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Effects of Donepezil on Amyloid- β and Synapse Density in the Tg2576 Mouse Model of Alzheimer's Disease

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Abstract

Donepezil, an acetylcholinesterase inhibitor, is an approved drug for the treatment of Alzheimer's disease (AD). Although extensive studies have demonstrated the symptomatic efficacy of donepezil treatment in patients with AD, the effects of donepezil, if any, on the AD process are not known. In this study, we sought to determine whether long-term administration of donepezil would slow amyloid plaque deposition or confer neuronal protection in a mouse model of AD. We used quantitative light and electron microscopy to investigate the effects of long-term administration (from 3 to 9 months of age for 6 months of treatment) of donepezil (1, 2, 4 mg/kg, in drinking water) on tissue amyloid- β (A β) protein, plaque deposition, synaptic protein (synaptophysin) and synapse density in the hippocampus of Tg2576 mice. Administration of the 4 mg/kg dose of donepezil, as compared to vehicle and lower doses of donepezil, significantly reduced brain tissue soluble A β ₁₋₄₀ and A β ₁₋₄₂, A β plaque number, and burden at the study endpoint in Tg2576 mice. 4mg/kg of donepezil also significantly increased synaptic density in the molecular layer of the dentate gyrus in Tg2576 mice. However, a significant change of the synaptophysin-positive bouton in the hippocampus was not observed. These results suggest that a higher dose of donepezil may have a measurable impact on tissue level of A β protein, plaque deposition, and may prevent synapse loss in the Tg2576 mouse model of AD.

Keywords

donepezil; amyloid; synaptophysin; synapse density; Tg2576 mice; Alzheimer's disease

1. Introduction

Findings of reduced cholinergic activity and loss of cholinergic neurons in the brains of individuals with Alzheimer's disease (AD) formed the rationale for the development of acetylcholinesterase inhibitors as drugs to ameliorate the dementia associated with AD (Davis et al., 1992; Rogers et al., 1998; Francis et al., 1999; Tariot et al., 2000; Csernansky et al., 2002). Donepezil, an acetylcholinesterase inhibitor, is one of the most popular approved therapies for AD. Although extensive clinical studies have demonstrated the symptomatic

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benefits of donepezil treatment in AD patients with mild, moderate, and severe deficits in cognitive function, behavior, and activities of daily living (Birks and Harvey, 2006; Hansen, 2008), the drug's disease-modifying effects remain a matter of debate (Giacobini, 2001; Mori et al., 2006).

Because muscarinic receptor activation may regulate processing of the amyloid precursor protein (APP) (Nitsch et al., 1992) and cholinesterase inhibitors have been shown to decrease the production of amyloid- β (A β) (Inestrosa et al., 1996; Nilsberth et al., 2002), it is plausible that acetylcholinesterase inhibitors might slow the disease process of AD. Consistent with this hypothesis, acetylcholinesterase inhibitors have been shown to be neuroprotective in experimental models of ischemia- and glutamate-induced neuronal injury (Zhou et al., 2001; Akasofu et al., 2003; Takada et al., 2003).

To our knowledge, there are no published data on the effects of donepezil on more detailed measures of neuronal structure (e.g., synapse density) in an animal model of AD. The Tg2576 mouse is one of the most well characterized transgenic mouse models of AD that overexpresses the human amyloid precursor protein (hAPP) gene (Hsiao et al., 1996, 2001; Sturchler-Pierrat et al., 1997; Irizarry et al., 1997). At approximately 9 months of age, A β deposits appear in cortical and limbic brain regions and indications of cellular inflammation and behavioral deficits emerge in Tg2576 mice (Hsiao et al., 1996; Chapman et al., 1999; Barnes et al., 2005). However, neuronal loss, including the loss of cholinergic neurons, is not prominent in the Tg2576 mouse (Irizarry et al., 1997; Apelt et al., 2002). In a previous study, we found that sub-chronic administration of donepezil could improve learning and memory functions in Tg2576 mice (Dong et al., 2005).

In this study, we administered donepezil in drinking water to Tg2576 mice beginning at 3 months of age and ending at 9 months of age when A β deposits and behavioral deficits usually become apparent (Hsiao et al., 1996; Dong et al., 2007, 2008). We used quantitative light and electron microscopy to assess brain tissue A β , plaque deposition in the brain, synaptic protein (synaptophysin) and synapse density in the hippocampus following long-term administration of donepezil in Tg2576 mice. We focused our structural assessments on the hippocampal formation because this structure plays a major role in learning and memory (Knowles, 1992; Bannerman et al., 2001) and is a major site for A β deposition in both AD patients (Probst et al., 1983; West et al., 2004) and in several transgenic mouse models of AD (Su and Ni, 1998; Reilly et al., 2003). Furthermore, we focused on synapse loss as a measure of neurodegeneration because it is a plausible consequence of A β toxicity and a likely basis for the behavioral deficits observed in Tg2576 mice (Stern et al., 2004).

