Moreover, we recently reported that neonatal injection of polyI:C in mice results in schizophrenia-like behavioral alterations in adulthood [11]. In the publication, we discussed the rationale in choosing the timing of polyI:C injection during the mouse neonatal stage that corresponds to the human second trimester [11]. Accordingly, neonatal DN-DISC1 mice were repeatedly injected with polyI:C for 5 days from postnatal day 2 to 6, which correspond to post conception day 128–158 for cortical events and 93–115 for limbic events of brain development in humans (http://translatingtime.net; see also [7]).

In the present study, we demonstrate that combined effect of neonatal polyI:C treatment and DN-DISC1 affects some behavioral and anatomical phenotypes in adulthood. Of note, as far as we are aware, this is the first experimental demonstration that "neonatal" interaction of major genetic and environmental susceptibility factors for schizophrenia results in the dramatic change in the parvalbumin-positive interneurons in the medial prefrontal cortex (mPFC), one of the best hallmarks for schizophrenia [17,20].

2. Materials and methods

2.1. Animals

Transgenic mice expressing a dominant-negative mutant DISC1 under the expression control of CaMKII promoter (DN-DISC1; line 10) [10] were used in this study. Littermates (both males and females) generated by cross breeding of wild-type (WT, C57BL/6N) female and DN-DISC1 male mice were used for experiments. They were housed under a standard 12-h light/dark cycle (light phase 9:00–21:00) at a constant temperature of 23 ± 1 °C, with free access to food and water throughout the experiments.

The animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Treatment

All litters were randomly divided into two groups: vehicle and polyI:C-treated mice. Mice were treated by subcutaneous injection with either pyrogen-free saline or 5 mg/kg polyI:C (Sigma–Aldrich, St. Louis, MO) daily between postnatal days 2 and 6. Animals were weaned at postnatal day 21, and divided along gender lines at postnatal day 28. Four groups [e.g., vehicle-treated WT (vehicle/WT), polyI:C-treated WT (polyI:C/WT), vehicle-treated DN-DISC1 (vehicle/DN-DISC1) and polyI:C-treated DN-DISC1 (polyI:C/DN-DISC1)] were derived from multiple (at least 3) litters to preclude possible differences in individual maternal behaviors as a mitigating factor in any subsequent long-lasting changes induced in the offspring. Behavioral analyses were started at 8 weeks old, and carried out in the following orders: Y-maze test, novel object recognition test, prepulse inhibition test, fear conditioning test, social interaction test and MK-801-induced hyperactivity.

2.3. Behavioral assays

2.3.1. Y-maze test

Y-maze test was carried out as described previously [10]. Each arm is 40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The arms converge in an equilateral triangular central area that is 4 cm at its longest axis. Each mouse is placed individually at the center of the apparatus and allowed to move freely through the maze during an 8-min session. The series of arm entries is recorded visually. Alternation is defined as successive entries into the three arms, on overlapping triplet sets. The percent alternation is calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus 2) multiplied by 100. Spontaneous alternation (%) defined as successive entries into the three arms on overlapping triplet sets is associated with the capacity of short-term memory.

2.3.2. Novel object recognition test

A novel object recognition test was carried out as described previously [11]. Mice were individually habituated to an open-box (30 cm × 30 cm × 35 high cm) for 3 days. During the training session, two novel objects were placed into the open field and the animals were allowed to explore for 10 min. The time spent exploring each object was recorded. During retention sessions, the animals were placed back into the same box 1 h after the training session, in which one of the familiar objects used during training was replaced by a novel object, and allowed to explore freely for 5 min. A preference index in the retention session, a ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as a ratio of the time spent exploring the object that was replaced by the novel object in the retention session, over the total exploring time.

2.3.3. Prepulse inhibition test

The prepulse inhibition (PPI) test was carried out as described previously [11]. After the animals were placed in the chamber under moderately bright light conditions (180 lux) (San Diego Instruments, San Diego, CA), they were allowed to habituate for 10 min, during which 65 dB background white noise was present. The animals then received 10 startle trials, 10 no-stimulus trials and 40 PPI trials. The intertrial interval was between 10 and 20 s and the total session lasted 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 ms. PPI trials consisted of a prepulse (20 ms burst of white noise at 69, 73, 77 or 81 dB intensity) followed, 100 ms later, by the startle stimulus (120 dB, 40 ms white noise). Each of the four prepulse trials (69, 73, 77 or 81 dB) was presented 10 times. Sixty different trials were presented pseudo-randomly, ensuring that each trial was presented 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured for 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified and fed into a computer, which calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 10 startle trials. PPI was calculated according to the formula: $100 \times [1 - (PPx/P120)]$ %, in which PPx was the mean of the 10 PPI trials (PP69, PP73, PP75 or PP80) and P120 was the basal startle amplitude.

2.3.4. Fear conditioning test

To examine contextual memory in polyI:C/DN-DISC1 mice, we used context-dependent conditioned fear test according to previous report [1]. In the conditioning phase, each mouse is placed in the training cage (30 cm × 30 cm × 40 cm) equipped with a metal floor, and a 15-s tone (85 dB) is delivered (conditioned stimulus). During the last 5 s of the tone stimulus, a foot shock of 0.8 mA is delivered as an unconditioned stimulus through a shock generator. This procedure is repeated four times with 15 s intervals. Twenty-four hours after the conditioning, context-dependent test was carried out. For context-dependent test, mouse is placed in the training cage, and the freezing response is measured for 2 min in the absence of the conditioned stimulus. Two hours after context-dependent test, tone-dependent test was carried out. For tone-dependent test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioned stimulus using mice which had been subjected to context-dependent test.

2.3.5. Social interaction test

We used the experimental paradigm described by Ibi et al. [11] to measure social behavior (e.g., social interaction, aggression and escape behavior). Vehicle/WT and polyI:C/DN-DISC1 mice were individually housed in a home cage (29 cm × 18 cm × 12 cm) for 2 days before the trial. We used 10–15-week-old male C57BL mice as intruders which had not shown aggressive behavior. In the first trial (5 min duration), an intruder mouse was introduced into the resident's home cage. The duration of social interaction (close following, inspection, anogenital sniffing, and other social body contacts except aggressive behavior), aggression (attacking/biting and tail rattling) and escape behavior were analyzed. Four trials, with an inter-trial interval of 30 min, were used to analyze social behavior using the same intruder mouse.

2.4. MK-801-induced hyperactivity

To investigate the possible changes in sensitivity of glutamate N-methyl D-aspartate (NMDA) receptor in polyI:C/DN-DISC1 mice, locomotor activity induced by MK-801 (Sigma–Aldrich) was measured as described previously [11]. Each mouse was placed in a standard transparent rectangular rodent cage (25 cm × 30 cm × 18 cm) and allowed a 120-min habituation period before MK-801 (0.3 mg/kg, i.p.) treatment. Locomotor activity was then measured for 180 min immediately after MK-801 treatment, using an infrared sensor (NS-AS01; Neuroscience, Tokyo, Japan) placed over the cage.

2.5. Histological analyses

In histological analysis, 8-week-old mice that had not previously subjected to behavioral analysis were deeply anesthetized with diethyl ether and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Their brains were removed, post-fixed in the same fixative, and then cryoprotected. Thirty micrometer-thick coronal brain sections were cut on a cryostat and mounted on slides. The section used for the analysis of the mPFC was collected between stereotaxic coordinates bregma 1.78 and 1.54 according to the brain atlas [21] and those in the hippocampus was collected according to Ibi et al. [12].

2.6. BrdU-staining

5-Bromo-2′-deoxyuridine (BrdU, Sigma–Aldrich) at a dose of 75 mg/kg was injected i.p. 3 times at 2 h intervals at 4 weeks old, and the number of BrdU-

labeled cells in the hippocampus was counted at 8 weeks [12]. Sections were treated overnight with 0.1% Nonidet P-40/0.01 M PBS (pH 7.2) at 4 °C and denatured by microwave oven in 0.01 M citrate buffer (pH 6.0). After blocking in 10% goat serum/PBS with 0.1% NP-40 for 30 min, BrdU-positive cells in the sections were detected using a BrdU labeling and detection kit 2 (Roche Diagnostics GmbH, Germany) according to the manufacture's instructions.

2.7. Ki67 and parvalbumin staining

Sections were incubated with 10% goat serum/PBS with 0.1% Triton X-100, and then incubated with rabbit anti-Ki67 antibody (1:2000; Novocastra, Newcastle, UK) or rabbit anti-parvalbumin antibody (1:500; Calbiochem, San Diego, CA) overnight at 4 °C. They were washed with 0.01 M PBS and incubated with biotinylated goat anti-rabbit antibody (1:200; BA-1000, Vector Laboratories, Burlingame) at room temperature for 1 h. The sections were washed and processed with avidin-biotinylated horseradish peroxidase complex (Vector ABC kit, Vector Laboratories), and action was visualized using diaminobenzidine.

For double staining of parvalbumin/Nissl in the mPFC, sections were incubated with blocking solution (10% donkey serum/PBS with 0.1% Triton X-100) and then rabbit anti-parvalbumin antibody (1:500; Calbiochem) diluted in blocking solution was applied to sections, which were then incubated overnight at 4 °C and for 30 min at room temperature. After washing in PBS, donkey anti-rabbit Alexa 488 (1:1000; Invitrogen, Eugene, OR) was added to sections for 2 h at room temperature. NeuroTracer Fluorescent Nissl Stains (1:100; Invitrogen) diluted in PBS was applied to sections, which were then incubated for 20 min at room temperature.

2.8. Quantification for BrdU, Ki67 and parvalbmin-positive cells

All BrdU and Ki67-labeled cells in the subgranular zone (SGZ), hilus and granule cell layer (GCL) were assessed as described by Ibi et al. [12]. Parvalbumin-positive cells in the hippocampus and mPFC were counted under ×10 magnification using a light microscope (Axio Imager; Zeiss, Jene, Germany) and confocal-laser scanning microscope (LSM 510; Zeiss), respectively.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) followed by the Bonferroni test was used for multiple-group comparisons. Two-tailed Student's *t*-test was used for two-group comparisons.

3. Results

3.1. Behavioral evidence of gene–environment interactions

Neonatal WT and DN-DISC1 mice were treated with polyI:C or saline, and their behaviors were analyzed after being 8 weeks old. In the Y-maze test, neither genetic manipulation (vehicle/DN-DISC1) nor an environmental factor (polyI:C/WT) affected behavioral phenotypes compared with controls (vehicle/WT). Nonetheless, polyI:C/DN-DISC1 mice showed marked reduction in spontaneous

