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Liquiritigenin Attenuates Alzheimer's-Like Neuropathology in an Amyloid Protein Precursor Transgenic Mouse Model and the Underlying Mechanisms

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1. Introduction

Estrogen plays a key regulatory role in a number of biological processes and, in addition to its classic function as a sex hormone, it has been linked to neurodegenerative diseases, including Alzheimer's disease (AD) (Wickelgren, 1997; Brann et al., 2007; Zhang et al., 2007). However, long-term compliance with estrogen therapy is often estimated to be no more than 15%–40%, due to its undesirable side-effects, such as an increased risk of developing breast and uterine cancer (Warren, 2004). Therefore, the body of recent research has focused on finding neuro-selective estrogen receptor agonists (Zhao et al., 2005) that mimic the beneficial effects of estrogen in the brain but which also exert negligible adverse effects on non-neural estrogen-responsive tissues. For instance, propylpyrazole triol and diarylpropionitrile – each of which exhibits relative specificity for the estrogen receptors ERα and ERβ respectively – have been proven to differentially regulate AD-like changes in female AD model mice (Carroll and Pike, 2008). Additionally, some phytoestrogens with fewer side-effects and potential neuroprotective effects have been developed for use in alternative treatment strategies (Zhao et al., 2002; Bang et al., 2004).

Liquiritigenin (7, 4'-dihydroxyflavanone) is a flavonoid extracted from the radix of Glycyrrhiza, an herbal that is frequently used to treat injury or swelling, or for detoxification in traditional Oriental medicine. Liquiritigenin is also one of the major active compounds of MF101 (Kupfer et al., 2008), an herbal extract currently used in clinical trials for the treatment of hot flushes and night-sweats in post-menopausal women. Our interest in liquiritigenin is based upon the following observations. First, liquiritigenin is shown to be a selective agonist of estrogen receptor-β (ERβ) (Mersereau et al., 2008). ERβ is expressed in the brain centre related to learning and memory, but it is unlikely to be related to sex (Shughrue et al., 1997; Gustafsson et al., 2003). Second, studies have already proven that liquiritigenin exerts cytoprotective effects in vitro and in vivo (Kim et al., 2004; Kim et al.,

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2006; Kim et al., 2008); in particular, we have previously observed that liquiritigenin inhibits amyloid β-peptide-induced neurotoxicity, not only in hippocampal neurons (Liu et al., 2009), but also in rats (Liu et al. 2010a). Third, we have found that liquiritigenin does not induce the proliferation of MCF-7 or T47D breast cancer cells (unpublished observations), which is consistent with the results of other studies (Gustafsson et al., 2003). Finally, pharmacokinetics data has demonstrated that liquiritigenin is absorbed well by the intestine amongst rats, with an in vitro blood-brain barrier penetration rate of (29.7±6.8)% within 90 min, similar to that of chloramphenicol (34.0±4.9)%, a known blood-brain barrier and highly penetrative drug (Kupfer et al., 2008; Lu et al., 2008; Kang et al., 2009). Taken together, these observations suggest that liquiritigenin may be a potentially effective therapy for AD.

2. Methods

2.1 Animals

Male and female 10-month-old transgenic 2576 (Tg2576) mice expressing the human 695 aa isoform of amyloid protein precursor (APP) and containing the mutation (K670N and M671L) were purchased from the Chinese Academy of Medical Sciences. Tg2576 mice and age- and strain-matched wild type (WT) mice (C57/BL6J) were housed with free access to standard food and water at a room temperature of 21±2°C, relative humidity of 45±15%, and 12 h light/dark cycles. All experimental manipulations and data analysis in this study were conducted in a blinded fashion.

All animal experiments were conducted in accordance with NIH guidelines under a protocol adhering to the guidelines of the Chinese Society of Laboratory Animal Sciences.

2.2 Experimental design

Liquiritigenin was synthesised at the Beijing Institute of Radiation Medicine (Beijing, China) and it was shown to be >95% pure by HPLC. Tg2576 mice – a widely used AD model (Spires et al., 2005; Howlett et al., 2009) – were randomly assigned to one of four liquiritigenin treatment groups (n= 5 male and 5 female/group): 30 mg/kg/d, 10 mg/kg/d, 3 mg/kg/d and 0 mg/kg/d (control vehicle treatment). Each treatment group received liquiritigenin continuously for 90 d (i.g.); the WT and vehicle-treated Tg2576 mice were treated with an identical volume (20 ml/kg/d) of vehicle (0.1% sodium carboxymethyl cellulose).

Animal behaviour was assessed from the 91st day from the beginning of treatment, with the tests carried out sequentially in accordance with the experimental schedule shown in Fig. 1.
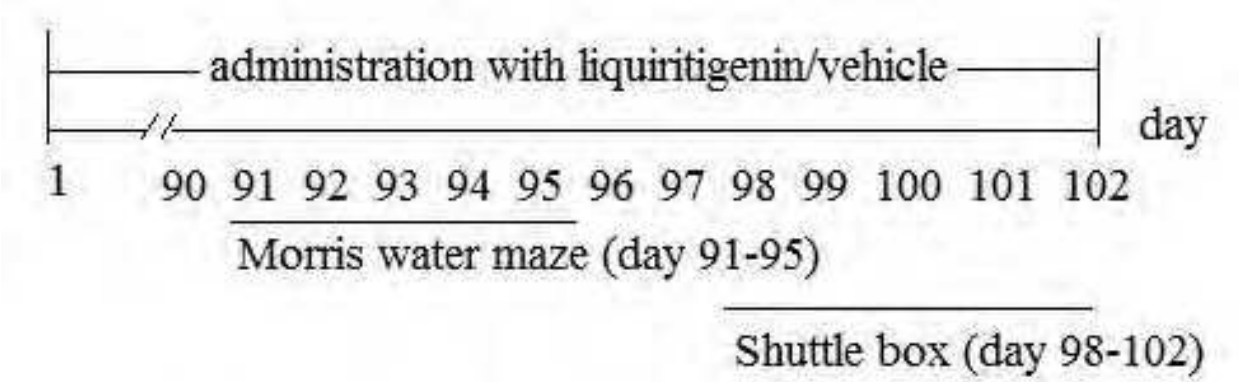

Fig. 1. Experimental schedule of behaviour tests.

2.3 Morris water maze test

A circular water tank (100-cm diameter, 40 cm tall, purchased from Chinese Academy of Medical Sciences) was divided into four equally spaced quadrants. A transparent platform was set at the east quadrant of the tank, 20 cm from the wall and 1 cm below the surface of the water.

Reference memory task: The task was conducted twice a day for 4 consecutive days. In each trial, the mouse was placed in the water at one of three starting positions (which were spaced equally around the rim of the tank), with the sequence of the positions selected at random. The mouse was allowed to swim until it found the platform or until 90 s had elapsed. In this last case, the trial was terminated and the animal was put on the platform for 30 s. The latency of escape onto the platform was recorded using Morris system software (Chinese Academy of Medical Sciences).

Probe task: On day 95, the platform was removed from the pool and the animals underwent a 90 s spatial probe trial. The time taken to reach the place where the platform had been located during training and the length of time spent in that quadrant was recorded.

2.4 Two-way shuttle avoidance task

The two-way shuttle avoidance box, placed in a dimly-lit, ventilated, sound-attenuated cupboard, is a rectangular chamber (44×28×23 cm, purchased from Chinese Academy of Medical Sciences) used to condition animals with a shock stimulus. During the conditioning stimulus (5 s of singing on a loudspeaker located on the ceiling), the animals had to move to the other side of the shuttle box in order to avoid a 2 s electrical shock of the foot (unconditioned stimulus). Between trials, the animals were able to move with impunity for 40 s. Every mouse was subjected to 10 trials per day for 4 consecutive days, and the time spent in avoidance (shuttling to the other part of the apparatus while the tone was on so as to avoid the shock), the frequency of the shock, and the length of time in shock were recorded.

