

Cuprizone-induced demyelination in mice: age-related vulnerability and exploratory behavior deficit

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ABSTRACT

Schizophrenia is a mental disease that mainly affects young individuals (15 to 35 years old) but its etiology remains largely undefined. Recently, accumulating evidence indicated that demyelination and/or dysfunction of oligodendrocytes is an important feature of its pathogenesis. We hypothesized that the vulnerability of young individuals to demyelination may contribute to the onset of schizophrenia. In the present study, three different age cohorts of mice, i.e. juvenile (3 weeks), young-adult (6 weeks) and middle-aged (8 months), were subjected to a 6-week diet containing 0.2% cuprizone (CPZ) to create an animal model of acute demyelination. Then, age-related vulnerability to CPZ-induced demyelination, behavioral outcomes, and myelination-related molecular biological changes were assessed. We demonstrated: (1) CPZ treatment led to more severe demyelination in juvenile and young-adult mice than in middle-aged mice in the corpus callosum, a region closely associated with the pathophysiology of schizophrenia; (2) the higher levels of demyelination in juvenile and young-adult mice were correlated with a greater reduction of myelin basic protein, more loss of CC-1-positive mature oligodendrocytes, and higher levels of astrocyte activation; and (3) CPZ treatment resulted in a more prominent exploratory behavior deficit in juvenile and young-adult mice than in middle-aged mice. Together, our data demonstrate an age-related

vulnerability to demyelination with a concurrent behavioral deficit, providing supporting evidence for better understanding the susceptibility of the young to the onset of schizophrenia.

Keywords: schizophrenia; oligodendrocytes; age; demyelination; cuprizone

INTRODUCTION

Schizophrenia is a common mental illness, and increasing data show that it is a neurodevelopmental disorder attributed to genetic and environmental factors^[1–3]. Its global annual incidence rate is 0.4–1%^[4], and a large proportion occurs in young adults^[5,6]. Studies of the pathogenesis of schizophrenia suggest that abnormalities in the development, differentiation and functions of oligodendrocytes (OLs) may be associated with the occurrence of schizophrenia^[7–9].

Diffusion tensor imaging provides consistent evidence showing white-matter (WM) pathology in schizophrenic brains^[10–12]. Postmortem histological examination has shown dysmyelination or OL apoptosis in schizophrenic patients^[13–15]. The major WM myelin membrane produced by the OLs wraps axons, suggesting that OLs may be involved in the pathogenesis of schizophrenia. Animal studies also offer evidence for roles of OLs in the pathophysiology of schizophrenia. In a recent study, C57BL/6 mice with WM damage induced by cuprizone (CPZ, 0.2%) added to the diet showed behavioral changes reminiscent of schizophrenic symptoms^[16]. CPZ is a copper chelator, which spe-

cifically kills central nervous system OLs without affecting peripheral nerves. More and more research shows that this model can be used to study the pathological mechanisms associated with schizophrenia^[17–19].

Therefore, in the present study, we focused on the relationships between the onset of schizophrenia-like symptoms and the susceptibility of OLs to demyelination in animals at different ages. We hypothesize that young animals are more vulnerable to demyelination, and thus are more susceptible to the onset of schizophrenia-like behavior. To evaluate whether OLs in young mice are more vulnerable to demyelination and whether the susceptibility of OLs to demyelination is linked to the development of schizophrenic endophenotypes, we used a CPZ-induced demyelination model in different-aged mice and determined the pathological outcomes at three levels: (1) pathological demyelination in the corpus callosum (CC); (2) cellular expression of the myelination-related molecular marker myelin basic protein (MBP) and the mature OL marker CC-1, as well as astrocyte activation; and (3) a behavioral test of exploratory activity in the open field - a schizophrenia-related endophenotype.

MATERIALS AND METHODS

Animals and Cuprizone-Treatment Paradigm

All animal procedures were performed in accordance with protocols and guidelines approved by the Institutional Animal Care and Use Committee of the Third Military Medical University. All animals were group-housed with water and food available *ad libitum* in temperature- and humidity-controlled rooms ($22 \pm 1^\circ\text{C}$ and 60% humidity) with a 12-h light/dark cycle throughout the entire experimental period.

