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RESEARCH PAPER

Promotion of axonal maturation and prevention of memory loss in mice by extracts of *Astragalus mongholicus*

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Background and purpose: Neurons with atrophic neurites may remain alive and therefore may have the potential to regenerate even when neuronal death has occurred in some parts of the brain. This study aimed to explore effects of drugs that can facilitate the regeneration of neurites and the reconstruction of synapses even in severely damaged neurons.
Experimental approach: We investigated the effects of extracts of *Astragalus mongholicus* on the cognitive defect in mice caused by injection with the amyloid peptide Aβ(25-35). We also examined the effect of the extract on the regeneration of neurites and the reconstruction of synapses in cultured neurons damaged by Aβ(25-35).
Key results: *A. mongholicus* extract (1 g kg^{-1} day^{-1} for 15 days, p.o.) reversed Aβ(25-35)-induced memory loss and prevented the loss of axons and synapses in the cerebral cortex and hippocampus in mice. Treatment with Aβ(25-35) (10 μM) induced axonal atrophy and synaptic loss in cultured rat cortical neurons. Subsequent treatment with *A. mongholicus* extract (100 μg/ml) resulted in significant axonal regeneration, reconstruction of neuronal synapses, and prevention of Aβ(25-35)-induced neuronal death. Similar extracts of *A. membranaceus* had no effect on axonal atrophy, synaptic loss, or neuronal death. The major known components of the extracts (astragalosides I, II, and IV) reduced neurodegeneration, but the activity of the extracts did not correlate with their content of these three astragalosides.
Conclusion and implications: *A. mongholicus* is an important candidate for the treatment of memory disorders and the main active constituents may not be the known astragalosides.
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Keywords: dementia; axon; synapse; neuronal death; Astragali radix; *Astragalus mongholicus*; astragalosides; morris water maze; A*β*(25–35)

Abbreviations: NF-H, neurofilament-H; NGF, nerve growth factor

Introduction

In addition to the death of neurons, atrophy of neurites and loss of synapses are the major causes of dysfunctions of the brain including Alzheimer's (DeKosky and Scheff, 1990; Terry *et al*., 1991; Dickson and Vickers, 2001), Parkinson's, Huntington and Creutzfeldt-Jakob diseases (Jackson *et al*., 1995; Liberski and Budka, 1999; Mattila *et al*., 1999). Neurons with atrophic neurites may remain alive and therefore may have the potential to regenerate even when neuronal death has occurred in some parts of the brain. We have hypothesized that reconstructing neuronal networks in the injured brain is essential for the recovery of brain function (Tohda *et al*., 2005). To reconstruct neuronal networks, neurites must be regenerated and synapses must be reconstructed. In the current studies, we explored the *in vitro* and *in vivo* effects of drugs that can facilitate the regeneration of neurites and the reconstruction of synapses even in severely damaged neurons.

Astragali radix (the root of *Astragalus mongholicus* Bunge or *A. membranaceus* Bunge) is used mainly as a tonic agent in traditional Chinese and Japanese Kampo medicine. A few reports show that Astragali Radix extract or its components can affect brain function. For example, Astragali Radix extract can upregulate muscarinic acetylcholine receptors in senile rats (Shi *et al*., 2001). Also, a mixture of astragalosides, the major components of Astragali Radix, can improve

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memory in aged mice (Lei *et al*., 2003), and astragaloside IV can reduce brain infarction in mice after focal ischemia (Luo *et al*., 2004). Despite these reports, the effect of Astragali Radix extract in Alzheimer's disease models has not been assessed and how it reverses memory dysfunction has not been determined.

Amyloid β (Aβ) is thought to be a major pathological cause of Alzheimer's disease. Although not found in Alzheimer's brain tissue, Aβ(25–35) is a partial fragment of Aβ that similarly forms a β-sheet structure (Pike *et al*., 1995) and induces neuronal cell death (Yankner *et al*., 1990; Pike *et al*., 1995), neurite atrophy (Grace *et al*., 2002; Tohda *et al*., 2004), synaptic loss (Grace *et al*., 2002; Tohda *et al*., 2003, 2004) and memory impairment (Maurice *et al*., 1996; Tohda *et al*., 2003, 2004). We previously showed that mice injected with Aβ(25–35) suffer from memory impairment and neurite and synaptic atrophy (Tohda *et al*., 2003, 2004; Kuboyama *et al*., 2005, 2006).

In this study, we have investigated the effects of *A. mongholicus* extract on the cognitive defect in mice caused by injection of Aβ(25–35). We also examined the effect of the extract on the regeneration of neurites and the reconstruction of synapses in cultured neurons damaged by Aβ(25–35).

Methods

Preparation of extracts

The aqueous extract of *A. mongholicus* (Uchida Wakan-yaku, Tokyo, Japan) was prepared as follows. Fifty grams of powdered Astragalis radix were placed in 600 ml water, and boiled for 1 h. The decoction was evaporated under reduced pressure and freeze-dried to obtain 16.23 g of extract powder. This extract was dissolved in tap water for animal experiments. The extract was given orally (1 g kg^{-1}, 400 μl).

The methanol extracts of *A. mongholicus* (Uchida Wakan-yaku) and *A. membranaceus* (Tochimoto Tenkaido, Osaka, Japan) were prepared as follows. Fifty grams of the preparation of radix from each plant were extracted in methanol (500 ml $\times$ 2) for (36 h and 24 h) at room temperature. The combined supernatants were evaporated to obtain the methanol extracts. The extracts were dissolved in dimethyl sulphoxide (DMSO) for primary culture experiments.

Animals

Conditions. Male ddY mice (8 weeks old, Japan SLC, Shizuoka, Japan) were housed with free access to food and water, and were kept in a constant environment (22 $\pm$ 2°C, 50 $\pm$ 5% humidity, 12-h light cycle starting at 07:00). Animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama, and all protocols were approved by the Animal Care Committee of the University of Toyama.