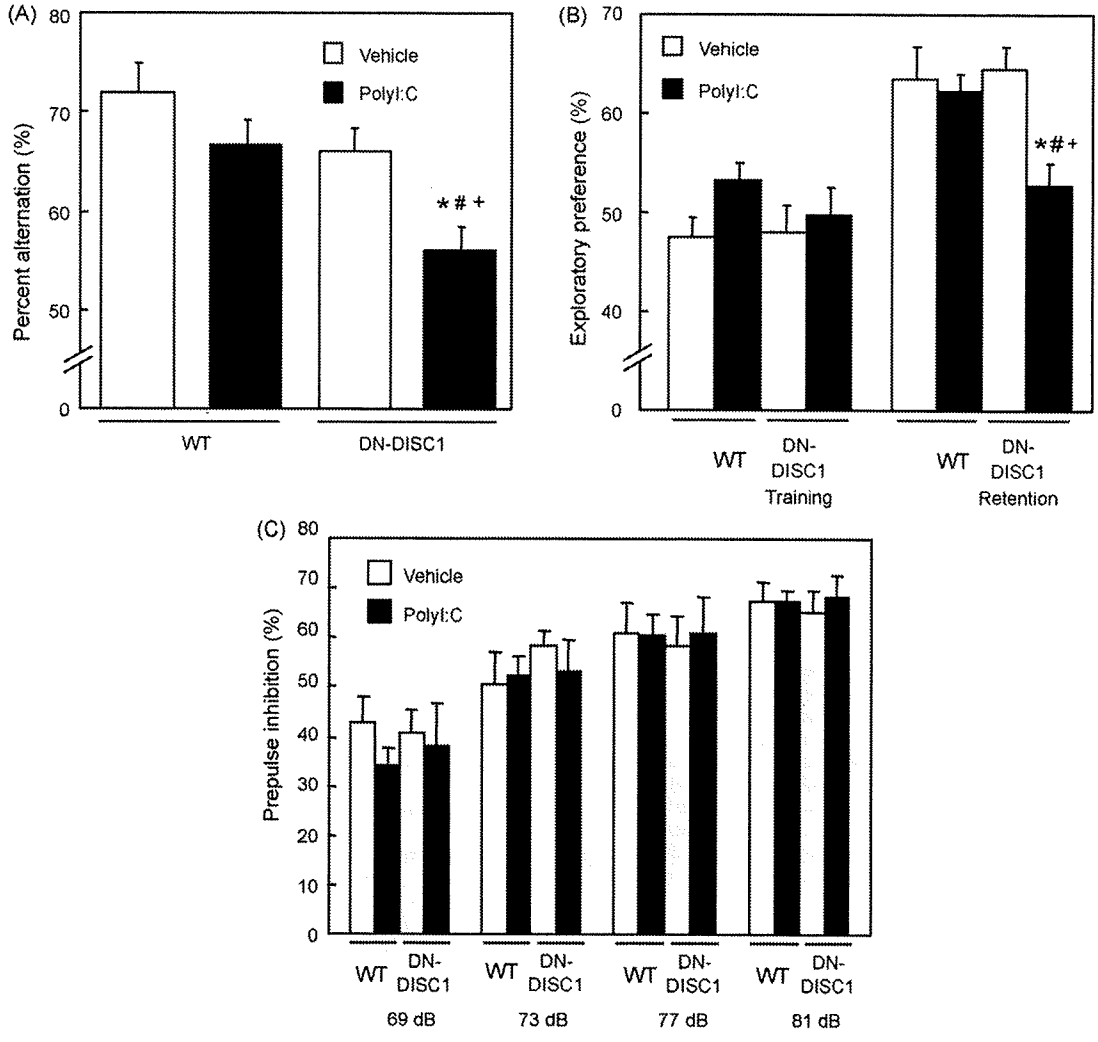

Fig. 1. Changes in short-term and recognition memories, and prepulse inhibition in polyI:C/DN-DISC1 mice. (A) Spontaneous alternation behavior in Y-maze test. Vehicle/WT, $n = 13$; polyI:C/WT, $n = 11$; vehicle/DN-DISC1, $n = 17$; polyI:C/DN-DISC1, $n = 13$. Percent alternation (%): $F_{(3,50)} = 6.529$, $p < 0.001$. (B) Exploratory preference in novel object recognition test. Vehicle/WT, $n = 12$; polyI:C/WT, $n = 11$; vehicle/DN-DISC1, $n = 10$; polyI:C/DN-DISC1, $n = 14$. Exploratory preference (%): $F_{(3,43)} = 5.427$, $p < 0.01$. (C) PPI (%) at four different prepulse intensities (69, 73, 77 and 81 dB) in PPI test. Vehicle/WT, $n = 9$; polyI:C/WT, $n = 14$; vehicle/DN-DISC1, $n = 11$; polyI:C/DN-DISC1, $n = 9$. Prepulse inhibition (%): $F_{(3,42)} = 0.0562$, $p = 0.982$. Values indicate the mean ± SE. $^{*}p < 0.05$ vs. vehicle/WT, $^{\#}p < 0.05$ vs. polyI:C/WT, $^{+}p < 0.05$ vs. vehicle/DN-DISC1.

alternation (Fig. 1A). There was no difference in the total number of arm entries among the four groups [vehicle/WT, 21.1 ± 1.0; polyI:C/WT, 24.7 ± 2.8; vehicle/DN-DISC1, 21.1 ± 0.9; polyI:C/DN-DISC1, 23.4 ± 1.7]. These results indicate a combined action of genetic (DN-DISC1) and environmental (polyI:C) factors, resulting in an impairment of short-term memory.

Similar effects were also observed as a marked decrease in exploratory preference to a novel object in the retention session of novel object recognition test, in which only polyI:C/DN-DISC1 mice displayed the deficit, whereas the other three groups showed no difference (Fig. 1B). There was no difference in total time exploring two objects among the four groups in the training [vehicle/WT, 30.0 ± 2.4 s; polyI:C/WT, 33.6 ± 3.2 s; vehicle/DN-DISC1, 33.6 ± 3.8 s; polyI:C/DN-DISC1, 33.0 ± 2.8 s]. Thus, polyI:C/DN-DISC1 mice have an impairment of object recognition memory.

On the contrary, in the PPI test of the startle reflex, no changes were observed in either polyI:C/WT, vehicle/DN-DISC1 or polyI:C/DN-DISC1 mice compared to vehicle/WT mice, indicating no impairment of sensorimotor gating in polyI:C/DN-DISC1 mice (Fig. 1C). There was also no difference in the acoustic startle amplitude [vehicle/WT, 215 ± 36; polyI:C/WT, 232 ± 36; vehicle/DN-DISC1, 162 ± 30; polyI:C/DN-DISC1, 184 ± 28].

We then focused on the characterization of schizophrenia-like behavioral abnormality in polyI:C/DN-DISC1 mice. Thus, in the following behavioral assays, behavioral changes in polyI:C/DN-DISC1 mice were compared with those in vehicle/WT mice without testing behaviors in polyI:C/WT or vehicle/DN-DISC1 mice.

In the fear conditioning memory test, polyI:C/DN-DISC1 mice showed a significant decrease in context-dependent freezing time compared to vehicle/WT mice (Fig. 2A). However, there was no difference in tone-dependent freezing time (35.5 ± 2.1 s in vehicle/WT mice, 33.0 ± 2.3 s in polyI:C/DN-DISC1 mice) or sensitivity to electric footshock (0.14 ± 0.02 mA in vehicle/WT mice, 0.15 ± 0.02 mA in polyI:C/DN-DISC1mice), suggesting an impairment of hippocampus-dependent fear memory in polyI:C/DN-DISC1mice.

In the social interaction test, time of interaction was markedly decreased, while escape and aggressive behaviors were increased, in polyI:C/DN-DISC1 mice, compared with vehicle/WT mice (Fig. 2B). MK-801-induced hyperactivity was significantly augmented in polyI:C/DN-DISC1 mice, compared to vehicle/WT mice (Fig. 2C).

3.2. Histological analyses in polyI:C/DN-DISC1 mice

Selective reduction in the immunoreactivity of parvalbumin, an indicator of a set of interneurons in the cerebral cortex, has frequently been reported in autopsied brains from patients with schizophrenia [17]. Very interestingly, polyI:C treatment in the neonatal stage developed the selective reduction in the immunoreactivity of parvalbumin, specific to the mPFC in DN-DISC1 mice (Fig. 3A and B).

Because polyI:C/DN-DISC1 mice showed a disturbance of the hippocampus-dependent fear memory, we examined whether there were histological alterations in the hippocampus of polyI:C/DN-DISC1 mice. We conducted Nissl-staining, BrdU staining, and immunohistochemistry for Ki67 (a marker of cell proliferation) [2,8]. Although there were no changes in Nissl-staining (data not shown) and immunostaining of Ki67 (Fig. 4A and B), a significant increase in the number of BrdU-positive cells in the GCL of the hippocampus was observed (Fig. 4A and C).

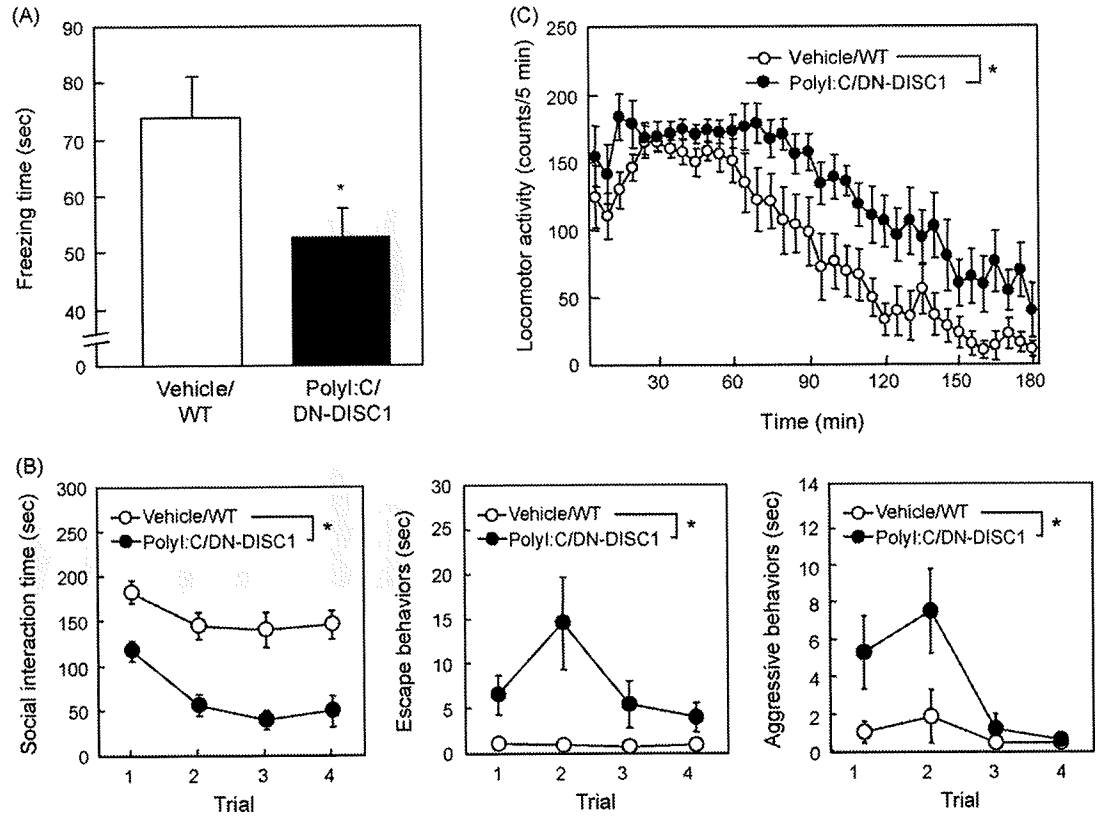

Fig. 2. Changes in fear memory, social behaviors and MK801-induced hyperactivity in polyI:C/DN-DISC1 mice. (A) Context-dependent memory in fear conditioning test. Vehicle/WT, $n = 11$; polyI:C/DN-DISC1, $n = 13$. (B) Social behaviors in social interaction test. Vehicle/WT, $n = 8$; polyI:C/DN-DISC1, $n = 8$. Social interaction time: $F_{(1,14)} = 41.172$; $p < 0.0001$, escape behaviors: $F_{(1,14)} = 7.012$; $p < 0.05$, aggressive behaviors: $F_{(1,14)} = 7.316$; $p < 0.05$. (C) MK-801-induced hyperactivity. Vehicle/WT, $n = 9$; polyI:C/DN-DISC1, $n = 10$. $F_{(1,17)} = 11.232$, $p < 0.01$. Values indicate the mean ± SE. *$p < 0.05$ vs. vehicle/WT.

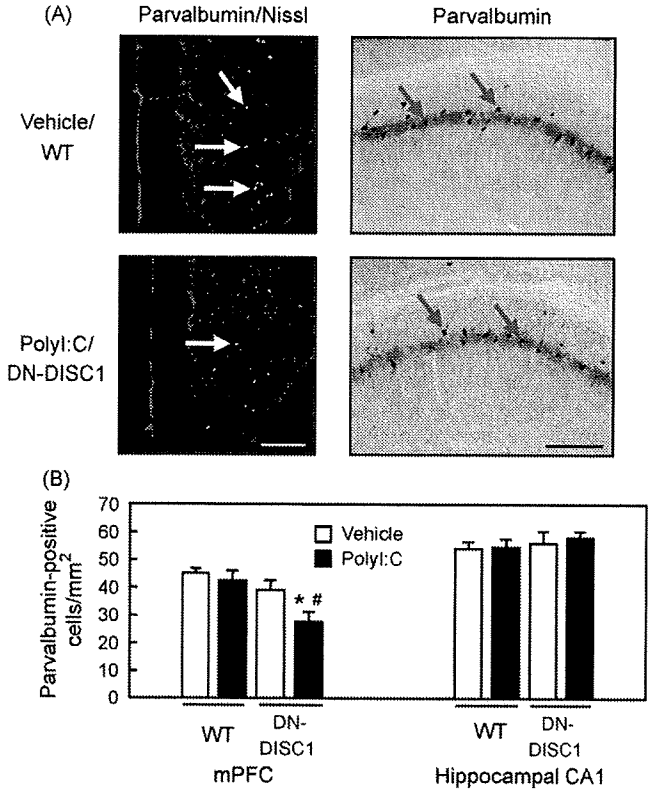

Fig. 3. Changes in parvalbumin-positive interneurons in polyI:C/DN-DISC1 mice. (A) Representative photographs showing parvalbumin-positive cells in the mPFC [left (parvalbumin-positive cell, green; Nissl-positive cell, red)] and hippocampal CA1 region (right). Upper panels, vehicle/WT mice; lower panels, polyI:C/DN-DISC1 mice. Scale bar: 200 μm. (B) Changes in the number of parvalbumin-positive interneurons. mPFC: vehicle/WT, $n = 10$; polyI:C/WT, $n = 12$; vehicle/DN-DISC1, $n = 8$; polyI:C/DN-DISC1, $n = 8$. $F_{(3,35)} = 4.996$, $p < 0.001$. Hippocampal CA1 region: vehicle/WT, $n = 3$; polyI:C/WT, $n = 4$; vehicle/DN-DISC1, $n = 3$; polyI:C/DN-DISC1, $n = 4$. $F_{(3,10)} = 0.386$, $p = 0.766$. Values indicate the mean ± SE. $^{*}p < 0.05$ vs. vehicle/WT, $^{\#}p < 0.05$ vs. polyI:C/WT.

