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RESEARCH PAPER

T-817MA, a neurotrophic agent, ameliorates the deficits in adult neurogenesis and spatial memory in rats infused i.c.v. with amyloid-β peptide

Tatsuo Kimura[1,2], Phuong Thi Hong Nguyen[1,2], Son Anh Ho[1,2], Anh Hai Tran[1,2,3], Taketoshi Ono[1,2] and Hisao Nishijo[1,2]

[1]*System Emotional Science, Graduate School of Medicine, University of Toyama, Toyama, Japan,* [2]*CREST, JST, Tokyo, Japan, and* [3]*Department of Physiology, Military Medical University, Ha Dong, Hanoi, Vietnam*

Background and purpose: Adult neurogenesis occurs throughout life in the subgranular zone and the dentate gyrus of the hippocampus. Deficient neurogenesis may be responsible for deficient hippocampal functions in neurodegenerative disorders such as Alzheimer's disease (AD). T-817MA [1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate] is a newly synthesized agent for AD treatment with neuroprotective effects against toxicity from amyloid-β peptide (Aβ) and actions promoting neurite outgrowth *in vitro*. Furthermore, systemic administration of T-817MA ameliorated cognitive dysfunctions caused by neurodegeneration in a rat model of AD, induced by intracerebroventricular (i.c.v.) infusion of Aβ. The present study investigated quantitative relationships between spatial memory performance in Aβ-infused rats and hippocampal neurogenesis, and the effects of T-817MA on neuronal proliferation *in vivo*.
Experimental approach: Seven weeks after infusion of Aβ (peptide 1–40; 300 pmol·day^{-1}; i.c.v.), rats were tested in a place learning task in which they were required to alternately visit two reward places in an open field to obtain intracranial self-stimulation as rewards.
Key results: Rats given Aβ infusions for 10 weeks displayed spatial memory impairments and a decrease in neurogenesis compared with those infused with vehicle. Treatment of the Aβ-infused rats with T-817MA (8.4 mg·kg^{-1}·day^{-1}, p.o.) significantly increased hippocampal neurogenesis and ameliorated spatial learning impairments. Furthermore, spatial learning in the task was significantly correlated with neurogenesis.
Conclusions and implications: These results suggest that defective hippocampal neurogenesis is a new target for AD treatment. The neurotrophic compound T-817MA increased hippocampal neurogenesis in an AD model and might be useful for treatment of AD patients.
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Keywords: T-817MA; neurogenesis; hippocampus; memory; Alzheimer's disease; dementia; animal model; adult rat dentate gyrus; CNS

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; BrdU, bromodeoxyuridine; DMT, distance movement task; ICSS, intracranial self-stimulation; IGF-1, insulin-like growth factor-1; PLT, place learning task; RRPST, random reward place search task; SMA, spontaneous motor activity

Introduction

In most brain regions of highly developed mammals, the majority of neurogenesis is terminated soon after birth. However, new neurons are continually generated throughout life in the subgranular zone and the dentate gyrus of the hippocampus. In the adult mammalian hippocampus, new neurons are continuously generated; about half of them die, while the surviving cells mature and are incorporated into neural circuits (Dayer *et al.*, 2003). Over the last few years, many neural, endocrine and experimental factors regulating different hippocampal neurogenetic steps have been identified. For example, oestrogens, environmental complexity and learning are positive regulators, whereas adrenal steroids, excitatory inputs and stressful experiences have negative effects (Ambrogini *et al.*, 2000; Lemaire *et al.*, 2000; Snyder *et al.*, 2001; Malberg and Duman, 2003; Rizk *et al.*, 2006;

Correspondence: Dr Hisao Nishijo, System Emotional Science, Graduate School of Medicine, University of Toyama, Sugitani 2630, Toyama 930-0194, Japan. E-mail: nishijo@med.u-toyama.ac.jp
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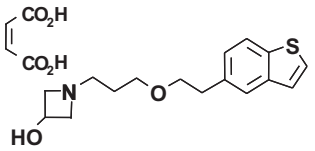

Figure 1 Chemical structure of T-817MA [1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate].

Chiba *et al*., 2007). Alzheimer's disease (AD)-related pathology is one of the important factors that affects hippocampal neurogenesis. It is reported that neurogenesis is increased in the hippocampus of some patients with AD (Jin *et al*., 2004b) and in transgenic mice that express human mutant amyloid precursor protein (APP) found in some familial AD pedigrees (Hale and Good, 2005). In other animal models of AD, neurogenesis was also disturbed (Feng *et al*., 2001; Haughey *et al*., 2002; Wen *et al*., 2002; Dong *et al*., 2004; Wang *et al*., 2004; Donovan *et al*., 2006). These findings suggest that an increase in neurogenesis reflects the organism's responses towards self-repair and that AD may be one of the diseases appropriate for neuron replacement therapy. If neurons produced by adult neurogenesis can replace those that are lost through the disease, then compounds that can stimulate neurogenesis might be beneficial to AD patients.

T-817MA, 1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate (Figure 1), a newly synthesized neurotrophic compound, protected neurons against neurotoxicity induced by amyloid-β peptide (Aβ) and hydrogen peroxide, and promoted neurite outgrowth in hippocampal slice cultures and reaggregation cultures of rat cortical neurons (Hirata *et al*., 2005). We recently reported the effects of T-817MA on place learning deficits in rats with hippocampal damage (Nguyen *et al*., 2007). In that study, to induce granule cell loss in the dentate gyrus of the hippocampus, Aβ (peptide 1–40) was continuously infused (300 pmol·day^{-1}) into the cerebral ventricle by using a mini-osmotic pump for 5 weeks. Three weeks after the Aβ infusion, the rats were tested in a place learning task (PLT), which required them to alternately visit two diametrically opposed areas in an open field to obtain intracranial self-stimulation (ICSS) reward. The results indicated that the Aβ-infused rats without treatment with T-817MA displayed learning impairment in the task and their performance level was significantly worse than that of the vehicle rats. Chronic treatment with T-817MA (8.4 mg·kg^{-1}·day^{-1}, p.o.), which was started on the same day the Aβ infusion was started, significantly improved the performance of the Aβ-infused rats and prevented granule cell loss due to Aβ infusion. These results suggest that T-817MA works as a neurotrophic factor, such as insulin-like growth factor-1 (IGF-1) that has neuroprotective effects by promoting neurogenesis (Carro *et al*., 2003; Hodge *et al*., 2004; Zhang *et al*., 2007).

To investigate the possible effects of T-817MA on neurogenesis in the hippocampus and its relations to learning and memory, we studied performance of Aβ-infused rats with and without T-817MA treatment in the same place learning paradigm using ICSS as reward. Previous studies reported that performance of aged rats and young rats with selective hippocampal lesions were disturbed in this paradigm (Zhong *et al*., 2000; Hori *et al*., 2002; Kurimoto *et al*., 2004). To investigate neuron replacement effects of T-817MA, the drugs were administered 4 weeks after Aβ infusion started when neuronal degeneration had already taken place. Cell proliferation and survival of newly generated cells were evaluated at different times after injection of the mitotic marker bromodeoxyuridine (BrdU), and newly generated cell phenotypes were investigated. Immature granule cells were quantified by detection of the expression of specific markers, such as the polysialylated form of the adhesion molecule (PSA-NCAM; Seki and Arai, 1991; Seki, 2002).

Methods

Animals

All animal care and experimental protocols were in accordance with the guide for care and use of laboratory animals at University of Toyama and the NIH Guide for the Care and Use of Laboratory Animals. Male Crj : Wistar rats (7 weeks, $n = 47$) were used. All rats were given food and water *ad libitum* in a clear cage and handled on three consecutive days before start of the experiments. The housing area was provided a temperature-controlled environment under a 12/12 h light cycle (on at 08:00 h, off at 20:00 h). These rats were divided into five groups: vehicle ($n = 11$), Aβ infusion control ($n = 10$), Aβ infusion + high-dose T-817MA (8.4 mg·kg^{-1}) ($n = 11$), Aβ infusion + low-dose T-817MA (0.84 mg·kg^{-1}) ($n = 9$) and Aβ infusion + donepezil (0.5 mg·kg^{-1}) ($n = 7$).