Three cohorts of C57BL/6 mice of different ages (3 weeks, 6 weeks, and 8 months) were used. Animals in each age-cohort were divided into two groups (8–10/group) with either a normal diet or an acute CPZ treatment paradigm. Briefly, CPZ (Cat # 14690, Sigma-Aldrich, St. Louis, MO) was mixed in with normal milled chow (0.2% by weight) to feed animals for a period of 6 weeks as previously described^[20].

Open-Field Assessment of Exploratory Activity

To assess exploratory activity, all CPZ and control diet-treated animals were tested for locomotor responses to a novel environment in an open field, consisting of a 25×25

cm square surrounded by 35-cm high walls as previously described^[16]. The movement track of each animal was recorded by a video camera placed above the arena. A video-tracking program (BW-OF302, Shanghai Biovill Co., Ltd, China) was used to measure: (1) locomotor activity (total distance traveled) and (2) exploratory activity (distance traveled in the central area, reflecting anxiety-like behavior). The central area was defined as the inner section 10 cm from each wall.

Immunohistochemistry (IHC)

To evaluate myelination-related molecular changes, after behavioral testing, five of the mice in each group were used for IHC evaluation as previously described^[20]. Briefly, serial frozen coronal sections (20 μm) of the brains were cut after perfusion and post-fixation with 4% paraformaldehyde in PBS at 4°C overnight followed by dehydration in 3% sucrose in PBS. After blocking in a solution of 0.3% Triton X-100 and 5% bovine serum albumin in PBS at 37°C for 1 h, the sections were incubated with the primary antibody anti-MBP (1:200, Santa Cruz, Dallas, TX) or anti-CC-1 (1:50, Millipore, Billerica, MA) overnight at 4°C . For Immunofluorescent staining, this was followed by 30-min incubation at room temperature with a secondary antibody, either Alexa Fluor 568-conjugated donkey anti-goat IgG (1:200, Life Technologies, Grand Island, NY) for the detection of MBP or Alexa Fluor 568-conjugated donkey anti-mouse IgG (1:200, Life Technologies) for CC-1. The sections were then rinsed three times with PBS and mounted on gelatin-coated slides and coverslipped with Dako fluorescent mounting medium (Carpentaria, CA). For IHC DAB-staining, sections were first incubated with anti-GFAP primary antibody (1:200; Boster, Wuhan, China), washed, and incubated with horseradish peroxidase-linked secondary antibody (1:200, Dako) at 37°C for 1 h. After PBS rinses, the antigen-antibody complexes were visualized using DAB (Boster) as the chromogen. The control specimens were subjected to the same procedures but without primary antibody. Luxol fast blue (LFB) staining was also performed as previously described^[20] to evaluate demyelination.

Western Blot Assessment

To confirm the myelination-related molecular changes evaluated by IHC, three of the tested mice in each group were sacrificed and their CC was dissected for Western blot as

previously described^[20]. After protein determination, 50 µg protein from each sample was loaded on 10% SDS-PAGE gels for electrophoresis and then transferred onto PVDF membranes. After blocking in 5% milk in PBST for 1 h, the membranes were incubated with the primary antibody anti-MBP (1:1 000; Santa Cruz) or anti-GFAP (1:2 000; Boster) at 4°C for 24–48 h. This was followed by 2-h incubation with the corresponding horseradish peroxidase-linked secondary antibody (1: 2 000; Dako) at room temperature. Immunoreactive bands were detected using an ECL-plus detection kit (Amersham Biosciences, Buckinghamshire, UK). The protein loading control was performed by re-probing membranes with an antibody against β-actin (1:2 000; Santa).

Image Analysis

To evaluate the expression levels of CC-1, GFAP and MBP in the CC, five coronal sections from each animal were chosen for optical density (OD) measurement as previously described with slight modification^[20]. Briefly, identical images of the CC were captured, using a digital camera (Nikon, Japan) mounted on a 90i fluorescence microscope (Nikon) for the fluorescent staining of MBP and CC-1, or an Olympus BX-51 light microscope (Olympus, Japan) equipped with a digital capture system (SPOT, Diagnostic Instruments Inc., Sterling Heights, MI) for the DAB-staining of GFAP. The OD of MBP-positive staining was measured using Image-Pro Plus software (version 5.1, Media Cybernetics, Inc., Silver Spring, MD). Conversely, the numbers of CC-1- or GFAP-positive cells were counted using the same software.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical differences between age groups were evaluated using analysis of variance (ANOVA). The two-tailed paired Student's *t*-test was used for comparisons between treatment groups. *P* < 0.05 was considered statistically significant.