2.5 Brain slice and homogenate preparation

After the above behavioural assessment was completed, all the mice were anesthetised with pentobarbital (50 mg·kg-1, i.p.), transcardially perfused with cold physiological saline, and killed by decapitation. Each cerebrum was divided into 3 parts. One part of the cerebral hemispheres was collected immediately and immersion-fixed in 4% paraformaldehyde for paraffin sections. Half of the remaining cerebral hemisphere were weighed and immediately used to make a 10% homogenate with saline by centrifugation at 3500 rpm for 10 min at 4 °C, and which were stored in -80°C until use. The remaining half of the hemispheres were lysed with 4°Ccell lysis buffer (Beyotime, P0013, China) for protein extraction – as described by the manufacturer – and stored at -80°C.

2.6 Detection of acetylcholinesterase and choline acetyltransferase activity

Acetylcholinesterase (AchE) and choline acetyltransferase (Chat) activity was detected in the 10% brain homogenate using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The absorbencies were determined with a spectrophotometer (DU-640, Beckman, USA).

2.7 Aβ/A4 immunostaining

Fixed hemibrains were blocked, sectioned (4 µm) exhaustively in the horizontal plane, and then processed for immunohistochemistry using a standard avidin-biotin peroxidase

complex protocol. Briefly, every section was immunostained using an antibody directed against Aβ/A4 (Santa Cruz, sc-28365, 1:100 dilution) at 4°C overnight, followed by signal development using an avidin-biotin peroxidase complex immunohistochemistry kit (Zhongshan Goldenbridge Bio Co., Ltd., PV-9002, China), diaminobenzidine for colour substrate reactions, and haematoxylin for counterstaining. The level of Aβ/A4 immunoreactivity in the hippocampus CA1, subiculum, amygdala, and cortex was quantified. In the hippocampus CA1, three adjacent but non-overlapping fields were captured for load analysis. In the subiculum and amygdala, we similarly captured two fields per section; in the cortex, six adjacent, non-overlapping fields were captured. The fields of immunolabelled sections were imaged and digitised using a video-capture system (a CCD camera coupled to an Olympus Optical Microscope, Tokyo, Japan) and the density of the images was quantified with Motic Images Advanced 3.2 software (on the internet at http://www.microscopeworld.com), using stereological approaches. All these assessments were performed by experimenters who were blinded to the genotypes of each brain section.

2.8 Determination of Aβ/A4 protein levels by Western blotting

Western blotting was performed to further validate the results of immunostaining. Briefly, protein concentrations were measured using a bicinchoninic acid protein determination kit (Walterson, H10020, China), and 40 µg protein per lane was analysed. SDS-PAGE and Western blotting were performed according to standard protocols (Zhang et al., 2007). The same Aβ/A4 antibody was used (1:500 dilution) and β-tubulin (Walterson, China, 1:1000 dilution) served as an internal control. A goat anti-mouse IgG-HRP secondary antibody was used (Zhongshan Goldenbridge Bio Co., Ltd., China, 1:5000 dilution). Levels of Aβ/A4 were expressed as a ratio of Aβ/A4 to β-tubulin signal, which was determined with Scion Image software (Scion Image for Windows β 4.02, on the internet at http://www.scioncorp.com).

2.9 Nissl staining for neurons

Nissl staining was directly used for routine histology with cresyl violet (C 1791, Sigma, USA). Briefly, sections were dipped into an aqueous solution of cresyl violet (0.5%) at 37°C for 20 min and then washed with distilled water followed by 70% ethanol. The density of CA1 and cortex pyramidal neurons was quantified with Motic Images Advanced 3.2 software using stereological approaches.

2.10 Detection of astrogliosis by immunostaining

Polyclonal rabbit anti-mouse glial fibrillary acidic protein (GFAP, Zhongshan, ZA-0117, China, 1:100 dilution) antibody was used for the immunostaining of astrocytes. The staining and analysis were conducted as described in Aβ/A4 immunostaining; the level of GFAP immunoreactivity was quantified in the hippocampus CA1 only.

2.11 Determination of Notch-2 expression by Western blotting

SDS-PAGE and Western blotting were performed according to standard protocols (Zhang et al., 2007) with an antibody against Notch-2 intracellular domains (Notch-2 IC, ab-52302, Abcam, UK, 1:300 dilution); 100 µg protein per lane was analysed. Otherwise, the procedure and reagents used were as described with Western blotting.

2.12 Aβ peptide oligomerisation assay in vitro

Soluble Aβ1-42 (100 μM) was dissolved in saline with 0, 0.02, 0.2 or 2 μM liquiritigenin (doses chosen based upon our preliminary tests) or 100 μM melatonin (positive control) and incubated at 37°C for 7 d so as to induce fibril formation. We then added 10 μl of this, conditioned Aβ1-42 to 990 μl 5 μM thioflavin-T (Th-T, Sigma, USA) dissolved in 50 mM glycine-NaOH (pH 8.5), and measured the fluorescent intensity of the liquid with a fluorescence spectrophotometer (excitation=435 nm, emission=485 nm).

Primary cerebrum neuron cells were obtained from newborn (<12 h postnatal) C57 mice following methods already reported (Zhao et al., 2002). These neurons were seeded into poly-D-lysine-coated 96-well plates at a density of 1.0×10^5 cells per well. To evaluate the toxicity of these, conditioned Aβ1-42, 7 day-old neuron cells were treated with the conditioned Aβ1-42 for 72 h. Cell viability was measured with a 3-[4,5-dimethyl-thiazolyl]-2,5- diphenyl-2- tetrazolium bromide (Sigma, USA) assay (Zhao et al., 2002), and cellular membrane penetrability was evaluated by measuring the amount of cytoplasmic lactate dehydrogenase released into the medium (Zhao et al., 2002) with a commercial kit (Biosino Bio-technology and Science Inc., Beijing, China).

2.13 Investigation of the mechanism underlying Notch-2 inhibition in vitro

We used the specific ER antagonist ICI 182 780 (Faslodex) to ascertain whether there was an inhibitory effect of liquiritigenin on Notch-2 involved ER. Primary cerebrum stem/precursor cells were obtained from newborn (<12 h postnatal) C57 mice using reported methods (Ray and Gage, 2006); these were grown for 7 days and seeded into 6-well plates at a density of 1.5×10^6 per well. The cells were treated with either vehicle ICI 182 780 (200 nM) alone or liquiritigenin alone (0.02, 0.2 or 2 μM), or else 2 μM liquiritigenin plus 200 nM ICI 182 780. ICI 182 780 was added 1 h prior to the liquiritigenin treatment, and liquiritigenin was incubated with ICI 182 780 for 5 d. Throughout the treatment, the medium was changed every other day; each change of media contained the appropriate drug concentrations.

Reverse transcriptase-polymerase chain reaction analysis and Western blotting were performed in order to determine changes of Notch-2 mRNA (GenBank accession no. NM_024358) and protein expression. Total RNA was extracted and reverse-transcribed to yield cDNA with commercial kits, as indicated by the manufacturer (Promega, A3500, USA). Polymerase chain reaction was performed using Notch-2 specific primers (forward: 5′-GCA TCC TGG TCA TCG TGGT-3′, reverse: 5′- GAG CCT ATT ATC TCC TGT TCC TG-3′). The predicted size of the amplified product was 229 bp. As an internal control, specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′-CAT GAC CAG AGT CCA TGC CAT CACT-3′, reverse: 5′-TGA GGT CCA CCA CCC TGT TGC TGTA-3′) were used. Amplification was carried out for 40 cycles: 30 s at 94°C, 30 s at 59°C and 30 s at 72°C in a thermal cycler (Gene Amp 2400, USA). The PCR products were visualised after 1% agarose gel electrophoresis.

