been previously located was used as an index of spatial reference memory.

Prepulse Inhibition of acoustic startle response

The rats were tested for their acoustic startle responses (ASR) in a startle chamber (SR-LAB, San Diego Instruments, CA, USA). The sessions consisted of five trial types: 1) pulse alone, a 40-millisecond broadband burst; 100 milliseconds preceding the pulse, a 20-millisecond prepulse (PP) that was either 2) 4 dB (PP74), 3) 8 dB (PP78), or 4) 16 dB (PP86) over the background (70 dB), and 5) background only (no stimulus). The amount of prepulse inhibition (PPI) is expressed as the percentage decrease in the amplitude of the startle response caused by presentation of the prepulse (%PPI).

To examine the effects of clozapine on PPI deficits in irradiated rats, vehicle (0.8% acetic acids; 1 ml/kg for 3 weeks) or clozapine (5 mg/kg/day for 3 weeks) were i.p. administered into control and irradiated rats (control/vehicle = 6, control/clozapine = 6, irradiated/vehicle = 6, irradiated/clozapine = 6). After the chronic (3 weeks) administration of vehicle or clozapine, PPI of acoustic startle response was examined as described above.

Statistical analysis

Data are expressed as means±standard errors of the means (SEM). The data from two experimental groups were compared by unpaired t-test, except for PPI analysis, which was performed by a two-way (irradiation and prepulse intensity) analysis of variance (ANOVA). The level of significance was set at $p<0.05$.

Results

Three months after fractionated ionizing irradiation, the total numbers of BrdU-positive cells in both the subventricular (SVZ: **Figure 1B**) and subgranular (SGZ: **Figure 1D**) zones of irradiated rats were significantly lower than those (SVZ: **Figure 1A**, SGZ: **Figure 1C**) of control (sham-irradiated) rats (SVZ: **Figure 1E**, SGZ: **Figure 1F**). These findings are consistent with those of previous reports [9,10,12]. In contrast, the cumulative numbers of granule cells in the granule layer were not different between the two groups (**Figure 1G**).

As shown in **Figure 2A**, the nocturnal spontaneous locomotion of irradiated rats was significantly ($t = 2.34$, $df = 38.1$, $p = 0.025$) lower than that of control rats. Furthermore, locomotor activity after administration of methamphetamine (2.0 mg/kg, i.p.) to irradiated rats was significantly ($t = -2.26$, $df = 32$, $p = 0.031$) higher than that of control (sham-irradiated) rats (**Figure 2B**). HPLC analysis revealed that levels of dopamine and its major metabolite DOPAC, and dopamine turnover (DOPAC/dopamine ratio) in the frontal cortex and striatum of irradiated rats were not different from those of sham-control rats (**Figure 3**). In contrast, locomotor activity after administration of the NMDA receptor antagonist dizocilpine ((+)-MK-801, 0.03 mg/kg, i.p.) to irradiated rats was not different from that of sham-control rats (**Figure 2C**). Furthermore, levels of amino acids (glutamate, glycine, glutamine, D-serine, L-serine) related with the NMDA receptor neurotransmission in the frontal cortex, hippocampus, and striatum, and cerebellum of irradiated rats were not different from those of sham-control rats (**Figure 4**).

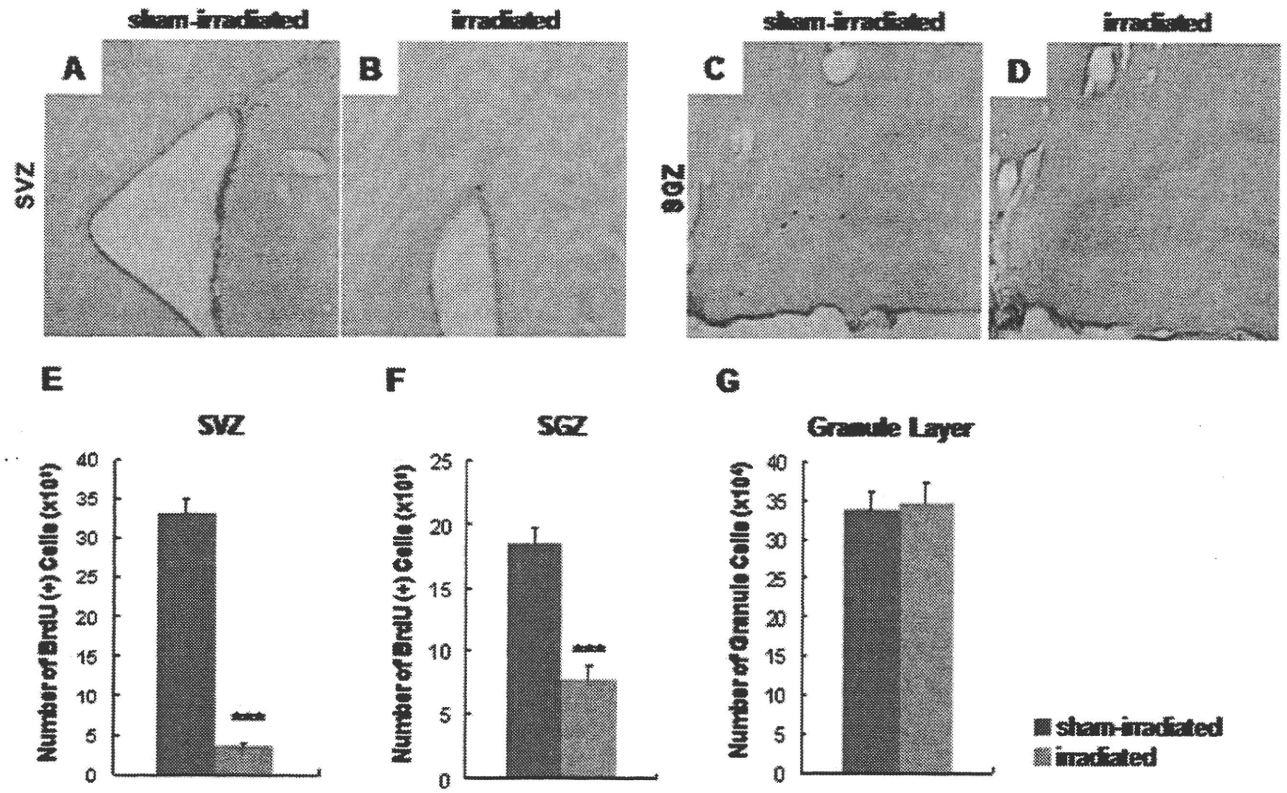

Figure 1. Decreased neurogenesis in the irradiated adult rats. The BrdU-positive cells in both SVZ (B and E) and SGZ (D and F) of the irradiated rats (n = 8) were significantly fewer than those (SVZ, A and E; SGZ, C and F) of control (sham-irradiated) rats (n = 8). Data are given as means±SEM. ***$p<0.001$ as compared with controls. (G) The total numbers of granule cells in the dentate gyrus in irradiated rats (n = 6) and control (sham-irradiated) rats (n = 6) were not different.
doi:10.1371/journal.pone.0002283.g001