RESULTS

CPZ Treatment Induced Severe Demyelination in Young Mice

To evaluate whether OLS in young mice are more vulnerable to demyelination, we performed immunohistological evaluation of the CC, a pivotal brain region affected in schizophrenia^[21,22]. The LFB staining demonstrated that

CPZ treatment led to prominent demyelination in the CC (Fig. 1A, d–f, B) of CPZ mice at 3 and 6 weeks, whereas demyelination was absent in mice with the control diet (Fig. 1A, a–c, B). Conversely, demyelination was absent in the middle-aged (8 months) CPZ-treated mice, similar to the 8-month controls (Fig. 1A, c, f). Together, our data showed that CPZ treatment induces prominent demyelination and

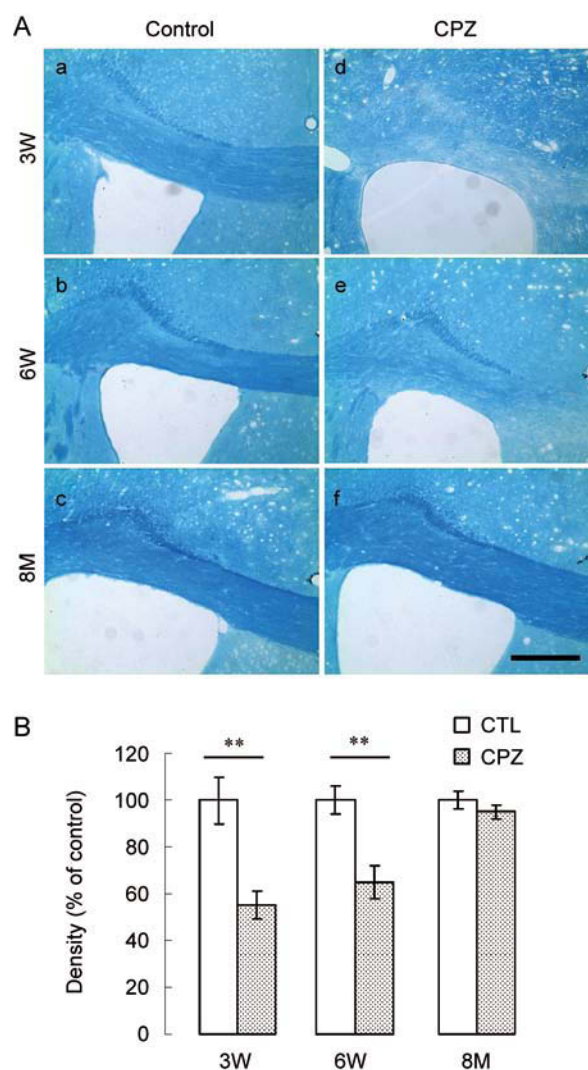


Fig. 1. Cuprizone (CPZ)-induced demyelination in mice at different ages. A: Representative luxol fast blue (LFB) staining of sections from CPZ-treated or control (CTL) mice at 3 weeks (3W), 6 weeks (6W) and 8 months (8M) of age. Scale bar, 200 µm. B: Quantitative analysis of LFB-stained myelin density of midline corpus callosum sections, indicating severity of demyelination. Data represent mean ± SEM. ***P* < 0.01, *n* = 5/group.