2 Results

2.1. Drug administration

To ensure the consistency of drug dosing and that the presence of donepezil did not influence daily water consumption, we measured the amount of water consumed by each mouse weekly and calculated the total and mean volume of drinking water with and without the drug. The mean amount of consumption between groups was not significantly different [$F(1,36) = 0.22$; $p = 0.64$].

2.2 Soluble A β -40 and A β -42

The analysis of tissue A β ₁₋₄₀ and A β ₁₋₄₂ levels in the cortex and hippocampus revealed a significant effect of the drug on A β ₁₋₄₀ [$F(2,13) = 6.03$; $p = 0.01$] and A β ₁₋₄₂ [$F(2,13) = 6.58$; $p = 0.01$]. Post-hoc tests show that the 4mg/kg dose of donepezil significantly reduced tissue A β ₁₋₄₀ (4mg vs. vehicle, $p = 0.005$; 4mg vs. 2mg, $p = 0.03$) and A β ₁₋₄₂ levels (4mg vs. vehicle,

$p = 0.005$; 4mg vs. 2mg, $p = 0.01$) as compared to the vehicle and 2mg/kg doses. The 2mg/kg dose of donepezil did not significantly influence brain tissue $A\beta_{1-40}$ and $A\beta_{1-42}$ in Tg2576 mice (Fig. 1). Soluble human $A\beta_{1-40}$ and $A\beta_{1-42}$ were not detected in the wild-type littermate control (WT) animals (data not shown).

Since the 2mg/kg dose of donepezil did not affect soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in Tg2576 mice, we predicted that donepezil would have no effect on soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ at the 1 mg/kg dose either. The amyloid- β plaque density analysis confirmed this expectation (see below). Consequently, we excluded the 1mg/kg dose group from the soluble $A\beta$ analysis and synaptic density measurements.

2.3 Amyloid- β Plaque density

In Tg2576 mice at 9 months of age, small $A\beta$ plaques ($<50 \mu\text{m}$ in diameter) were observed in the hippocampus and overlying cortex, consistent with previous findings (Dong et al., 2008). No $A\beta$ plaques were observed in WT animals and therefore, WT mice were not included in further analyses for this variable. Since we did not find a ratio difference between the drug treated and control groups (data not shown) in the brain subregions, i.e. the cortex and hippocampus, we compared the total number of plaques found in the whole brain. Analysis of amyloid deposition in the brains of Tg2576 mice after donepezil administration indicated there was a significant effect of the drug on plaque number [$F(3,16) = 3.59$; $p = 0.04$] and plaque burden [$F(3,16) = 3.84$; $p = 0.03$]. Post-hoc tests show that the 4mg/kg dose of donepezil significantly reduced plaque number and burden as compared to vehicle, 1mg/kg, and 2mg/kg doses ($p < 0.02$). Doses of 1mg/kg and 2mg/kg of donepezil did not significantly influence amyloid deposition in Tg2576 mice (Figs. 2, 3).

2.4. Synaptophysin-positive bouton density

Synaptophysin immunostaining was dense in the neuropil of the hippocampus and the cortex in both Tg2576 and WT mice. Within the hippocampal formation, the pyramidal cell layer and granule cell layer of the dentate gyrus showed very light staining, while the mossy fiber area showed intense clusters of staining. At a higher magnification, the synaptophysin immunostaining appeared distinctly granular and synaptic boutons were apparent (Fig. 4, Panels A and B). Stereological measurement showed there was no significant effect of genotype [$F(1, 21) = 1.96$; $p = 0.18$] or drug [$F(2,21) = 0.18$; $p = 0.19$], nor was there a significant genotype by drug interaction [$F(2,21) = 0.18$; $p = 0.84$] on the synaptophysin-positive bouton density within the molecular layer of the dentate gyrus (Fig. 4, Panel C).

2.5. Synapse density

We previously found that synapse density was decreased in the molecular layer of the dentate gyrus in Tg2576 mice at 6-9 months of age (Dong et al., 2007); therefore, we selected the molecular layer of the dentate gyrus as the measurement area to examine whether chronic administration of donepezil would prevent synapse loss. In this study, we found a significant effect of drug [$F(2,29) = 3.52$; $p = 0.042$] and trends toward an effect of genotype [$F(2,29) = 3.44$; $p = 0.074$] but no genotype by drug interaction [$F(2,29) = 0.65$; $p = 0.53$]. Post-hoc tests showed that the 4mg/kg but not the 2 mg/kg dose significantly increased synaptic density ($p < 0.01$) in Tg2576 mice. These results suggest that the higher dose of donepezil (4mg/kg) could prevent synapse loss induced by amyloid over-expression (Figs. 5, 6).