Surgical procedures

The detailed procedures have been described previously (Nguyen *et al*., 2007). Briefly, the rats were anaesthetized with pentobarbital (40 mg·kg^{-1}, intraperitoneal, i.p.). An infusion cannula for Aβ delivery was implanted into the left cerebral ventricle (A: –0.3 mm, L: 1.2 mm, V: 3.6 mm), and then monopolar stimulating electrodes (polyurethane-insulated, 0.1 mm diameter, stainless steel) for ICSS reward were implanted into the medial forebrain bundle at the level of the lateral hypothalamus (4.3, 1.6 and 8.7–8.9 mm) according to the atlas of Paxinos and Watson (1986). Aβ (1–40) (AnaSpec Inc., San Jose, CA, USA) was dissolved in 30% acetonitrile/0.1% trifluoroacetic acid. Continuous infusion of Aβ (1–40) (300 pmol·day^{-1}) was maintained for 10 weeks by a mini-osmotic pump, Alzet 2002 (Alza Corporation, Palo Alto, CA, USA) connecting to an infusion cannula (Nabeshima and Nitta, 1994; Nitta *et al*., 1994; Yamada *et al*., 1998). The vehicle-treated rats were infused with an equal volume of 30% acetonitrile/0.1% trifluoroacetic acid.

ICSS training

Five weeks after the start of Aβ infusion (1 week from the start of drug administration), the rats were screened for self-stimulation in an operant chamber equipped with a lever on one wall. Each lever press triggered the delivery of a 0.5 s train of 0.3 ms negative square wave pulses at 100 Hz. The current intensity for ICSS was determined to produce 40–70 lever presses per minute in the operant chamber. All rats used in the

present study met this criterion. The rats were trained for ICSS for 7 days (15–30 min·day^{-1}).

The use of ICSS as a reward provided the following advantages over natural reinforcements, such as food and water: (i) rapid learning of task; (ii) lack of satiation; and (iii) absence of external sensory information (visual, auditory, olfactory, etc.) (Fukuda *et al.*, 1992).

Experimental set-up for behavioural testing

The apparatus used for the behavioural testing in the present study was substantially the same as described previously (Fukuda *et al.*, 1992; Kobayashi *et al.*, 1997; Zhong *et al.*, 2000; Tamura *et al.*, 2001; Hori *et al.*, 2002; Kurimoto *et al.*, 2004; Nguyen *et al.*, 2007). A diagram of the apparatus is provided in Figure 2B.

For the three spatial tasks noted below, an open field (150 cm diameter) was used. The open field was enclosed by a black curtain. The ceiling of the enclosure contained four small speakers mounted near the circumference, spaced 90° apart, four light bulbs individually mounted near the inner edge of each speaker and a charge-coupled device (CCD) video camera at the centre (Figure 2B). Usually a light bulb at the 3 o'clock position and a white noise speaker at the 9 o'clock position on the ceiling of the enclosure were turned on. A CCD camera viewed the open field containing a rat from top centre, to signal the rat's position in Cartesian coordinates. The video signal was sent to a conventional TV monitor and a digital interface, which sent the X and Y coordinates of a miniature light bulb attached to the head of the rat to a computer. The computer plotted the trail of the rat, compared the rat's behaviour with preset criteria and gated ICSS delivery as reward to the medial forebrain bundle at the level of the lateral hypothalamus from a stimulator when the criteria were met.

Behavioural tests

After ICSS training, the rats were first tested in the open field to measure spontaneous motor activity (SMA), and then tested in the three kinds of spatial tasks: distance movement task (DMT), random reward place search task (RRPST) and PLT in the open field. In each of these spatial tasks, the small electric bulb on the head of the rat was lit at the start of a trial, and a train of ICSS current was delivered to activate the rat. Each trial was terminated after 50 rewards had been delivered or 10 min had elapsed, whichever occurred first. The rats were given training of five trials per day.

Spontaneous motor activity (SMA). SMA was measured in the open field on the first training day in the DMT. A rat was put on the centre of the open field. SMA of the rat was monitored by the CCD camera, and distance travelled by the rat for 10 min was computed. There was no ICSS reward given to the rats during this testing. Then, the rat was subsequently trained with the DMT.

Distance movement task (DMT). The rats learned to move in the open field on this task (Figure 2Ca). A computer programme computed the cumulative distance travelled by the rat from the rats' trail. The rat acquired ICSS rewards if it had moved the fixed distance (i.e. 120, 160, 200 or 240 cm). The predetermined fixed distance to acquire ICSS reward was progressively increased by 40 cm from 120 cm to 240 cm when the rat acquired 50 ICSS rewards within 10 min of one trial. If the rat could not acquire 50 rewards, the trial was also terminated at the end of the 10 min. If the rat could acquire 50 rewards within a trial, the distance was also increased by 40 cm from the next trial. When the rat passed the criterion in the DMT (i.e. distance of 160 cm), training in the DMT was completed. Thus, rats learned to associate ICSS rewards with movements in the DMT.

Random reward place search task (RRPST). In this protocol, rats must learn to navigate randomly in the open field. A computer programme delimited a circular reward place (70 cm diameter, thick line circle); its centre was chosen randomly within a square circumscribed around the open field (Figure 2Cb). The rat was rewarded with ICSS when it entered the reward place, which was then made inactive (changed to thin line circle). After a 5 s interval, the reward place was moved to a different location and reactivated. The rat was trained until it could achieve a criterion of 25 rewards over three trials in a day.

Place learning task (PLT). Two 40 cm diameter reward places were located diametrically opposite to one another in the open field. The rat was rewarded in both reward places, when it returned to one of them after a visit to the other one (Figure 2Cc). Furthermore, the rats were required to stay in the reward place for more than 1 s in order to acquire ICSS reward; otherwise, they could not receive the reward even though they had entered the reward place. So, in this task the rat was required to learn and memorize the places where ICSS rewards were delivered. The task was terminated when the rat had acquired 50 rewards or when 10 min had elapsed, whichever came first. The rats were tested for 14 days with this protocol.

BrdU injection and tissue preparation

The rats were given an i.p. injection of BrdU (Sigma, St. Louis, MO, USA) dissolved in 0.9% NaCl (50 mg·kg^{-1} body weight) once daily, for 5 days. BrdU injection started from the first day of the PLT. After 1 week from completion of the PLT (i.e. after 3 weeks from the first injection of BrdU), the rats were deeply anaesthetized with pentobarbital sodium (50 mg·kg^{-1}, i.p.). They were then perfused transcardially with 0.9% saline containing heparin followed by 10% buffered formalin. The whole brains were then removed and immersed in the same fixative for 1 day. After fixation, the brains were dehydrated with graded ethanol, passed through xylene and embedded in paraffin. Serial, 5 μm thick, coronal sections, which included the dorsal hippocampal formation, were made.

Antibodies

The mouse IgM monoclonal antibody 12E3 was donated generously by Dr T Seki (Juntendo University, Tokyo, Japan). It was found to recognize the PSA portion of PSA-NCAM (Seki