4. Discussion

In agreement with the previous report by Hikida et al. [10], behavioral and histological abnormalities in DN-DISC1 mice were mild and subtle. In the present study, we demonstrated that combination of neonatal polyI:C treatment with DN-DISC1 resulted in the deficits of short-term, object recognition, and hippocampus-dependent fear memories in adulthood, although polyI:C treatment by itself had little influence on WT mice. Furthermore, polyI:C/DN-DISC1 mice exhibited signs of impairment of social recognition and interaction and augmented susceptibility to MK-801-induced hyperactivity. DN-DISC1 mice is reported to display a small, but significant, deficit in PPI of the startle reflex in the previous study [10], whereas there was no significant difference between vehicle/WT and vehicle/DN-DISC1 mice in the present study. The discrepancy in the PPI deficit of DN-DISC1 mice may be explained by the difference in experimental schedule. Behavioral analysis was carried out at the age of 12–32 weeks in the previous report [10] while we started the analysis from 8-week old mice. Accordingly, it is possible that the changes in PPI response in DN-DISC1 mice may be undetectable at younger ages as we selected [24].

The present study demonstrated a significant impact of DN-DISC1 expression on parvalbumin-positive interneurons in the mPFC (Fig. 3A and B), which is consistent with the previous findings [10]. Importantly, the post hoc analysis revealed the additive effect of neonatal immune activation induced by polyI:C treatment and genetic impact of DN-DISC1, leading to a marked decrease in parvalbumin-positive interneurons in the mPFC of polyI:C/DN-DISC1 mice in adulthood. This pathological change is currently regarded as the best hallmark for the pathophysiology of schizophrenia, which is likely to underlie the cognitive dysfunction in patients with schizophrenia [17,20].

For the past couple of years, many lines of genetic mouse models based on susceptibility genes for schizophrenia have become available and characterized mainly by behavioral alterations [10,13,18,19,25]. Nonetheless, only a few studies have addressed for possible gene-environmental interactions in the context of schizophrenia [16]. We believe that the present study has two major strengths: first, a most promising pair of genetic and environmental factors for schizophrenia is tested together for the phenotypic assessment; second, of most importance, the present study addresses a specific interneuron deficit, well-accepted histological hallmark for schizophrenia, and examine how gene-environmental interactions during the "critical neurodevelopmental period" can result in this objective phenotype in "adulthood". Furthermore, we also demonstrated that such gene-environmental interactions specifically affect some, but not all, types of behaviors elicited by the genetic factor. The concept of neonatal "critical neurodevelopmental period" proposed in the

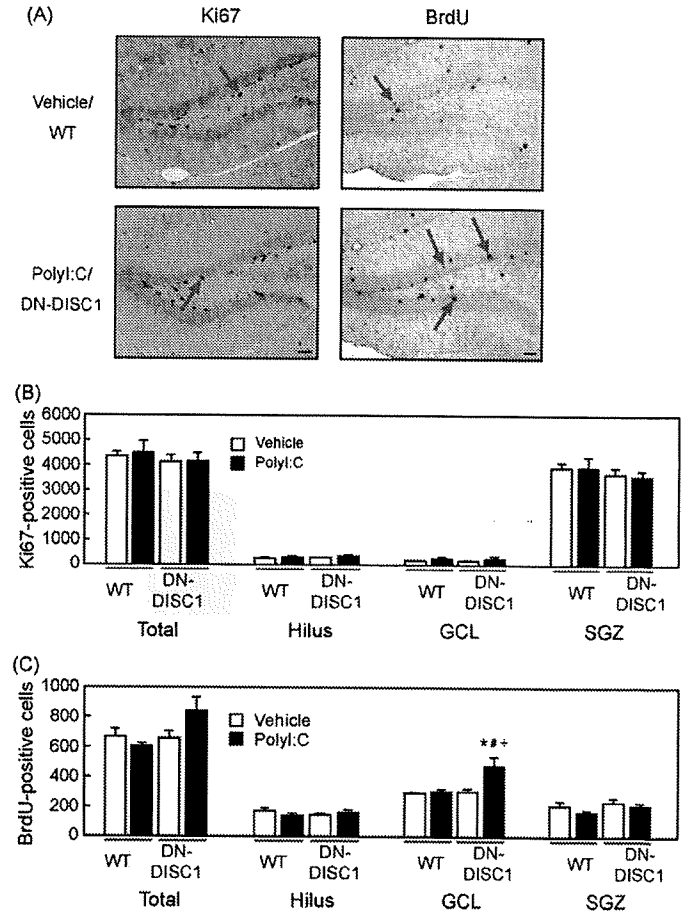

Fig. 4. Changes in Ki67 and BrdU-positive cells in polyI:C/DN-DISC1 mice. (A) Representative photographs showing Ki67-positive, and BrdU-positive cells in the DG of hippocampus. Upper panels, vehicle/WT mice; lower panels, polyI:C/DN-DISC1 mice. Scale bar: 200 μm. (B) Changes in the number of Ki67-positive cells in the hippocampus. Vehicle/WT, $n = 3$; polyI:C/WT, $n = 4$; vehicle/DN-DISC1, $n = 3$; polyI:C/DN-DISC1, $n = 4$. Total: $F_{(3,10)} = 0.231$, $p = 0.873$, Hilus: $F_{(3,10)} = 0.318$, $p = 0.812$, GCL: $F_{(3,10)} = 0.926$, $p = 0.463$, SGZ: $F_{(3,10)} = 0.358$, $p = 0.785$. (C) Changes in the number of BrdU-positive cells in the hippocampus. Vehicle/WT, $n = 5$; polyI:C/WT, $n = 5$; vehicle/DN-DISC1, $n = 5$; polyI:C/DN-DISC1, $n = 5$. $F_{(3,16)} = 6.406$, $p < 0.01$. There were no significant differences in other brain areas. Values indicate the mean ± SE. $^{*}p < 0.05$ vs. vehicle/WT, $^{\#}p < 0.05$ vs. polyI:C/WT, $^{+}p < 0.05$ vs. vehicle/DN-DISC1.

present study is consistent with the observation by the Cannon and Silva's group [19], in which their group demonstrated the critical requirement of DISC1 function in neonatal days by using inducible transgenic mice for mutant DISC1. On the other hand, from the clinical point of view, "critical development period" of schizophrenia is postulated not only in early (pre- and peri-natal) stage but also in late (pubertal) stage in human [26]. Further studies are required to clarify the effect of pubertal exposure to psychosocial stresses or polyI:C in DN-DISC1 mice on phenotypic changes.

To elucidate a genuine gene-based effect, it is important to use more than two independent lines of transgenic mice. The present study investigated the gene-environmental interaction by using only one line of DN-DISC1 mice (line 10) because basic characterization of these behavioral deficits has already conducted in two independent lines (lines 10 and 37) which showed that the behavioral and histological phenotypes in DN-DISC1 mice are due to the disruption of DISC1 gene [10]. However, to completely exclude a possibility that another, unknown gene may participate in the interaction, we need to use another line of DN-DISC1 (line 37).

In summary, taking effects in several behavioral and pathophysiological deficits into consideration, we propose that neonatal polyI:C treatment in DN-DISC1 mice may provide a model for schizophrenia that reflects gene-environmental interactions.

Conflict of interest

The authors declare that there is no conflict of interest in the publication of the present work.

Acknowledgements

We thank Drs. Pamela Talalay and Akiko Takagi-Hayashi for critical reading of this manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research (No. 19390062) from the Japan Society for the Promotion of Science, Research on Risk of Chemical Substances, Health and Labor Science Grants supported by Ministry of Health, Labour and Welfare, Academic Frontier Project for Private Universities; matching fund subsidy from MEXT, 2007–2011, Takeda Science Foundation, AstraZeneca Research Grant 2008, and by JST, CREST. NIH grants of MH-084018, MH-069853, as well as grants from Stanley, CHDI, HighQ, S-R, and NARSAD support AS.

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Behavioural Brain Research 207 (2010) 387–393

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Silibinin attenuates cognitive deficits and decreases of dopamine and serotonin induced by repeated methamphetamine treatment

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ARTICLE INFO

Article history:
Received 23 August 2009
Received in revised form 14 October 2009
Accepted 16 October 2009
Available online 24 October 2009

Keywords:
Methamphetamine
Silibinin
Dopamine
Serotonin
Memory

ABSTRACT

Cognitive deficits are a core feature of patients with methamphetamine (METH) abuse. It has been reported that repeated METH treatment impairs long-term recognition memory in the novel object recognition test (NORT) in mice. Recent studies indicate that silibinin, a flavonoid derived from the herb milk thistle, has potent neuroprotective effects in cell cultures and several animal models of neurological diseases. However, its effect on the cognitive deficit induced by METH remains unclear. In the present study, we attempt to clarify the effect of silibinin on impairments of recognition memory caused by METH in mice. Mice were co-administered silibinin with METH for 7 days and then cognitive function was assessed by NORT after 7-day withdrawal. Tissue levels of dopamine and serotonin as well as their metabolites in the prefrontal cortex and hippocampus were measured 1 day after NORT. Silibinin dose-dependently ameliorated the impairment of recognition memory caused by METH treatment in mice. Silibinin significantly attenuated the decreases in the dopamine content of the prefrontal cortex and serotonin content of the hippocampus caused by METH treatment. We also found a correlation between the recognition values and dopamine and serotonin contents of the prefrontal cortex and hippocampus. The effect of silibinin on cognitive impairment may be associated with an amelioration of decreases in dopamine and serotonin levels in the prefrontal cortex and hippocampus, respectively. These results suggest that silibinin may be useful as a pharmacological tool to investigate the mechanisms of METH-induced cognitive impairments.

1. Introduction

Methamphetamine (METH) is a globally popular and highly addictive drug that can cause neuropsychiatric complications such as hallucination and delusions [14,28,40]. Recent studies in humans have also demonstrated that chronic use of METH causes cognitive deficits after withdrawal [14,39,41]. In rodents, repeated METH treatment induces spatial working memory impairment in the radial arm maze test [26], recognition impairment in the object recognition test [15,27], and cognitive impairment in the five-choice serial reaction time test [9]. Therefore, the METH-induced cognitive impairment in rodents may be useful as an animal model of the cognitive deficits in METH abusers.

Several studies have revealed that the cognitive impairments produced by METH are associated with disruptions of the dopaminergic and serotonergic systems [14,15,17,22,28]. Disturbances of the dopaminergic system have been widely reported in METH abusers and animals, and are associated with behavioral deficits [17]. For example, our group has reported that repeated METH treatment results in a dysfunctional dopamine D_1 receptor-extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in the prefrontal cortex, which is associated with memory deficits after drug withdrawal [15,27]. The serotoninergic system also plays a critical role in cognitive behavior [7,24] and is associated with METH abuse [17]. Moreover, the serotonergic system interacts with the dopaminergic system, implicating it in cognitive function and drug abuse [46]. Although the interactions involved are complicated, a report has confirmed that dysfunction of either

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doi:10.1016/j.bbr.2009.10.024

dopaminergic or serotonergic system alone is insufficient to produce the impairments seen in the novel object recognition test (NORT) following METH treatment [3].

Silibinin (silybin), a flavonoid derived from the herb milk thistle (*Silybum marianum*), has been shown to have anti-oxidative and anti-inflammatory properties [38]. Further, silibinin also has potential effects on monoamine transmission and cognitive function. For example, high-performance liquid chromatography (HPLC) analyses have shown increased serotonin levels in the cortex and increased dopamine and norepinephrine levels in the cerebellum after 5-day repeated treatment with silymarin, a mixture of flavonoids present in milk thistle and whose main component is silibinin, in BALB/c mice [29]. An *in vitro* study has demonstrated that silibinin inhibits the activity of monoamine oxidase (MAO) that catalyzes the oxidative deamination of monoamines [23]. These studies suggest that silibinin may be beneficial/neuroprotective effect on METH-induced dysfunction. However, it remains unclear whether silibinin prevents METH-induced cognitive deficits.

In this study, we investigated the effects of silibinin on the impairment of recognition memory in NORT and decrease of dopamine and serotonin levels in the prefrontal cortex and hippocampus in repeated METH-treated mice.

2. Materials and methods

2.1. Animals

ICR male mice (Japan SLC Inc., Shizuoka, Japan), aged 6 weeks at the beginning of experiments, were used. They were housed in plastic cages and kept in a regulated environment (23 ± 0.5 °C, 50 ± 5% humidity) with a 12/12-h light/dark cycle (lights on from 08:00 to 20:00). Mice received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum. Behavioral experiments were carried out in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Faculty of Pharmaceutical Sciences of Meijo University.