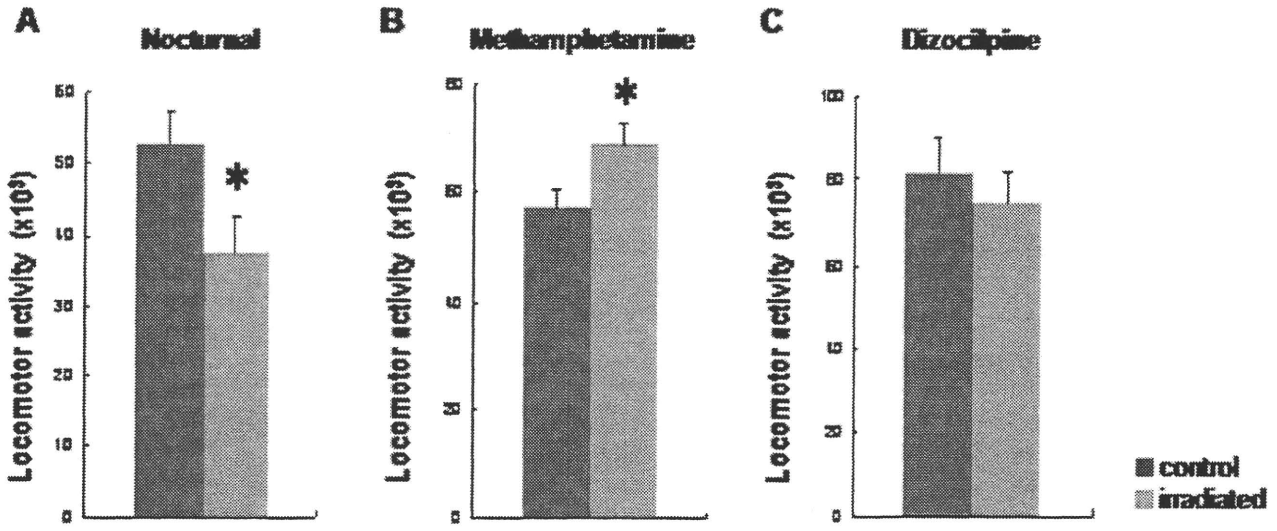

Figure 2. Spontaneous locomotion and response to methamphetamine and dizocilpine in irradiated adult rats. (A) Nocturnal spontaneous locomotion (21:00–3:00) in the irradiated rats (n = 17) was significantly lower than that of control (sham-irradiated) rats (n = 17). (B) Horizontal locomotor activity during the 120-min period after administration of the psychostimulant drug methamphetamine (2 mg/kg, i.p.) in irradiated rats (n = 17) was significantly higher than that of control rats (n = 17). (C) Horizontal locomotor activity during the 120-min period after administration of dizocilpine (0.03 mg/kg, i.p.) in the irradiated rats (n = 18) was not different from that of control rats (n = 18). Data are given as means ± SEM.
doi:10.1371/journal.pone.0002283.g002

In the sensorimotor gating test, two-way ANOVA revealed a significant effect [F (1, 42) = 47.1, $p<0.001$] of irradiation exposure on prepulse inhibition (PPI) (**Figure 5A**), while acoustic response amplitude in the two groups was not different (**Figure 5B**). PPI deficits in irradiated rats were shown at each level of prepulse intensity (72, 76, and 84 dB) (**Figure 5A**). In the social interaction test, the time spent in social behavior in the irradiated rats was significantly ($t = 3.73$, $df = 10$, $p = 0.004$) lower than that that in the control rats (**Figure 6**). In the eight-arm radial maze test, the number of working memory errors in the irradiated rats was significantly ($t = -3.63$, $df = 27.3$, $p = 0.001$) higher than that of control rats (**Figure 7A**). In contrast, the two groups' times in the probe test of a Morris water maze as an index of spatial reference memory were not different (**Figure 7B**).

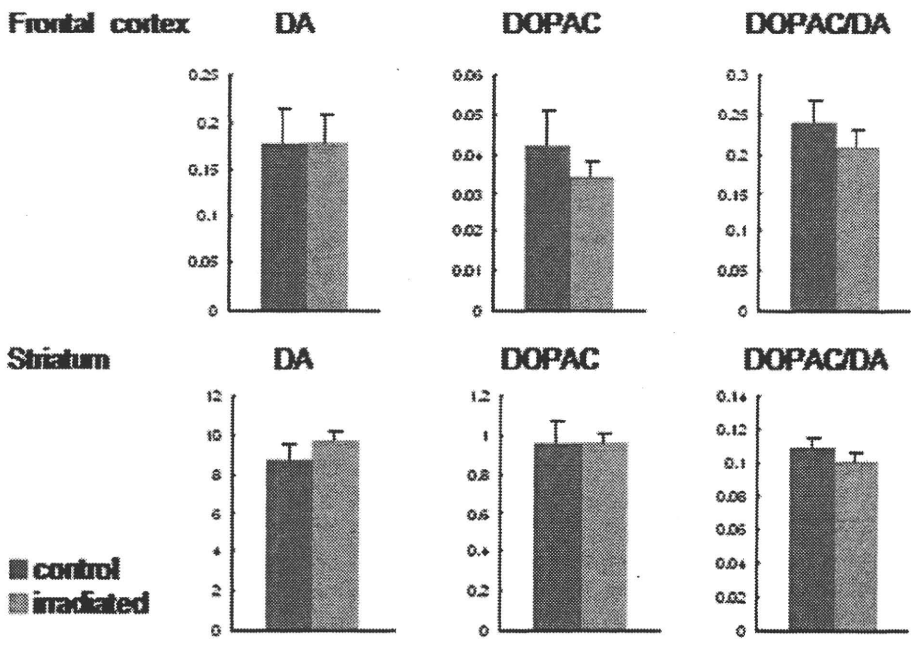

Figure 3. Dopamine and its major metabolite DOPAC levels in the frontal cortex and striatum of rat brain. Levels of dopamine and its major metabolite DOPAC, and dopamine turnover (DOPAC/dopamine ratio) in the frontal cortex and striatum were determined by HPLC analysis. There are no differences between irradiated rats (n = 6) and sham-control rats (n = 6).
doi:10.1371/journal.pone.0002283.g003