Treatments. Aβ(25–35) was dissolved in saline at a concentration of 5 mM and incubated at 37°C for 4 days to allow for fibril formation. The mice were anesthetized with chloral hydrate (375 mg kg^{-1}, i.p.), and Aβ(25–35) (25 nmol) or the vehicle (saline) was injected into the right ventricle, with stereotaxic coordinates from the Bregma being, in mm, A −0.22, L −1.0 and V 2.5. Seven days after an intracerebroventricular (i.c.v.) injection of Aβ(25–35), the aqueous water extract of *A. mongholicus* (1 g kg^{-1} day^{-1}) or the vehicle (tap water) was administered orally once daily for 15 days.

Water maze test

White-colored water was poured into a circular pool (diameter, 120 cm; height, 28 cm), and a white platform (diameter, 12 cm) was placed 1.2 cm below the water level in the middle of a fixed quadrant. The water temperature was adjusted to 21–23°C. Training trials were performed four times daily for 7 days to reach a steady escape latency. Briefly, at 1.5 h after p.o. administration of the drug, the mice were allowed to swim freely for 60 s and were left for an additional 30 s on the platform. The interval during four trials was 90 min. The pattern of the start positions in each trial was changed every day. Mice failing to find the platform were manually placed on the platform.

Memory-retention tests were performed 7 days after the last training session, that is, 7 days after the discontinuation of p.o. administration of the drugs. The platform was removed, and each mouse was allowed a free 60-s swim. The number of crossings over the point where the platform had been located was counted by video recorder replay.

Immunohistochemistry

Three days after the retention test, the mice were killed by decapitation. The brains were quickly removed from the skull, and frozen in powdered dry ice. The brains were cut in 12-μm coronal sections using a cryostat (CM3050S, Leica, Heidelberg, Germany). The slices were fixed by 4% paraformaldehyde and stained with a monoclonal antibody to phosphorylated neurofilament-H (NF-H) or synaptophysin. Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin (Ig)G was used as the secondary antibody. The fluorescence images were captured using a fluorescence microscope (AX-80, Olympus, Tokyo, Japan) at 662 $\times$ 880 μm, and three sets of serial brain slices from three mice were used to capture the images for each treatment. The areas positive for phosphorylated NF-H or synaptophysin were measured in five brain regions (ATTO densitograph, ATTO, Tokyo, Japan). In each region, the measuring points were randomly selected as 20 squares of 41.5 $\times$ 41.5 μm.

Primary culture of cortical neurones

Embryos were removed from pregnant Sprague–Dawley rats (Japan SLC) at 18 days of gestation. The cortices were dissected and the dura mater was removed. The tissues were minced and dissociated, and were then grown in cultures with Neurobasal medium including 12% horse serum on eight-well chamber slides (Falcon, Franklin Lakes, NJ, USA) coated with poly-D-lysine at 37°C in a humidified incubator with 10% CO_2. When Aβ(25–35) or other compounds were added, half of the medium in each well was replaced with fresh medium containing 2% B-27 supplement without serum. In cases of long-term culture (for synaptophysin

staining), half of the medium in each well was replaced every 7 days with serum-free medium containing the 2% B-27 supplement after initiation of the culture period. The time schedules of the experiments are shown below each Figure.

Analysis of axonal outgrowth

Rat cortical neurons were cultured in eight-well chamber slides at a density of 1.18×10^5 cells cm^{-2}. The cells were then treated with 10 μM Aβ(25–35) for 3 days, followed by addition of the extract, a compound, mouse β-nerve growth factor (NGF) or vehicle (0.1% DMSO). Twelve days later, the cells were fixed by 4% paraformaldehyde, and were immunostained with a monoclonal antibody to phosphorylated NF-H (1:1000) as an axonal marker. Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200) was used as a second antibody. The fluorescence images were captured by a fluorescence microscope (AX-80) at 450×600 μm, and four images were captured per treatment. The lengths of neurites which were positive for phosphorylated NF-H were measured using an image analyzer (Scion Image, Scion, Frederick, MD, USA) for each cell.

Analysis of synaptic formation

Rat cortical neurons were cultured in eight-well chamber slides at a density of 1.18×10^5 cells cm^{-2} for 28 days. The cells were treated with 10 μM Aβ(25–35) for 4 days, and then test compounds were administered. Four days after administration, half of the medium in each well was replaced with fresh medium containing each test compound. Then, 10 days after administration of compounds, the cells were fixed and double-immunostained with a combination of a monoclonal antibody to synaptophysin (1:500) as a presynaptic marker and an antiserum to microtubule-associated protein 2 (MAP2) (1:1000). Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:200) were used as secondary antibodies. The fluorescence images were captured using a confocal laser scanning microscope (Radiance 2100, Bio-Rad, Hercules, CA, USA) at 205×205 μm, and four images were captured per treatment. The area of positive puncta to synaptophysin on each dendrite was measured using an image analyzer (ATTO densitograph, ATTO). The length of the dendrites was measured with Scion Image software (Scion).