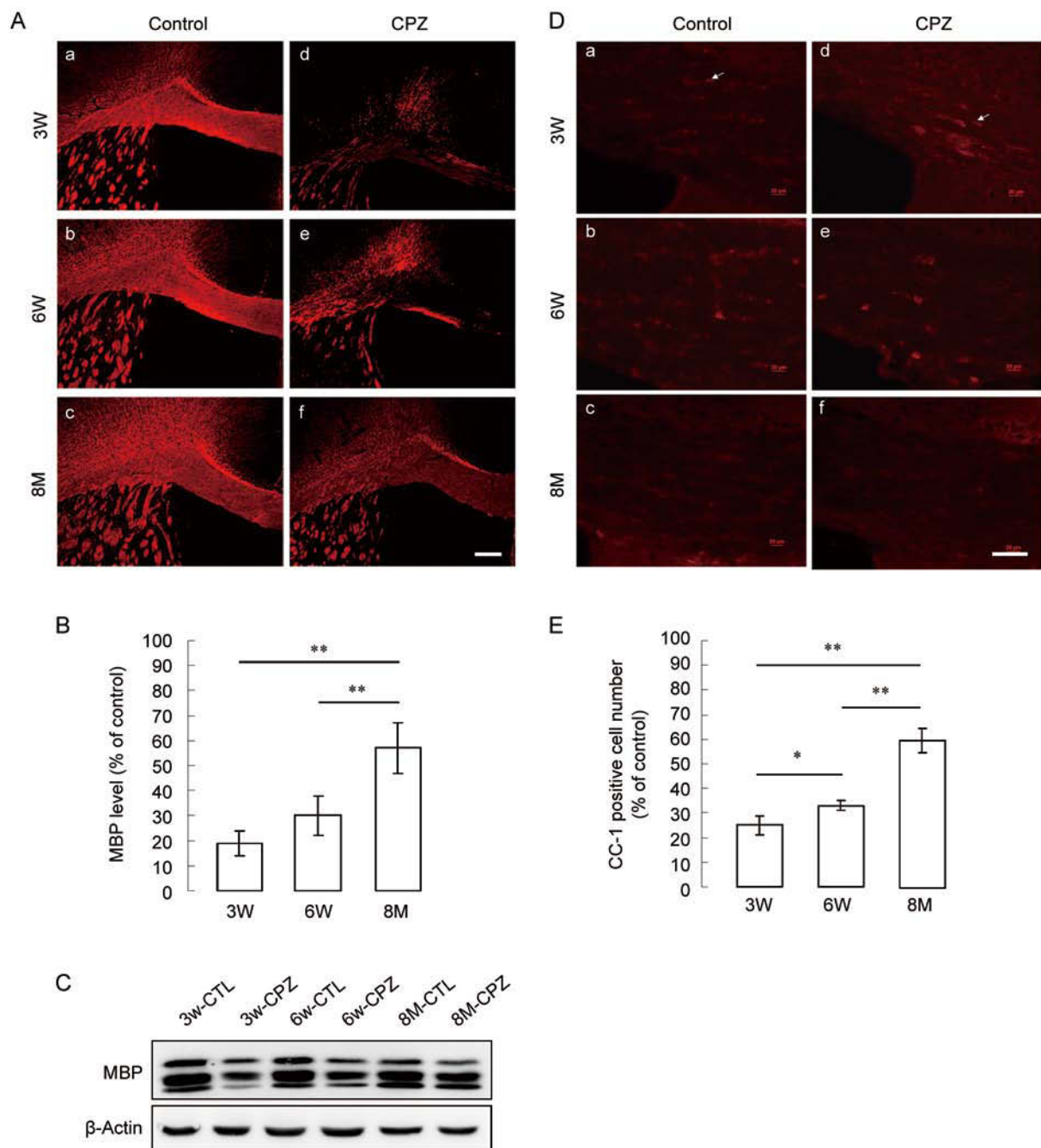


Fig. 2. Cuprizone (CPZ)-treatment induced loss of myelin basic protein and mature OLs. **A:** Representative myelin basic protein (MBP) staining of the corpus callosum from CPZ-treated and control mice at 3 weeks (3W), 6 weeks (6W) and 8-months (8M) of age. Scale bar, 200 μ m. **B:** Quantitative analysis of MBP-positive fibers in the corpus callosum. Data show the percentage of optical density of MBP-positive fibers *versus* corresponding control mice at the same age ($n = 5/\text{group}$; $*P < 0.05$; $**P < 0.01$). **C:** Representative Western blots of MBP in the corpus callosum from different groups. **D:** Representative CC-1 staining of the corpus callosum of mice with a CPZ or control diet, at 3 weeks (3W), 6 weeks (6W) and 8 months (8M) of age. Scale bar, 50 μ m. **E:** Quantitative analysis of CC-1-positive cell numbers in the corpus callosum. Data show percentage of CC-1-positive cells in corresponding control mice at the same age ($n = 5/\text{group}$; $*P < 0.05$; $**P < 0.01$).

juvenile and young-adult mice are more vulnerable.