For Western blotting, the procedures and reagents used were similar to those described above, with an antibody against the Notch-2 intracellular domains (Notch-2 [IC], ab-52302, Abcam, UK, 1:300 dilution), and 100 μg protein per lane was analysed. Otherwise, the procedures and reagents used were as already described.

2.14 Data analysis

All data was expressed as mean ± SEM. Assays in vitro were repeated in at least three independent experiments, each of which was performed in triplicate. Behavioural results

were analysed with a two-way ANOVA followed by Dunnett's test; other raw data was compared among groups by one-way ANOVA followed by Dunnett's test. P<0.05 was assumed so as to indicate statistical significance.

3. Results

3.1 Liquiritigenin treatment rescued behavioural impairment in the Morris water maze test

Changes of escape latency in the reference memory task are shown in Fig. 2(A). The escape latency of mice in every group decreased gradually with increased training time, but the vehicle-treated Tg2576 mice exhibited significantly prolonged escape latency from day 2 to day 4, compared with the WT mice. Compared with the vehicle-treated Tg2576 mice, the liquiritigenin-treated mice required less time to reach the platform. A 90-s spatial probe trial was carried out after the 8th training trial. As shown in Fig. 2(B), the time required to reach the platform for the WT mice was 11.55±2.78 s. The vehicle-treated Tg2576 mice required a considerably longer time (23.65±4.84 s, P<0.05 vs. control), while the liquiritigenin-treated mice (10 and 30 mg/kg/d) performed better than the vehicle-treated mice (10 mg/kg/d: 17.43±3.08 s, 30 mg/kg/d: 16.76±4.02 s, P<0.05). The vehicle-treated Tg2576 mice (16.8±2.1 s) also showed a significant decrease in the length of time spent in the quadrant where the platform had been located during training as compared to the WT mice (23.7±2.6 s). Only the highest dose of liquiritigenin treatment increased this parameter (20.8±2.7 s), as shown in Fig. 2(C).

3.2 Liquiritigenin-treated mice perform better in the shuttle box task

The results of the shuttle box test are shown in Fig. 2(E), (F) and (G). Compared with the WT mice, the vehicle-treated Tg2576 mice were shocked more times and for longer periods, and their initiative escaping time was significantly decreased. Compared with the vehicle-treated Tg2576 mice (shocks: 6.7±1.3, shock time: 9.9±1.8 s, escaping time: 7.9±1.2 s), the liquiritigenin-treated mice (10 and 30 mg/kg/d) were shocked fewer times (10 mg/kg/d: 3.7±0.6, 30 mg/kg/d: 2.3±0.4, P<0.05) and spent less time being shocked (10 mg/kg/d: 4.6±0.8 s, 30 mg/kg/d: 3.0±0.5 s, P<0.05); their escaping time also increased (10 mg/kg/d: 16.6±1.8 s, 30 mg/kg/d: 16.9±2.2 s, P<0.05). The performance of mice treated with a lower dose of liquiritigenin (3 mg/kg/d) was similar to that of the vehicle-treated mice.

3.3 Changes in AchE and Chat activities upon liquiritigenin treatment

The results of AchE and Chat activity determination are shown in Table 1. The activities of AchE and Chat in the vehicle-treated Tg2576 mice were set as 100% and used as the baseline. The results in Table 1 show that the vehicle-treated Tg2576 mice exhibited nearly a 1.7-fold increase in AchE activity and a 0.6-fold decrease in Chat activity compared to WT. The AchE activity of mice treated with the middle and high doses of liquiritigenin decreased markedly to 88.0±7.9% and 67.9±5.9% respectively compared with that of the vehicle-treated Tg2576 mice. Chat activity in these mice increased to 119.0±12.7% (middle dose) and 150.9±11.2% (high dose) compared with the vehicle-treated mice.

3.4 Liquiritigenin inhibited the expression of Aβ/A4 in different brain regions

Aβ/A4 load was quantified in the hippocampus CA1, subiculum, cortex and amygdala. Fig. 3(A) shows representative images of Aβ/A4 immunostaining in the brain. Not all the

liquiritigenin-treatment groups are shown due to limitations of space. According to a report by Carroll (Carroll et al., 2007), amyloid A4 exhibits a different staining pattern to that of Aβ; amyloid A4 localises to the periphery of the cell body, while Aβ deposits localised throughout the cell body, often with a punctuate distribution. The localisation of Aβ/A4 is shown in Fig. 3(A), and some dense extracellular accumulation of Aβ could also be seen, especially in the cortex. However, because it is difficult to distinguish between different types of immunoreactive cells when doing quantitative analysis, we counted all positive staining in. For the quantitative analysis of immunostaining, the Aβ/A4 load of the vehicle-treated Tg2576 mice was set as 100%. We found that the vehicle-treated Tg2576 mice had a higher Aβ/A4 load in the hippocampus CA1, subiculum, cortex and amygdala, as compared with the WT mice. Liquiritigenin treatment attenuated the increase in the Aβ/A4 load to some extent. The Aβ/A4 load in the hippocampus was 93.3±14.6% (low dose), 65.3±7.1% (medium dose) and 55.1±9.0% (high dose) of that observed in the vehicle-treated Tg2576 mice; in the cortex, the load decreased to 91.4±13.2%, 72.1±9.1% and 67.2±7.5%, and in the amygdala, the load decreased to 87.6±8.7%, 66.9±6.1% and 53.7±5.7%, as shown in Fig. 3(B). The Aβ/A4 in the subiculum was not markedly changed by liquiritigenin treatment.
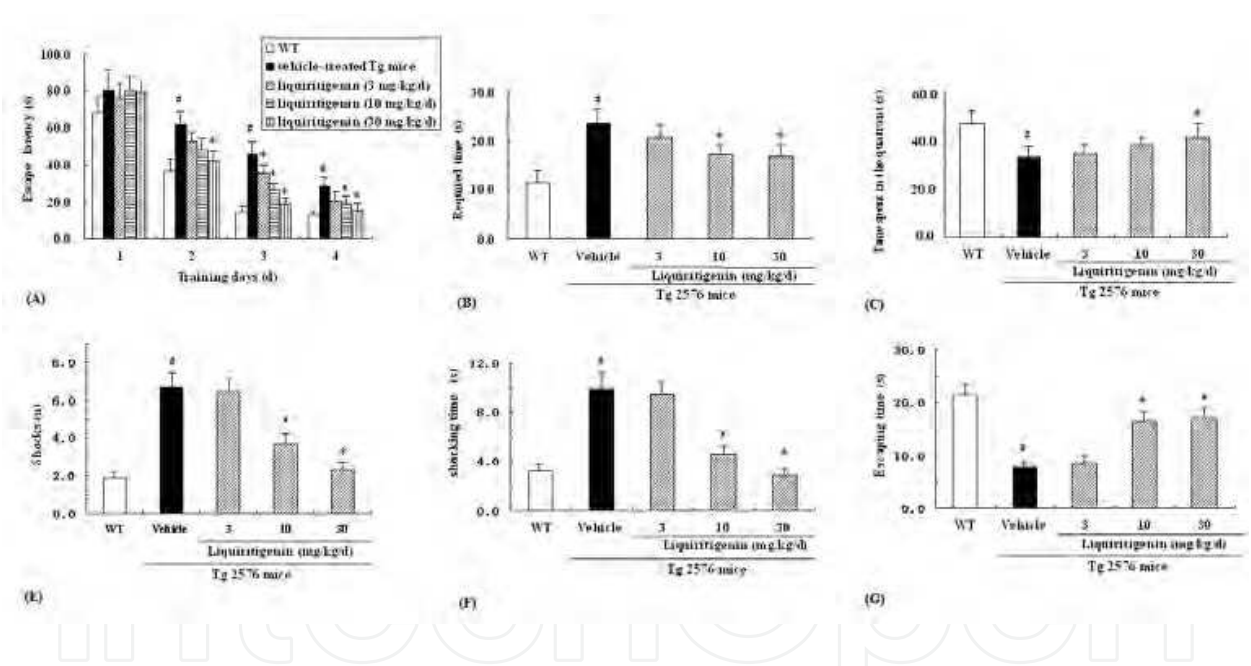