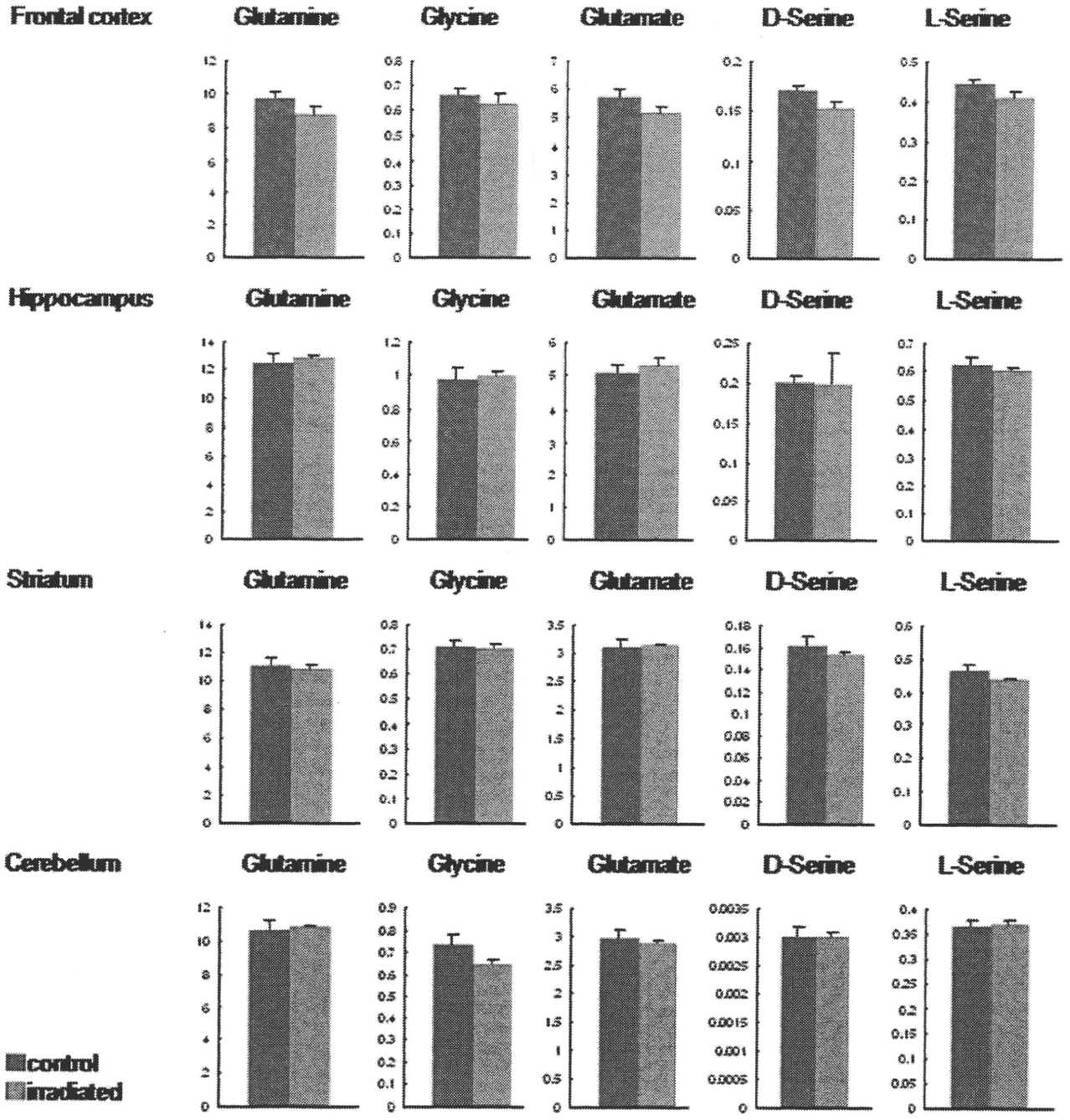

Figure 4. Levels of amino acids in the brain. Levels of amino acids (glutamate, glycine, glutamine, D-serine, L-serine) related with the NMDA receptor neurotransmission in the frontal cortex, hippocampus, and striatum, and cerebellum were determined by HPLC analysis. There are no differences between irradiated rats (n = 6) and sham-control rats (n = 6).
doi:10.1371/journal.pone.0002283.g004

We examined whether the antipsychotic drug clozapine could improve the reduction of neurogenesis and PPI deficits in irradiated rats. Subsequent chronic administration of clozapine (5 mg/kg/day for 3 weeks) did not alter the reduction of neurogenesis in the irradiated (data not shown). Furthermore, subsequent chronic administration of clozapine (5 mg/kg/day for 3 weeks) did not alter PPI in control rats (**Figure 8A**). However, we found that chronic administration of clozapine (5 mg/kg/day for 3 weeks) slightly improved PPI deficits in irradiated rats although a statistical analysis was not significant (**Figure 8B**).

Discussion

The major findings of the present study are that fractionated ionizing irradiation to the adult male rat brain causes schizophrenia-relevant abnormal behaviors (e.g., methamphetamine-induced

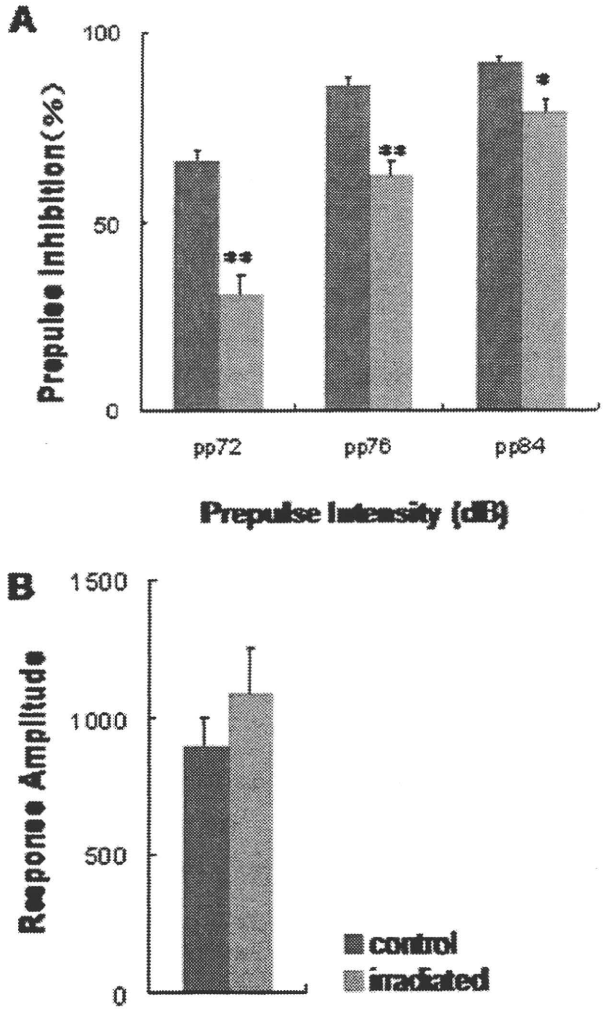

Figure 5. Sensorimotor gating deficits in the irradiated adult rats. (A) Auditory sensorimotor gating test: The irradiated rats (n = 23) show significant PPI deficits as compared with control (sham-irradiated) rats (n = 21). (B) Amplitude (in arbitrary units) of acoustic startle responses to the 120 dB auditory stimuli without prepulse in both groups was not different. Data are given as means±SEM. *p<0.05, **p<0.01 as compared with control (sham-irradiated) rats.
doi:10.1371/journal.pone.0002283.g005