Loss of MBP and Mature Oligodendrocytes Contributed to CPZ-Induced Demyelination

To evaluate the underlying mechanism of age-related vulnerability to CPZ-induced demyelination, loss of MBP was evaluated by IHC staining. In line with the LFB findings and previous studies^[23], MBP staining also demonstrated that CPZ treatment induced a prominent reduction in the CC of all CPZ-treated mice (Fig. 2A, d-f), indicating CPZ-induced demyelination. Importantly, the CPZ mice at 3 and 6 weeks developed much more severe MBP loss. In contrast, MBP loss in middle-aged (8 months) CPZ mice was significantly lower than that in the 3 and 6 week-old mice with CPZ-treatment (Fig. 2B). Furthermore, the results of Western blot confirmed the reduction of MBP levels in the CC of CPZ-treated animals *versus* control (Fig. 2C), consistent with the different severity of MBP loss in the IHC study (Fig. 2B).

Furthermore, to evaluate the contribution of myelin-forming cells (mature OLs) in CPZ-induced demyelination,

we counted the number of mature OLs using the marker CC-1. The CC-1 staining demonstrated that the number of CC-1-positive cells in the CC was significantly decreased across all CPZ-treated groups compared with the corresponding controls (Fig. 2D, E). Importantly, the CC-1-positive cell loss was more severe in the CPZ mice at 3 and 6 weeks than in the middle-aged (8 months) CPZ mice (Fig. 2D, E). Together, our data showed that MBP and mature OLs were reduced in CPZ-induced demyelination while the pathological changes were more severe in juvenile and young-adult mice.

Activation of Astrocytes Involved in the Vulnerability to CPZ-Induced Demyelination

To evaluate the involvement of astrocyte activation in the age-related vulnerability to demyelination, we performed immunostaining for GFAP, the cellular marker of astrocyte activation. The GFAP staining showed that CPZ treatment significantly increased the number of GFAP-positive cells in the CC (Fig. 3A, d-f). Interestingly, this increase

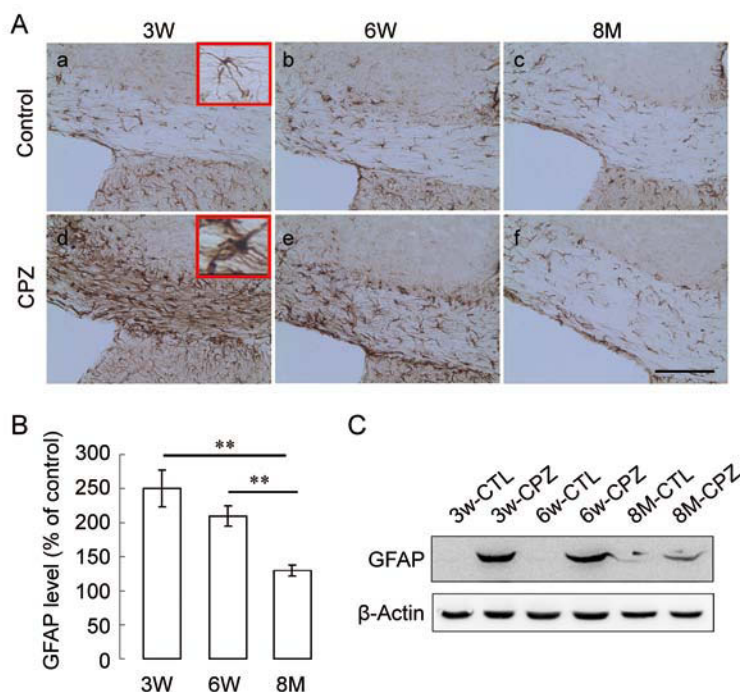


Fig. 3. (CPZ)-induced demyelination involved astrocyte activation. **A:** Representative GFAP staining of the corpus callosum of mice with the CPZ or control diet at 3 weeks (3W), 6 weeks (6W) and 8 months (8M) of age. Scale bar, 100 μ m. Inserts (A, a and d) indicate GFAP-positive astrocytes under higher magnification (100 $\times$). **B:** Quantitative analysis of GFAP-positive cell numbers in the corpus callosum. Data show mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$. **C:** Representative Western blots of GFAP in the corpus callosum from mice at different ages with the CPZ or control (CTL) diet.