Quantifying the content of astragalosides in Astragalus extracts

Each standard astragaloside (10 mg) was accurately weighed into a 10 ml volumetric flask and dissolved in methanol. To obtain calibration curves, a series of standard solutions were prepared from the stock solution and filtered through a 0.2 μm Millipore filter (Advantec, Tokyo, Japan). One gram of methanol extract powder was accurately weighed and dissolved in 50 ml water. After extraction with 20 ml ethyl acetate twice, the aqueous phase was further extracted with 20 ml *n*-butanol three times. The combined extracts were evaporated and the residue dissolved in 2 ml of 43% aqueous acetonitrile. After filtration through a 0.45 μm Milllipore filter (Advantec), 10 μl was injected into the high-pressure liquid chromatography (HPLC) system for analysis. The JASCO HPLC system (Jasco, Tokyo, Japan) is composed of a CO-1580 intelligent HPLC pump, a DG-1580-53 3-line degaser, a LG-1580-02 ternary gradient unit, a CO-1565 intelligent column oven, an AS-2057 plus intelligent sampler and a MD-1510 diode array detecter. Comparative analysis was carried out using a Mightysil RP-18GP (15 mm, 250×4.6 mm i.d.) with column temperature at 40°C. The mobile phase was acetonitrile:water = 43:57. The flow rate was 1.0 ml/min and detection wavelength was 205 nm. The chromatographic data were collected and processed using BORWIN-PDA Application and BORWIN chromatography Software (version 1.5, Jasco).

Cell viability

Viability of cortical neurons was measured by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Statistical analysis

Statistical comparisons were carried out using one-way analysis of variance followed by Dunnett's *post hoc* test. Values of $P < 0.05$ were considered significant. The means of the data are presented together with the s.e.m.

Materials

The amyloid peptide Aβ(25–35) (Sigma, Saint Louis, MO, USA) was dissolved in sterile distilled water at a concentration of 5 mM and 1 mM, respectively, and were incubated at 37°C for 4 days to allow fibril formation. Neurobasal media and B-27 supplement were purchased from Gibco BRL (Rockville, MD, USA). Mouse β-NGF was purchased from Astral Biologicals (San Ramon, CA, USA). A monoclonal antibody to phosphorylated NF-H was purchased from Sternberger Monoclonals Incorporated (Lutherville, MD, USA). An antiserum to MAP2 was purchased from Chemicon (Temecula, CA, USA). Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR, USA).

Astragalosides I, II and IV were isolated from *A. mongholicus*, and were identified by ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR.

Results

Aqueous extract of A. mongholicus *ameliorates the impairment of spatial memory caused by injection of Aβ(25–35)*

We previously confirmed that neurites and synapses are lost in the hippocampus and cerebral cortex of mice 7 days after the i.c.v. administration of Aβ(25–35) and that the losses continue for at least 14 days (unpublished data). We also confirmed that a spatial memory deficit occurs 14 days after the i.c.v. administration of Aβ(25–35) (data not shown). Therefore, mice were given the aqueous extract of *A. mongholicus* (p.o.) starting 7 days after the i.c.v. administration of Aβ(25–35), when neuronal and synaptic loss had already begun (see the lower panel of Figure 1 for a schematic

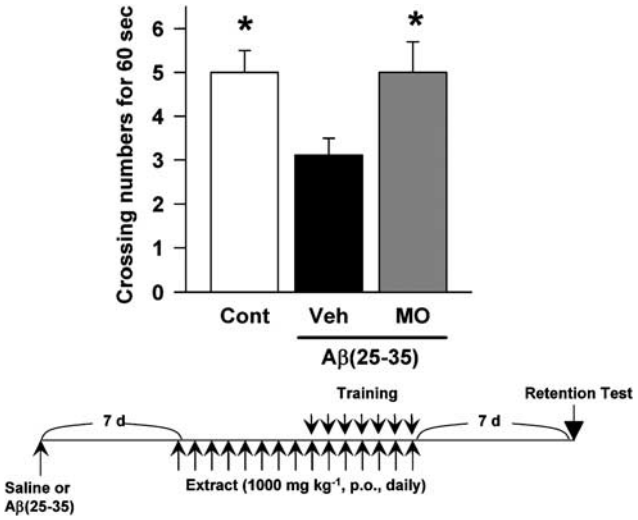


Figure 1 Effect of *A. mongholicus* on spatial memory deficit induced by Aβ(25–35). Aβ(25–35)-injected mice were given vehicle (Veh) or the aqueous extract of *A. mongholicus* (MO) for 15 days. The control mice (Cont) were injected with saline and then given vehicle. Seven days after the last drug treatment and the last training in a Morris water maze, the number of crossings over the position where the platform had been located was measured for 60 s. The results indicate the means$\pm$s.e.m. of 9–11 mice. *$P<0.05$ vs Veh.

illustration of the experimental design). The mice continued to receive the extract every day for 7 days, after which the mice were trained in a Morris water maze and continued to receive the extract every day for 7 days. After the training was completed (i.e., 21 days after the i.c.v. administration of the Aβ peptide), the mice were subjected to a memory retention test, wherein the number of crossings over the platform position was counted. The number of crossings was significantly lower in the Aβ(25–35)-injected mice than the control (saline-treated) mice (upper panel of Figure 1). In addition, the number of crossings was significantly higher in the Aβ(25–35)-injected mice receiving the *A. mongholicus* water extract than those receiving vehicle. In contrast, the locomotory activities of the mice did not differ between the groups (data not shown).

Following the memory retention test, we measured the levels of phosphorylated NF-H and synaptophysin in the brains of the mice by immunohistochemistry. We examined two cortical regions (parietal cortex and temporal cortex) and three hippocampal regions (CA1, CA3 and dentate gyrus) which show neuronal degeneration in both Alzheimer's disease patients (DeKosky and Scheff, 1990; Heinonen *et al*., 1995) and a mouse model of Alzheimer's disease (Games *et al*., 1995). We found that, in Aβ(25–35)-injected mice, the number of NF-H-positive areas in all regions except for CA3 was higher in extract-treated mice than in vehicle-treated mice (Figure 2). As we reported previously (Kuboyama *et al*., 2005), the dentate gyrus was resistant to Aβ(25–35)-induced neuronal loss, although the mechanism of this resistance is not clear. Injection with Aβ(25–35) also reduced the expression of synaptophysin in all five-brain areas (Figure 3b). Finally, the loss and the synaptic density in Aβ(25–35)-injected mice was almost completely reversed by treatment with *A. mongholicus* extract.