2.2. Drugs

Methamphetamine hydrochloride (METH; Dainippon Sumitomo Pharma Co Ltd, Osaka, Japan), was dissolved in 0.9% physiological saline. Silibinin was purchased from Panjin Green Biological Development Co., Ltd (Panjin, China) and suspended in 0.3% carboxymethyl cellulose (CMC). Mice were divided into five groups: (I) Saline/CMC, (II) METH/CMC, (III) METH/100 mg/kg silibinin, (IV) METH/200 mg/kg silibinin, (V) saline/200 mg/kg silibinin. METH (1 mg/kg, s.c.) or saline were administered to mice in combination with silibinin (100 or 200 mg/kg, p.o.) once daily for 7 days. The doses were selected based on previous report that at the dose of 200 mg/kg silibinin has significant effect on amyloid-β-induced learning and memory impairments in mice [20,21] in addition to potential effects on monoamine transmission in the brain [29]. All drugs were administered in a volume of 0.1 ml/10 g body weight. NORT was performed on day 15–19 after the first administration of METH and silibinin (Fig. 1). Ten mice per group were randomly selected and sacrificed 1 day after the test to measure the amounts of dopamine, serotonin and their metabolites (Fig. 1).

2.3. Novel object recognition test (NORT)

NORT was performed according to a previous report [20]. The task consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box (W30 × L30 × H30 cm) with 10 min of exploration in the absence of objects for 3 days (days 15–17). In the training session, two objects (e.g. object A: wooden block; object B: golf ball) were placed in the middle of the box. Each mouse was then placed midway at the front of the box and total time spent exploring the two objects was recorded for 10 min (day 18). Exploratory behavior was defined as directing the nose to the object at a distance of less than 2 cm and/or touching it with the nose. In the retention session, the mouse was placed back into the same box 24 h after the training session, in which one (e.g. object A) of the familiar objects used during training was replaced with a novel object C. The animal was then allowed to explore freely for 5 min and the time spent exploring each object was recorded (day 19). Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. Therefore, inherent object preferences might be equally distributed among the treatment groups. Preference index was defined as a ratio of the amount of time spent exploring any one of the two objects over the total time spent exploring both objects in the training session. Recognition index, a ratio of the amount of time spent exploring a novel object over the total time spent exploring both objects in the retention session, was used to measure cognitive function.

2.4. Dopamine, serotonin and their metabolites

Usually, we use less than 10 rodents to do monoamines analysis, whereas behavioral studies need more animals [47]. Ten mice per group were randomly selected 1 day after NORT and sacrificed to measure the amounts of dopamine, serotonin and their metabolites. Brains were rapidly removed and the prefrontal cortex and hippocampus were dissected out on an ice-cold plate. Each tissue sample was quickly frozen with dry ice and stored in a deep freezer at −80 °C until assayed. The amounts of dopamine (DA), serotonin (5-HT) and their metabolites (3,4-dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA; and 5-hydroxyindoleacetic acid, 5-HIAA) were determined using a HPLC system with an electrochemical detector (HTEC-500, Eicom Co. Ltd., Kyoto, Japan). Briefly, each frozen tissue sample was weighed, and then homogenized with an ultrasonic processor (Heat Systems Inc., New York, USA) in 350 μl of 0.2 mol/l perchloric acid containing isoproterenol (internal standard). The homogenate was placed in ice for 30 min and then centrifuged at 20,000 × *g* for 15 min at 4 °C. The supernatant was mixed with 1 mol/l sodium acetate to adjust the pH to 3 and then injected into a liquid chromatography system equipped with a reversed-phase ODS-column (Eicompak MA-5 ODS, 4.6 mm × 150 mm, Eicom). The column temperature was maintained at 25 °C and the detector potential was set at 500 mV. The mobile phase consists of 0.1 mol/l citric acid and 0.1 mol/l sodium acetate, pH 3.9, containing 14% methanol, 160 mg/l sodium-l-octanesulfonate and 5 mg/l EDTA; the flow rate was 0.5 ml/min.

2.5. Statistical analysis

Results are expressed as the mean ± SEM. Statistical differences among the experimental groups were tested using the one-way or two-way analysis of variance (ANOVA) for behavioral tests and monoamines assay and Tukey's post hoc test was employed for multiple comparisons. Pearson's correlation analysis was used to identify relationships between memory performance and dopamine or serotonin levels in the prefrontal cortex and hippocampus. *p*-values less than 0.05 were considered significant.

3. Results

3.1. Effect of silibinin on recognition memory impairment induced by METH in NORT

In the training session, the mice spent similar time in the exploration of each object (e.g. object A: wooden block, object B: golf ball; $F_{Object\ (1,\ 146)} = 0.002$, $p = 0.996$; $F_{Group\ (4,\ 146)} = 0.499$, $p = 0.737$; $F_{Object \times Group\ (4,\ 146)} = 0.199$, $p = 0.938$, Supplementary Fig. 1) and showed a similar preference to them ($F_{(4,\ 72)} = 1.165$, $p = 0.334$, Fig. 2A), suggesting that there was no biased exploratory preference in the groups and the mice did not show any inherent preference for each object. In addition, the total time spent in the exploration of two objects ($F_{(4,\ 72)} = 0.274$, $p = 0.894$, Fig. 2B) in the training session did not differ among groups.

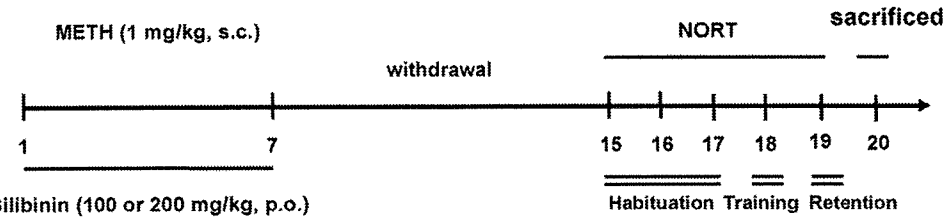

Fig. 1. Experiment schedule.

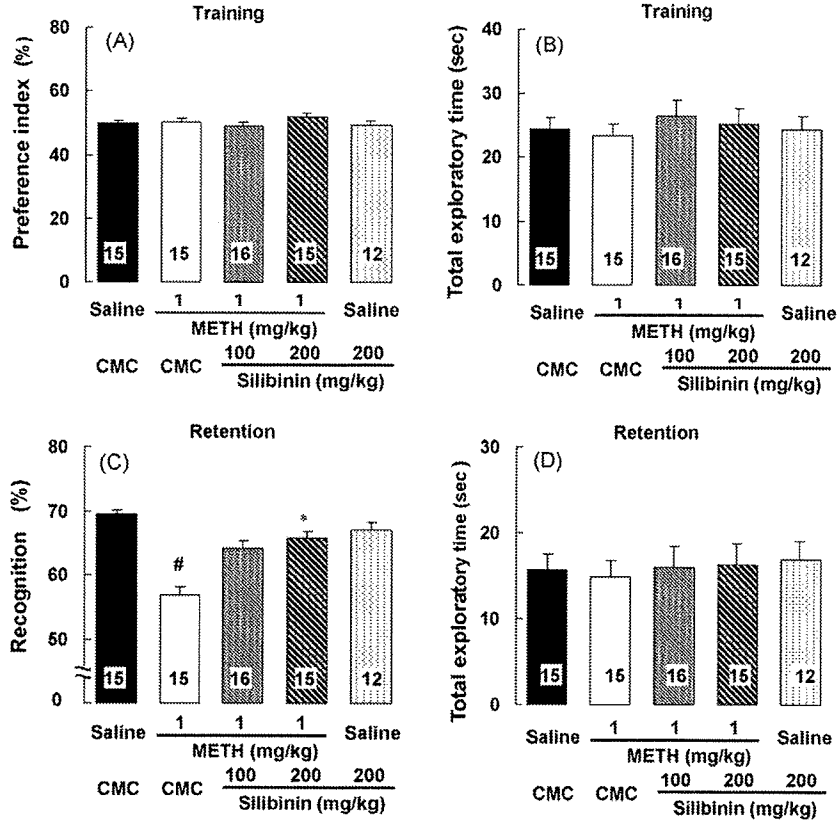

Fig. 2. Effects of silibinin on recognition memory impairments induced by repeated methamphetamine (METH) treatment in the novel object recognition test. Preference index (A) and total exploratory time (B) in the training session; recognition index (C) and total exploratory time (D) in the retention session. Data were expressed as the mean ± SEM. The numbers of mice are shown in the columns. #$p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice; *$p < 0.05$ vs CMC + METH-treated mice.

In the retention session, the level of exploratory preference for a novel object in the METH-injected mice was significantly smaller than that in the saline-injected mice ($p < 0.05$, Fig. 2C). Silibinin attenuated the memory impairment in METH-injected mice, with a significant change at a dose of 200 mg/kg ($F_{(4,72)} = 4.929$, $p < 0.05$, Fig. 2C). Silibinin itself did not affect the level of exploratory preference for the objects and the total exploration time in either the training or retention session in saline-injected mice (Fig. 2A–D).

3.2. *Effect of silibinin on the levels of dopamine, serotonin and their metabolites in the prefrontal cortex and hippocampus*

To clarify the mechanism that silibinin (200 mg/kg) prevents METH-induced learning and memory impairments, we measured the tissue contents of dopamine, serotonin and their metabolites in the prefrontal cortex and hippocampus. We found that repeated METH treatment caused decreases in the dopamine content of the prefrontal cortex (Fig. 3A) and serotonin content of the hippocampus (Fig. 4B). Treatment with silibinin (200 mg/kg) significantly attenuated the decreases of dopamine content of the prefrontal cortex and serotonin content of the hippocampus ($F_{(3,39)} = 8.324$, $p < 0.01$ for dopamine content of the prefrontal cortex, Fig. 3A; $F_{(3,39)} = 6.408$, $p < 0.01$, for serotonin content of the hippocampus, Fig. 4B) induced by repeated METH treatment but did not have a significant effect on serotonin content of the prefrontal cortex or dopamine content of the hippocampus of METH-injected mice ($F_{(3,39)} = 1.947$, $p = 0.140$ for serotonin content of the prefrontal cortex, Fig. 3B; $F_{(3,39)} = 1.086$, $p = 0.367$, for dopamine contents in the hippocampus, Fig. 4A). Silibinin itself did not affect the levels of dopamine and serotonin or their metabolites in the prefrontal cortex and hippocampus of saline-injected mice (prefrontal cortex: $p = 0.848$ for dopamine contents, $p = 0.947$ for DOPAC, $p = 0.727$ for HVA, $p = 0.976$ for serotonin, $p = 0.998$ for 5-HIAA; hippocampus: $p = 0.999$ for dopamine, $p = 0.992$ for DOPAC, $p = 0.998$ for HVA, $p = 0.994$ for serotonin, and $p = 0.998$ for 5-HIAA, in Table 1A). We also found a correlation between the recognition value and dopamine or serotonin contents of the prefrontal cortex and hippocampus ($p < 0.05$ for the dopamine content of the prefrontal cortex, Fig. 3C; $p < 0.05$ for the serotonin content of the prefrontal cortex, Fig. 3D; $p < 0.05$ for the dopamine content of the hippocampus, Fig. 4C; $p < 0.05$ for the serotonin content of the hippocampus, Fig. 3D).

As shown in Table 1B, silibinin significantly attenuated the increase in the of HVA/DA ratio and DOPAC + HVA/DA ratio in the prefrontal cortex (HVA/DA: $F_{(3,39)} = 6.717$, $p < 0.001$; DOPAC + HVA/DA: $F_{(3,39)} = 6.824$, $p < 0.001$) and the 5-HIAA/5-HT ratio in the hippocampus ($F_{(3,39)} = 6.317$, $p < 0.001$). However, there was no significant difference in the DOPAC/DA or 5-HIAA/5-HT ratio in the prefrontal cortex (DOPAC/DA: $F_{(3,39)} = 1.859$, $p = 0.154$; 5-HIAA/5-HT: $F_{(3,39)} = 2.407$, $p = 0.083$) and the DOPAC/DA, HVA/DA or DOPAC + HVA/DA ratio in the hippocampus (DOPAC/DA: $F_{(3,39)} = 0.335$, $p = 0.800$; HVA/DA: $F_{(3,39)} = 1.357$, $p = 0.274$; DOPAC + HVA/DA: $F_{(3,39)} = 0.675$, $p = 0.573$) among groups.