Fig. 2. Effects of liquiritigenin on the performance of Tg2576 mice in the Morris water maze task and shuttle box task. (A) Escape latency with reference to the memory task in the Morris water maze test. (B) The time required to reach the previous platform location in the Morris water maze test probe task. (C) Time spent in the platform quadrant in the Morris water maze test probe task. (D) Shocks received during training in the shuttle box task. (E) Length of shocks received in the shuttle box task. (F) Initiative escaping time in shuttle box task. Morris water maze tasks were given on days 91-95 and shuttle box tasks were given on days 98-102, following the administration of liquiritigenin. The values shown are mean±SEM for 10 animals. # p<0.05 vs. WT, * p<0.05 vs. vehicle-treated Tg2576 mice (Dunnett's test).

Group	Dose (mg/kg/d)	AchE (%)	Chat (%)
WT	-	61.4±6.2	171.4±14.2
Vehicle	-	100.0±8.5#	100.0±11.7#
liquiritigenin	3	102.0±10.2	105.8±13.1
	10	88.0±7.9*	119.0±12.7*
	30	67.9±5.9*	150.9±11.2*

Table 1. Effect of liquiritigenin on brain AchE and Chat activity in Tg2576 mice (mean±SEM, n=10/group). Acetylcholinesterase (AchE) and choline acetyltransferase (Chat) activity. The activities in the vehicle-treated Tg2576 mice were defined as 100%. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

3.5 Liquiritigenin treatment alters the protein levels of Aβ but not amyloid A4

Aβ/A4 levels were further detected by Western blotting. As shown in Fig. 4(A), the two bands near 100 kDa are amyloid A4 and the band near 40 kDa is oligomeric form of Aβ, according to the manufacturer. The protein level was calculated as a ratio against the loading control β-tubulin. As shown in Fig. 4(B), the vehicle-treated Tg2576 mice exhibited a higher level of both amyloid A4 (2.14-fold increase) and Aβ (2.8-fold increase) load compared with the WT mice. There was no difference in amyloid A4 load between the liquiritigenin-treated groups and the vehicle-treated Tg2576 group. However, the oligomeric form of Aβ protein levels were significantly lower in mice treated with the middle and high doses of liquiritigenin, decreasing to 0.71- and 0.57-fold of the level in the vehicle-treated Tg2576 mice, respectively.

3.6 Liquiritigenin treatment promotes an increase in neuronal cell number

Neuronal cell loss was examined by nissl staining with cresyl violet. Fig. 5(A) is a representative image of nissl staining, which shows that the vehicle-treated Tg2576 mice exhibited a loss of neurons in the hippocampus CA1 and cortex, and that liquiritigenin treatment attenuated this loss to a degree. Quantitative analysis in Fig. 5(C) shows that the number of neurons in the vehicle-treated Tg2576 mice decreased to 54.5±8.5% of WT mice in the hippocampus and 63.2±9.2% in the cortex. Liquiritigenin treatment markedly increased the neuron numbers compared with the vehicle-treated Tg2576 mice: in the 10 mg/kg/d dosing group, the number of neurons increased to 122.5±14.7% in the hippocampus and 117.5±15.0% in the cortex while, in the 30 mg/kg/d dosing group, the numbers increased to 163.8±17.7% in the hippocampus and 134.1±15.0% in the cortex.

3.7 Liquiritigenin treatment attenuates astrocytosis

In AD, deposition of Aβ in senile plaques is associated with astrocytic proliferation. Thus, we investigated whether liquiritigenin would decrease astrogliosis. Astrocytes were identified by GFAP immunoreactivity. We quantified the GFAP load specifically in the hippocampus CA1 due to the importance of this region in memory, and we have found in this study that astrocytosis was apparent in CA1. Fig. 5(B) shows representative images of GFAP immunohistochemistry in CA1. Quantitative analysis of CA1 subfields in Fig. 5(D) shows that the vehicle-treated Tg2576 mice had a 2.43-fold increase in GFAP load compared

with the WT mice, and that GFAP load was decreased significantly in mice subjected to middle and high doses of liquiritigenin compared to the vehicle-treated Tg2576 mice (10 mg/kg/d: 85.5±9.0%, 30 mg/kg/d: 73.8±7.6%).
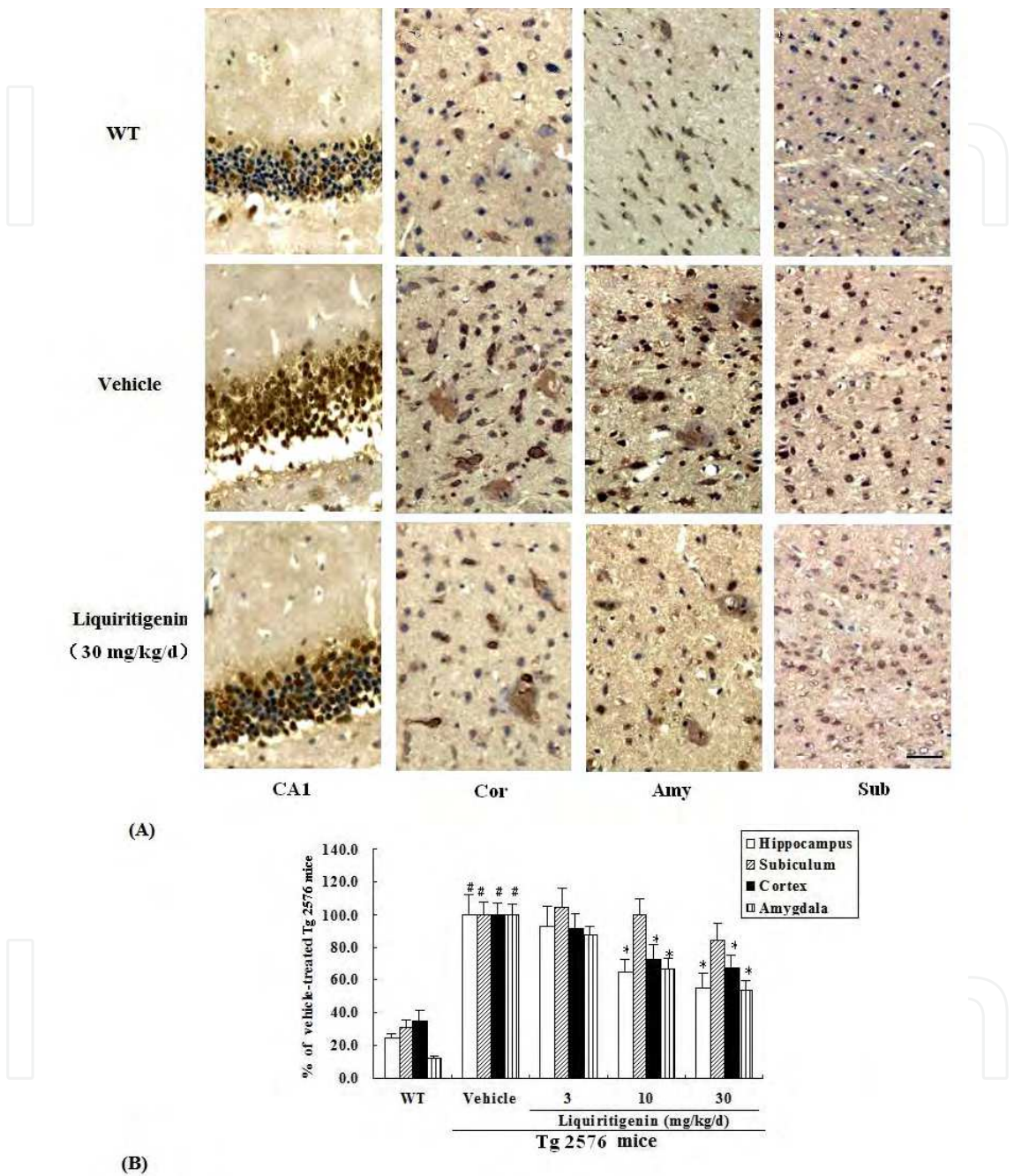