Recovery from axonal atrophy and synaptic loss by treatment with the aqueous extract of *A. mongholicus* was particularly apparent in the cerebral cortex. Memory retention seems to be related to the consolidation of memory, which is thought to be carried out in the cerebral cortex rather than the hippocampus. Therefore, we next investigated the effects of *A. mongholicus* on axonal maturation in cortical neurons *in vitro*.

Methanolic extract of A. mongholicus *promotes axonal regeneration in damaged neurons*

We next examined the effect of methanolic extracts of *A. mongholicus* on neurite regeneration. Previously, we confirmed that the methanolic and aqueous extracts of *A. mongholicus* have the same abilities to promote neurite regeneration in rat cortical neurons (data not shown). In this set of experiments, we examined the effect of the methanolic extract. We also compared its effects with the methanolic extract of another type of Astragali Radix, from *A. membranaceus*. Furthermore, because astragalosides are known to be the main components of the Astragali Radix preparations, we also examined the effects of astragalosides I, II and IV (Figure 4). We measured the length of neurites after a 5-day treatment of rat cortical neurons with Aβ(25–35) or the test compounds (Figure 5a). The methanol extract of *A. mongholicus* (100 μg/ml), astragalosides I, II and IV (100 μM), and NGF (100 ng ml^{-1}) all significantly increased the axon length compared with neurons treated with Aβ(25–35) alone.

We next examined the ability of the extracts and pure components to promote axonal regeneration after axonal atrophy had already occurred. Therefore, the compounds were administered 3 days after treatment with Aβ(25–35), and axon lengths were measured after an additional 9 days (Figure 5b). We found that the axon lengths were shorter in the cells treated with Aβ(25–35) followed by vehicle than in control cells. The axon lengths were significantly longer in the Aβ(25–35)-treated cells when they were also treated with *A. mongholicus* extract and astragaloside II than when they were treated with vehicle alone. The extract of *A. membranaceus*, however, did not significantly enhance the extension of axons when added simultaneously (Figure 5a) or after treatment with Aβ(25–35) (Figure 5b). Dose–response experiments indicated that 100 μg/ml of *A. mongholicus* extract and 100 μM of astragalosides resulted in maximal axonal outgrowth (data not shown).

Synaptic reconstruction in damaged neurons

Determining whether regenerated neurites can also reconstruct synapses is essential. As *A. mongholicus* extract was able to regenerate axons in Aβ(25–35)-treated neurons (Figure 5), we next examined its effect on synaptic maturation. Rat cortical neurons were cultured for 28 days to allow development of mature synapses *in vitro* as described previously (Zhang and Benson, 2001). The cultures were treated for 4 days with Aβ(25–35). After that, cells were treated with test compounds for 10 days. Dendritic shafts were visualized by double-immunostaining with antibodies to synaptophysin