4. Discussion

The object recognition task is based on the spontaneous behavior of rodents to explore a novel object. It has been proposed that this task has a close analogy with recognition tests that are widely used in humans to test memory and to characterize amnesic syndromes by providing an accurate index of the overall severity of declarative memory impairment [10,33]. Several studies revealed that this task is dependent on the prefrontal cortex [15,26,27] and hippocampus [5]. In the present study, repeated METH treatment

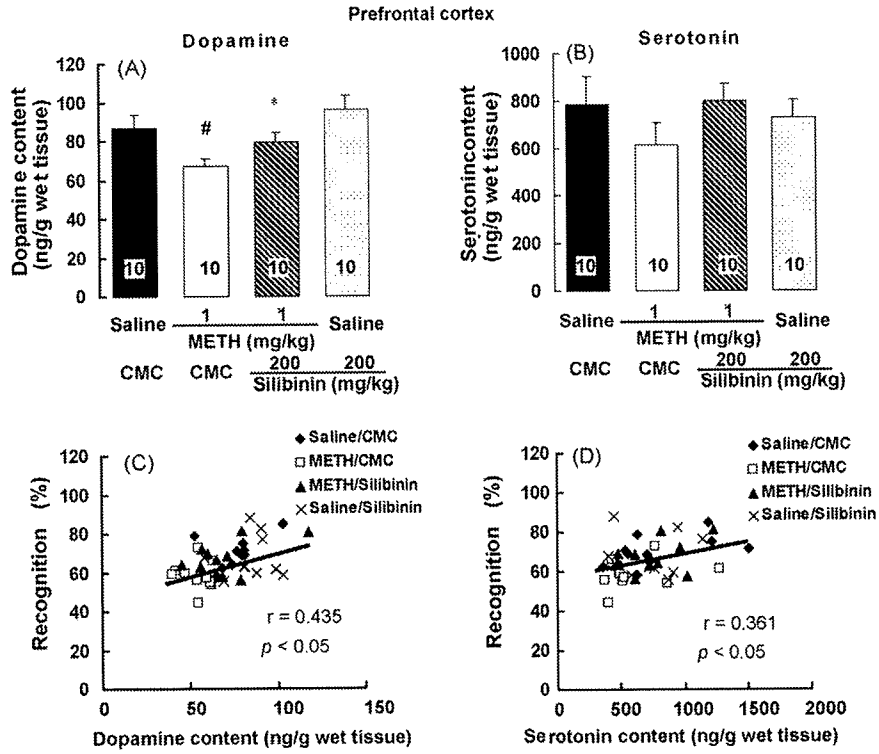

Fig. 3. Effects of silibinin on the decrease in dopamine and serotonin contents in the prefrontal cortex induced by repeated methamphetamine (METH) treatment. (A and B) The levels of dopamine (A) and serotonin (B); (C and D) the correlation of memory performance with dopamine (C) and serotonin (D) levels. Data are expressed as the mean ± SEM. The numbers of mice are shown in the columns. #$p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice, *$p < 0.05$ vs CMC + METH-treated mice.

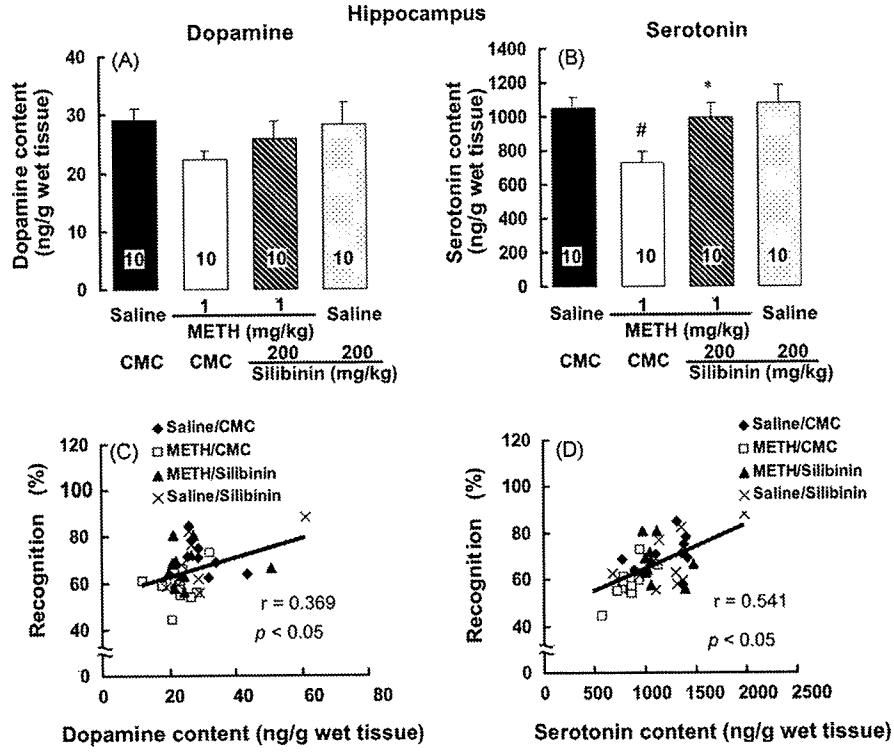

Fig. 4. Effects of silibinin on the decrease in dopamine and serotonin contents in the hippocampus induced by repeated methamphetamine (METH) treatment. (A and B) The levels of dopamine (A) and serotonin (B); (C and D) the correlation of memory performance with dopamine (C) and serotonin (D) levels. Data are expressed as the mean ± SEM. The numbers of mice are shown in the columns. #$p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice, *$p < 0.05$ vs CMC + METH-treated mice.

Table 1
Effect of silibinin on the levels of dopamine, serotonin and their metabolites (A) and turnover of dopamine and serotonin (B) in the prefrontal cortex and hippocampus.

Region	Treatment	DA	DOPAC	HVA	5-HT	5-HIAA
(A)						
Prefrontal cortex	Saline/CMC	86.58 ± 6.988	54.83 ± 4.867	88.86 ± 5.343	780.3 ± 121.7	227.4 ± 14.76
	METH/CMC	67.05 ± 3.762#	55.48 ± 9.707	101.1 ± 5.402	615.1 ± 88.85	257.7 ± 12.85
	METH/silibinin	79.59 ± 4.542*	51.46 ± 7.508	83.31 ± 6.196	798.3 ± 70.57	253.2 ± 16.80
	Saline/silibinin	96.10 ± 7.082	60.74 ± 7.751	97.38 ± 6.143	727.8 ± 78.29	231.1 ± 14.76
Hippocampus	Saline/CMC	28.98 ± 2.071	35.13 ± 4.362	92.25 ± 5.606	1050 ± 65.58	507.5 ± 43.11
	METH/CMC	22.28 ± 1.504	30.17 ± 3.417	79.43 ± 8.837	729.7 ± 69.93#	500.7 ± 30.07
	METH/silibinin	25.85 ± 3.051	35.15 ± 2.434	64.08 ± 4.005	991.3 ± 85.40*	391.7 ± 40.89
	Saline/silibinin	28.22 ± 3.810	33.71 ± 3.713	78.29 ± 3.966	1079 ± 108.5	498.2 ± 40.31

Region	Treatment	DOPAC/DA	HVA/DA	DOPAC + HVA/DA	5-HIAA/5-HT
(B)					
Prefrontal cortex	Saline/CMC	0.638 ± 0.047	1.074 ± 0.090	1.713 ± 0.097	0.352 ± 0.047
	METH/CMC	0.957 ± 0.190	1.716 ± 0.105#	2.673 ± 0.226#	0.484 ± 0.061
	METH/silibinin	0.662 ± 0.086	1.156 ± 0.148*	1.818 ± 0.190*	0.334 ± 0.026
	Saline/silibinin	0.645 ± 0.073	1.079 ± 0.123	1.724 ± 0.169	0.350 ± 0.036
Hippocampus	Saline/CMC	1.242 ± 0.175	3.259 ± 0.192	4.502 ± 0.296	0.493 ± 0.044
	METH/CMC	1.431 ± 0.216	3.564 ± 0.273	4.995 ± 0.336	0.715 ± 0.059#
	METH/silibinin	1.452 ± 0.139	2.618 ± 0.199	4.070 ± 0.328	0.401 ± 0.038*
	Saline/silibinin	1.264 ± 0.152	3.057 ± 0.300	4.322 ± 0.383	0.495 ± 0.065

Data are expressed as ng/g of wet weight tissue (A) or a ratio (B) as the mean ± SEM for 10 mice from each group.
# $p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice.
* $p < 0.05$ vs CMC + methamphetamine (METH)-treated mice.

resulted in memory impairment in NORT in mice, which is consistent with our previous reports [15,25–27]. Silibinin at 200 mg/kg attenuated the recognition memory impairments induced by METH treatment. Since silibinin had no effect on total time spent exploring objects in NORT, it is unlikely that the protective effect of silibinin is due to changes in motivation or sensorimotor function.

The dopaminergic system exhibits modulatory effects on many cognitive functions, including memory, attention, task switching, and response inhibition [8]. Attention-associated recognition memory (latent learning) was impaired by dopamine reuptake inhibitors, and recovered by a dopamine receptor antagonist [11]. It has been confirmed that disturbances of dopamine transmission play an important role in METH-induced memory deficits. For example, the dopamine D_1 receptor antagonist SCH23390 blocks novelty-induced place preference [4]. Repeated METH treatment disrupts the novelty-induced activation of dopamine D_1 receptors as well as the downstream signal, ERK1/2, in the prefrontal cortex, which is associated with memory deficits after withdrawal [15,27]. This memory impairment is improved by chronic treatment with clozapine [15], and another study has shown that chronic treatment with clozapine increases basal concentration of dopamine in the prefrontal cortex [48]. In the present study, we found that silibinin attenuated the loss of dopamine content in the prefrontal cortex and the memory deficits correlated with the degree of the decrease in dopamine in METH-treated mice. Thus, it is likely that dopaminergic hypofunction contributes to recognition memory deficits caused by repeated METH treatment and silibinin attenuates the memory deficits partly by ameliorating the dopaminergic hypofunction.

Serotonergic systems also play a critical role in cognitive function [7,24,31,34]. Serotonergic terminal deficits have been observed in METH abusers, as evidenced by a loss of serotonin transport and the depletion of serotonin [17]. Serotonin deficiencies have been implicated in the cognitive impairments associated with the abuse of 3,4-methylene-dioxymethamphetamine, a derivative of METH [44]. METH-dependent subjects show cognitive patterns that are similar to those of subjects who have been on tryptophan-depleted diets that cause a low level of serotonin in the brain [35]. In the present study, silibinin attenuated the decrease in the serotonin content of the hippocampus induced by METH. Furthermore, we observed a correlation between cognitive deficits and the degree of decrease in serotonin. These results suggest inhibition of the loss of serotonin to be involved in the protective effect of silibinin on METH-induced cognitive impairment.

It has been suggested that the prefrontal cortex plays a role in object recognition in human [2]. In rodents, the dopamine D_1 receptors in the prefrontal cortex are necessary for the protein synthesis-dependent long-term retention of recognition memory [27]. On the other hand, the hippocampus has shown a greater response to recollection, a component of recognition [13]. In this study, METH treatment produced a more serious decrease in serotonin content (−30.5% vs control) in the hippocampus than the decrease of dopamine contents (−22.1% vs control) in the prefrontal cortex. It is likely that the damage of serotonergic system contributes more to the memory impairment. However, a report has confirmed that damage of either dopaminergic or serotonergic system alone is insufficient to produce the impairments of novel object recognition memory following METH treatment [3]. In addition, it has been reported that both the hippocampus and prefrontal cortex are implicated in a visual object recognition circuit [36]. Therefore, it is possible that both of the two impairments contribute to a disruption of the visual object recognition circuit, which undergoes the recognition deficit induced by METH.

Although the exact mechanisms by which silibinin prevents the METH-induced decreases in dopamine and serotonin remain to be determined, there are several possible explanations. First, it is known that MAO catalyzes the oxidative deamination of monoamines, resulting in decreased levels of monoamines such as dopamine and serotonin. Inhibitors of MAO effectively blocked METH-induced behavioral changes in rodents [16]. An *in vitro* study demonstrated that silibinin inhibits the activity of MAO [23]. Here, silibinin attenuated the increases in the ratios of metabolites of dopamine and serotonin to those in METH-treated mice, suggesting that the increase in the metabolism of dopamine and serotonin induced by METH was blocked by silibinin. Therefore, it is possible that silibinin prevents METH-induced decreases in dopamine and serotonin by inhibiting the activation of MAO.

Second, peroxynitrite ($ONOO^-$) has been implicated as a causative factor in the toxicity resulting from exposure to METH [12]. $ONOO^-$ is a potent oxidant that can modify proteins and affect their functions [30]. It has been shown that peroxynitrite not only inactivates dopamine or serotonin transporters [6,32], but also inhibits the activities of tyrosine hydroxylase and tryptophan hydroxylase [1,17,18]. Since our and other groups have reported that silibinin has strong free radical-scavenging activity [20,21,43], a decrease in peroxynitrite levels caused by silibinin may recover in part the levels of dopamine and serotonin in the brain.

Third, it has been reported that silibinin has anti-inflammatory properties. For example, silymarin shows neuroprotective effects against lipopolysaccharide-induced neurotoxicity and microglial activation [45], which initiates a pro-inflammatory cascade that results in the release of potentially inflammatory cytokines. Microglial activation is an early response to METH treatment [19,42], and is observed in the midbrain, striatum, thalamus, and orbitofrontal and insular cortices of human abusers [37]. METH-induced impairment of recognition memory was ameliorated by minocycline, an inhibitor of microglial activation [25]. Thus, it is possible that silibinin attenuates the impairment of METH-induced cognitive dysfunction in part by inhibiting the activation of microglia.

In addition, one issue should be paid attention. Five-day repeated treatment with silymarin produces an increase in serotonin levels in the cortex, but no change of dopamine, in the normal mice [29]. Consistent with this report, silibinin did not affect dopamine contents in the non-METH-treated mice, whereas inconsistent effect was observed in serotonin contents, which may be due to the different experiment schedule. It suggests that silibinin might have an acute effect on the metabolism of monoamines by itself, but the effect did not last for long time. To be noted, silibinin may have modulating effects only on abnormal conditions such as METH treatment.