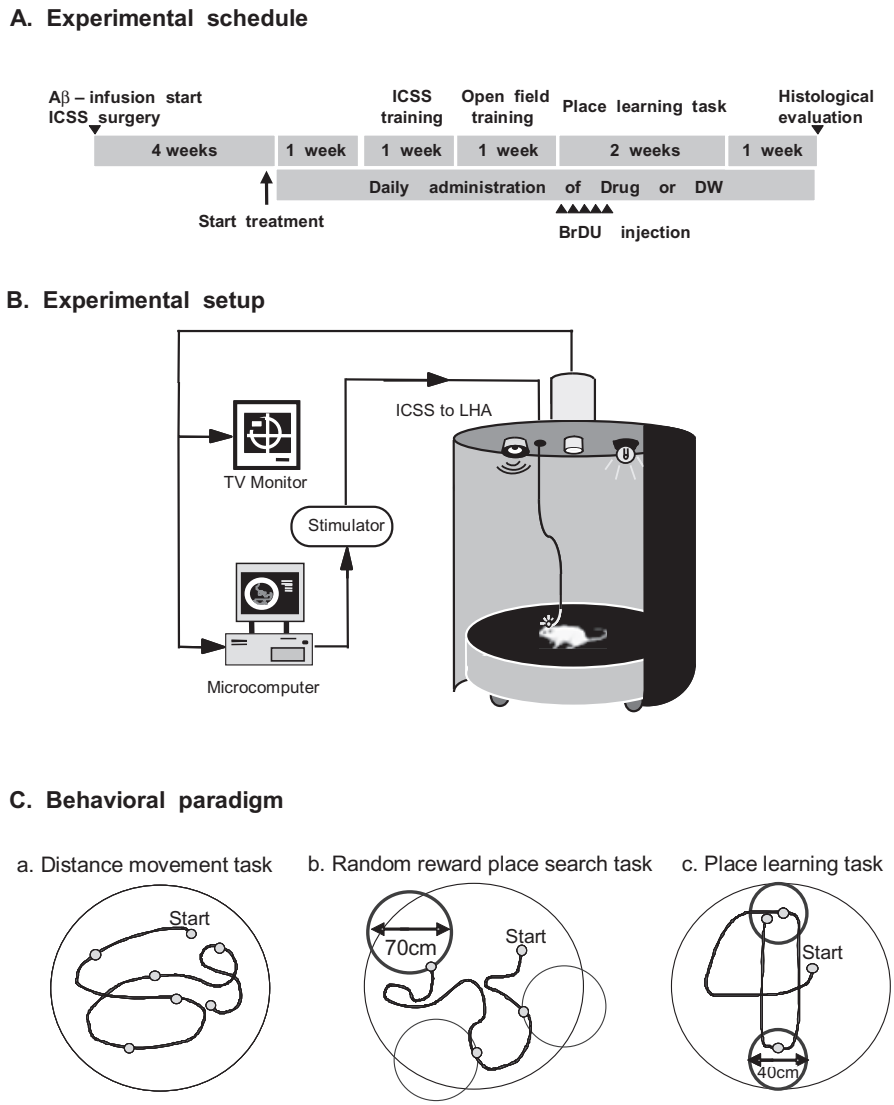

Figure 2 Outline of experimental schedule (A); experimental set-up (B) and behavioural paradigms (C). (A) 4 weeks after the start of amyloid-β peptide (Aβ) infusion and intracranial self-stimulation (ICSS) surgery, drugs or distilled water (DW) were given throughout the subsequent experimental period. After 5 weeks from the start of Aβ infusion, the rats were screened for ICSS in an operant box. After the ICSS training, rats were trained to perform spatial tasks in the open field. Injection of bromodeoxyuridine (BrdU) (once daily, for 5 days) synchronized with start of the place learning task (PLT). After 1 week from the end of the PLT, histological evaluation was performed. (B) An open field (150 cm diameter) containing a rat was viewed from top centre by a charge-coupled device video camera with brightness tracking interface that signalled the rat's position in Cartesian coordinates. The open field was enclosed by a black curtain. The incandescent lamp at the 3 o'clock position and a white noise speaker at the 9 o'clock position on the ceiling of the enclosure were turned on. The video signal was sent to a conventional TV monitor and a digital interface sent the X and Y coordinates of a miniature light bulb attached to the head of the rat to a computer. The computer plotted the trail of the rat, compared the rat's behaviour with preset criteria and gated rewarding ICSS from a stimulator to the lateral hypothalamic area (LHA) when the criteria were met. (C) Behavioural paradigm. In distance movement task (a), a computer programme computed distance travelled from a trail. The rat acquired ICSS rewards if it moved the fixed distance (i.e. 120, 160, 200 and 240 cm). In random reward place search task (b), a computer programme delimited a circular reward place (thick line circle; 70 cm diameter) at some randomly selected coordinate. The rat was rewarded with ICSS when it entered the reward place, which was then made inactive (changed to thin line circle). After a 5 s interval, the reward place was moved to a different location randomly and reactivated. In PLT (c), the rat received rewards in two target areas (two thick line circles; 40 cm diameter) when it returned to one reward place after a visit to the other reward place. Large circle, open field; Start, location of the rat at start of a trial; line, trail of the rat; each small open circle, the location of reward delivery.

and Arai, 1991). In addition, the following antibodies were used as the primary antibodies: rat IgG monoclonal anti-BrdU (Becton Dickinson, Mountain View, CA, USA, 1:200) and mouse IgG monoclonal anti-NeuN (Chemicon, Temecula, CA, USA, 1:200). As the secondary antibodies, the following antibodies were used: biotinylated anti-mouse IgM antibody (Vector Laboratories, Burlingame, CA, USA, 1:200), Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA, 1:200) and Cy2-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 1:200). The antibodies were diluted in phosphate-buffered saline containing 1% bovine serum albumin.

Immunohistochemistry

To examine proliferation of neural progenitor cells in the dentate gyrus, the immunostaining of PSA-NCAM, which is a marker of proliferating cells, was performed. Sections were treated with phosphate-buffered saline containing 1% bovine serum albumin at room temperature for 30 min, then in methanol for 30 min and incubated with mouse IgM monoclonal antibody 12E3 (1:500) overnight at 4°C. They were incubated for 1 h with biotinylated anti-mouse IgM antibody followed by the incubation with avidin–biotin complex (ABC Elite kit, Vector Laboratories). The labelled cells were visualized with diaminobenzidine by using a diaminobenzidine substrate kit (Vector Laboratories). Lastly, neurons were counterstained with methyl green (Vector Laboratories).

For double labelling of BrdU and the neuronal marker, neuronal nuclei (NeuN), the sections were subsequently treated with 2 mol·L^{-1} HCl at 37°C for 30 min and neutralized with 0.1 mol·L^{-1} borate buffer (pH 8.5) for 10 min. Then, the sections were incubated with rat monoclonal anti-BrdU (1:200) and mouse monoclonal anti-NeuN antibody (1:200) overnight at 4°C. The sections were further incubated at room temperature for 1 h with a mixture of secondary antibodies, Cy3-conjugated donkey anti-rat IgG (1:200) and Cy2-conjugated goat anti-mouse IgG (1:200). Finally the specimens were mounted on slide glasses.

Data analysis

To evaluate the influence of Aβ infusion and/or T-817MA treatment on motivational level and spontaneous activity, the data of: (i) ICSS current intensity; (ii) SMA for 10 min; and (iii) number of rewards acquired and the navigation distance travelled in the last day of the RRPST training, were analysed by one-way ANOVA.

Then, the behavioural data in the PLT were analysed in terms of the following four parameters: (i) navigation pattern; (ii) mean number of rewards acquired in a trial; (iii) mean trial duration taken for a rat to complete the PLT; and (iv) mean navigation distance between the two reward places. A two-way ANOVA was performed to compare performance in the PLT among the five groups. Bonferroni's post hoc test was employed for pair wise comparisons.

Quantitative analysis of immunohistological data was based on cell counts. Histological evaluation was performed by different experimenters in order to avoid biases by individual differences in the selection of animals. For quantification of PSA- or BrdU- and NeuN-positive cells in the dentate gyrus, an average of four sections per rat was used. Adjacent sections were not used for the cell counting to avoid double counting. For quantification of BrdU- and NeuN-positive cells in the dentate gyrus, all of the counting was performed under a TCS-SP5 confocal laser-scanning microscope (Leica, Mannheim, Germany) and using 40× objective in stacks of five optical sections.

To measure density of normal dentate granule cells, the sections were stained with haematoxylin and eosin (H&E), and then examined under a BX-50 light microscope (Olympus, Tokyo, Japan). The number of normal granule cells was counted separately in the four regions of the left and right sides of the dentate gyrus at the AP level 4.0 mm and 5.0 mm posterior from the bregma. Normal granule cells were defined as those displaying cell profiles with pale nuclei and a granular background, surrounded by a ring of visible cytoplasm and displaying one or more clearly visible nucleolus (Mignini *et al.*, 2004). The granule cell density was defined as the number of normal granule cells per 1 mm strip along the granular cell layer, and granule cell densities in both the sides were averaged in each rat. These data from the different animal groups were compared by one-way ANOVA. Bonferroni's test was used for post hoc comparisons. Relationships between behavioural scores and immunohistochemical data were evaluated by simple linear regression analysis.

These statistical tests were performed by using StatView 5.0J (Abacus Concepts, Berkeley, CA, USA). Significance level employed for all tests was $P < 0.05$.

Materials

T-817MA and donepezil was provided by the Research Laboratory of Toyama Chemical Co., Ltd. (Toyama, Japan). T-817MA (high-dose: 8.4 mg·kg^{-1} and low-dose: 0.84 mg·kg^{-1}) and donepezil (0.5 mg·kg^{-1}) were dissolved in distilled water in a volume of 5 mL·kg^{-1}. Suspensions of these drugs or water were given orally, via a gastric tube, daily, 4 weeks after the start of Aβ infusion until the end of the experiment (see Figure 2A).

Results

ICSS behaviour and SMA

Because effectiveness of ICSS and SMA of the rats could strongly affect performance in the PLT, these parameters were assessed before training for the PLT. There were no significant group differences in ICSS current intensities [one-way ANOVA; $F(4, 42) = 0.085$, $P > 0.05$] (Figure 3A), nor in 10 min SMA [$F(4, 42) = 0.368$, $P > 0.05$] (Figure 3B). These results indicated that there were no significant differences in basic motivation and motor functions among the five groups.

