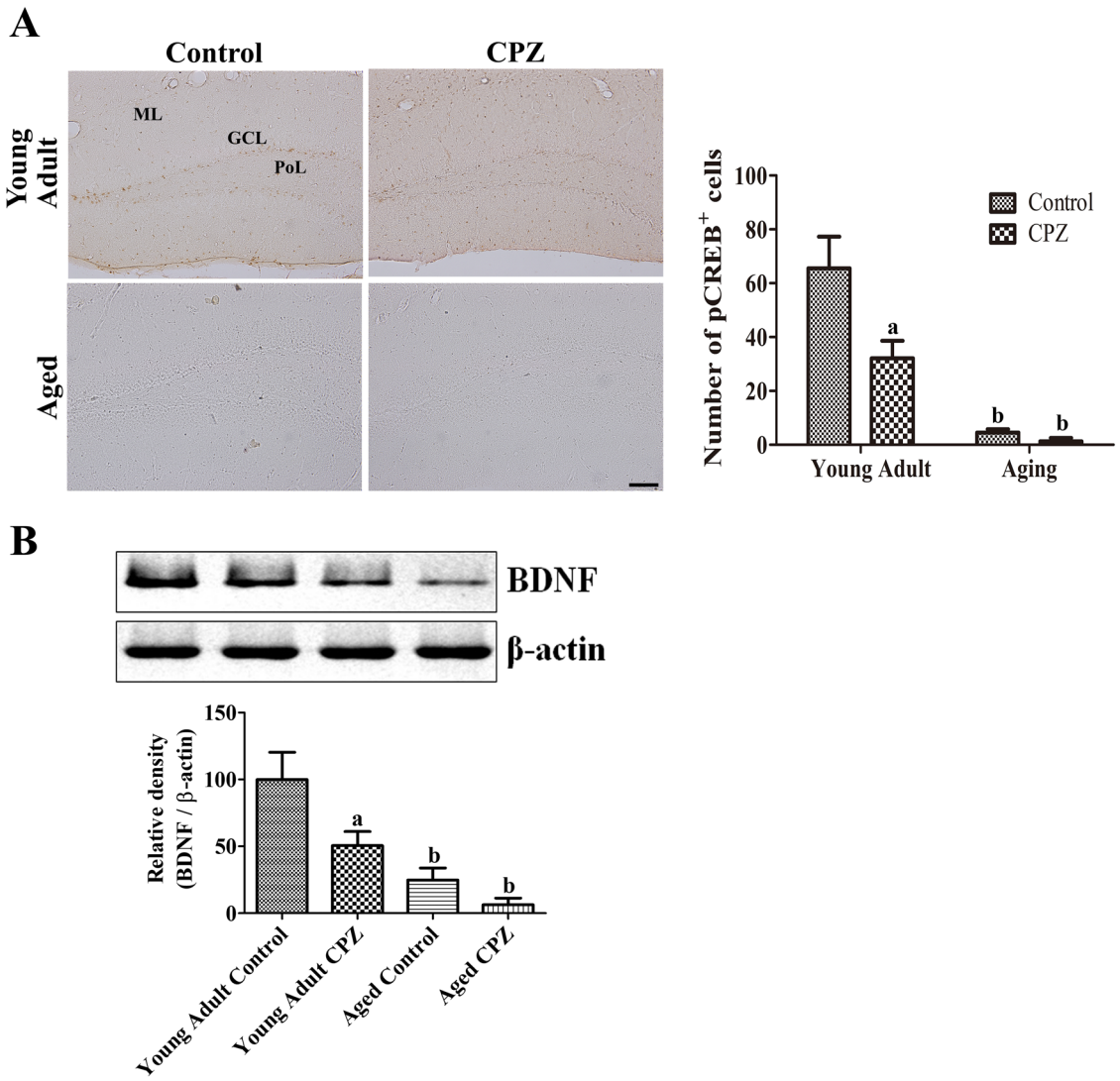

Fig. 4 Immunohistochemistry for Ser133-phosphorylated cAMP response element-binding protein (pCREB) in the hippocampal dentate gyrus (**A**) and western blot analysis of brain-derived neurotrophic factor (BDNF, **B**) of vehicle-treated young adult (young adult control), cuprizone-treated young adult (young adult CPZ), vehicle-treated aged (aged control), and CPZ-treated aged (aged CPZ) mice. GCL, granule cell layer; ML, molecular layer; PoL, polymorphic layer. Scale bar = 50 μm. The number of pCREB-positive cells per section and relative optical densities of BDNF bands normalized with β-actin bands in all groups are also shown (n = 7 per group; $^{a}p < 0.05$, significantly different between control and CPZ group; $^{b}p < 0.05$, significantly different between young adult and aged group). All data are presented as the means ± standard deviation