In conclusion, silibinin had an ameliorating effect on METH-induced memory impairment possibly by preventing of the loss of dopamine and serotonin in the prefrontal cortex and hippocampus, respectively. Silibinin may be useful as a pharmacological tool to investigate the mechanisms of METH-induced cognitive impairments or useful for the prevention of cognitive deficits induced by METH abuse.

Acknowledgements

This work was supported by Grants-in-Aid for "Academic Frontier Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; Grants-in-Aid for Scientific Research (B) from MEXT of Japan [Grant 20390073]; Grants-in-Aid for Exploratory Research from Sprout Society for the Promotion of Science [Grant 19659017]; Grants-in-Aid for Regional Joint Research Program for Private Universities to Cover Current Expenses from MEXT; Research Science of Pharmaceutical and Medical Devices and Research on the Risk of Chemical Substances from the Ministry of Health, Labor and Welfare (MHLW) of Japan; Research on Regulatory Science of Pharmaceutical and Medical Devices from MHLW, International Research Project Supported by the Meijo Asian Research Center (MARC); Research grant from Japan China Medical Association, Japan; and Research grants from the Takeda Science Foundation and from the Nagai Foundation Tokyo.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2009.10.024.

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British Journal of Pharmacology (2009), 157, 1270–1277

0007-1188/09
www.brjpharmacol.org

別刷 No.5

RESEARCH PAPER

Silibinin prevents amyloid β peptide-induced memory impairment and oxidative stress in mice

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Background and purpose: Accumulated evidence suggests that oxidative stress is involved in amyloid β (Aβ)-induced cognitive dysfunction. Silibinin (silybin), a flavonoid derived from the herb milk thistle (*Silybum marianum*), has been shown to have antioxidative properties; however, it remains unclear whether silibinin improves Aβ-induced neurotoxicity. In the present study, we examined the effect of silibinin on the memory impairment and accumulation of oxidative stress induced by $A\beta_{25-35}$ in mice.
Experimental approach: Aggregated $A\beta_{25-35}$ (3 nmol) was intracerebroventricularly administered to mice. Treatment with silibinin (2, 20 and 200 mg·kg^{-1}, once a day, p.o.) was started immediately after the injection of $A\beta_{25-35}$. Locomotor activity was evaluated 6 days after the $A\beta_{25-35}$ treatment, and cognitive function was evaluated in a Y-maze and novel object recognition tests 6–11 days after the $A\beta_{25-35}$ treatment. The levels of lipid peroxidation (malondialdehyde) and antioxidant (glutathione) in the hippocampus were measured 7 days after the $A\beta_{25-35}$ injection.
Key results: Silibinin prevented the memory impairment induced by $A\beta_{25-35}$ in the Y-maze and novel object recognition tests. Repeated treatment with silibinin attenuated the $A\beta_{25-35}$-induced accumulation of malondialdehyde and depletion of glutathione in the hippocampus.
Conclusions and implications: Silibinin prevents memory impairment and oxidative damage induced by $A\beta_{25-35}$ and may be a potential therapeutic agent for Alzheimer's disease.
British Journal of Pharmacology (2009) **157,** 1270–1277; doi:10.1111/j.1476-5381.2009.00295.x; published online 22 June 2009

Keywords: amyloid β; silibinin; memory deficits; oxidative stress

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; CMC, carboxymethylcellulose; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); GSH, glutathione; MDA, malondialdehyde; MES buffer, 0.2 M 2-(N-morpholino)ethanesulphonic acid buffer; TBARS, thiobarbituric acid-reactive substance

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with global mental dysfunction and impairment of cognitive function (Palmer, 2002). Common pathological features of AD are senile plaques, neurofibrillary tangles and neuronal loss in the medial temporal lobe structures and cortical areas of the brain (Blennow *et al.*, 2006). The deposition of amyloid β (Aβ) peptide in neuritic plaques is the defining feature for diagnosis of AD, and the level of Aβ peptide correlates well with the extent of cognitive impairment (Naslund *et al.*, 2000). Aβ, a spontaneously aggregating peptide of 39–43 amino acids, is the primary protein component of senile plaques, a pathological hallmark of AD in the brain (Shen *et al.*, 2002). Several studies have demonstrated that oxidative stress is involved in Aβ-induced neurotoxicity and the progression of AD (Schubert *et al.*, 1995; Yankner, 1996). In particular, Aβ fragment 25–35 ($A\beta_{25-35}$) seems to be responsible for toxic and oxidative events leading to brain damage, such as oxidative stress-mediated changes in hippocampal long-term potentiation (Trubetskaya *et al.*, 2003), protein nitration, induction of inducible nitric oxide synthase (Tran *et al.*, 2001; Alkam *et al.*, 2008) and protein oxidation in fibroblasts derived from AD patients (Choi *et al.*, 2003).

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Received 19 August 2008; revised 30 January 2009; accepted 11 March 2009

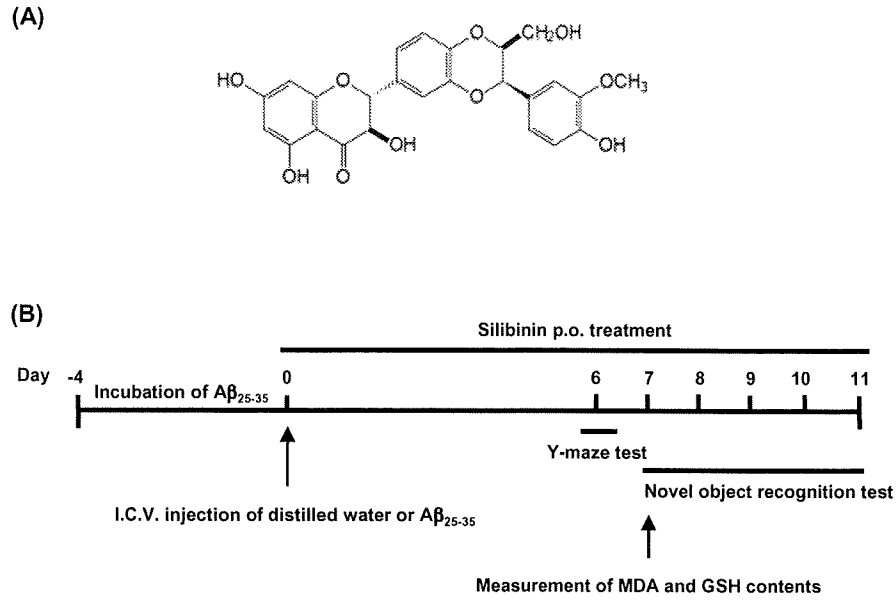

Figure 1 (A) Chemical structure of silibinin and (B) protocol used in this study.

In animal experiments, intrahippocampal or i.c.v. injections of $A\beta_{25-35}$ induced histological and biochemical changes, learning deficits (Maurice *et al.*, 1996; Meunier *et al.*, 2006; Alkam *et al.*, 2008) and dysfunction of the cholinergic system, which plays an important role in the cognitive deficits associated with aging and neurodegenerative diseases (Tran *et al.*, 2001). Thus, $A\beta_{25-35}$-injected animals are useful models for understanding the pathogenesis and progression of AD, and for evaluating new therapeutic agents for AD (Maurice *et al.*, 1996).

Several synthetic antioxidants are available, but there is a growing trend towards the use of natural products (polyphenols, flavonoids, vitamins, carotenes and lycopenes) as antioxidants. Of these, bioflavonoids are a ubiquitous group of polyphenolic substances present in most plants (Nijveldt *et al.*, 2001). Silibinin (silybin) is a flavonoid derived from the herb milk thistle (*Silybum marianum*) (Kren and Walterová, 2005; Figure 1A) and used as a hepatoprotectant in the clinical treatment of liver disease (Kren and Walterová, 2005). Silibinin seems to protect against oxidative stress as it has been reported to decrease lipid peroxidation, a sensitive marker of oxidative lipids, in liver microsomes and isolated hepatocytes (Bosisio *et al.*, 1992). Furthermore, it has been demonstrated that the antioxidative activity of silibinin is related to the scavenging of free radicals (De Groot and Rauen, 1998; Trouillas *et al.*, 2008) and activation of antioxidative defenses: increases in cellular glutathione (GSH) content (Valenzuela *et al.*, 1989) and superoxide dismutase levels (Müzes *et al.*, 1991). These findings suggest that silibinin could attenuate oxidative stress-induced brain dysfunction. In fact, there are a few reports that silymarin, a mixture of flavonoids present in milk thistle and whose main component is silibinin, has protective effects on the central nervous system against ethanol-induced brain injury (La Grange *et al.*, 1999) and lipopolysaccharide-induced neurotoxicity (Wang *et al.*, 2002). However, it remains unclear whether silibinin protects against Aβ-induced neurotoxicity.

In the present study, to confirm the usefulness of silibinin against Aβ-induced neurotoxicity, we investigated whether silibinin prevents memory impairment in an $A\beta_{25-35}$-injected animal model of AD. Moreover, we examined the protective effect of silibinin on oxidative events indicated by an increase in malondialdehyde (MDA), the end product of lipid peroxidation, and decrease in GSH, an endogenous antioxidant, in the hippocampus of $A\beta_{25-35}$-injected mice.

Methods

Animals

Male ICR mice, 5 weeks old at the beginning of experiments, were obtained from Japan SLC Inc. (Shizuoka, Japan). They were housed in plastic cages and kept in a regulated environment (23 ± 0.5°C, 50 ± 5% humidity) with a 12/12 h light/dark cycle (lights on from 08.00 h to 20.00 h). The mice received food (CE2; Clea Japan Inc., Tokyo, Japan) and water *ad libitum*. Behavioural experiments were carried out in a sound-proof and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Faculty of Pharmaceutical Sciences Meijo University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (2006). The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

I.c.v. injection

$A\beta_{25-35}$ was dissolved in distilled water at a concentration of 1 mM as a stock solution and stored at –20°C. In the present study, the concentration and dose were selected according to a previous study (Maurice *et al.*, 1996). It has been shown that

i.c.v. administration of $A\beta_{25-35}$ at the dose of 9 nmol leads to memory impairment with severe neurotoxicity in mice, and at the dose of 3 nmol produces mild brain dysfunction, whereas it fails to cause cognitive deficits at the dose of 1 nmol (Maurice *et al.*, 1996). The $A\beta_{25-35}$ (3 nmol = 3 μg) is greatly diluted by cerebrospinal fluid and interstitial fluid to a level of about 6000 $ng \cdot g^{-1}$ tissue, which is in the range of tissue levels (21.0–89.1 $ng \cdot g^{-1}$ tissue: water-soluble Aβ; 377.3–3000.0 $ng \cdot g^{-1}$ tissue: water-insoluble Aβ) found in brains of AD patients (Kuo *et al.*, 1996), when it diffuses throughout the whole brain (average weight is about 0.5 g). Moreover, a certain amount of $A\beta_{25-35}$ is cleared or moved out from brain, which further decrease its concentration. Therefore, $A\beta_{25-35}$ at the dose of 3 nmol was used in this study. The $A\beta_{25-35}$ was aggregated, or 'aged', by incubating it in distilled water at 37°C for 4 days. Aggregated $A\beta_{25-35}$ [3 $nmol \cdot (3\ \mu L)^{-1}$] or distilled water (3 μL) was injected i.c.v., as described previously (Maurice *et al.*, 1996). Briefly, a microsyringe with a specially made 28-gauge stainless steel needle, 3 mm in length, was used for microinjections. Mice were anaesthetized lightly with ether, and the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull (A, –0.22 mm; L, 1 mm from the bregma; V, 2.5 mm from the skull). $A\beta_{25-35}$ [3 $nmol \cdot (3\ \mu L)^{-1}$] or distilled water (3 μL) was delivered gradually within ~3 s. Mice exhibited normal behaviour within 1 min after injection. In preliminary experiments the injection site was confirmed by injecting Indian ink. Neither insertion of the needle nor injection of the distilled water had a significant influence on survival, behavioural responses or cognitive functions.

Drug treatment

Silibinin was suspended in a 0.3% carboxymethylcellulose solution. Mice were administered silibinin (2, 20 and 200 $mg \cdot kg^{-1}$, p.o.) or the 0.3% carboxymethylcellulose solution by oral gavage 60 min before the Y-maze test and the training session of the novel object test. All compounds were administered systemically in a volume of 0.01 $mL \cdot g^{-1}$ body weight (Figure 1B).

Behavioural procedures

Previously it has been shown that acute exposure to aged $A\beta_{25-35}$ induces apoptosis-mediated neuronal toxicity during 6 days of incubation in hippocampal cultures, and cognitive dysfunction in several learning and memory tests in mice (Maurice *et al.*, 1996). The behavioural tests were started 6 days after the $A\beta_{25-35}$ injection and were carried out sequentially according to the experimental schedule shown in Figure 1B. The present study was conducted in a blind manner.