DMT and RRPST training

All rats successfully passed the criterion in the DMT. Figure 3C shows the mean numbers of training trials for the rats to reach the criteria in the DMT. There were no significant differences in the mean number of training trials among these five groups [one-way ANOVA: $F(4, 42) = 0.663$, $P > 0.05$].

Figure 3Da shows the mean numbers of training sessions for the rats to reach the criteria in the RRPST. There were no significant differences in the training sessions among the five groups of the rats [one-way ANOVA: $F(4, 42) = 0.182$, $P > 0.05$]. On the final day of RRPST training, each group rats, which passed the criterion, moved randomly in the open field. There were no significant differences among the five groups in mean numbers of rewards per trial [one-way ANOVA; $F(4, 42) = 0.456$, $P > 0.05$] (Figure 3Db), nor in the mean navigation distance travelled per trial [$F(4, 42) = 0.885$, $P > 0.05$] (Figure 3Dc) on the final day of RRPST training.

Navigation trails in the PLT

Figure 4 indicates typical examples of navigation trails in the vehicle (Figure 4A), Aβ infusion control (Figure 4B), Aβ

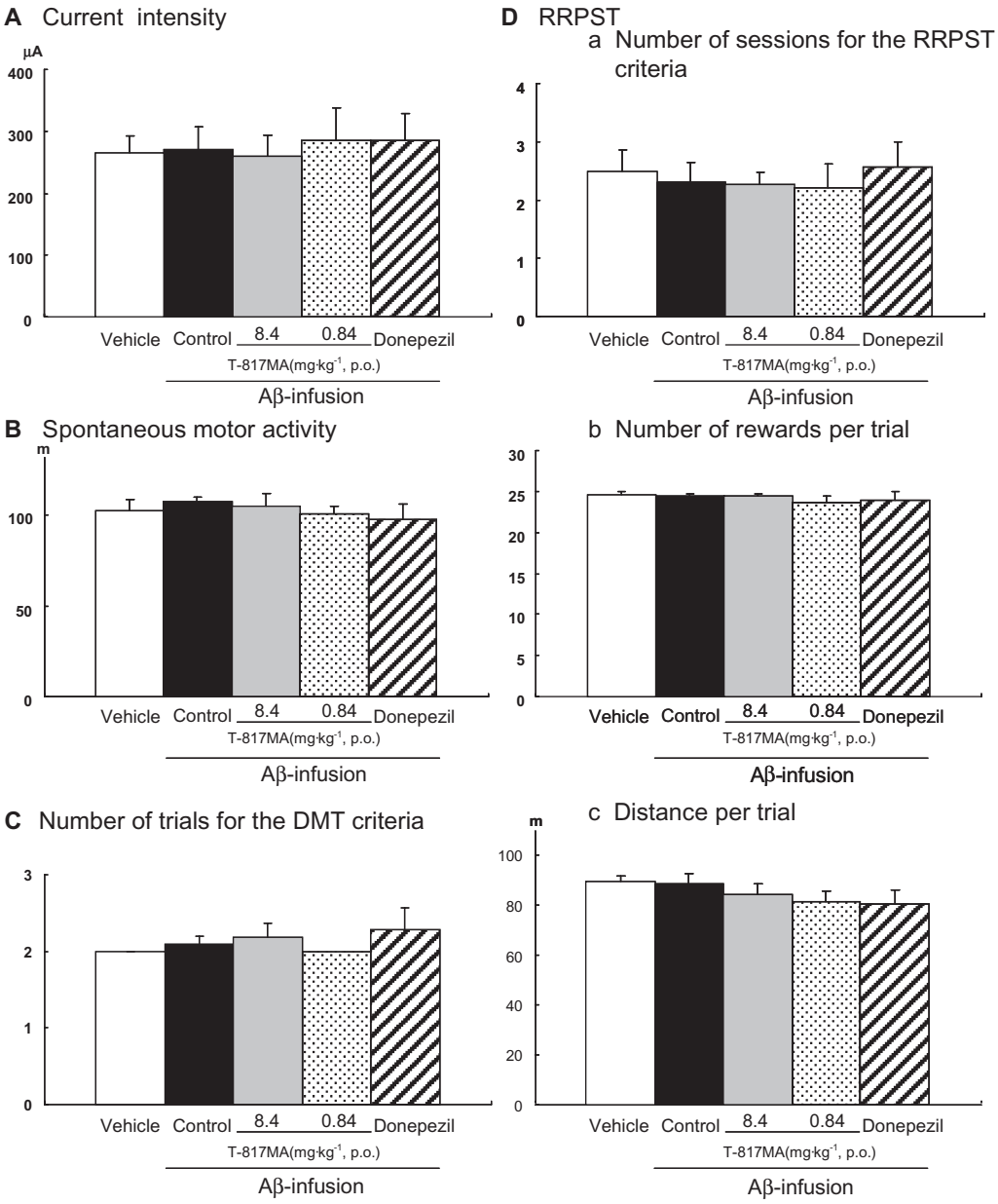

Figure 3 Comparisons of current intensity for intracranial self-stimulation (ICSS) (A), spontaneous motor activity (B), and performance in the distance movement task (DMT) (C) and random reward place search task (RRPST) (D) among the five groups of the rats. No significant differences were observed in the mean current intensity for ICSS (A), spontaneous motor activity (B) and the mean number of training trials for the rats to pass the criteria in the DMT (C) (one-way ANOVA, $P > 0.05$). In the RRPST (D), there were no significant differences in the mean number of sessions to pass the criteria (a). Furthermore, there were no differences in the mean number of rewards (b) and mean distance (c) per trial in the last session of the RRPST (one-way ANOVA, $P > 0.05$). T-817MA, 1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate.

infusion + high-dose T-817MA (Figure 4C), Aβ infusion + low-dose T-817MA (Figure 4D) and Aβ infusion + donepezil (Figure 4E) groups during the PLT. On the first day of the PLT (Day 1), the rats in these groups randomly travelled in the open field to the same extent and acquired only a small number of rewards. On the fifth day (Day 5), the vehicle group rats began to show shuttle behaviour, direct back-and-forth movements between the two reward places, and this behaviour dominated progressively in the course of the training (Figure 4A).

In contrast to the vehicle group, the rats in the Aβ infusion control group did not show shuttle behaviour during the 14 day test period (Figure 4B). Rather, they developed a navigation pattern of moving along the wall of the open field, although they did show a weak tendency to make shortcuts, later in the training period. The rats in the Aβ infusion + donepezil group showed a similar behavioural pattern (Figure 4E).

The rats in the Aβ infusion + high-dose T-817MA group showed a pattern resembling that of the vehicle-treated rats (Figure 4C). The rats began to show shuttle behaviour around the fifth day and established it during the rest of the training period. The rats in the Aβ infusion + low-dose T-817MA group

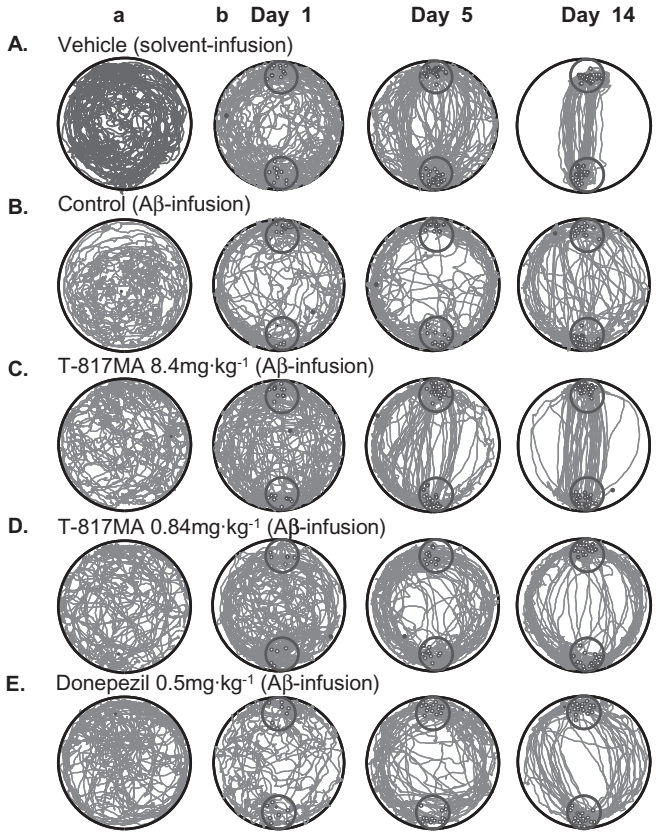

Figure 4 Examples of changes in navigation patterns of the five groups of the rats. (A–E) Trails of the rats in the vehicle (A), amyloid-β peptide (Aβ) infusion control (B), Aβ infusion + high-dose T-817MA [1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate] (C), Aβ infusion + low-dose T-817MA (D) and Aβ infusion + donepezil (E) are indicated. (a), trails in the last trial of the random reward place search task; (b), trails in the place learning task on the indicated days. Other descriptions as for Figure 2B.

behaved similarly to those in the Aβ infusion control group (Figure 4D).