Fig. 3. Liquiritigenin attenuated Aβ/A4 accumulation in different brain regions. (A) Representative images of Aβ/A4 immunohistochemistry of different brain regions. Scare bar: 50 μm. (B) Quantification of Aβ/A4 load. The level of Aβ/A4 immunoreactivity was quantified in the hippocampus CA1, subiculum, amygdala and cortex, by Motic Images Advanced 3.2 software. The average Aβ/A4 load of the vehicle-treated Tg2576 mice was defined as 100%. The values shown are mean±SEM for 10 animals. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).
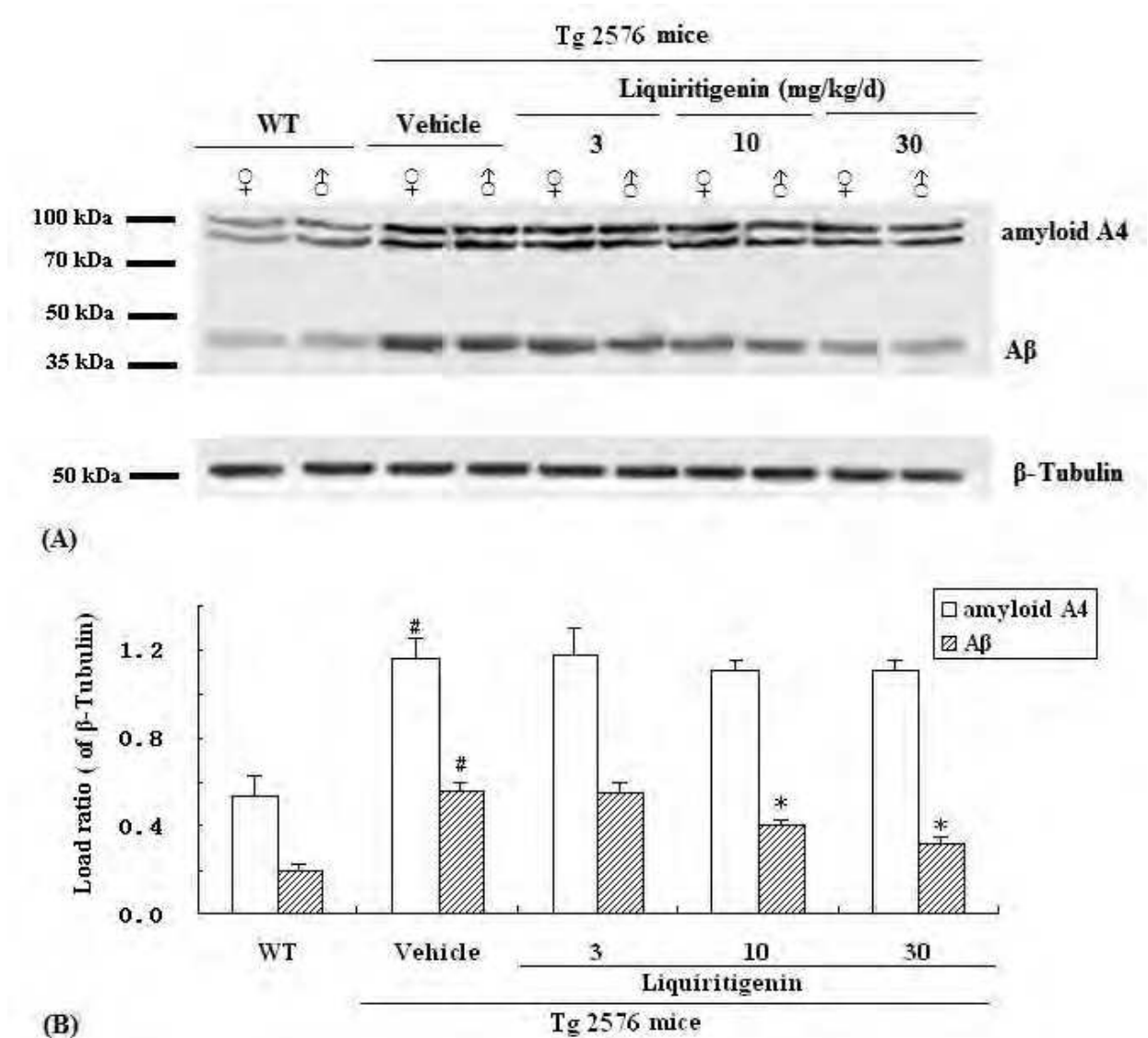
Fig. 4. The effect of liquiritigenin on Aβ/A4 accumulation, assayed by Western blotting. (A) Representative Western blots. The two bands near 100 kDa are amyloid A4 and the single band near 40 kDa is oligomeric form of Aβ. (B) Quantification of Western blotting. The optical density of Aβ bands was quantified with Scion Image software. ♀ indicates female and ♂ indicates male. The values shown are mean±SEM for 10 animals. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

3.8 Liquiritigenin decreased the expression of Notch-2 in vivo and in vitro

Notch signalling is involved in many critical cellular processes, such as neurogenesis, proliferation and apoptosis etc. The preliminary experiment results of a microarray assay performed in our lab suggested that liquiritigenin had a potential inhibitory activity on Notch-2 mRNA (down-regulated for 5.34 times) and protein expression (down-regulated for 3.12 times) in normal rat hippocampal neurons (unpublished data). Thus, we investigated whether liquiritigenin had a similar inhibitory effect on Notch-2 in vivo and in vitro. As shown in Fig. 6(A), Notch-2IC (the active fragment) appears as a band near 100 kDa. Fig. 6(B) shows that the vehicle-treated Tg2576 mice expressed a 1.73-fold higher level of Notch-2IC