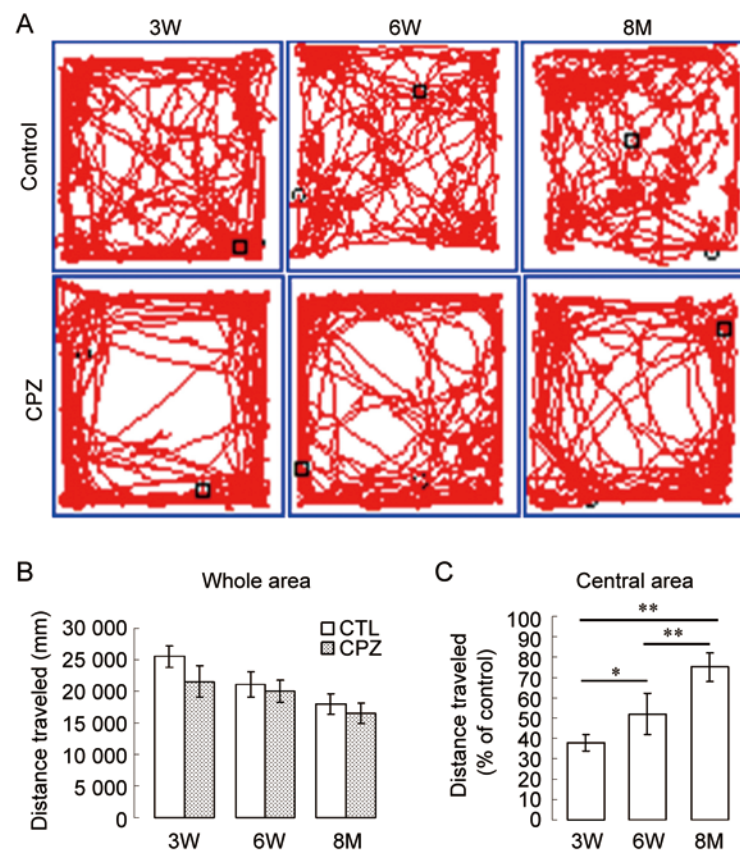


Fig. 4. Cuprizone (CPZ) treatment reduced exploratory activity in the open field. **A:** Typical patterns of locomotor activity in mice with a CPZ or control diet at 3 weeks (3W), 6 weeks (6W) and 8 months (8M) of age. **B and C:** Quantitative analysis of open-field test showing the total distance traveled (**B**) and distance traveled in the central area (**C**). Data in **C** show the percentage of corresponding control mice of the same age. * $P < 0.05$; ** $P < 0.01$ ($n = 8-10$ /group).

was greater in the 3 and 6 week-old than in the 8 month-old CPZ mice (Fig. 3B). Furthermore, the Western blot data confirmed the GFAP change found with IHC, showing significant enhancement of GFAP expression in the CC of CPZ-treated animals, particularly in the 3 and 6 week-old mice (Fig. 3C). Together, our data indicate the involvement of astrocyte activation in the CPZ-induced demyelination as well as the age-related vulnerability.

CPZ-Induced Demyelination Led to Dysfunction in Exploratory Behavior

To evaluate whether the vulnerability of juvenile and young-adult mice to demyelination is linked to behavioral deficits in the CPZ model, the open-field test was used (Fig. 4A). Two sets of behavioral data, locomotor activity and exploratory activity, were measured in the open field. The locomotor activity data showed that the total distance traveled was

similar in mice with CPZ treatment and control mice (Fig. 4B), suggesting intact motor function in the CPZ-treated animals. Notably, CPZ treatment led to a deficit in exploratory activity. The distance traveled in the central area was significantly reduced in the CPZ-treated mice at all ages studied (Fig. 4C). Importantly, this reduction was more prominent in CPZ mice at 3 and 6 weeks than in those at 8 months, while the reduction in the youngest mice (3 weeks) was greater than that in the 6 week-old mice (Fig. 4C). Together, the open-field data suggested that CPZ-induced demyelination led to a deficit of exploratory activity, and the vulnerability of juvenile and young-adult mice resulted in a more severe deficit.