Mean numbers of rewards acquired per trial in the PLT

In the present study, the maximum time for a trial was 10 min. Therefore, the more efficiently and quickly the rats moved between the two reward places in the PLT, the more rewards they acquired. Furthermore, the maximum number of rewards that could be acquired in a trial was set at 50. Consequently, the rats that learned the task more efficiently and quickly took less time to gain the maximum number of rewards. We used this parameter (number of rewards) as an index to evaluate learning of the rats. As shown in Figure 5A, the number of rewards acquired per trial increased progressively in the vehicle-treated rats, from the first day to the maximum number by the seventh day, and they then maintained this level (50 rewards) throughout the rest of the testing period.

This parameter increased slowly in the Aβ infusion control group from the first day to the fourteenth day. In contrast with this group, the number of rewards increased in the Aβ infusion + high-dose T-817MA group, as quickly as the vehicle

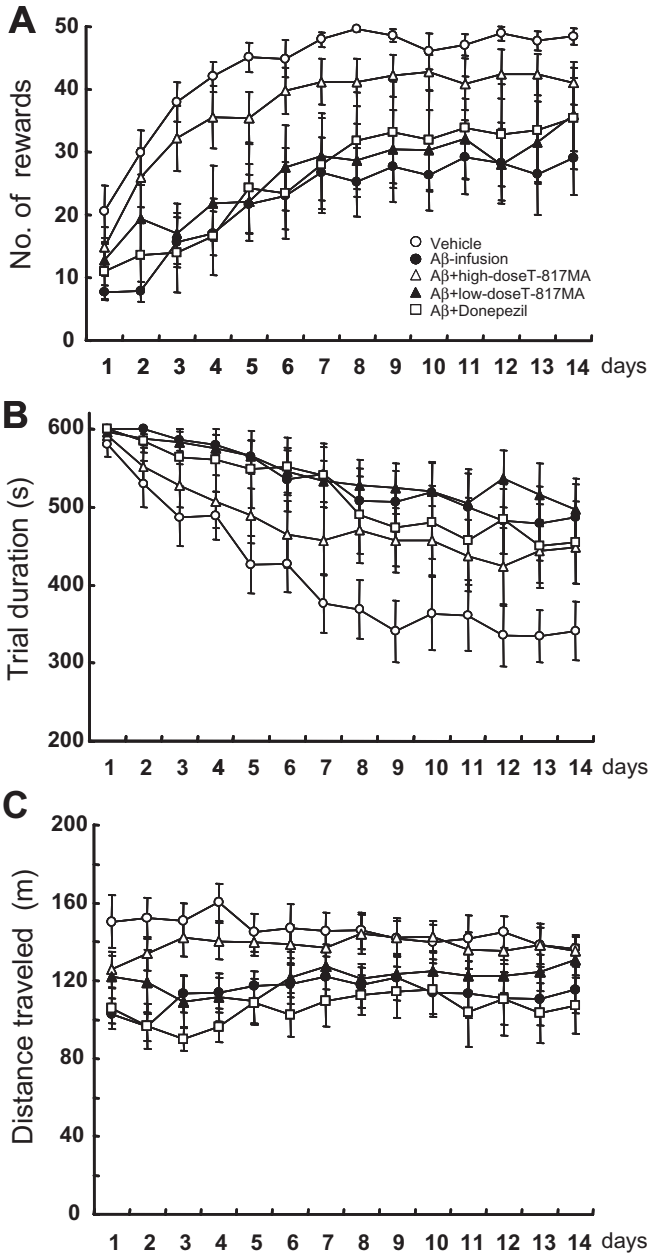

Figure 5 Effects of T-817MA on behavioural performance in the PLT. (A–C) Mean number of rewards acquired per trial (A), mean trial duration (B) and mean navigation distance travelled per trial (C) in the PLT. Each point with vertical bars indicates the mean ± SEM. Abscissa, experimental days. Aβ, amyloid-β peptide; PLT, place learning task; T-817MA, 1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate.

group. The rats in the Aβ infusion + low-dose T-817MA group acquired less rewards than the Aβ infusion + high-dose T-817MA group. Meanwhile, the rats in Aβ infusion + donepezil group showed a similar pattern to that in the Aβ infusion control group.

Statistical analyses by two-way ANOVA (group × day) indicated significant main effects of group [$F(4, 42) = 4.683$, $P < 0.05$] and day [$F(13, 546) = 40.342$, $P < 0.01$], with no significant interaction between group and day [$F(52, 546) = 0.852$, $P > 0.05$]. Post hoc comparisons revealed that the vehicle and

Aβ infusion + high-dose T-817MA groups acquired significantly more rewards than the Aβ infusion control group respectively (Bonferroni's test, $P < 0.01$, $P < 0.05$, respectively).

Mean trial duration in the PLT

As the maximum number of rewards in one trial was set at 50, the trial duration became shorter if the rats performed the PLT more efficiently and quickly. As shown in Figure 5B, the rats in the vehicle-treated group learned the task rapidly, that is, the trial duration decreased steeply from the first day to the fourteenth day. However, the trial durations for the Aβ infusion control, the Aβ infusion + donepezil and the Aβ infusion + low-dose T-817MA groups of rats showed much less reduction over the 14 days. The rats in the Aβ infusion + high-dose T-817MA displayed an intermediate rate of reduction in trial duration

Statistical analyses by two-way ANOVA (group × day) indicated significant main effects of group [$F(4, 42) = 3.062$, $P < 0.05$] and day [$F(13, 546) = 29.901$, $P < 0.01$]. There was no significant interaction between group and day [$F(52, 546) = 1.282$, $P > 0.05$]. Post hoc comparisons revealed that trial duration in the Aβ infusion control group was significantly longer than those in the vehicle group (Bonferroni's test, $P < 0.01$), but was not significantly different from the Aβ infusion + high-dose T-817MA group.

Mean navigation distance travelled per trial in the PLT

Figure 5C shows the mean distance travelled during the 14 days of navigation learning. The mean distance gradually decreased from the fourth day to the fourteenth day in the vehicle group. In the Aβ infusion + high-dose T-817MA group, this parameter also gradually decreased throughout the testing period from fourth day to fourteenth day. On the other hand, in the Aβ infusion control, the mean navigation distance slightly increased from the fourth day to the fourteenth day. In the Aβ infusion + low-dose T-817MA and Aβ infusion + donepezil groups, the mean distance also increased from the fourth day to the fourteenth day.

Statistical analyses compared by two-way ANOVA (group × day) indicated a significant main effect of group [$F(4, 42) = 3.769$, $P < 0.05$] with no significant main effect of day [$F(13, 546) = 0.932$, $P > 0.05$], nor significant interaction between group and day [$F(52, 546) = 0.985$, $P > 0.05$]. Post hoc comparisons revealed that the mean distance travelled was significantly larger in the vehicle group and Aβ infusion + high-dose T-817MA group than the Aβ infusion control group respectively (Bonferroni's test, $P < 0.01$, $P < 0.05$, respectively).

Effects on hippocampal neurogenesis

To confirm the involvement of Aβ toxicity in proliferation of neural progenitor cells in the dentate gyrus, immunostaining for PSA was performed and positive cells were counted. To also examine the effects of T-817MA and donepezil against Aβ toxicity, the effects of chronic administration of these drugs were analysed. Figure 6A–E shows typical immunostaining of PSA in the dentate gyrus. Fewer PSA-positive cells were found in the dentate gyrus of the Aβ infusion control group (Figure 6B), compared with the vehicle group (Figure 6A) and Aβ infusion + high-dose T-817MA group (Figure 6C). The mean density of PSA-positive cells in the dentate gyrus is shown in Figure 6F. The statistical analysis by one-way ANOVA indicated that there were significant differences in the mean density of PSA-positive cells among the five groups [$F(4, 40) = 7.855$, $P < 0.01$]. The post hoc test indicated that the mean density of PSA-positive cells was significantly larger in the vehicle and Aβ infusion + high-dose T-817MA groups than that in the Aβ infusion control group (Bonferroni's test, $P < 0.01$). However, there were no significant difference in the mean density of PSA-positive cells between the Aβ infusion control and Aβ infusion + low-dose T-817MA groups, nor between the Aβ infusion control and Aβ infusion + donepezil groups (Bonferroni's test, $P > 0.05$).