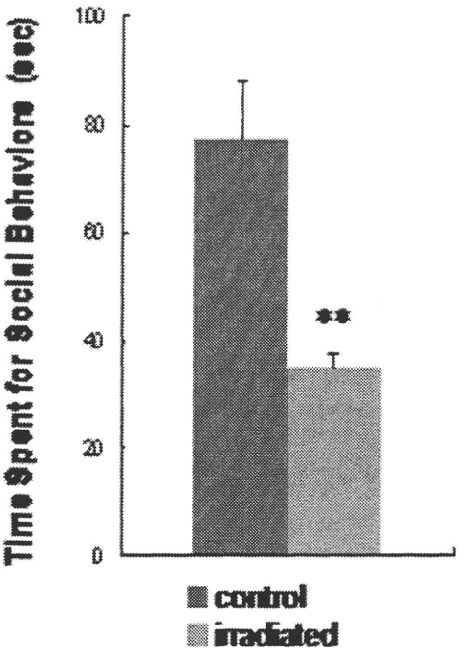

Figure 6. Social withdrawal in the irradiated adult rats. Social interaction test: Total time (sec) spent in social behaviors for 10 min in the irradiated rats (n = 6) was significantly lower than that of control rats (n = 6). Data are given as means±SEM. **p<0.01 as compared with control (sham-irradiated) rats.
doi:10.1371/journal.pone.0002283.g006

hyperactivity, sensory motor gating deficits, social interaction deficits, and working memory deficits) at three months after the irradiation. To the best of our knowledge, this is the first report demonstrating an animal model of schizophrenia by irradiation at adulthood. Although the irradiated adult rats may show essential features (positive and negative symptoms as well as cognitive deficits) relevant to schizophrenia, the pathophysiological mechanism underlying these behavioral changes remains unclear. A recent study using postmortem brain samples demonstrated that proliferation of hippocampal neural stem sells was significantly reduced in patients with schizophrenia, but not unipolar depression [16], suggesting that reduced neural stem cell proliferation may contribute to the pathogenesis of schizophrenia. Moreover, it has been reported that the reduction of cell proliferation in the SGZ after repeated administration of the NMDA receptor antagonist phencyclidine (PCP) may occur in tandem with PCP-induced behavioral changes in rats [22]. In this regard, it is likely that reduction of adult neurogenesis by irradiation may be involved in the schizophrenia-like behavioral abnormalities in rats. Recently, the association between neurogenesis dysfunction and schizophrenia has been also demonstrated [23].

Monje et al. [11] observed that irradiation of the brains of adult rats produced neural progenitor cell dysfunction within the neurogenic zones of the hippocampus, regions plausibly implicated in cognitive deficits. Furthermore, it has been suggested that irradiation-induced cognitive deficits in animals may be associated with a decrease in hippocampal proliferation and a decrease in adult neurogenesis [9–15]. In the eight-arm radial maze test, irradiated rats showed a deficit in working memory, which is also shown in schizophrenic patients [24]. It has been suggested that adult neurogenesis may serve an important role in hippocampal-dependent memory processes [25,26]. First, exposure to an enriched environment or increased physical activity leads to increased hippocampal neurogenesis and improved spatial memory [26–28]. Second, the comprehensive loss of hippocampal-dependent memory function in old age is related to decreased neurogenesis [29]. Taken together, it seems that cognitive impairment in irradiated rats may be due to reduction of hippocampal neurogenesis.

In this study, we found that methamphetamine-induced hyperactivity was significantly enhanced in the irradiated rats, suggesting hyperdopaminergic activity. The precise mechanisms underlying the hyperdopaminergic states in irradiated rats could not be explained, as we found no alteration of dopamine or its major metabolite DOPAC in the irradiated rat brains. Since mesolimbic dopaminergic neurons innervate the SGZ of the dentate gyrus [30], the dopaminergic activities of these neurons may be involved in the regulation of hippocampal neurogenesis. Furthermore, we previously reported that cell destruction of

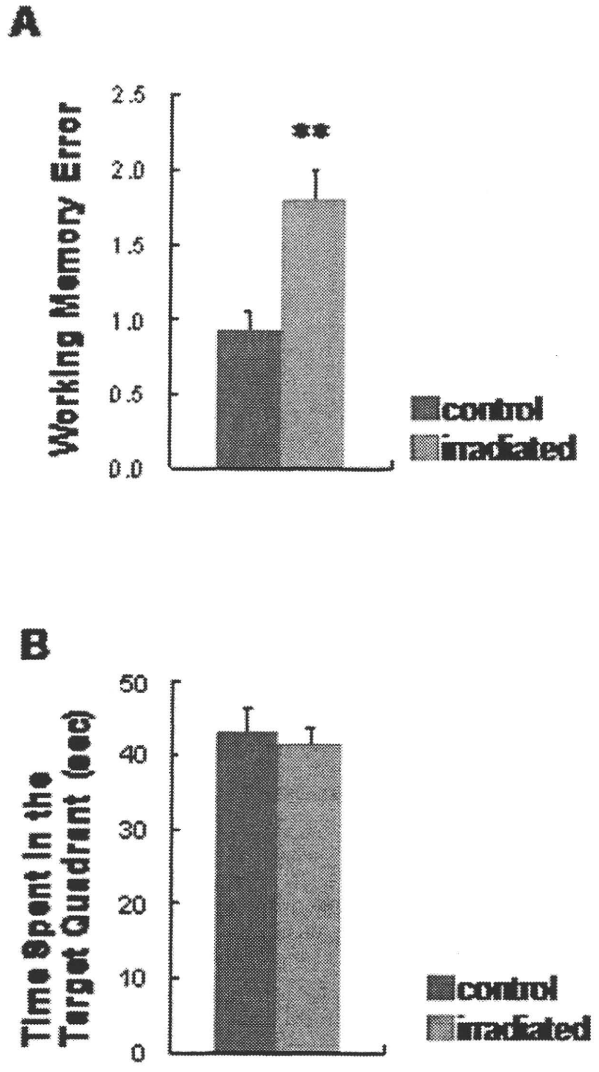

Figure 7. Cognitive impairments in the irradiated adult rats. (A) Spatial working memory in the eight-arm radial maze with 30-sec delay. Total number of revisits to arms from which pellets had already been taken, (i.e., working memory error) is represented as mean±SEM. Irradiated rats (n = 17) showed a higher number of working memory errors than control (sham-irradiated) rats (n = 15). (B): In the probe trials of the Morris water maze, spatial reference memory was intact in the irradiated rats (n = 12). Data are given as means±SEM. **p<0.01 as compared with control (sham-irradiated) rats (n = 12).
doi:10.1371/journal.pone.0002283.g007

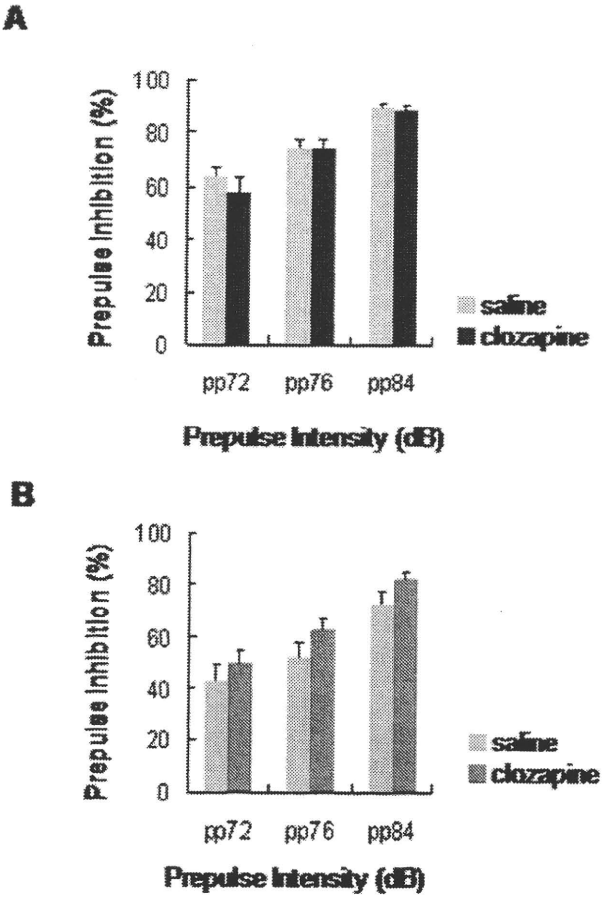

Figure 8. Effects of chronic clozapine administration on PPI deficits. (A) Control (sham-irradiated) rats: Chronic administration of clozapine (5 mg/kg/day for 3 weeks, i.p.) did not alter PPI deficits in the control rats (clozapine: n = 6; vehicle: n = 6). (B) Irradiated rats: Chronic administration of clozapine (5 mg/kg/day for 3 weeks, i.p.) significantly did not alter PPI deficits in the irradiated rats (clozapine: n = 6; vehicle: n = 6). Data are given as means±SEM.
doi:10.1371/journal.pone.0002283.g008