than WT mice. The treatment of Tg2576 mice with liquiritigenin resulted in a mild but significant decrease (10 mg/kg/d: 18.4%; 30 mg/kg/d: 23.7%) in Notch-2[IC] expression compared with vehicle-treated ones, while the dose of 3 mg/kg/d liquiritigenin did not seem to influence Notch-2 levels.
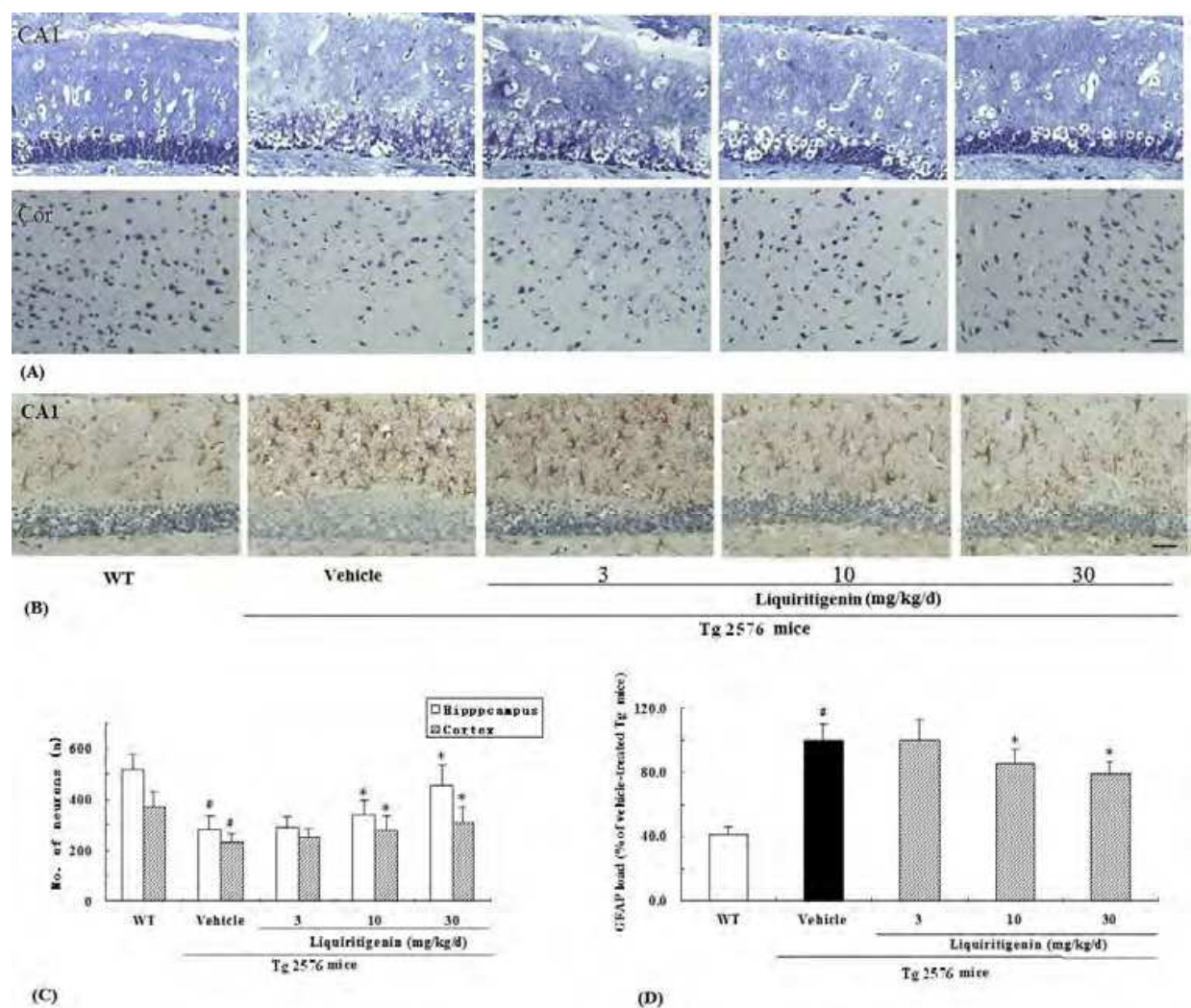

Fig. 5. Liquiritigenin attenuated neuron loss and excess proliferation of astrocytes in the brains of Tg2576 mice. (A) Representative image of nissl staining. (B) Representative image of GFAP immunostaining. (C) Quantification of nissl staining. (D) Quantification of GFAP load. Quantification was performed with Motic Images Advanced 3.2 software. The values shown are mean±SEM for 10 animals. # $p < 0.05$ vs. WT, * $p < 0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

In vitro studies showed similar results. The treatment of primary neurons obtained from newborn C57 mice with 0.2 µM or 2 µM liquiritigenin resulted in a 37.1% to 48.6% reduction in Notch-2[IC] as assayed by Western blotting. The introduction of the ER inhibitor ICI 182 780 blocked the effects of liquiritigenin, as shown in Fig. 6(C). RT-PCR showed that the treatment of primary neurons with 0.2 or 2 µM liquiritigenin decreased levels of Notch-2 mRNA by 30%-60% compared with the control. Treatment with ICI 182 780 blocked the effect of liquiritigenin completely, but had no independent effect on Notch-2 expression, as shown in Fig. 6(D).
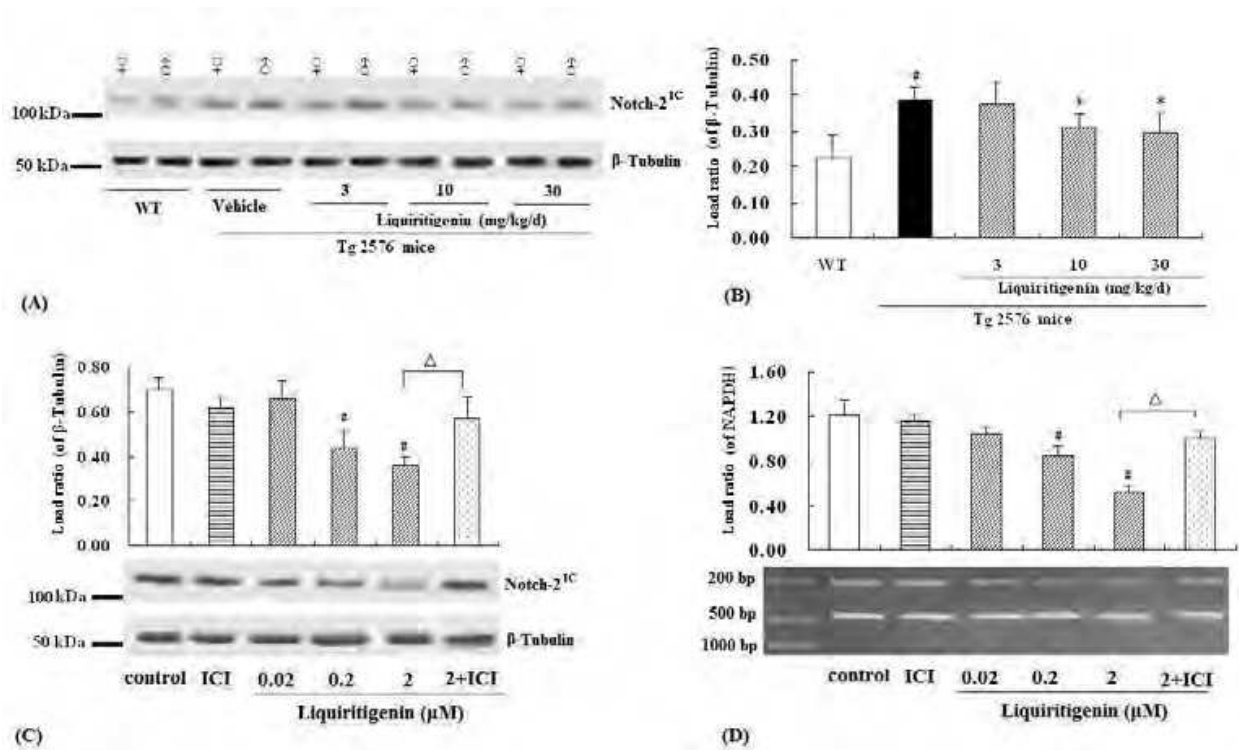
Fig. 6. Liquiritigenin inhibited Notch-2 expression both in vivo and in vitro.
(A) Representative Western blots from tissue samples. (B) Quantification of Western blots.
(C) Effect of liquiritigenin on Notch-2 protein expression in vitro shown by Western blotting. (D) Effect of liquiritigenin on Notch-2 mRNA expression in vitro shown by RT-PCR. Band densities were quantified with Scion Image software. The values shown are mean±SEM. # $p < 0.05$ vs. WT or control, * $p < 0.05$ vs. vehicle-treated Tg2576 mice, $\Delta p < 0.05$ vs. liquiritigenin (0.2 μM)-treated cells (Dunnett's test).