We also performed double staining of BrdU and NeuN to examine the effects of T-817MA and donepezil on the differentiation to neurons after Aβ infusion. Most BrdU-positive cells were co-localized with NeuN in the five groups of the rats (Figure 7A–C). The mean density of BrdU- and NeuN-double positive cells in the dentate gyrus is shown in Figure 7D. The statistical analysis by one-way ANOVA indicated that there were significant differences in the mean density of BrdU- and NeuN-double positive cells among the five groups [$F(4, 40) = 3.431$, $P < 0.01$]. The post hoc test indicated that the mean density of BrdU- and NeuN-double positive cells was significantly larger in the vehicle and Aβ infusion + high-dose T-817MA groups than that in the Aβ infusion control group (Bonferroni's test, $P < 0.01$). However, there were no significant differences in the mean density of BrdU- and NeuN-double positive cells in the dentate gyrus between the Aβ infusion control and Aβ infusion + low-dose T-817MA groups, nor between the Aβ infusion control and Aβ infusion + donepezil groups (Bonferroni's test, $P > 0.05$).

Relations between task performance and neurogenesis

The above results indicated the vehicle and Aβ infusion + high-dose T-817MA groups displayed efficient learning in the PLT, while these two groups also displayed vigorous neurogenesis. This implied a relationship between task performance and neurogenesis. Therefore, we analysed the correlation between behavioural performance tested in the PLT and cell proliferation using simple regression analysis. A positive correlation was found between the mean PLT reward number and the density of PSA-positive cell in the granule cell layer (Figure 8A). Analysis of variance indicated that the linear regression line fit to the data was statistically significant [$F(1, 43) = 17.207$, $P < 0.01$]. Similar results were obtained for the BrdU- and NeuN-double positive cells (Figure 8B). Analysis of variance indicated that the linear regression line fit to the data was statistically significant [$F(1, 43) = 12.887$, $P < 0.01$]. These results indicated that the animals with a higher level of neurogenesis displayed higher performance in place learning.

Granule cell count analysis

Histological changes in the hippocampus are shown in Figure 9. At a high magnification on a light microscope, the granule cell layer of the dentate gyrus was clearly observed in

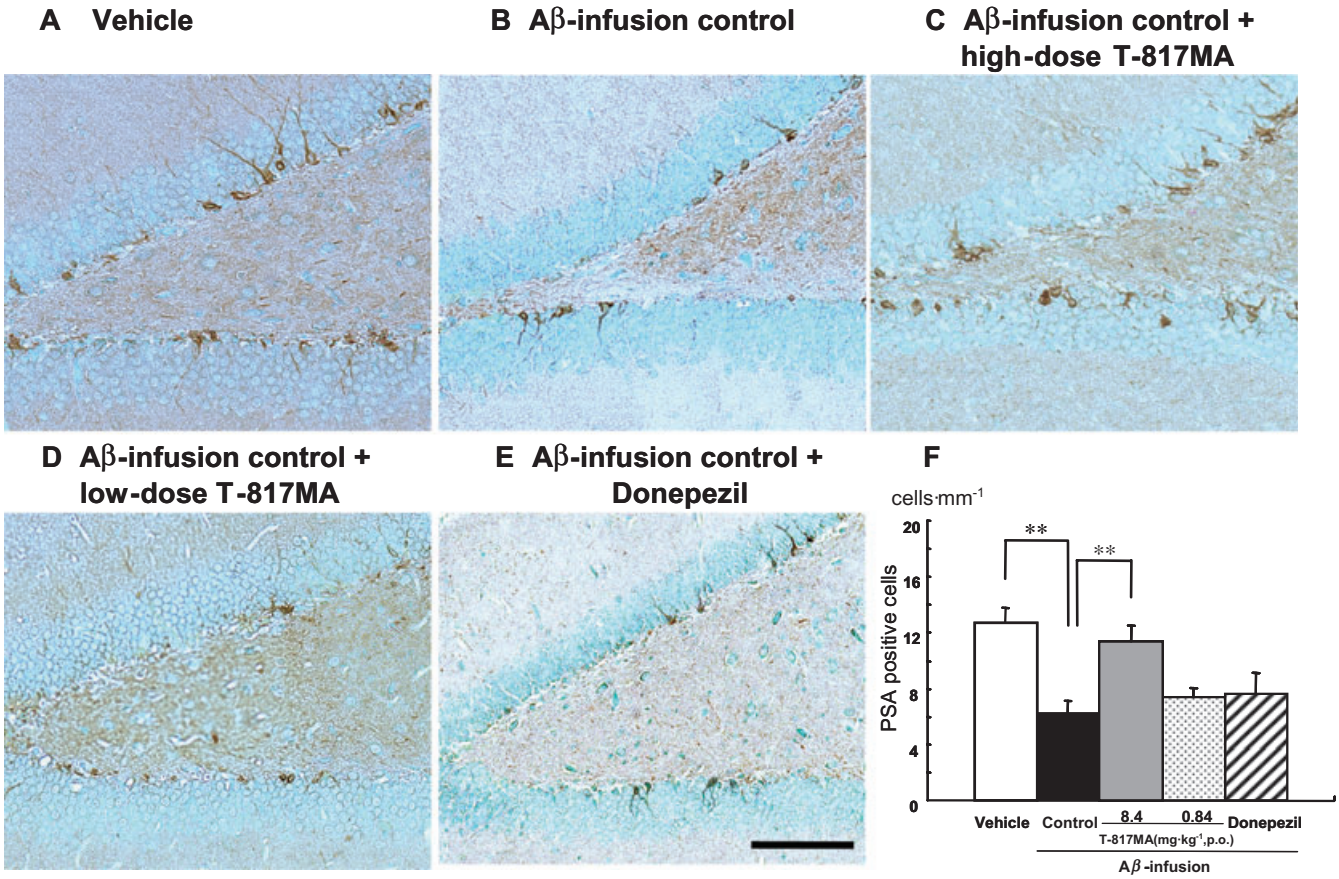

Figure 6 PSA expressing cells in the rat dentate gyrus. (A–E) PSA-positive cells in the vehicle (A), amyloid-β peptide (Aβ) infusion control (B), Aβ infusion + high-dose T-817MA [1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate] (C), Aβ infusion + low-dose T-817MA (D) and Aβ infusion + donepezil (E) groups. PSA-positive cells are located in the border of the granule cell layer. They extend apical dendrites ahead to the molecular layers. A horizontal line below the photomicrographs (E) shows scale bar: 200 μm. (F) Comparison of PSA-positive cell density in the dentate gyrus between the experimental groups. There were significant differences in granule cell density in the dentate gyrus. $^{**}P < 0.01$ versus control, Bonferroni's test.

the all groups (Figure 9A–E). The mean density of normal granule cells in the dentate gyrus is shown in Figure 9F. The statistical analysis by one-way ANOVA indicated that there were significant differences in the mean granule cell density among the five groups [$F(4, 40) = 16.908$, $P < 0.01$]. The post hoc test indicated that the mean density of granule cells was significantly larger in the vehicle group than that in the Aβ infusion control group (Bonferroni's test, $P < 0.01$). On the other hand, treatment with T-817MA and donepezil did not increase the mean density of normal granule cells; there were no significant differences in the mean granule cell density among the Aβ infusion control, Aβ infusion + high-dose T-817MA, Aβ infusion + low-dose T-817MA and Aβ infusion + donepezil groups (Bonferroni's test, $P > 0.05$).

Discussion

Effects of T-817MA and donepezil on place learning

In the present study, the Aβ infusion control rats with neuronal loss in the dentate gyrus showed slow spatial learning and low navigation efficacy, findings similar to those in our previous study using rats with dentate lesions by Aβ infusion (Nguyen *et al.*, 2007). In that study, chronic T-817MA administration was started from the same day when Aβ infusion started, and T-817MA completely blocked Aβ toxicity on granule cell survival. In the present study, we administered the drugs 4 weeks after the start of Aβ infusion, and the granule cell density was decreased even in the Aβ infusion + high-dose T-817MA group to the same degree as the Aβ infusion control group. These findings suggest that histological degeneration had already occurred before drug treatment. However, there were significant differences in task performance in the PLT between the Aβ infusion control and Aβ infusion + high-dose T-817MA groups, while there were no significant differences between the vehicle and Aβ infusion + high-dose T-817MA groups in any behavioural parameters. These findings indicated that the Aβ infusion + high-dose T-817MA rats performed as well as the vehicle-treated rats in the PLT. Thus, the present study demonstrated that treatment with high-dose T-817MA significantly ameliorated cognitive impairments of the Aβ infused rats.