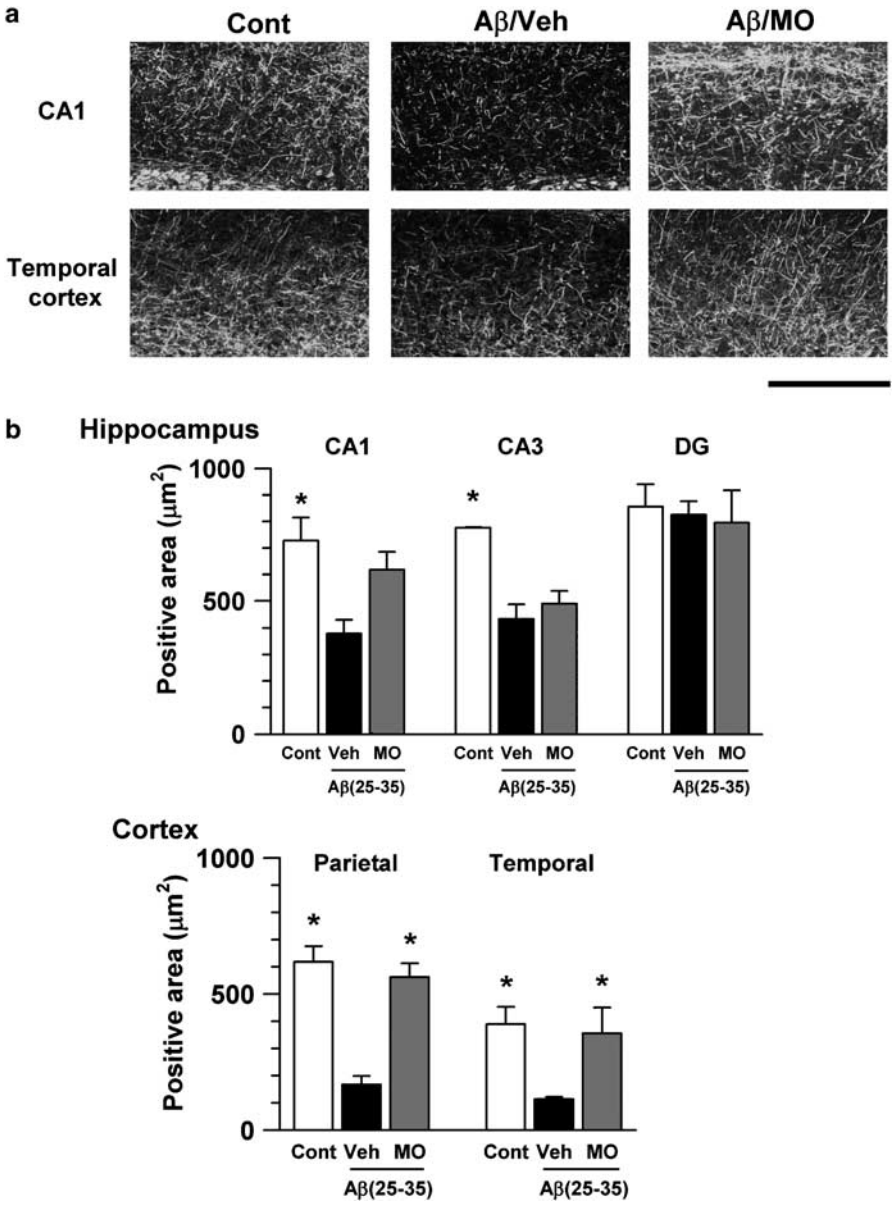


Figure 2 Effect of *A. mongholicus* on Aβ(25–35)-induced axonal atrophy *in vivo*. Three days after the retention test (Figure 1), mice were decapitated, and their brains were cut into coronal sections. The slices were immunostained with phosphorylated NF-H. (**a**) Representative images are shown from the hippocampal CA1 (upper) and the temporal cortex (below). Scale bar = 200 μm. (**b**) Quantitation of phosphorylated NF-H-positive areas in CA1, CA3, dentate gyrus (DG) and the parietal and temporal cortices. Cont, control; Aβ/Veh, mice injected with Aβ(25–35) and then treated with vehicle; Aβ/Veh, mice injected with Aβ(25–35) and then treated with the aqueous extract of *A. mongholicus*. The results represent the means $\pm$ s.e.m. of three mice. $^*P < 0.05$ vs Aβ/Veh.

and MAP2. Synaptophysin-positive puncta were present at the edge of the dendritic shafts (Figure 6a). The number and fluorescence intensity of synaptophysin-positive puncta on dendrites was visually decreased by treatment with Aβ(25–35). Quantification of the area synaptophysin-positive puncta confirmed that Aβ(25–35) caused a significant decrease in synapses (59.0% of control; Figure 6b). These results indicate that Aβ(25–35) caused the loss of synaptic structures in long-term cultured cortical neurons. This was reversed by treatment with *A. mongholicus* extract and astragaloside I; specifically, the number of synaptophysin-positive puncta was 119 and 94% of control, respectively. The extract from *A. membranaceus* and NGF had no effect on the synaptic density. Astragalosides II and IV showed similar abilities to promote axonal formation but no effect on synaptic formation. In contrast, astragaloside I enhanced only synaptic formation.

Protection from neuronal death

Aβ(25–35) is known to induce neuronal cell death, especially *in vitro* (Yankner *et al*., 1990). We therefore investigated the effects of the extracts and astragalosides on cell survival (Figure 7). Treatment of cortical neurons with 10 μM Aβ(25–35) for 3 days caused substantial cell death. Addition of *A. mongholicus* extract along with Aβ(25–35) completely