Measurement of locomotor activity

The measurement of locomotor activity in a novel environment was carried out on day 6 after the start of silibinin administration. Spontaneous locomotor activity was measured as previously reported with a minor modification (Miyamoto *et al.*, 2001). Mice were placed individually in a transparent acrylic cage with a black frosted Plexiglas floor (45 × 26 × 40 cm) for 90 min, and locomotor activity was measured in 5 min intervals by using digital counters with infrared sensors. The system was equipped with photosensor frames in the side walls. Locomotor activity was defined as the total number of beam cuts due to horizontal movement measured by the photosensors. The acrylic cage was wiped with a paper towel between uses and kept clean.

Spontaneous alternation in a Y-maze test

This behavioural test was performed 6 days after the $A\beta_{25-35}$ injection, according to a previous study (Mouri *et al.*, 2007), with minor modifications. The maze was made of black painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at the centre of the apparatus and allowed to move freely through the maze for 8 min. The series of arm entries was recorded visually. Alternation was defined as successive entry into the three arms on overlapping triplet sets. Alternation behaviour (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two) multiplied by 100.

Novel object recognition test

The novel object recognition test was performed 7–11 days after the $A\beta_{25-35}$ injection, according to a previous study (Mouri *et al.*, 2007), with minor modifications. The task consisted of three sessions: habituation, training and retention. Each mouse was individually habituated to the box (30 × 30 × 30 high cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed in the middle of the box. A mouse was then placed midway at the front of the box, and total time spent exploring the two objects was recorded for 10 min. During the retention session, animals were placed back into the same box 24 h after the training session, in which one of the familiar objects used during training was replaced with a novel object. The animals were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function.

Determination of lipid peroxidation level

Malondialdehyde was measured with a thiobarbituric acid-reactive substance (TBARS) assay kit. Briefly, the hippocampus was isolated and homogenized in cooled RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS,1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail). Tissue homogenate from the

hippocampus was incubated with 8.1% sodium dodecylsulphate for 10 min followed by the addition of 20% acetic acid (pH 3.5). The reaction mixture was incubated with 0.6% thiobarbituric acid at 99°C for 1 h. After being cooled, the mixture was centrifuged at 1600× *g* for 10 min at 4°C. The absorbance was measured by using a plate reader (Bio-Rad 550) at 540 nm. MDA content was expressed as μmol·g^{-1} tissue.

Determination of glutathione content

A GSH assay kit was used for detecting GSH in the hippocampus. Briefly, the hippocampal tissues were isolated and homogenized in cold buffer (0.05 M phosphate, pH 6–7, containing 1 mM EDTA). The homogenates were centrifuged at 10 000× *g* for 15 min at 4°C, and the supernatant was subsequently incubated with MES buffer (0.2 M 2-(N-morpholino)ethanesulphonic acid buffer, 0.1 M phosphate and 2 mM EDTA, pH 6.0), cofactor mixture, enzyme mixture and DTNB [5,5′-dithiobis (2-nitrobenzoic acid)] in the dark on the orbital shaker at room temperature for 30 min. Absorbance at 405 nm was measured by using a plate reader (Bio-Rad 550), and GSH content was calculated as μmol·g^{-1} tissue.

Statistical analysis

All data are expressed as the means ± s.e.mean. Statistical differences among the experimental groups were tested by using a one- or two-way analysis of variance (ANOVA) for behavioural tests, and Tukey's *post hoc* test was employed for multiple comparisons. *P*-values less than 0.05 were accepted as significant.

Materials

$A\beta_{25-35}$ was purchased from Bachem (Bubendorf, Switzerland); silibinin from Panjin Green Biological Development Co., Ltd. (Panjin, China); digital counters with infrared sensors Scanet SV-10 (Melquest Ltd., Toyama, Japan); TBARS assay kit and GSH assay kit, Cayman (Ann Arbor, MI, USA).

Results

Effect of silibinin on locomotor activity

The counts of spontaneous locomotor activity of mice were measured on day 6 after $A\beta_{25-35}$ injection. There were no significant differences in the time course of locomotor activity [$F_{group}(5, 756) = 1.556$, $P = 0.170$; $F_{time}(17, 756) = 108.92$, $P < 0.01$; $F_{group \times time}(85, 756) = 0.347$, $P = 0.999$] and total locomotor activity [$F(5, 47) = 0.202$, $P = 0.96$] among the groups (Figure 2A).

Effect of silibinin on short-term memory impairment induced by $A\beta_{25-35}$ in Y-maze test

We evaluated the effects of silibinin on impairment of short-term memory 6 days after the $A\beta_{25-35}$ injection in a Y-maze test. $A\beta_{25-35}$-injected mice showed significantly reduced spontaneous alternation behaviour compared with vehicle-injected mice ($P < 0.05$, Figure 2B). Treatment with silibinin (2, 20 and 200 mg·kg^{-1}) dose-dependently attenuated the impairment of spontaneous alternation behaviour in $A\beta_{25-35}$ injected mice [$F(5, 66) = 6.325$, $P < 0.05$, Figure 3]. Silibinin doses of 20 and 200 mg·kg^{-1} significantly prevented $A\beta_{25-35}$-induced memory impairment ($P < 0.05$, Figure 3). There was no significant difference in the number of arm entries among the groups [$F(4, 66) = 0.463$, $P = 0.763$: data not shown]. Silibinin did not affect spontaneous alternation (Figure 2B) and the number of arm entries (data not shown) in mice injected with distilled water.

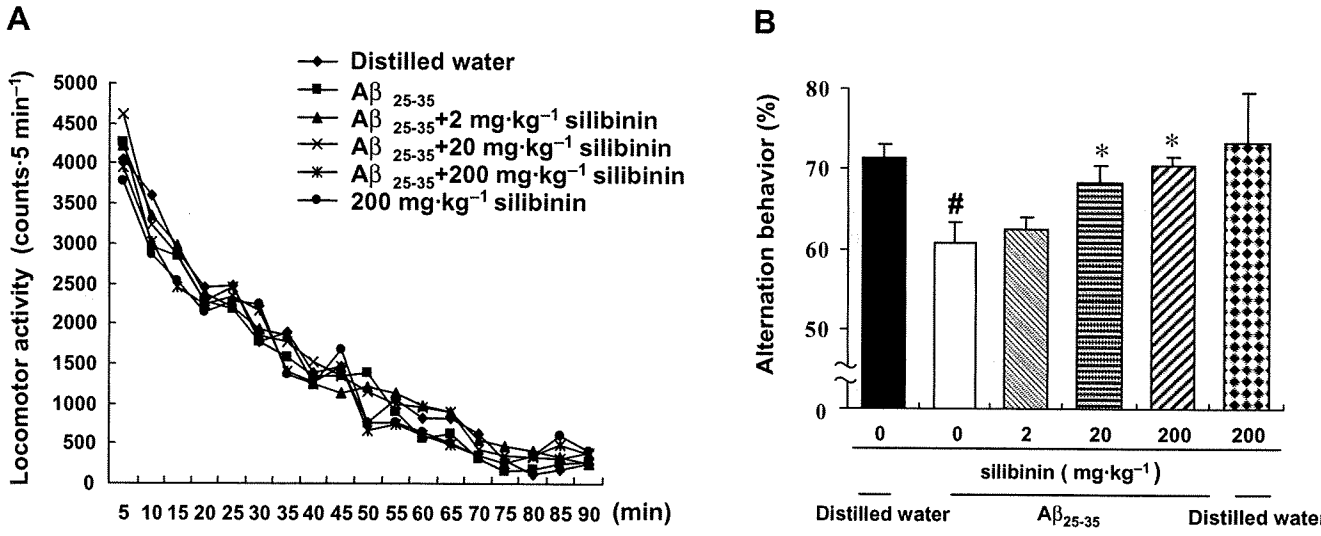

Figure 2 Effects of silibinin on locomotor activity (A) and impairment of short-term memory in Y-maze test (B) in amyloid β ($A\beta)_{25-35}$-injected mice. Results are expressed as the means ± s.e.mean (A: $n = 8$, B: $n = 13–15$) and were analysed by a one or two-way ANOVA, followed by Tukey's test for multiple comparisons. #$P < 0.05$ versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *$P < 0.05$ versus CMC-treated, $A\beta_{25-35}$-injected mice.

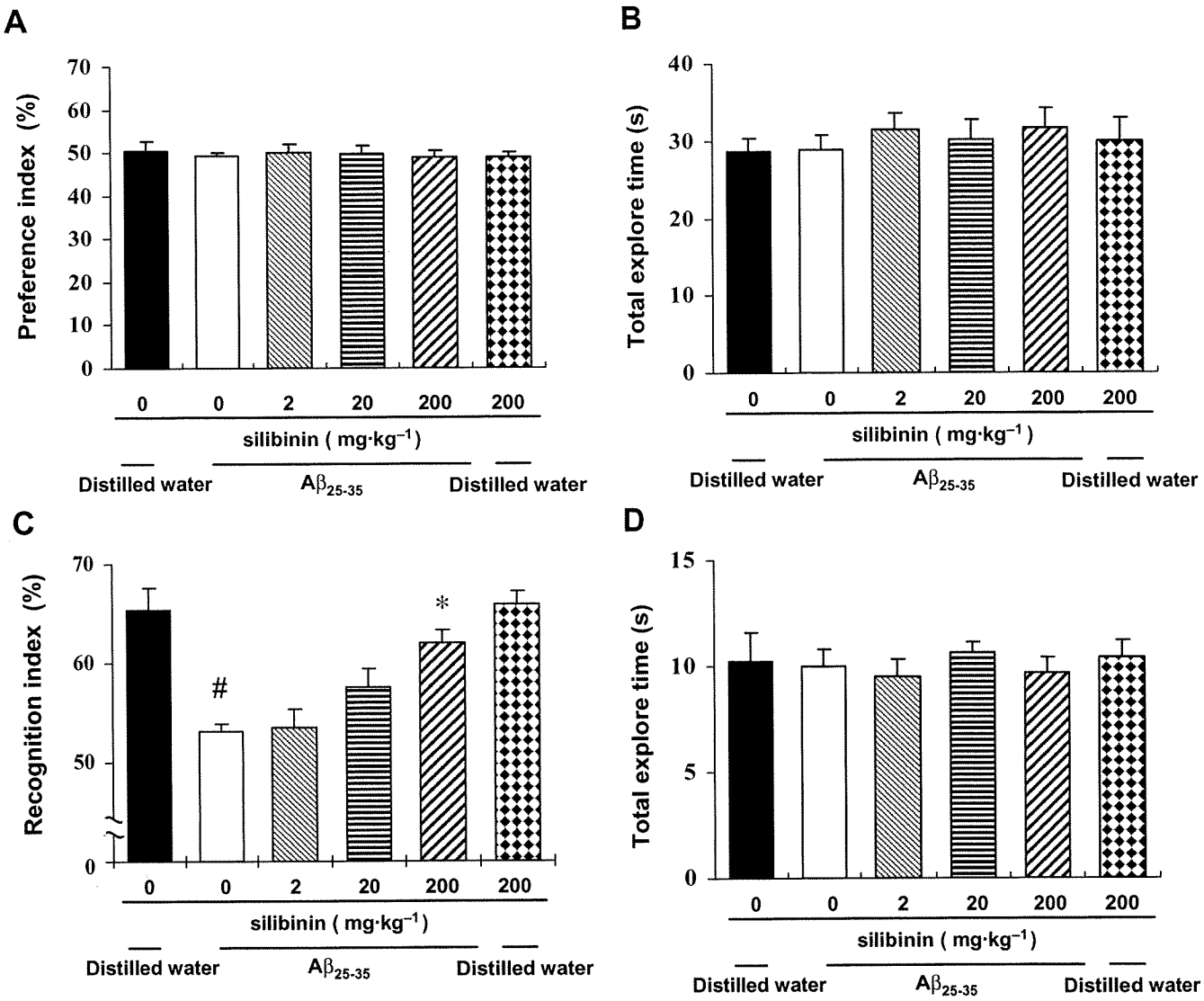

Figure 3 Effect of silibinin on recognition memory impairments induced by amyloid β $(Aβ)_{25-35}$ in the novel object recognition test. (A) Preference index in training session. (B) Total exploration time in training session. (C) Recognition index in retention session. (D) Total exploration time in retention session. Results are expressed as the means ± s.e.mean (n = 13–15) and analysed by a one-way ANOVA, followed by Tukey's test for multiple comparisons. #$P < 0.05$ versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *$P<0.05$ versus CMC-treated, $Aβ_{25-35}$-injected mice.

Effect of silibinin on recognition memory impairment induced by $Aβ_{25-35}$ in the novel object recognition test

We evaluated the effect of silibinin on impairment of visual recognition memory 7 days after the $Aβ_{25-35}$ injection in a novel object recognition test. In the training session, mice injected with $Aβ_{25-35}$ or distilled water spent equal amounts of time exploring either of the two objects (Figure 3A), showing there was no biased exploratory preference in either group of animals. In addition, total time spent in the exploration of objects in the training and retention sessions did not differ between the groups injected with $Aβ_{25-35}$ or distilled water (Figure 3B and D).