dentate granules by intrahippocampal injection of colchicine enhanced methamphetamine-induced hyperactivity in rats [31], suggesting that dentate granule cells may regulate methamphetamine-induced behavioral changes. Taken together, the evidence suggests that the decrease in hippocampal neurogenesis by irradiation may, in part, be implicated in the hyperdopaminergic activity of irradiated rats although the cumulative numbers of granule cells in the granule layer were not altered in irradiated rats.

Accumulating evidence suggests that hypofunction of the NMDA receptors may play a role in the pathophysiology of schizophrenia [32–34]. However, we did not find any alteration in dizocilpine-induced hyperactivity and levels of amino acids related to NMDA receptor neurotransmission in irradiated rat brains. Therefore, it is unlikely that alteration in the NMDA receptors is involved in the behavioral abnormalities in irradiated rats, although further studies are necessary.

The idea that antipsychotic drugs may increase neurogenesis in the rat hippocampus has not been consistently supported [17]. In this study, we found that chronic administration of clozapine (5 mg/kg/day for 3 weeks) did not alter the reduction of neurogenesis in the irradiated and control rats. In addition, we found that chronic administration of clozapine (5 mg/kg/day for 3 weeks) significantly did not improve PPI deficits in irradiated rats although a slight improvement by clozapine was shown. Therefore, it is likely that the inefficiency of clozapine treatment on PPI deficits in irradiated rats may be dependent upon the reduction of adult neurogenesis, although a further study will be necessary.

The total numbers of BrdU-positive cells in both SVZ and SGZ were significantly lower than those of sham-irradiated rats three months after fractionated irradiation. The static BrdU-positive cell count may reflect neurogenesis and/or survival of the recent born cells. Monje et al. [10] have demonstrated that normal number of neural progenitors was surviving two months after radiation exposure although proliferative activity was reduced. They also

have shown that the neural stem/precursor cells isolated from irradiated hippocampi failed to expand only 2–3 passages. These findings suggest that the reduction of proliferating cells in the present study might be due to ablated neurogenesis without reduction of cell survival. Actually, it is reported that the decline of proliferating cells lasted at 15 months after irradiation [35].

In the present study, we have regarded the neurogenesis dysfunction as a possible mechanism underlying the radiation induced abnormal behaviors associated with schizophrenia, based on the findings suggesting the link between neurogenesis dysfunction and schizophrenia. However, it has been reported that irradiation also induces apoptosis [9], neuroinflammation [11], and loss of oligodendrocyte precursor [35]. Further detailed investigation is required to discriminate the involvement of these factors on irradiation induced abnormal behavior relevant to schizophrenia. Further studies on the optimization of radiation dose, phenotypic alteration by the exposure age, and sex differences are also needed.

In conclusion, the present findings suggest that irradiation in adulthood might provide a new animal model of schizophrenia. Further understanding the molecular and cellular mechanisms underlying the behavioral abnormalities in irradiated rats would contribute to the pathophysiology of schizophrenia.

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Author Contributions

Conceived and designed the experiments: KH NM YI KSuzuki. Performed the experiments: KH KN YI KSuzuki TW NS IT HM TM TU SM TS SS SY KT GS KSato. Analyzed the data: KH YI NT. Contributed reagents/materials/analysis tools: KH YI. Wrote the paper: KH YI.

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老年病態モデル編

マウスを用いた学習記憶試験法

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我が国は超高齢化社会を迎え、アルツハイマー病などの認知症の患者は増加の一途をたどっている。基礎研究領域ではアルツハイマー病発症メカニズムの解明や治療薬の開発のために様々なモデルマウスが生み出され、そうしたモデル動物や新規化合物の有用性を評価するための行動薬理学的手法（学習記憶試験）が数多く開発されてきた。ここでは我々の研究室で使用している方法を中心に、手軽に実施できる方法で多くの論文で採用されている方法について紹介する。

行動薬理学的試験では実験環境は実験結果に直接影響する非常に重要な要因である。我々は室温25℃に設定し5～7畳ほどの半防音性の実験室で、天井の照明用蛍光灯の光を直接マウスや実験装置に当てないように厚紙や黒色ビニールで明るさを調節し、実験装置付近の明るさをおよそ30ルクスに保って実験している。

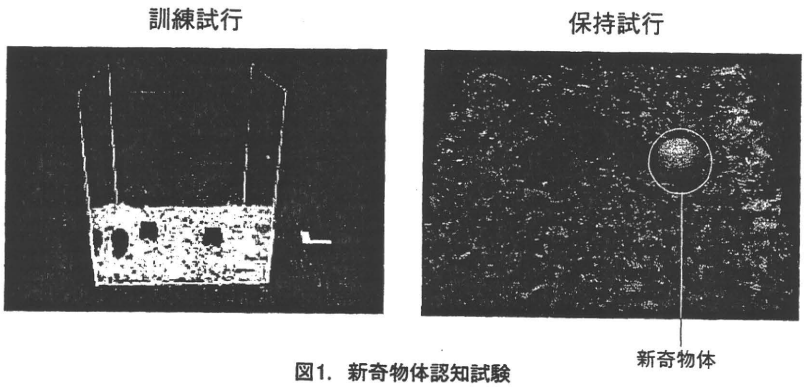

図1. 新奇物体認知試験

3日間の馴化期間中から装置内に軽く床敷を敷いておくとマウスは比較的物体に注目するようになる（見やすくするために透明の装置で撮影した）。

1. 新奇物体認知試験[1,2]（図1）

この試験は4〜5日からなり、短期および長期記憶能力を評価する方法として利用している。マウスが新奇なものに興味を示すという習性を利用するため強化因子は不要である。

マウスを黒色アクリル製の装置（縦30×横30×高さ35 cm）に慣らすために1匹ずつ3日間（1日15分）装置内を探索させる。4日目に訓練試行として装置内に2つの同じオブジェクトを置き、各オブジェクトに接触する時間および回数を5分間記録し、マウスを静かに飼育ケージに戻す（訓練試行）。保持試行としてその直後（あるいは1時間後、24時間後など）に一方のオブジェクトを新奇オブジェクトと取替え、再びマウスを装置に戻し、各オブジェクトに接触する時間および回数を5分間記録する。