3. Discussion

In this study, we evaluated the effects of chronic donepezil administration on soluble $A\beta$ protein and plaque deposition, synaptic protein synaptophysin, and synaptic density in Tg2576 mice, a commonly used animal model of AD. We found that administration of the highest selected

dose of donepezil (4 mg/kg) for 6 months significantly decreased both soluble and insoluble A β in the brain and prevented synapse loss in the molecular layer of the dentate gyrus in Tg2576 mice. However, we did not find a significant change in synaptophysin after donepezil administration. Also, we did not observe neuronal degeneration, which we had previously observed after chronic memantine administration to Tg2576 mice (Dong et al., 2008).

We chronically administered donepezil to an AD animal model to mimic the therapy used clinically on human patients. The doses of donepezil used in this study were selected based upon the literature on animal studies and clinical doses. Previous studies indicate that the most common dosages of donepezil are 3-5mg/kg by oral administration (Spowart-Manning and van der Staay, 2004; Saxena et al., 2008) or 0.1-1mg/kg via IP injection (Dong et al., 2005; Yuede et al., 2007). Clinical studies show the mean ($\pm$ SEM) donepezil plasma concentrations at the study end point were 25.9 $\pm$ 0.7 ng/ml and 50.6 $\pm$ 1.9 ng/ml in the groups receiving doses of 5 mg/d and 10 mg/d, respectively (Rogers et al., 1998). In fact, we measured the plasma donepezil concentration at different doses and routes: Thirty minutes after animals received a 1 mg/kg or 4 mg/kg intraperitoneal donepezil injection, their plasma levels of donepezil were 34 ng/ml and 256 ng/ml, respectively. However, when we administered 1 mg/kg and 4 mg/kg doses in drinking water, the plasma levels of donepezil were 6 ng/ml and 61 ng/ml two months later. The doses utilized in this study produced similar plasma donepezil concentrations as those found in clinical studies. Drug administered through drinking water is a common approach to investigate its effects, especially for a chronic administration paradigm, which avoids the stress associated with daily injections or oral gavage. Our previous studies and studies of others indicate that administering drugs by drinking water is a reliable method (Dong et al., 2008; Frisch et al., 2009; Handattu et al., 2009). Indeed, the use of oral administration is also to be commended since oral treatments of donepezil are used clinically. We did not observe any significant changes in body weight, behavior, or an increase in animal death following donepezil treatment.

The most important finding was the apparent reduction of both A β ₁₋₄₀ and A β ₁₋₄₂ and amyloid plaque deposition in the brain of Tg2576 mice receiving the highest dose of donepezil (4 mg/kg). This finding is consistent with the results of studies suggesting that cholinesterase inhibitors may regulate amyloid metabolisms *in vitro* (Nitsch et al., 1992; Bartolini et al., 2003; Zimmermann et al., 2004; Greig et al., 2005; Akasofu et al., 2008). The neuroprotective effects of donepezil counteract both A β ₁₋₄₀ and A β ₁₋₄₂ toxicity in septal neurons (Akasofu et al., 2008). Recent investigations have revealed that acetylcholinesterase promotes aggregation of A β peptides, and donepezil can inhibit the A β ₁₋₄₀ aggregation induced by human recombinant acetylcholinesterase (Bartolini et al., 2003). Acetylcholinesterase has been shown to promote assembly of A β ₁₋₄₀ into amyloid filaments through its peripheral anionic site (PAS) (Inestrosa et al., 1996). Donepezil at 100 μ M was shown to inhibit acetylcholinesterase-induced A β aggregation by 22% (Bartolini et al., 2003), which suggests that when donepezil interacts with PAS, it can block the promoting activity of acetylcholinesterase on A β aggregation (Inestrosa et al., 1996; Bartolini et al., 2003; Piazz et al., 2003). Recently accumulated evidence also strongly suggests that acetylcholinesterase inhibitors possess neuroprotective properties whose mechanism is independent of acetylcholinesterase inhibition and suggested Alpha4 and Alpha7-nicotinic receptors may play important roles in acetylcholinesterase inhibitor-induced neuroprotection (Takada-Takatori et al., 2009). The effect of donepezil administration on plaque deposition was not observed at lower doses in our study, or in prior studies of sub-chronic (two week) donepezil treatment conducted by our group (Dong et al., 2005) or others (Capsoni et al., 2004). Studies indicated that a higher concentration of donepezil was necessary for neuroprotection than expected, given donepezil's IC₅₀ for AChE inhibition (Akaike, 2006) and an *in vivo* report demonstrates that a 12mg/kg dose of donepezil protects the brain from neuronal death and cognitive impairment induced by concussive head injury (Fujiki et al., 2008). Therefore, it is possible that lower doses (1-2mg/

kg in drinking water) and shorter treatment periods of donepezil are insufficient to block the activity of acetylcholinesterase in A β aggregation. Another possibility is that lower doses or shorter treatment periods of donepezil may influence the level of soluble A β but not yet A β plaque deposition. Also, in this study, we began the administration of donepezil at 3 months of age, which is long before the plaque deposition is expected in Tg2576 mice. Thus, our study design allows us to determine whether donepezil has the capacity to prevent the formation of new A β plaques but not to determine whether donepezil has capacity to reduce the number of plaques already formed. To test whether donepezil could retard ongoing pathological changes, treatment of donepezil starting at 9 months of age or later, and work on older mice are necessary in the future.