Donepezil is an acetylcholinesterase inhibitor used for treatment of AD. In the present study using Aβ infusion model, daily administration of donepezil at a dose of 0.5 $mg \cdot kg^{-1} \cdot day^{-1}$ did not show significant amelioration of

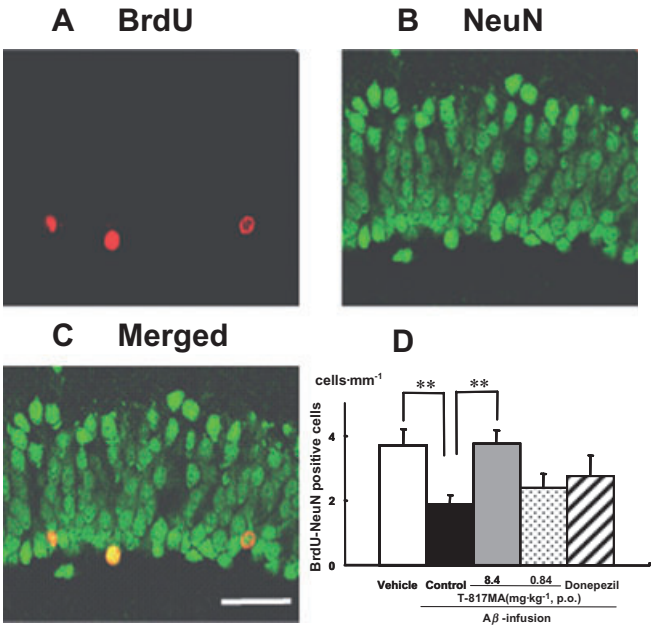

Figure 7 Double staining of bromodeoxyuridine (BrdU) and NeuN in the rat dentate gyrus. (A–C) Representative photomicrographs of BrdU-positive cells (red) (A), NeuN-positive cells (B) and NeuN- and BrdU-double positive cells (yellow) (C) in the vehicle group. A horizontal line below the photomicrographs (C) shows scale bar: 50 μm. (D) Comparison of mean density of BrdU- and NeuN-double positive cells in the dentate gyrus among the five groups (D). There were significant differences in granule cell density in the dentate gyrus. $^{**}P < 0.01$ versus control, Bonferroni's test. T-817MA, 1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate.

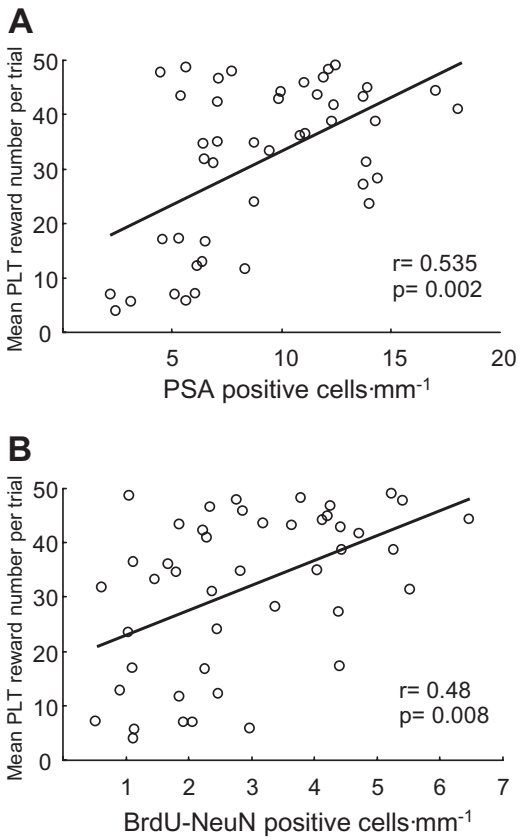

Figure 8 Relationship between task performance in the PLT and neurogenesis in the dentate gyrus. Task performance (mean number of ICSS rewards/trial) in the PLT was significantly correlated to cell proliferation in the dentate gyrus as measured by PSA- (A) and BrdU- and NeuN-double (B) positive cell density. BrdU, bromodeoxyuridine; ICSS, intracranial self-stimulation; PLT, place learning task.

behavioural deficits in the PLT nor neurogenesis in the dentate gyrus. Kotani *et al.* (2006) observed neurogenic effects of donepezil in normal rats at a dose of 0.5 mg·kg^{-1}·day^{-1} for 4 weeks, in that this treatment enhanced neurogenesis and scopolamine suppressed the survival of newly generated cells in the dentate gyrus. This discrepancy might be ascribed to differences between the normal animals and the animals with Aβ infusion, or those in other experimental procedures such as treatment schedule.

Effects of Aβ infusion on adult neurogenesis

In the present study, dividing cells were labelled with BrdU just before starting the PLT task. The number of BrdU-positive cells in the dentate gyrus was increased by T-817MA against chronic Aβ infusion. PSA-NCAM is a marker of proliferating cells, and the number of immuno-reactive cells was increased after T-817MA treatment, indicating that it stimulated proliferation of neural progenitor cells in the dentate gyrus. On the other hand, the increase in BrdU-positive cells induced by T-817MA treatment indicates that survival of the newly generated cells was enhanced. Continuous infusion of Aβ decreased PSA-positive and BrdU-positive cells, indicating that it suppressed both proliferation of neural progenitor cells and survival of newly generated cells in the dentate gyrus.

These results are consistent with previous reports that injection of Aβ(25–35) inhibited neurogenesis and significantly decreased the number of BrdU-positive cells in the dentate gyrus of the hippocampus (Li and Zuo, 2005), and that neurogenesis was disturbed in the PDAPP mouse, an animal model of AD with age-dependent accumulation of amyloid-42 (Aβ42)-containing plaques (Haughey *et al.*, 2002; Dong *et al.*, 2004; Donovan *et al.*, 2006). Deficient adult hippocampal neurogenesis was also reported in other AD mouse models with mutations similar to human AD in APP or presenilin (Feng *et al.*, 2001; Wen *et al.*, 2002; Wang *et al.*, 2004). These findings are consistent with the idea that AD-related pathology including elevated brain levels of Aβ42 impairs adult neurogenesis. It is noted that the three learning tasks were imposed on the animals in the present study and several reports have demonstrated that some kinds of learning enhanced adult neurogenesis (Gould *et al.*, 1999; Snyder *et al.*, 2005). These findings suggest that Aβ might disturb learning, which would consequently decrease learning-induced neurogenesis in the present study. However, the previous studies reported that Aβ decreased adult neurogenesis, even in naive animals, under a control condition without any learning tasks (Feng *et al.*, 2001; Haughey *et al.*, 2002; Wen *et al.*, 2002; Dong *et al.*, 2004; Wang *et al.*, 2004; Li and Zuo, 2005; Donovan *et al.*, 2006). These findings suggest that Aβ might have a direct effect on progenitor cells, thereby preventing proliferation and differentiation.