訓練試行においては総探索時間におけるいずれかのオブジェクトへの探索時間の割合を、また保持試行においては総探索時間における新奇オブジェクトへの探索時間の割合を探索嗜好性（%）として算出し、後者を認知機能の指標とする。また、短期および長期記憶の測定のために保持試行は、訓練試行の直後、1時間後あるいは24時間後など研究目的に応じて変える。なお、オブジェクトは一般に積み木、小さい塩ビ性の人形などが用いられるが、我々は高い再現性から、フィルムケースを利用している。また、新奇オブジェクトにはゴルフボールや、単1乾電池などを利用している。訓練試行では探索嗜好性（%）が期待値の50%となるよう実験環境を工夫すべきである。また、保持試行では新奇物体に対して70%ほどであればおおむね良好な実験環境であり再現性は高い。

2. 水探索試験[3,4]

この試験は訓練試行および保持試行の2日間からなり、潜在学習能力を評価する方法である。とくに強化因子を必要としないこと、またヒトにも容易に外挿可能な課題として活用している。

装置は図2のような灰色で塩ビあるいは木製のオープンフィールド（縦30×横50×高さ15 cm）と小部屋（縦10×横10×高さ10 cm）からなり、

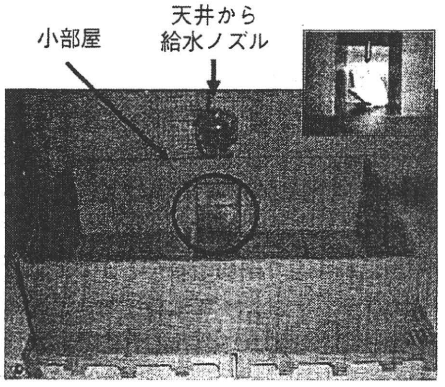

図2. 水探索試験

給水ノズルの長さを変えて見つけやすさを調節する。

給水ビンを逆さにして小部屋の天井から給水ノズルのみが小部屋内に出るように設置する。またオープンフィールドは10 cm四方となるように黒線で区切り、隣の升目に移動したときに行動量を1としてカウントする。訓練試行では実験直前まで給餌給水したマウスを装置に静かに入れ、3分間自由に探索させる。探索行動を始める時間（開始潜時：Starting latency）、給水ビンのある小部屋に入るまでの時間（進入潜時：Entering latency）および給水ノズルを見つけるまでの時間（発見潜時：Finding latency）をストップウォッチで計測する。給水ノズルを見つけてからマウスのオープンフィールド内での行動量と給水ノズルへのアプローチ回数をさらに3分間記録して、マウスの給水ノズルに対するモチベーションを確かめておく。3分以内に給水ノズルに接触しなかったマウスは以後の実験を行わず除外する。

訓練試行終了後、静かにマウスを飼育ケージに戻し24時間の絶水を行う。絶水後体重の減少を確認し、訓練試行と同じように各潜時を測定し、給水ノズルを発見するまで最大5分間観察する。発見潜時が小さいほど潜在学習能力が高いと判断できる。対照実験として訓練試行を行わず絶水したマウスを用いる。

なお、給水ノズルの床からの高さはマウスの系統や週齢など大きさにもよるが、ICRやddY系の6～7週齢（35 g前後）では訓練試行時には5 cm、

保持試行時には7cmで行い、訓練試行時の発見潜時が100～150秒くらいでまとまれば、潜在学習能力を亢進するかあるいは抑制するか比較する薬物の効果や遺伝子の影響について検討することができる。

3. 水迷路試験[5,6]

この試験は数日～10日からなり空間情報を手がかりにして、いかに早く水から逃れてゴールのプラットフォームに到達できるかを経時的あるいは経日的に計測し、空間学習記憶能力を調べる方法である。

図3に示したように直径120cmのプール（動物の毛色が白であれば、プールの色は黒色や青色にし、黒色、灰色、チョコレート色、茶色などの場合はプールの色を白にする）に水を張り、直径約7cmのゴール用のプラットフォームを水面下1cmに置く（水温は24～26℃）。目印としてプールの周辺に星型や丸型の絵を張ったりビンを置くが、目印が多くてもかえってマウスが混乱することもある。

水面下のプラットフォームを探索する訓練試行（獲得試行）では4分割

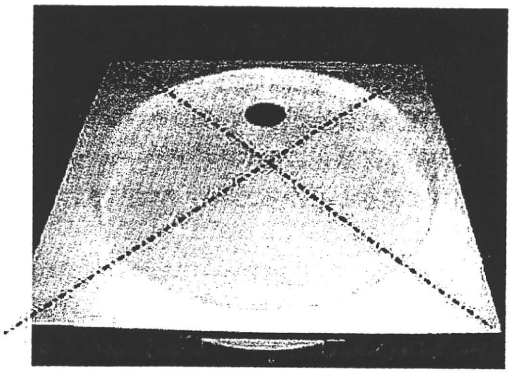

図3. 水迷路試験

黒色や茶色のマウスの測定のための白色のプールである。
点線のように4分画に区切り、ランダムに各領域からマウスを
入れて遊泳させる。中央の円はプラットフォームの位置を示している。

した任意の1区画の壁面付近からマウスを遊泳させ、60～90秒の観察時間内に、ゴールするのに要した遊泳時間（逃避潜時：Escape latency）を記録する。ゴールしたマウスは20秒間滞在させた後、静かに救い上げタオルで水をよくふき取っておく。ゴールできなかったマウスについてはプラットフォームに誘導し同様に20秒間滞在させてプラットフォームの位置を認識させる。この課題を1日3試行5～10日間行い、1日の平均逃避時間で表記する。逃避潜時が一定（15～30秒）となり、マウスがゴールの位置を記憶したと確認できたら、プローブ試行を行う。この試行はプラットフォームを取り除いた状態でマウスを泳がせたときにどれほどプラットフォームがあった区画を泳ぐか測定することで、記憶が保持されているかを見ることができる。

4. 恐怖条件付け学習試験[7,8]（図4）

この試験はパブロフ型条件付けを応用した試験で、電気刺激（無条件刺激）と、ブザー音やランプの光などの条件刺激を組み合わせて与え、一定

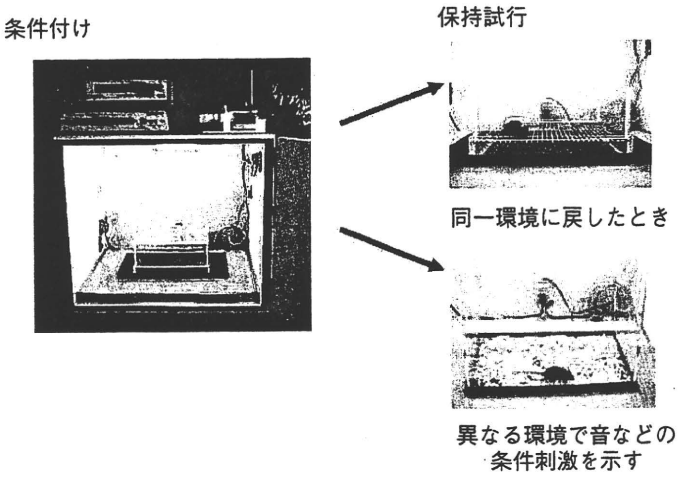

図4. 恐怖条件付け学習試験

条件付け時は半防音箱に入れる（撮影のために扉を開けている）。
保持試行で異なる環境下におく際には照明を調節するのもよい。

時間後に恐怖行動（すくみ行動：freezing behavior）を指標としておもに長期記憶能を評価する。すくみ行動は、条件刺激を与えた時と同一環境下に戻し観察する文脈的すくみ行動（contextual freezing）と、異なる環境下で条件刺激を提示し観察する音や光依存性のすくみ行動（tone/light dependent freezing）に分けられている。