DISCUSSION

The CPZ model of acute demyelination is widely used in

studies in relation to neuropsychiatric disorders^[24]. It has been reported that a higher dose of CPZ is required in aged mice to achieve a degree of demyelination similar to that in young mice^[25-27], but the underlying mechanisms are underexplored. In present study, we demonstrated that juvenile mice are more vulnerable to CPZ-induced demyelination. Our data showed that demyelination in the CC of juvenile mice was more severe than that in middle-aged and young-adult mice with CPZ treatment. Furthermore, we demonstrated that reduction of MBP and the concurrent loss of mature OLs play a crucial role in the age-related vulnerability to CPZ-induced demyelination. Finally, this age-related vulnerability resulted in a more severe exploratory deficit – a behavioral dysfunction linked to anxiety and schizophrenia phenotypes.

The mature OL is the myelin-forming cell in the CNS. Loss of the mature OL marker CC-1 in CPZ-treated animals was noted in our previous study^[20]. Recently, the demyelination hypothesis of schizophrenia has been proposed and has gained accumulating evidence^[28]. The CPZ-induced loss of mature OLs occurred in the demyelinated CC – a region closely linked to the pathophysiology of schizophrenia. We further found that the CPZ-induced dysfunction of OLs and demyelination were most severe in juvenile animals though the underlying mechanisms remain unknown. It has been shown that the metabolic demands during myelination are extraordinarily high. For example, the daily amount of protein synthesized at the peak of myelination in a single OL myelinating multiple axonal segments can be up to three times the weight of its perikaryon^[29]. Even when myelination is completed, mature OLs continuously require a high energy supply for the maintenance of their lipid-rich myelin membrane. Indeed, energy imbalance can, at the peak of inflammation, constitute an energy crisis leading to demyelination *via* oxidative damage. The metabolic demands of mature OLs seem to be greater in juvenile animals, and thus may result in their high vulnerability to demyelination. Further investigation is needed to validate this corollary. Nevertheless, the age-related feature is apparently coincident with the epidemiological feature of schizophrenia onset, which shows that juvenile and young adults comprise the major population affected by schizophrenia^[5,6]. Our data suggest that high vulnerability of demyelination in young population may be an etiological risk factor for schizophrenia.

Astrocytes compose the majority of glia in the CNS. It has been reported that the activation of astrocytes participates in demyelination as a cellular response to inflammation and injury^[30-32]. Recent findings suggest that astrocytes play an active role during demyelination and oligodendrocyte degeneration by controlling local inflammation in the CNS, which damages OLs and axons^[33]. Astrocyte production of IL-15 leads to the activation of CD8 T-lymphocytes that contribute to the exacerbation of tissue damage during MS^[34]. Recent studies also suggest that aberrant upregulation of astroglial ceramide potentiates OL injury^[35,36]. In the present study, we found hypertrophic and hyperplastic astrocytosis in the demyelinated region. For the first time, we demonstrated that, correlated with the severity of demyelination, the hypertrophic and hyperplastic astrocytosis was most prominent in CPZ-treated juvenile mice though the underlying mechanisms need further investigation.

The open-field test has been used to assess motor and psychiatric behavioral deficits, including spontaneous locomotion and exploratory activity in a novel environment^[37,38]. The exploratory activity has been linked with both anxiety and schizophrenia phenotypes^[23,39]. Here, we demonstrated that CPZ-induced demyelination led to a deficit in exploratory activity, and juvenile mice showed the most severe deficit. Our open-field data also ruled out the influence of spontaneous locomotor activity on the CPZ-induced exploratory deficit. However, additional schizophrenia-related behavioral tests are needed for further evaluation. Nevertheless, our data suggest a role of CPZ-induced demyelination in a schizophrenia-related deficit in mice, and the CPZ mouse model is of value in studies of schizophrenia.

Together, our findings provide insight into the age-related vulnerability of demyelination, which is correlated with etiological features of schizophrenia. This work contributes to a better understanding of the susceptibility of young adults to the onset of schizophrenia as well as the demyelination hypothesis of schizophrenia.

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