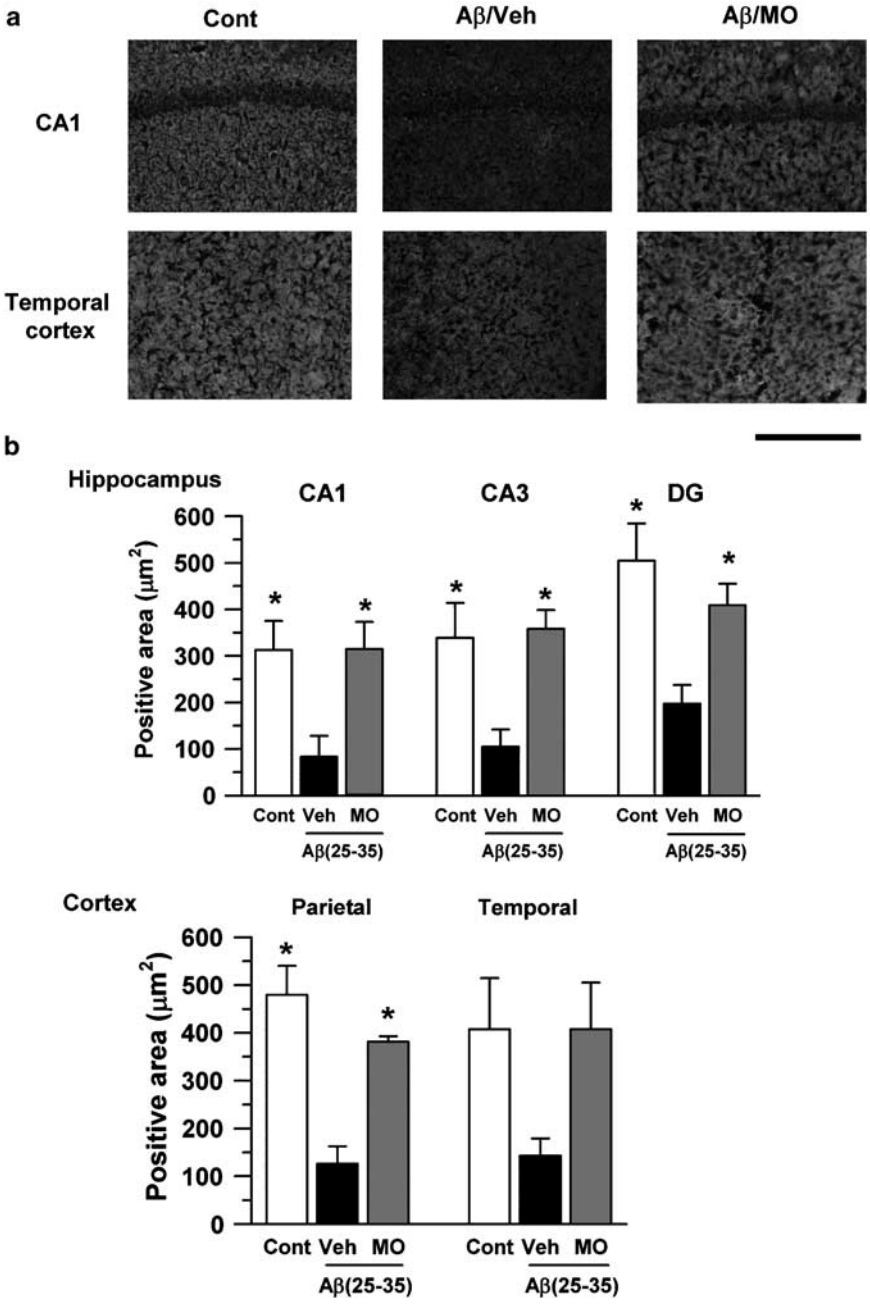


Figure 3 Effect of aqueous extract of *A. mongholicus* on Aβ(25–35)-induced synaptic loss *in vivo*. Mouse slices were immunostained with an antibody to synaptophysin. (**a**) Representative images are shown from the hippocampal CA1 (upper row) and the temporal cortex (lower row). Scale bar = 200 μm. (**b**) Quantitation of synaptophysin-positive areas in CA1, CA3, dentate gyrus (DG) and the parietal and the temporal cortices. Cont, control; Aβ/Veh, mice injected with Aβ(25–35) and then treated with vehicle; Aβ/Veh, mice injected with Aβ(25–35) and then treated with the aqueous extract of *A. mongholicus*. The results represent the means ± s.e.m. of three mice. *$P<0.05$ vs Veh.

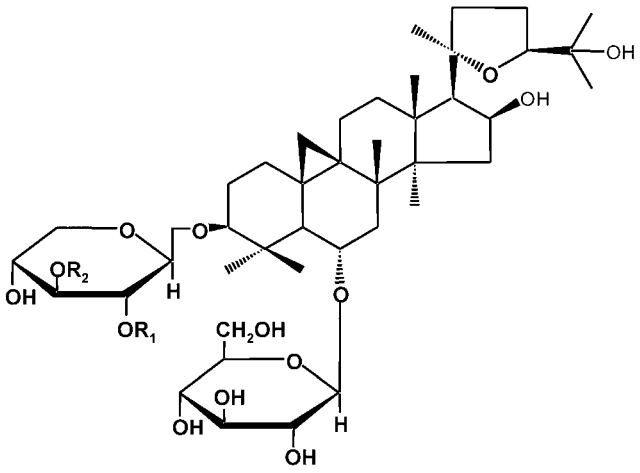
protected the neurons from cell death. In addition, astragaloside IV and NGF significantly protected the cells from Aβ(25–35)-induced neuronal death.

Contents of astragalosides in two species of Astragali Radix

We next investigated whether the difference in the effects of the *A. mongholicus* and *A. membranaceus* extracts is related to the astragalosides that they contain. We measured the levels of three astragalosides in the methanolic extracts by HPLC. Although there was more astragaloside I in the *A. mongholicus* extract than the *A. membranaceus* extract, the levels of astragalosides II and IV were higher in the *A. membranaceus* extract (Table 1).

Discussion and conclusions

In the current studies, we have demonstrated for the first time that administration of an aqueous extract of



	R1	R2
Astragaloside I	**Ac**	**Ac**
Astragaloside II	**Ac**	**H**
Astragaloside IV	**H**	**H**

Figure 4 Structures of astragalosides I, II and IV.

A. mongholicus improved memory deficit induced by Aβ(25–35) in mice. We also found that the extract reversed Aβ(25–35)-induced loss of axons and synapses in the cerebral cortex and hippocampus and that it promoted axonal extension and synapse formation in Aβ(25–35)-treated cortical neurons.

The effects of the extract on axonal extension by cortical neurons were examined using two experimental protocols. We found that the extract of *A. mongholicus* both prevented Aβ(25–35)-induced axonal atrophy (Figure 5a) and restored axonal outgrowth after atrophy (Figure 5b). In addition, the extract protected neurons from Aβ(25–35) induced cell death. The extract of another type of Astragali Radix, *A. membranaceus,* however, did not have a significant effect on neurite atrophy, synaptic loss or cell death.

The major components of Astragali Radix, astragalosides I, II and IV (100 μM), were also effective in the experiments using cortical neurons. Astragaloside I promoted axonal extension, synaptic formation and cell survival; astragaloside II promoted axonal extension; and astragaloside IV promoted axonal extension and cell survival. Each astragaloside was present at a concentration less than 1 μM in 100 μg/ml of *A. mongholicus* extract. Dose–response experiments, however, showed that astragalosides I, II and IV had weak but not significant effects at doses less than 1 μM (data not shown). In addition, the content of total astragalosides was higher in the extract from *A. membranaceus* than that from *A. mongholicus*, although the latter had no activity. We also isolated astraisoflavoneglucoside, astraisoflavanglucoside, astrapterocarpanglucoside and ononin from *A. mongholicus* to seek other active constituents and assayed their effects on axonal extension, synaptic formation, and cell survival. We found that they had no effect and were present at similar