実験は、床面に1cm間隔でステンレスグリッドを設置した透明アクリル製のチャンバー（縦30×横50×高さ15cm）内にマウスを入れ、ブザー音（85デシベル、30秒）を鳴らし最後の2秒間だけ電気刺激（0.6 mA）と組み合わせて条件付けを行う。この条件付けを1セットとし15秒間隔で3回繰り返す。電気刺激を与えている際にマウスのすくみ行動の有無を確認し、条件付けが成立しているかどうか確認をしておく。保持試行として条件付けの一定時間後（直後、1時間後、24時間後など）に、同じ装置内にマウスを静かに戻し、ブザー音や電気刺激のない環境下で3分間すくみ行動を観察し記録する。また、条件付けを行った環境とは全く異なる環境下で、ケージにマウスを静かにいれ、ブザー音を聞かせた際に起きるすくみ行動を3分間観察記録する。

データはそれぞれ全測定時間に対するすくみ行動時間の割合（%）で示す。我々の実験系でのすくみ行動は薬物未処置のICR系やC57BL系マウスにおいては、条件付けで20～30%前後、保持試行で60～70%である。

5. 最後に

行動実験のプロトコールは唯一ではなく、研究者によって最適な手順で行われており様々である。本項では代表的な行動実験について我々の実験手順を紹介した。紙面の都合上4課題しか紹介できなかったが、他にも評価法はあるので他書[9,10]も参考にして欲しい。ここで取り上げた試験法は、マウスの学習記憶機能を評価できると受け入れられているが、検討を行う際には必ず複数の試験を行った上で総合的に学習記憶機能における作用や役割について考察することが肝要である。また、実験者によってデータにばらつきが生じることもあるので充分に訓練した後に本試験を始めることをお勧めする。当然実験データはビデオカメラ等で記録し保存しておくべきである。客観的なデータの集積には各種機器を利用するのもよい。この場

合は、機器による測定では行動の一断面しか計測できないことを認識すべきであり、行動実験の基本は実験者の五感によることを忘れてはいけない。

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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.
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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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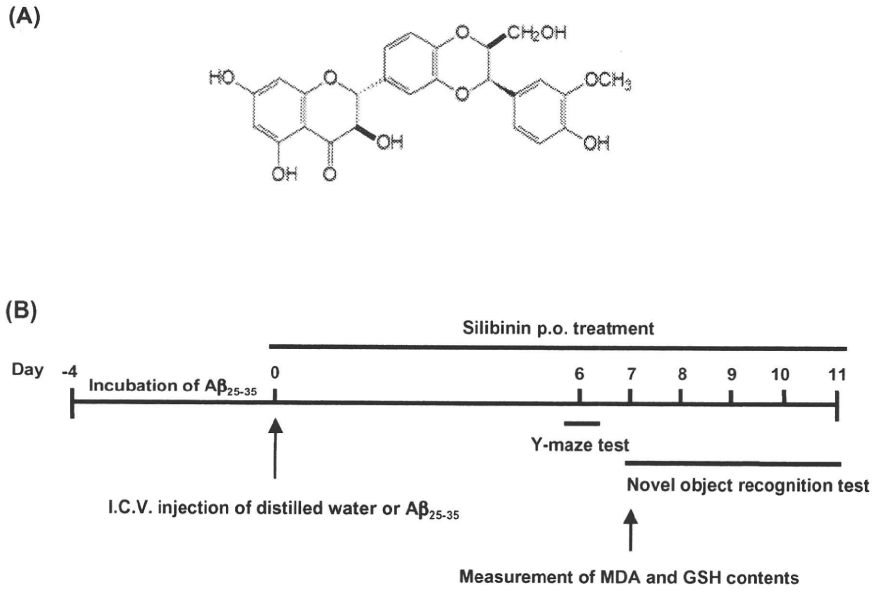

Figure 1 (A) Chemical structure of silibinin and (B) protocol used in this study.

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 $\mu mol \cdot g^{-1}$ 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 $\mu mol \cdot g^{-1}$ 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 $mg \cdot kg^{-1}$) 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 $mg \cdot kg^{-1}$ 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.

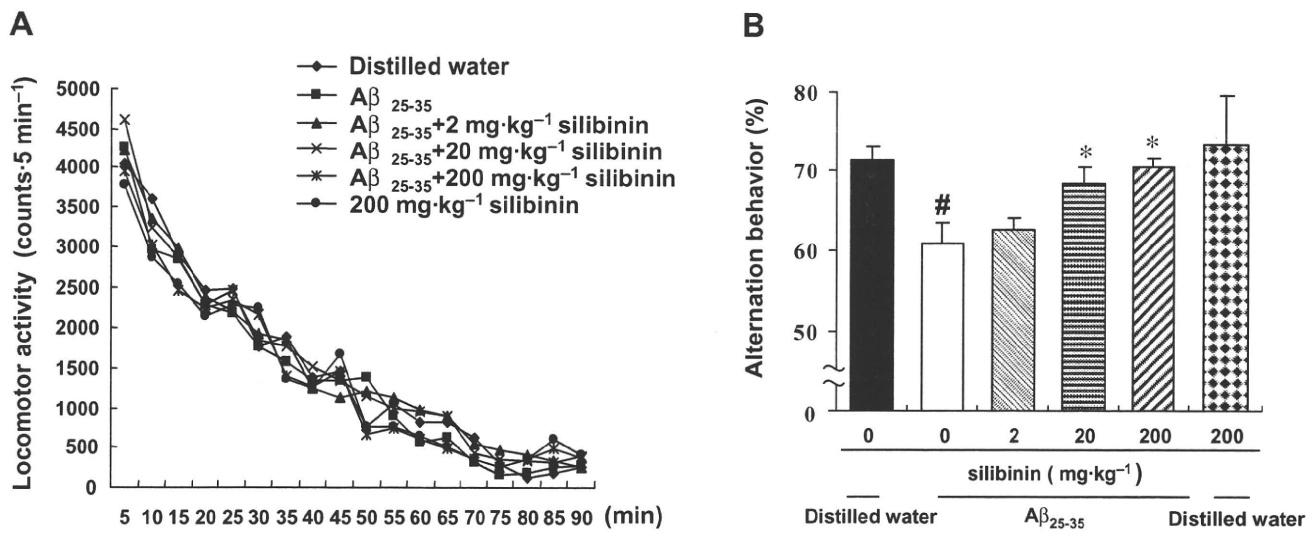

Figure 2 Effects of silibinin on locomotor activity (A) and impairment of short-term memory in Y-maze test (B) in amyloid β $(A\beta)_{25-35}$-injected mice. Results are expressed as the means ± s.e.mean (A: $n = 8$, B: $n = 13–15$) and were analysed by a one or two-way ANOVA, followed by Tukey's test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