corpus callosum of young adult mice (3 or 6 weeks old) compared with that in middle-aged mice (8 months old) [14, 24]. Similarly, gray matter volume in the hippocampus of adult MS patients (30.4 years old) has been found to be significantly reduced, whereas the changes in older MS patients (48.7 years old) were found to be non-significant [25]. In the present study, however, we used 24-month-old or older mice as an aged group, given that 24-month-old mice are considered to exhibit an aged phenotype equivalent to that of an 80-year-old human [26]. Compared with young adult mice, we observed significant reductions in MBP and myelinated fibers in the corpus callosum of the aged mice, and speculate that these differences may be attributable a critical time period associated with age-related reductions in oligodendrocyte lineage cells with aging [15, 27, 28], particularly between 12 and 16 months of age [15]. Contrastingly, we detected no appreciable differences between young adult and aged mice with respect to NeuN-positive mature neurons, which showed no significant reductions in the hippocampus, including the dentate gyrus, in either group of mice following CPZ treatment for 5 weeks. These observations thus tend to indicate although feeding mice a CPZ-supplemented diet

for 5 weeks does not cause damage to mature neurons, it can induce demyelination of the efferent fibers of pyramidal cells and afferent fibers, such as perforant path and mossy fibers such as perforant path and mossy fibers [5].

Aging is characterized by a reduction in the proliferation of neural progenitor cells [29, 30], and in the present study, we accordingly compared the effects of CPZ on neural progenitors in the dentate gyrus of young adult and aged mice. Immunohistochemical staining for Ki67 and DCX to detect proliferating cells and neuroblasts, respectively, in the dentate gyrus of young adult and aged mice revealed that CPZ treatment causes a significant reduction in the number of proliferating cells and neuroblasts in the dentate gyrus of these mice. Kuhn et al. used 5-bromodeoxyuridine labeling in senescent mice to conclude that senescence-associated attenuation of proliferation is specified for granular cell precursors in the dentate gyrus. The extents of the reductions in proliferating cells and neuroblasts were found to be more pronounced in the young adult mice than in the aged mice. These observations are consistent with those of previous studies showing that CPZ treatment significantly reduces proliferating cell nuclear antigen (PCNA)-positive M-phase proliferating cells or Ki67-positive proliferating cells and DCX-positive neuroblasts in the dentate gyrus of adult animals [5, 31]. Conversely, however, Klein et al. have demonstrated no significant increase in PCNA-positive M-phase proliferating cells during demyelination in the dentate gyrus [13], whereas Abe et al. showed a 2-fold increase in S-phase cells in the dentate gyrus [31]. Nevertheless, we suspect that these apparent discrepancies could be associated with the stages at which proliferating cells were detected and/or the concentration or route of CPZ treatment.

In the present study, we observed changes in the levels of BDNF protein and pCREB-positive nuclei in the dentate gyrus following CPZ treatment. These observations are consistent with observations that the promotion of CREB phosphorylation in primary hippocampal neurons is followed by the stimulation of BDNF and increased neuronal plasticity [32], and that phosphorylated CREB and BDNF are closely linked, contributing to neurogenesis [33]. The rate of degeneration in pathological aging is said to completely abolish the already diminished plastic function of neural networks [34, 35]. Since neuroplasticity is highly reduced during ageing, we tried to find out what difference in hippocampus in older and younger mice make after demyelination induced by CPZ.

We detected significant reductions in pCREB-positive nuclei and BDNF expression in young adult mice following CPZ treatment, which is consistent with the findings of previous studies showing that CPZ treatment reduces BDNF mRNA levels [31], BDNF protein levels [5], BDNF-positive cells [36], and pCREB-positive nuclei [5]. Reductions on the levels of BDNF and pCREB, which are important factors in the regulation of adult neurogenesis, have previously been reported in the hippocampus of aged mice [37–39], and in the present study, we similarly detected significantly lower levels of BDNF and pCREB-positive nuclei in aged mice compared with those seen in young adult mice. In the aged mice, however, only slight non-significant reductions were detected in the hippocampus following CPZ treatment, which can be ascribed to the fact that the pre-treatment basal numbers of pCREB-positive nuclei and levels of BDNF protein were already very low in the control and CPZ-treated aged mice.

In summary, CPZ treatment reduced the levels of myelin basic protein, myelinated fibers, proliferating cells, and neuroblasts, which are closely associated with reductions in BDNF and pCREB, in the hippocampi of both young adult and aged mice. However, the reductions in these cells and cell constituents tended to be more pronounced in the young adult hippocampus than in that of aged mice. It was also confirmed that the mice treated with CPZ had more reduction than the control group did. Our findings accordingly indicate that CPZ could be used as an animal model of multiple sclerosis in both young adult and aged animals, although the severity of damage appears to be weaker in aged mice.

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