3.9 Liquiritigenin inhibits amyloidosis in vitro

To explore the mechanisms underlying the liquiritigenin-mediated improvement in AD-type cognitive function and Aβ neuropathology, we investigated whether liquiritigenin influenced Aβ peptide oligomerisation in vitro. Aβ is a 39-43 amino acid peptide that derives from APP. $A\beta_{1-42}$ is the most easily amyloidogenic form, so it was used in the present study. Liquiritigenin was added to soluble $A\beta_{1-42}$ and these tubes were stored at 37 for 7d to allow fibril formation. The thioflavin-T assay shown in Fig. 7(A) revealed that the fluorescence of $A\beta_{1-42}$ incubated with 0.2, 2 and 20 μM liquiritigenin was decreased to 96.9±2.5%, 77.2±7.0% and 72.5±6.4% of the fluorescence observed for $A\beta_{1-42}$ incubated alone. Treatment with melatonin caused the fluorescence to decrease to 49.9%.

Primary cerebral neurons obtained from newborn C57 mice were treated with the conditioned $A\beta_{1-42}$ for 72 h. Cell viability was measured by 3-[4,5-dimethyl-thiazolyl]-2,5-diphenyl-2- tetrazolium bromid assay and LDH release. As shown in Fig. 7(B), incubation with $A\beta_{1-42}$ reduced cell viability to 69.5±3.8% ($P < 0.05$ vs. control) while neurons incubated with melatonin-treated $A\beta_{1-42}$ were 85.4±2.4% viable ($P < 0.05$ vs. Aβ25-35). When the $A\beta_{1-42}$ were treated with 0.2, 2 or 20 μM liquiritigenin, the cell viability was increased to 71.4±5.8 % ($P > 0.05$ vs. $A\beta_{25-35}$), 78.0±4.7% ($P < 0.05$ vs. $A\beta_{25-35}$) and 82.2±3.7% ($P < 0.05$ vs. $A\beta_{25-35}$), respectively. These results showed that $A\beta_{1-42}$ alone caused a 4.3-fold increase in LDH

leakage compared to controls (P<0.05 vs. control), and that LDH leakage in 0.2 and 2 µM liquiritigenin-treated groups decreased to 80.3±4.2% and 70.1±4.8% that of $A\beta_{1-42}$ alone, respectively (P<0.05 vs. $A\beta_{25-35}$). Treatment with 0.2 µM liquiritigenin did not affect LDH leakage, as shown in Fig. 7(C).

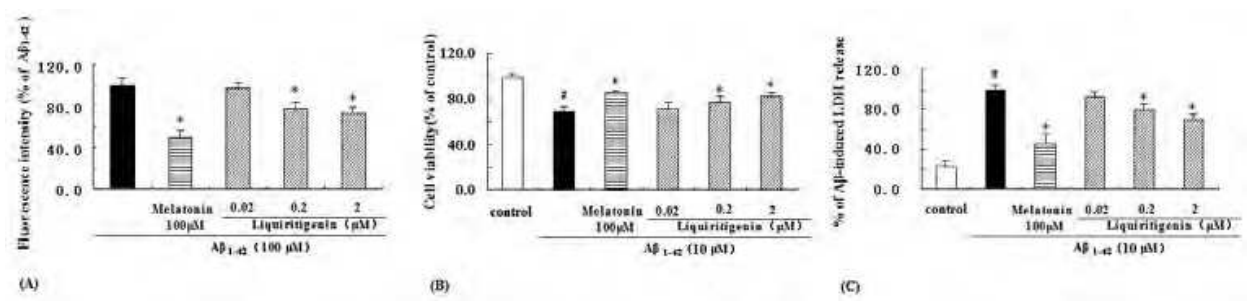Fig. 7. Liquiritigenin inhibited $A\beta_{1-42}$ fibril formation in vitro. Soluble $A\beta_{1-42}$ (100 µM) was incubated with liquiritigenin (0, 0.02, 0.2 and 2 µM) or 100 µM melatonin (positive control) at 37°C for 7 d so as to induce fibril formation. (A) The inhibitory effects of liquiritigenin on fibril formation were measured by thioflavin-T. The fluorescence intensity indicates fibril formation. (B) Cell viability and (C) LDH release were detected in primary neurons treated with the conditioned $A\beta_{1-42}$ for 72 h. Values shown are mean±SEM. # p<0.05 vs. control, * p<0.05 vs. $A\beta_{1-42}$-treated alone (Dunnett's test).

4. Discussion

A primary clinical concern regarding hormone replacement therapy in postmenopausal women is the increased risk of breast and uterine cancer associated with prolonged treatment with estrogenic compounds. However, hormone replacement therapy is also reported to be associated with a reduced incidence and severity of AD. Therefore, the development of neuro-selective estrogen receptor agonists that exert beneficial effects on the brain but minimal effects on other estrogen-responsive tissues is of critical importance. In the present study, we investigated the efficacy of the newly found selective estrogen receptor agonist liquiritigenin in regulating neuropathology in the Tg2576 mouse model of AD. We observed that liquiritigenin treatment significantly improved behavioural performance in such mice. At the molecular level, liquiritigenin treatment reduced $A\beta$ accumulation in some brain regions, regulated the function of the acetylcholine system, increased the average neuron number in the brain, and attenuated astrogliosis in the hippocampus. More interestingly, we have found that liquiritigenin can inhibit Notch-2 expression in an ER-dependent way. Previously, work from Carroll has shown that administration of propylpyrazole triol (an ERα agonist) but not diarylpropionitrile (an ERβ agonist) can improve behavioural performance of 3×Tg-AD model mice, and that both propylpyrazole triol and diarylpropionitrile can reduce $A\beta$ accumulation in the brain (Carroll and Pike, 2008). Our results are not inconsistent with these observations, and it may that, although liquiritigenin has been reported as a ERβ agonist, it also has been shown to exhibit some affinity to ERα (Hillerns et al., 2005) – a report that we are agree with, based on the results of a reporter gene-based cell screening method for ER subtype ligands in our lab (unpublished data). Besides, the difference in behavioural assays used in these studies might also cause some discrepancies in results.

Since the Morris water maze and shuttle box tests investigate distinct motivations, and different skills are required for a good performance in each task, we used both of these tests

to examine the memory skills of the mice in this study. In the Morris water maze test, the mice in all three liquiritigenin dosing groups exhibited a better performance in the reference memory task. The middle and high dosing groups also needed less time to reach their destination in the probe task, but only mice receiving the 30 mg/kg/d dose of liquiritigenin treatment showed improvement in the time spent in the quadrant where the platform had been located. In the shuttle box test, the mice that received the middle and high dosages of liquiritigenin performed better than the vehicle-treated mice, indicating that a 3 mg/kg/d dose may be too low to be effective in this test. Furthermore, a dose higher than 30 mg/kg/d is likely to be unnecessary as higher doses did not cause further improvement in other AD models that we used previously (unpublished observations).