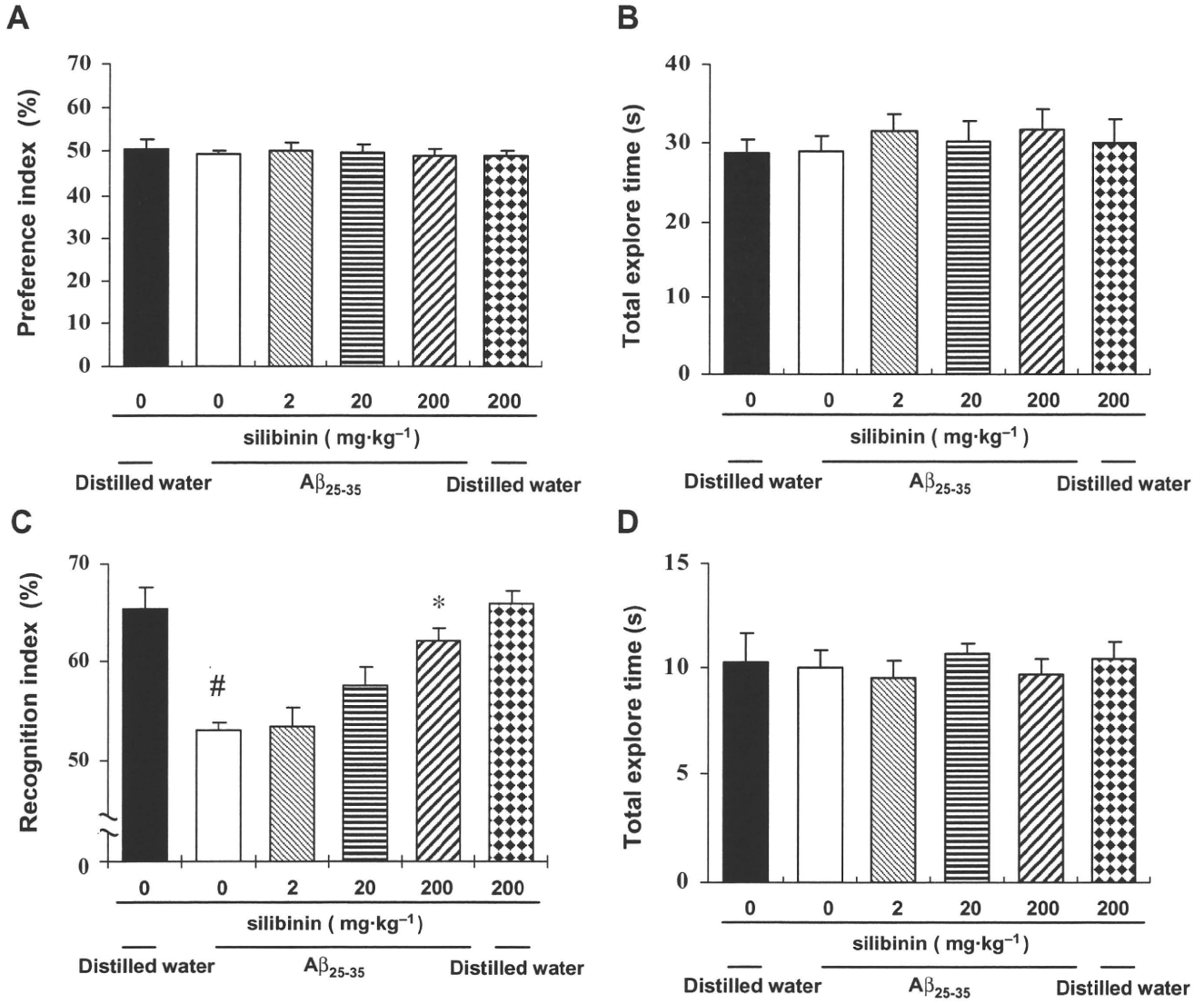

Figure 3 Effect of silibinin on recognition memory impairments induced by amyloid β $(Aβ)_{25-35}$ in the novel object recognition test. (A) Preference index in training session. (B) Total exploration time in training session. (C) Recognition index in retention session. (D) Total exploration time in 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

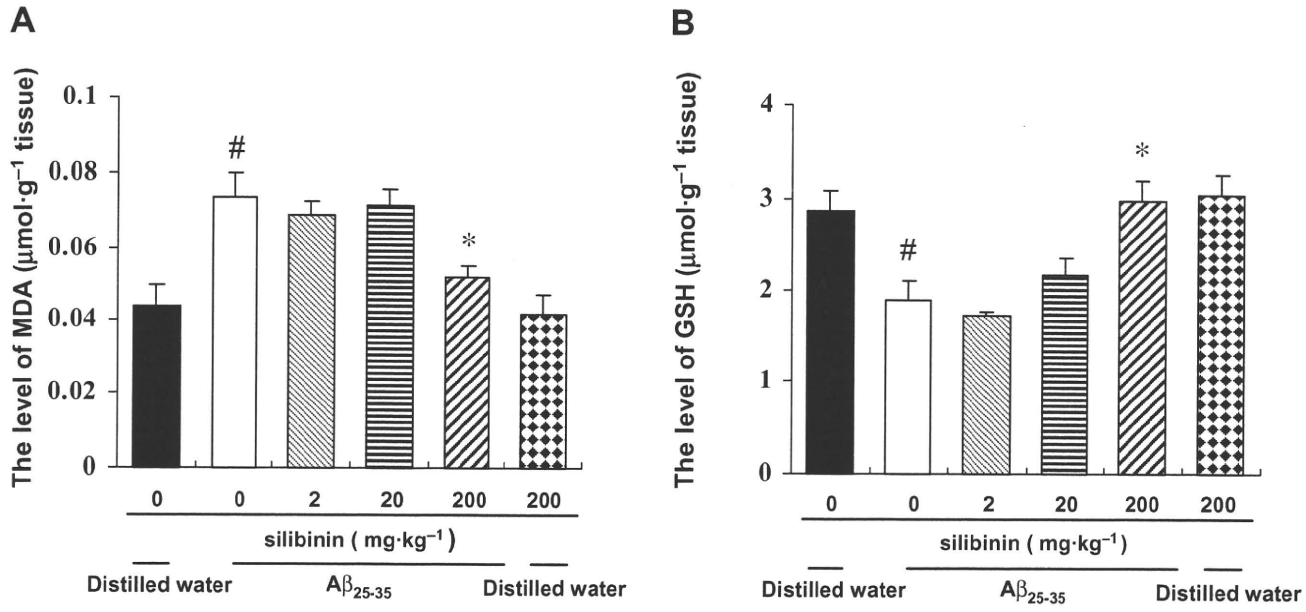

Figure 4 Effects of silibinin on amyloid β $(Aβ)_{25-35}$-induced increase in malondialdehyde (MDA) levels and decrease in glutathione (GSH) levels in the hippocampus. (A) MDA levels. (B) GSH levels. Results are expressed as the means ± s.e.mean (n = 6–7) and analysed by a one-way ANOVA, followed by 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.

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Conflict of interest

The authors state no conflict of interest.

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