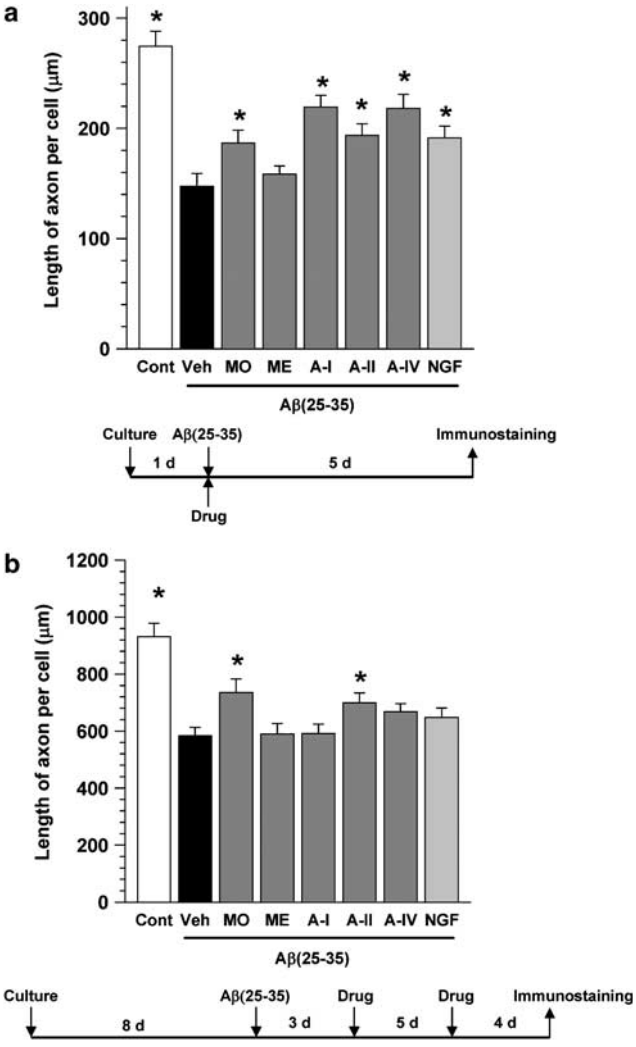


Figure 5 Effects of extracts and astragalosides on axonal regeneration following Aβ(25–35)-induced atrophy. (**a**) After culture for 1 day, the cortical neurons were treated with or without (Cont) Aβ(25–35). The cells were simultaneously treated with 100 μg ml^{-1} of the methanolic extract of *A. mongholicus* (MO) or *A. membranaceus* (ME); 100 μM of astragalosides I (A I), II (A II) or IV (A IV); 100 ng ml^{-1} of NGF; or vehicle (Veh). Five days after treatment, the cells were fixed and immunostained with an antibody to phosphorylated NF-H. The lengths of NF-H-positive neurites were measured for each treatment. The results represent the means$\pm$s.e.m. of 28–40 cells. *$P<0.05$ vs Veh. (**b**) Cortical neurons were cultured for 8 days and then treated with or without (Cont) 10 μM Aβ(25–35). Three days after the administration of Aβ(25–35), the cells were treated with 100 μg ml^{-1} of the methanolic extract of *A. mongholicus* (MO) or *A. membranaceus* (ME); 100 μM of astragalosides I (A I), II (A II) or IV (A IV); 100 ng ml^{-1} of NGF; or vehicle (Veh). Nine days after treatment, the cells were fixed and immunostained with an antibody to phosphorylated NF-H. The lengths of NF-H-positive neurites were measured for each treatment. The results represent the means$\pm$s.e.m. of 48–85 cells. *$P<0.05$ vs Veh.

concentrations in the *A. membranaceus* extract. These results suggest that unidentified components mediate the effects of the *A. mongholicus* extracts. Current experiments in our laboratory are attempting to identify which are the active compounds.

Although the extract of *A. mongholicus* inhibited Aβ(25–35)-induced neuronal death, this may not be the sole reason