The cholinergic system plays a crucial role in cognitive functions, and most AD patients exhibit a reduction in cholinergic functioning. Estrogen treatment has been found to directly enhance the activity of the cholinergic system by some researchers (Kompoliti et al., 2004; Bora et al., 2005). In the present study, we found that the selective ERβ agonist liquiritigenin enhanced the activity of Chat and decreased AchE activity, thus enhancing cholinergic functioning. Given that liquiritigenin is a kind of phytoestrogen, our observations are consistent with other research showing that phytoestrogens, such as soy isoflavones, can reverse the increase of AchE observed in ovariectomised rats (Monteiro et al., 2007). The effects of liquiritigenin on the cholinergic system may be related to its activity on ER, and further research will be required to verify this hypothesis.

Excessive deposition of Aβ in the brain is a key characteristic of AD patients. In APP Tg mice, Aβ deposits appears in a few months after birth and they progressively accumulate (Reilly et al., 2003). We chose to use 10-month old mice in this study because serious Aβ accumulation has already occurred within this population, and we wanted to observe the therapeutic effects of treatment with liquiritigenin, rather than any preventive effects. In this study, we used both immunohistochemistry and Western blotting to measure Aβ accumulation. The antibody that we used cross-reacted with amyloid A4 and oligomeric form of Aβ (toxic fragment), both of which are given rise to from APP, and they could both be clearly and easily discriminated in Western blots, while immunohistochemistry with this antibody could effectively detect Aβ /A4 load in different regions of brain. Accordingly, the two methods were used. We report in the present study that liquiritigenin treatment attenuates Aβ /A4 load in the hippocampus, cortex and amygdala, but not in the subiculum. This conclusion is consistent with what is known about ER distribution in the brain. Studies in both humans and rodents have demonstrated a differential distribution of ERα and ERβ in brain regions affected in AD, including the hippocampus, frontal cortex and the amygdala. *In situ* hybridisation studies have demonstrated a higher density of ERβ-expressing cells compared with ERα-expressing cells in the hippocampus (Mehra et al., 2005) and layers 4-6 of the cortex (Shughrue et al., 1997 ; Mitra et al., 2003), but similarly high levels of ERβ- and ERα- expressing cells were expressed in the amygdala (Shughrue et al., 1997). A recent study showed that ERβ in the hippocampus and/or cortex is required for enhanced performance in cognitive tasks and anti-anxiety behaviour in mice (Walf et al., 2009). Our results are also consistent with the hippocampus-dependent nature of the behavioural tasks. Further complicating this issue, the mechanisms by which estradiol or selective estrogen receptor agonists propylpyrazole triol, diarylpropionitrile and liquiritigenin reduce levels of Aβ have not been clearly elucidated. Here, we have shown that liquiritigenin can inhibit amyloidosis in a cell-free system in vitro, so it is possible that

the regulation of Aβ involves non-genomic cell signalling besides classic genomic pathways (Zhang et al., 2004), potentially involving the molecular structure of liquiritigenin itself. It is worth noting that liquiritigenin doesn't have a significant effect on amyloid A4, which is another form of APP, and it may indicate that liquiritigenin can regulate the abnormal cleavage of APP, to some extent, but that it does not influence the expression level of APP. Clearly, the underlying mechanisms of liquiritigenin should be explored further.

Nissl staining was used to assess whether liquiritigenin could attenuate the neuronal cell death observed in APP transgenic mice. We hypothesise that the positive results of this assay may be due to liquiritigenin treatment triggering neurogenesis, as we have previously observed that liquiritigenin can induce the differentiation of primary stem cells into neurons through the pathway of Notch in vitro (Liu et al., 2010b). Consistent with this, liquiritigenin inhibits Notch-2 expression both in vivo and in vitro in the present study. It has been shown that Notch signal activation can inhibit neuronal differentiation, whereas a glial progenitor derived from a stem cell differentiates into an astrocyte with the help of Notch signals (Louvi et al., 2006). Therefore, it is possible that when neurons are damaged, the Notch signal pathway may be inhibited with the help of liquiritigenin, and as a result, more endogenous stem cells in the brain may develop into neurons. Our finding that Notch-2 is increased in Tg2576 mice is supported by other researchers' observations that Notch-2 is upregulated in aged and cognitively impaired rats (Bowe et al., 2007), which indicates that elevated Notch-2 signalling may correlate with memory disorders and aging.

Recent research indicates that estradiol inhibited Notch activity in ERα-positive breast cancer cells, and the ER inhibitors tamoxifen and raloxifene, can block this effect by reactivating Notch, suggesting a model in which estrogen inhibits Notch via ERα (Rizzo et al., 2008). In the present paper, we propose that liquiritigenin may exert its inhibitory effect on Notch-2 through ERβ because liquiritigenin has a much higher transcriptional activation activity on ERβ than on ERα. However, as ICI 182 780 is an ER blocker on both ERα and ERβ, it is impossible to distinguish between these two possibilities in our culture system. We have begun to test our hypothesis with ERβ knockdowns, which are ongoing at the time of writing. In any case, we consider that this triggering of neurogenesis is of great importance, and may work as a universal, endogenous protective mechanism against neuronal insults in a damaged brain, because the newly generated neurons are not subject to immune rejection and may have a good chance of integration into the original neural circuits.

As an exploration of the possibility of AD treatment with selective estrogen receptor agonists, the present report shows that the ERβ agonist liquiritigenin has a promising future. Our data confirms the potential of selective estrogen receptor agonists, especially Neuro-selective estrogen receptor agonists, in protecting against AD neuropathology, and it supports continued development and investigation in this field. Interestingly, we found that liquiritigenin has effects on both male and female mice. This may be due to the fact that ERβ is less correlated with sex than is ERα (Shughrue et al., 1997 ; Gustafsson, 2003). However, more research is needed to confirm the effects of liquiritigenin. From a mechanistic standpoint, there is a significant need for targeted research in order to better elucidate the signalling involved in both estradiol and selective estrogen receptor agonists mediated activation of genomic and non-genomic kinase signalling pathways. Relative studies has shown recently that estradiol could work through any of several ERs, including the novel G-protein coupled receptor GPR30, to enhance the learning and memory ability in ovariectomised rats (Hammond et al., 2009). As such, and subsequently, in our gonadally

intact models, animals could be simultaneous treated with an antagonist of ER and examined as to whether the effects of liquiritigenin would be antagonised. Nevertheless, the following research should move this interesting field forward, and aim at answers to many of the key questions that remain.

5. Conclusion

In summary, as a newly found selective ERβ agonist, liquiritigenin could improve the behavioural performance of Tg mice and attenuated Aβ accumulation pathologies both in vivo and in vitro. The effects of liquiritigenin may be due to its inhibition of neuron loss and astrocytosis in the brain, which could be through the Notch signalling pathway. These findings provide evidence for the beneficial activities of the selective estrogen receptor agonist liquiritigenin in a mouse model of AD and support the continued investigation of selective estrogen receptor agonists as an alternative to estrogen-based treatment in reducing the risk of AD.

6. Acknowledgements

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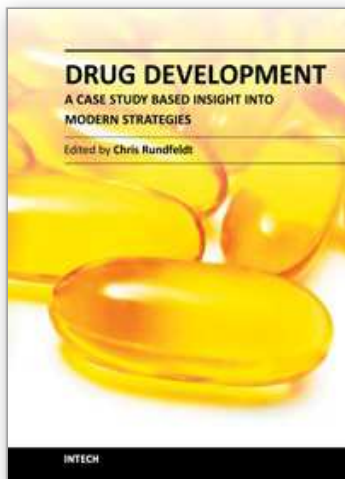
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