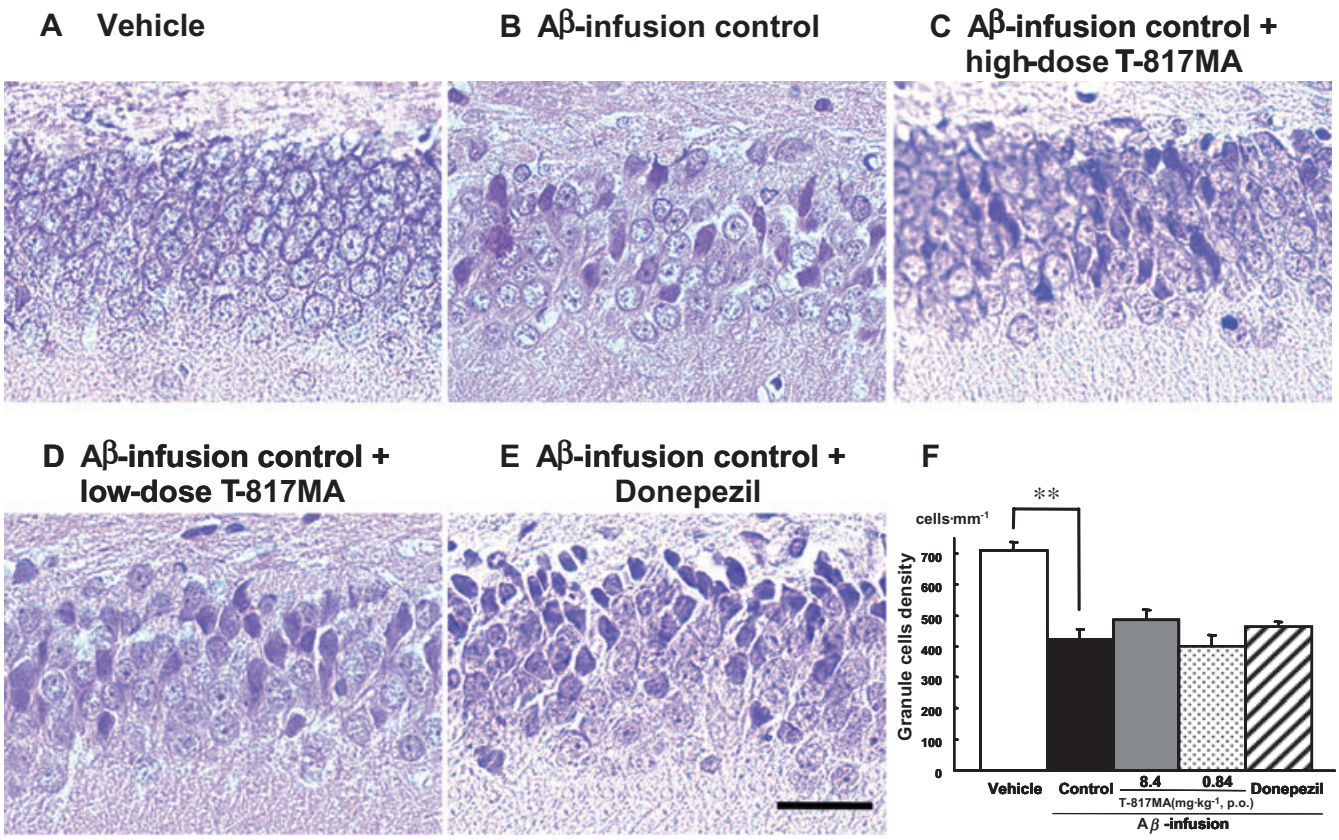

Figure 9 Histological evaluation by haematoxylin and eosin (H&E) staining of the rat dentate gyrus. (A–E) Photomicrographs of the dentate gyrus in vehicle (A), amyloid-β peptide (Aβ) infusion control (B), Aβ infusion + high-dose T-817MA [1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-azetidinol maleate] (C), Aβ infusion + low-dose T-817MA (D) and Aβ infusion + donepezil (E) groups. A horizontal line below the photomicrographs (E) shows scale bar: 50 μm. (F) Comparison of surviving (normal) granule cell density in the dentate gyrus among the five groups. There were significant differences in granule cell density in the dentate gyrus. $^{**}P < 0.01$ versus control, Bonferroni's test.

However, there are reports that neurogenesis was increased in the $APP_{Sw, Ind}$ mice (Jin *et al*., 2004a) and in AD post-mortem tissues (Jin *et al*., 2004b). This apparent inconsistency could be ascribed to methodological differences. For example, increases in progenitor cells in the dentate gyrus (both studies) and increased progenitor and other protein levels in whole hippocampal dissections (post-mortem study) were interpreted as evidence of enhanced adult neurogenesis. However, many immature neurons in the adult mammalian hippocampus never reach maturity (Gould, 1994). Therefore, assessment of adult neurogenesis requires quantification of survival as well as proliferation and differentiation. Further studies are required to determine whether neurogenesis is increased in AD patients.

Place learning and neurogenesis

Neural progenitor cells are multipotent. Depending upon composition of trophic and neurotrophic factors in their microenvironment, the neural progenitors can differentiate into the three cell types in the brain: neurons, astroglia and oligodendroglia. In the adult mammalian brain, two active centres for the generation of progenitor cells exist: the subgranular zone of the dentate gyrus of the hippocampal formation and the subventricular zone. The adult dentate gyrus has a unique property of persistent neurogenesis (Eriksson *et al*., 1998; Roy *et al*., 2000), which is detectable even at old age, but at a slower rate (Kuhn *et al*., 1996; Kempermann *et al*., 1997). Neurogenesis is required to maintain memory and associated learning (Gould *et al*., 1999; Shors, 2004; Snyder *et al*., 2005). Thus, depression of neurogenesis could be involved in an age-associated decline of hippocampal learning (Kuhn *et al*., 1996; Cameron and McKay, 1999).

In the present study, we examined whether proliferation of newly generated cells (neural progenitor cells) was associated with place learning performance. The results indicated a significant positive correlation between learning ability and the number of PSA-positive cells. Survival of newly generated cells and differentiation to a neuronal phenotype was also correlated with learning ability of the Aβ-infused rats. The animals showing the best behavioural performance also displayed the highest numbers of BrdU- and NeuN-positive cells that survived 3 weeks after their birth. That is, more cells differentiated to neurons in the Aβ-infused rats that showed better learning performance than in the vehicle group. These results indicate that a certain level of neurogenesis is required to maintain normal place learning.

The number of newly generated neurons was significantly different between the Aβ infusion control and Aβ infusion + high-dose T-817MA groups, while the mean density of

granule cells was the same in the both group rats. It is supposed that the number of granule cells newly produced by neurogenesis in adulthood is relatively small and quantitatively negligible, compared with a total pool of 1 or 2 million mature granule cells. These results suggest that addition of young granule cells to the hippocampal circuits rather than their total number itself is more important to maintain learning ability (Drapeau *et al*., 2003). However, further studies are required to determine how hippocampal neurogenesis influences the granule cell/mossy fibre/Ammon's horn neural networks and subsequently cognitive performance.

Possible mechanisms of T-817MA

Chronic oral treatment with T-817MA almost completely prevented toxicity due to Aβ infusion in the dentate gyrus (Nguyen *et al*., 2007). This was consistent with a previous study *in vitro* reporting that T-817MA protected cultured neurons from Aβ toxicity, through modulation of endogenous antioxidative mechanisms rather than scavenging of reactive oxygen radicals (Hirata *et al*., 2005).

Interestingly, Hirata *et al*. (2005) also suggested that T-817MA might work as a neurotrophic factor, such as IGF-1, to promote neurite outgrowth. In the adult rat brain, IGF-1 induces proliferation of hippocampal progenitors, followed by neurogenesis and enhancement of spatial memory (Aberg *et al*., 2000; Lichtenwalner *et al*., 2001; Emsley and Hagg, 2003; Tatebayashi *et al*., 2003). Furthermore, IGF-1 regulates hyperphosphorylation of tau via the PI3-kinase/GSK-3 signal transduction cascade (Hong and Lee, 1997; Lesort and Johnson, 2000; Avila, 2004) and metabolism and clearing of intracellular and extracellular Aβ from primary neurons and from the brains of Tg2576 transgenic mice (Gasparini *et al*., 2001; Carro *et al*., 2002). It is noteworthy that these transgenic mice displayed a lower blood levels of IGF-1 than their non-transgenic littermates (Carro *et al*., 2002), consistent with the data in individuals with familial AD (Mustafa *et al*., 1999). As T-817MA might work as a neurotrophic factor, such as IGF-1 (Hirata *et al*., 2005), these results suggest that neurotrophic effects of T-817MA might be beneficial by activating neurogenesis and thus allowing AD patients to recover from learning and memory deficits. This possibility is supported by our finding, in rats, that chronic T-817MA treatment increased the number of PSA-positive and BrdU-positive cells in the dentate gyrus of the hippocampus when they had been decreased by chronic Aβ infusion. The present results suggest that the central neurotrophic system is critically involved in neurogenesis in the adult dentate gyrus. It would be interesting to see whether T-817MA could increase neurogenesis in control naive animals. If T-817MA has neurotrophic effects *in vivo*, this compound should also increase neurogenesis in control animals. Further studies will be required to clarify mechanisms of T-817MA to stimulate neurogenesis.

In conclusion, the present results demonstrated that T-817MA, given orally over 4 weeks, improved place learning in the Aβ-infused rats and that this treatment enhanced neurogenesis in the dentate gyrus. Furthermore, the degree of neurogenesis in the dentate gyrus was correlated with learning ability in the spatial task. These results suggest that T-817MA appears to be a candidate for the treatment of memory disorders related to neurodegenerative diseases such as AD.

Conflict of interest

Dr Tatsuo Kimura is now an employee of Toyama Chemical Co. Ltd. that developed T-817MA.

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