When the retention session was performed 24 h after the training session, the level of exploratory preference for the novel objects in the $Aβ_{25-35}$-injected mice was significantly decreased compared with that in the distilled water-injected mice ($P < 0.05$, Figure 3C). Treatment with silibinin (2, 20 and 200 mg·kg^{-1}) dose-dependently and significantly reversed the decreased level of exploration preference in the retention session in $Aβ_{25-35}$-injected mice [$F(5, 66) = 16.195$, $P < 0.05$, Figure 3C]. Tukey's *post hoc* analysis revealed that silibinin at 200 mg·kg^{-1} significantly prevented the memory impairment in $Aβ_{25-35}$-injected mice ($P < 0.05$, Figure 3C). Treatment with silibinin did not affect either the level of exploratory preference for the objects or the total exploration time in either the training or retention sessions for the mice injected with $Aβ_{25-35}$ or those injected with distilled water (Figure 3A–D).

Effect of silibinin on the MDA and GSH levels in the hippocampus of $Aβ_{25-35}$-injected mice

To determine whether lipid peroxidation is involved in the ameliorating effect of silibinin in $Aβ_{25-35}$-injected mice, we

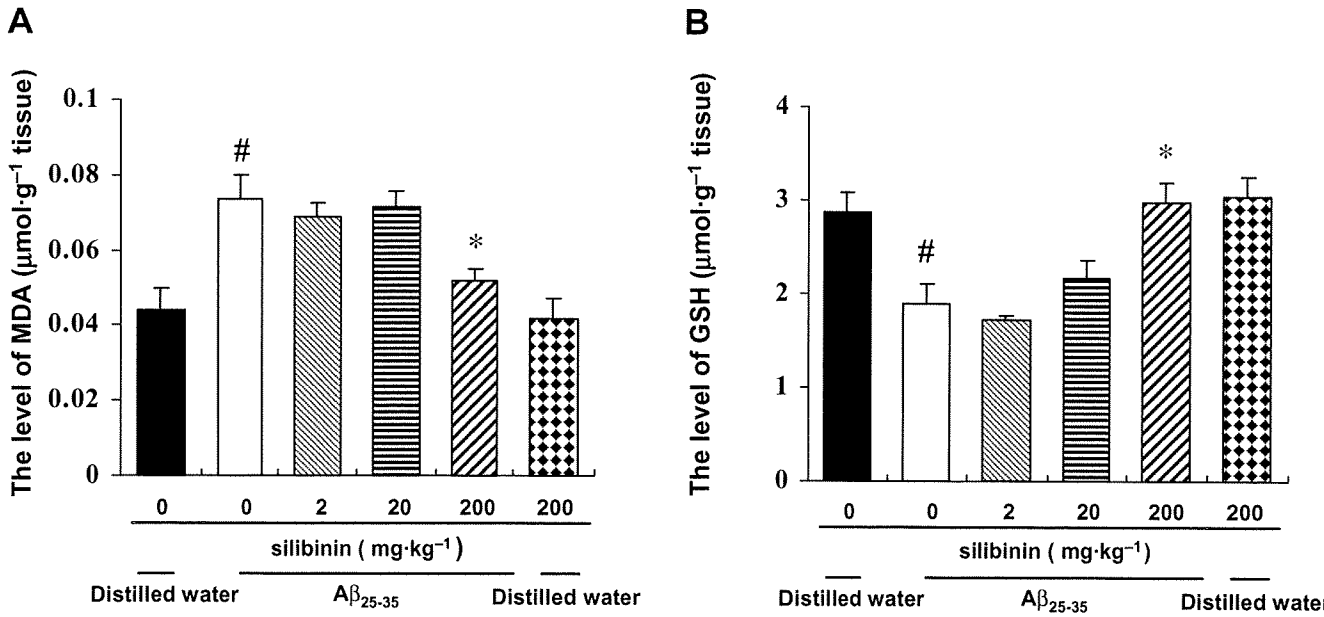

Figure 4 Effects of silibinin on amyloid β $(Aβ)_{25-35}$-induced increase in malondialdehyde (MDA) levels and decrease in glutathione (GSH) levels in the hippocampus. (A) MDA levels. (B) GSH levels. Results are expressed as the means ± s.e.mean ($n = 6–7$) and analysed by a one-way ANOVA, followed by Tukey's test for multiple comparisons. #$P < 0.05$ versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *$P < 0.05$ versus CMC-treated, $Aβ_{25-35}$-injected mice.

examined the effect of silibinin on the levels of MDA in the hippocampus 7 days after the $Aβ_{25-35}$ injection. A significant increase in the level of MDA was observed in the hippocampus of $Aβ_{25-35}$-injected mice as compared with levels in the control group [$F(5, 34) = 8.281$, $P < 0.05$, Figure 4A]. Treatment with silibinin (200 mg·kg^{-1}) significantly prevented the increase of MDA levels in the hippocampus of $Aβ_{25-35}$-treated mice ($P < 0.05$, Figure 4A). In contrast, $Aβ_{25-35}$-injected mice showed a significant decrease of GSH levels in the hippocampus compared with the control mice. Treatment with silibinin (2, 20 and 200 mg·kg^{-1}) dose-dependently prevented the decrease in GSH levels in the hippocampus of $Aβ_{25-35}$-injected mice [$F(5, 34) = 9.351$, $P < 0.05$, Figure 4B]. Tukey's *post hoc* analysis indicated a significant effect of silibinin at the dose of 200 mg·kg^{-1} ($P < 0.05$, Figure 4B). However, silibinin did not affect levels of MDA and GSH in the hippocampus of mice injected with distilled water (Figure 4A and B).

Discussion and conclusions

In the present study, we examined the effect of silibinin on the memory impairment induced by $Aβ_{25-35}$ in mice. Silibinin prevented $Aβ_{25-35}$-induced impairment of short-term and recognition memory in the Y-maze and novel object recognition tests respectively. Furthermore, silibinin prevented the accumulation of lipid peroxide (MDA) and decrease of antioxidant (GSH) in the hippocampus after the $Aβ_{25-35}$ treatment. To our knowledge, this is the first study to show that silibinin protects against Aβ-injected neurotoxicity by regulating oxidative stress in the brain.

The accumulation of Aβ proteins is highly toxic to primary and other cell lines (Kim *et al.*, 2007; Nie *et al.*, 2008). $Aβ_{25-35}$ is most toxic Aβ fragment that has been detected in the brain of AD patients (Pike *et al.*, 1995; Kubo *et al.*, 2002; Zameer *et al.*, 2006). $Aβ_{25-35}$ is the core fragment of full-length Aβ and possesses many of the characteristics of the full-length Aβ peptide, including aggregative ability and neurotoxic properties such as learning and memory impairment, morphological alterations and cholinergic dysfunction (Pike *et al.*, 1995; Tran *et al.*, 2001; Kubo *et al.*, 2002; Alkam *et al.*, 2008). Therefore, the effect of silibinin on Aβ-induced neurotoxicity was evaluated in $Aβ_{25-35}$-injected mice. In the present study, $Aβ_{25-35}$-injected mice showed memory impairment in both the Y-maze and the novel object recognition tests. These results are consistent with our previous findings that $Aβ_{25-35}$ induces cognitive impairment in mice (Alkam *et al.*, 2007; 2008; Tsunekawa *et al.*, 2008).

Silibinin dose-dependently and significantly prevented the impairment of short-term and recognition memory induced by $Aβ_{25-35}$. It is unlikely that the protective effect of silibinin is due to changes in motivation or sensorimotor function, as various motivations are involved in these behavioural tasks, and different skills are required for better performance in each task. Actually, silibinin had no effect on locomotor activity, number of arm entries in the Y-maze test and total time spent exploring objects in the novel object test. These results suggest that silibinin attenuates cognitive impairments in the $Aβ_{25-35}$-injected mice without affecting motor function, motivation and exploratory activity.

It has been proposed that oxidative stress plays a critical role in the development of AD (Smith *et al.*, 1996). Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages membranes and generates a number of secondary products including aldehydes, such as MDA, and 4-hydroxy-2-nonenal, ketones, etc. (Slater, 1984). Analysis of AD brains demonstrates an increase in lipid peroxidation products in the amygdala, hippocampus and parahippocampal gyrus of the AD brain compared with

age-matched controls (Markesbery and Lovell, 1998). Amyloid precursor protein transgenic mice, a genetic mouse model of AD, have shown a systemic increase in lipid peroxidation compared with wild-type littermates (Pratico *et al.*, 2001). MDA is the most abundant individual aldehyde resulting from lipid peroxidation and can be considered a marker of lipid peroxidation. To confirm the effect of silibinin on Aβ-induced oxidative stress, we measured the levels of MDA in the hippocampus. $A\beta_{25-35}$ increased the levels of MDA in the hippocampus, suggesting that it caused lipid peroxidation. Treatment with silibinin prevented the accumulation of MDA induced by $A\beta_{25-35}$. Accordingly, these results suggest that the protective effect of silibinin on $A\beta_{25-35}$-induced memory impairment is related to an accumulation of oxidative stress in the hippocampus.

It has been proposed that Aβ peptide impairs the antioxidative defenses in brain, which may contribute to the pathogenesis of AD (Mattson *et al.*, 1998). GSH is one of the most abundant intracellular non-protein thiols in the central nervous system, where it plays a major antioxidative role within both neurones and non-neuronal cells. In the present study, $A\beta_{25-35}$ decreased the level of GSH in the hippocampus, consistent with reports of the depletion of GSH in the brain of AD patients (Aksenov and Markesbery, 2001). Furthermore, the $A\beta_{25-35}$-induced decrease in the level of GSH was prevented by treatment with silibinin, indicating that the protective effect of silibinin on $A\beta_{25-35}$-induced cognitive impairment involves the activation of antioxidative defenses.

Although the mechanism by which silibinin regulates $A\beta_{25-35}$-induced oxidative stress remains to be determined, there are several possible explanations. Firstly, as a polyphenolic flavonoid, silibinin has strong free radical-scavenging activity (Trouillas *et al.*, 2008). Silibinin reacts with a damaging free radical and forms a flavonoid radical, which has greater stability, and then breaks the free radical chain reaction (Weber *et al.*, 2006). It is possible that silibinin prevents oxidative damage directly by scavenging free radicals.

Secondly, it has been demonstrated that silibinin has a metal-chelating effect (Borsari *et al.*, 2001). The neurotoxicity of Aβ is mediated by $A\beta Cu^{2+}$ or $A\beta Fe^{3+}$ forming H_2O_2, which generates lipid peroxidation adducts, protein carbonyl modifications and nucleic acid adducts in various cellular compartments. Thus, metal ions mediate the oxidative stress mechanism of Aβ toxicity. Incubation with the Fe^{3+} chelator desferrioxamine has been shown to decrease the toxicity of synthetic Aβ (Rottkamp *et al.*, 2001). It is possible that silibinin attenuates the Aβ-induced oxidative stress in the hippocampus by chelating metal ions.

Thirdly, oxidative stress occurs when the antioxidative defenses are broken down by the overproduction of reactive oxygen species. Expanding the antioxidant capacity of neurones will provide a potential strategy to protect neurones from oxidative damage (Ahlemeyer *et al.*, 2001; Alcaraz-Zubeldia *et al.*, 2001). Silibinin is known to induce the expression of antioxidative enzymes (Valenzuela *et al.*, 1989;Müzes *et al.*, 1991). Therefore, it is possible that silibinin prevents oxidative damage indirectly by activating antioxidative systems including GSH biosynthesis. It remains to be determined whether silibinin affects antioxidant enzymes in the hippocampus of Aβ-injected mice.

As another possible mechanism, silibinin may directly suppress aggregation of Aβ or stability of aggregated Aβ, so affecting Aβ conformation in the brain. Polyphenols, such as silibinin, have shown to inhibit aggregation of Aβ and exhibit significant destabilizing activity on aggregated Aβ (Ono *et al.*, 2003; Porat *et al.*, 2006; Shoval *et al.*, 2007). Because aggregated $A\beta_{25-35}$ was used in this study, it is possible that silibinin may affect the degradation of aggregated Aβ. However, further studies are needed to clarify the effect of silibinin on the conformation and levels of Aβ.

The results from the present study confirm, for the first time, that silibinin could alleviate the memory deficits induced by $A\beta_{25-35}$ in mice. The effect of silibinin may be attributed to the prevention of oxidative damage in the hippocampus, measured in terms of the amount of peroxidized lipid and the level of GSH. As a therapeutic agent, silibinin is well tolerated and largely free of adverse effects and has few negative drug interactions (Jacobs *et al.*, 2002). Therefore, silibinin is a potential candidate for further preclinical study aimed at the treatment of cognitive deficits in AD.

Acknowledgements

This study was supported in part by the Academic Frontier Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Research on Risk of Chemical Substances, Health and Labour Science Research Grants supported by the Ministry of Health, Labour and Welfare and the International Research Project Supported by the Meijo Asian Research Center.

Conflict of interest

The authors state no conflict of interest.

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