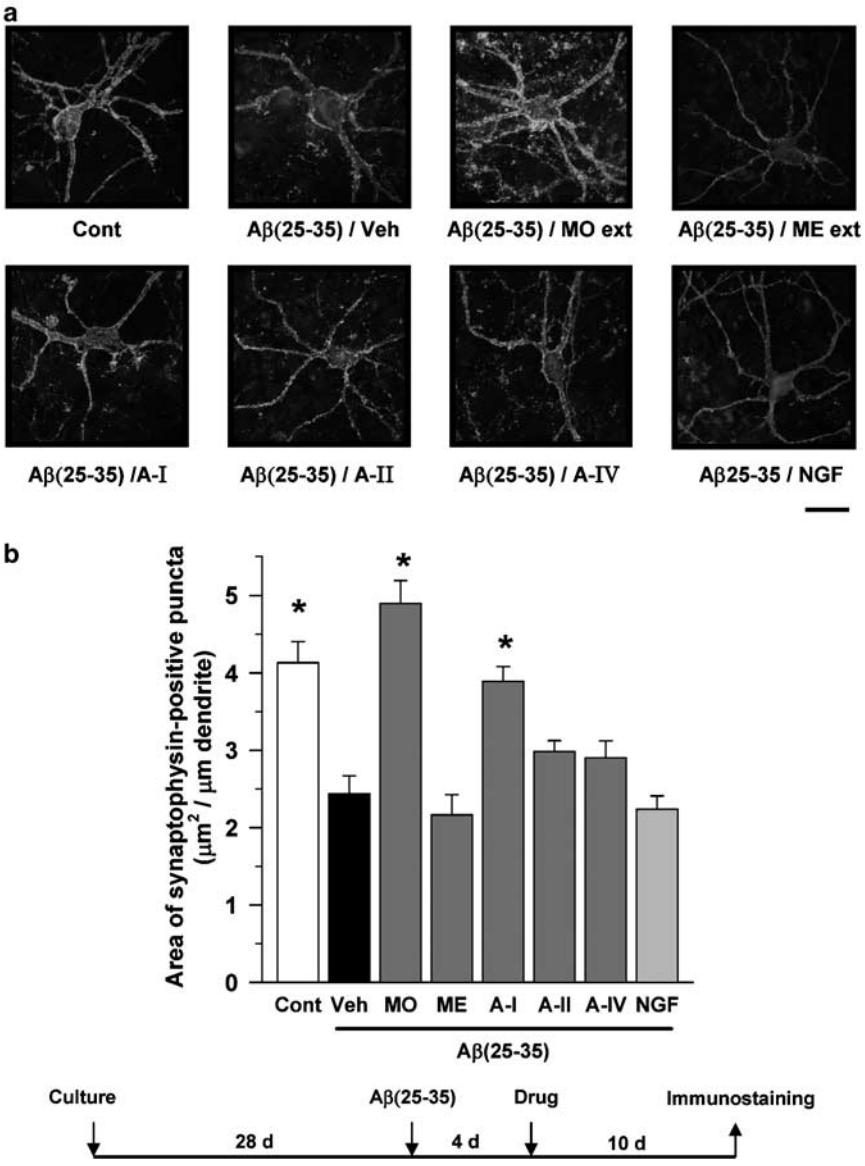


Figure 6 Effects of *Astragalis* extracts or astragalosides on synaptic reconstruction after Aβ(25–35)-induced synaptic loss. After culture for 28 days, cortical neurons were treated with or without (Cont) Aβ(25–35). The cells were then treated with 100 μg ml^{-1} of methanolic extracts of *A. mongholicus* (MO) or *A. membranaceus* (ME); 100 μM astragalosides I (A I), II (A II) or IV (A IV); 100 ng ml^{-1} NGF; or vehicle (Veh). (**a**) Ten days after treatment, the cells were double-immunostained for synaptophysin (green) and MAP2 (red). (**b**) The number of areas containing synaptophysin-positive puncta per micron of dendrites. The results represent the means$\pm$s.e.m. of 19–36 dendrites. *$P<0.05$ vs Veh. Scale bar = 50 μm.

that the extract promoted axonal extension and synapse formation because the drugs were administered to cells after treatment with Aβ(25–35) when the damage had already occurred (Kuboyama *et al*., 2005). Aβ has been reported to induce cell death via a variety of mechanisms (Yamada and Nabeshima, 2000), but differences in the time courses of cell death and neurite atrophy (Grace *et al*., 2002) and in Aβ formation (Postuma *et al*., 2000) suggest that Aβ induces cell death and neurite atrophy by different mechanisms. As described previously, Aβ-induced neurite atrophy is thought to be caused by impaired cell adhesion (Postuma *et al*., 2000; Grace and Busciglio, 2003). In addition, Aβ causes neurite deformation by interfering with the polymerization and aggregation of actins (Hiruma *et al*., 2003). Therefore, different components of *A. mongholicus* extract may affect cell adhesion, cytoskeletal molecules and cell survival. These multiple activities highlight the advantage of herbal medicines.

The amyloid peptide Aβ(25–35) is not found in brains of patients with Alzheimer's disease. However, several reports support that Aβ(25–35) is an active partial fragment of Aβ. This fragment also forms a β-sheet structure (Pike *et al*., 1995) and induces neuronal cell death (Yankner *et al*., 1990; Pike *et al*., 1995), neuritic atrophy (Grace and Busciglio, 2003; Tohda *et al*., 2004) and synaptic loss (141–147; Grace and Busciglio, 2003; Tohda *et al*., 2003, 2004). Also in our

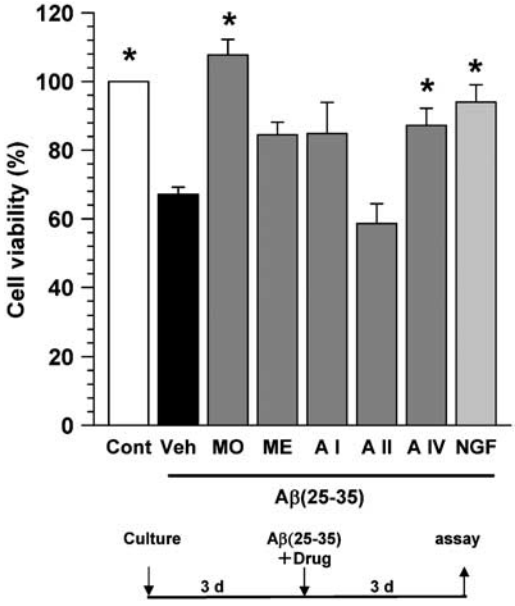


Figure 7 Effects of *Astragalis* extracts or astragalosides on Aβ(25–35)-induced neuronal death. After culture for 3 days, the cortical neurons were treated with or without (Cont) Aβ(25–35). The cells were simultaneously treated with 100 μg ml^{-1} of methanolic extract from *A. mongholicus* (MO) or *A. membranaceus* (ME); 100 μM of astragalosides I (A I), II (A II) or IV (A IV); 100 ng ml^{-1} of NGF; or vehicle (Veh). Three days after treatment, cell viability was measured. The results represent the means$\pm$s.e.m. of three independent experiments. *$P<0.05$ vs Veh.

Table 1 Contents of astragalosides in extracts

	Contents in methanol extracts (%)			
Extracts	*Astragaloside I*	*Astragaloside II*	*Astragaloside IV*	n
MO	0.100$\pm$0.001	0.534$\pm$0.010	0.126$\pm$0.003	9
ME	0.059$\pm$0.001	3.012$\pm$0.027	0.222$\pm$0.005	9

Abbreviations: MO, *A. mongholicus*; ME, *A. membranaceus*.

previous paper, we found that Aβ(25–35) and Aβ(1–42) had similar effects on neuritic atrophy and cell death and that 10 μM Aβ(1–42) induced the same axonal and dendritic atrophy as 10 μM Aβ(25–35). The degree of the atrophy induced by Aβ(1–42) was not different from that induced by Aβ(25–35).

In conclusion, we have shown that extracts of *A. mongholicus* can ameliorate the memory deficit in mice caused by Aβ(25–35) and that they can promote the regeneration of neurites and synapses in the cerebral cortex and hippocampus. These effects were shown only by extracts of *A. mongholicus* and not by those of *A. membranaceus*. Although astragalosides and polysaccharides are the most studied biologically active compounds in Astragali Radix, other compounds should be analyzed for their anti-dementia effects.

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Conflict of interest

The authors state no conflict of interest.

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