We also found that synapse density was significantly increased in the molecular layer of the dentate gyrus only in Tg2576 mice receiving the 4 mg/kg dose of donepezil for 6 months. The molecular layer of the dentate gyrus is one of the first regions to show amyloid deposition and synapse loss in the Tg2576 mouse model of AD (Su and Ni, 1998; Reilly et al., 2003; Dong et al., 2007). Recent studies suggest that glutamate receptors may be involved in A β -induced synaptic dysfunction (Snyder et al., 2005; Tyszkiewicz and Yan, 2005; Floden et al., 2005), and soluble A β ₁₋₄₀ can induce NMDA-dependent degradation of the postsynaptic density-95 protein within glutamatergic synapses (Roselli et al., 2005). However, donepezil has been shown to have a neuroprotective effect against glutamate-induced neurotoxicity (Zhou et al., 2001; Akasofu et al., 2003; Takada et al., 2003). We speculate that the neuroprotective effect of donepezil against synapse loss is due to a decrease in the toxic form of A β accumulation (Kimura et al., 2005a; 2005b).

Our results show there was no effect of donepezil on synaptophysin-positive boutons in Tg2576 mice although the synapse density was increased at higher dosages. These discrepancies might be due to the fact that synaptophysin labels a specific protein within the presynaptic terminal; thus, allowing us to count only synapses with a functional presynaptic terminal. A β may destabilize synaptic elements without completely destroying whole axons or dendrites (Dong et al., 2007). Spires et al. (2005) found decreases in dendritic spines but not axons in proximity to A β plaques. Our previous observation of synaptophysin clusters at the margins of A β plaques (Dong et al., 2007) supports this hypothesis. The presence of synaptophysin-positive clusters in the vicinity of A β plaques is also consistent with work done by other investigators (King et al., 2002). However, the molecular basis of these clusters is not clear. Such clusters may represent the rearrangement of presynaptic structural elements after the degeneration of postsynaptic elements.

Fortunately, we found no evidence of axonal degeneration or other histopathological changes associated with neuronal toxicity in the cortex and hippocampus at the light and electron microscope level after donepezil administration. In a previously conjoined study, we found that chronic administration of 20mg/kg of memantine, a NMDA antagonist, was associated with axonal degeneration in the hippocampus of Tg2576 mice (Dong et al., 2008). In addition, a study performed on normal rats indicated that donepezil could enhance memantine's neurotoxicity (Creeley et al., 2008), although the authors did not report if a higher dose of donepezil alone could induce these harmful effects. Since the combination of donepezil and memantine for the treatment of AD is extensively prescribed in AD patients, it is important to fully understand the collective effects of donepezil combined with memantine. For this reason, the higher dose (such as 8mg/kg) of donepezil alone or combined effects of donepezil and memantine should be assessed in Tg2576 mice.

In summary, chronic administration of donepezil was found to decrease brain tissue A β proteins and A β plaque deposition in Tg2576 mice that overexpress human APP. Moreover, increased synaptic density was observed in the molecular layer of the dentate gyrus in these mice. The

mechanisms by which donepezil confers its neuroprotective functions need to be further investigated, which may help us to optimize the drug treatment of patients with this disease.

4. Experimental procedures

4.1. Animals

The Tg2576 mouse strain, created by Hsiao et al., (1996), was used for this study. Tg2576 mice are derived from C57B6/SJL $\times$ C57B6 crosses, and contain the double mutation Lys670-Asn, Met671-Leu (K670N, M 671L). This mutation is driven by a hamster prion protein gene promoter in C57B6J $\times$ SJL. Brain levels of APP are more than 4 times higher and A β levels are 5-14 times higher in transgenic mice compared to control mice. The presence of the human APP gene was confirmed in each animal by a post-weaning tail biopsy and DNA genotyping using primers as previously described (Hsiao et al., 1996). The breeding and maintenance of the Tg2576 mouse colony were conducted in consultation with the veterinary staff in the Center for Comparative Medicine at Northwestern University Feinberg School of Medicine. All animal procedures were done in accordance with the National Institutes of Health and Institutional Guidelines.

Transgenic mice expressing the human APP gene (Tg2576) and their non-transgenic littermates (WT) were randomly assigned to various groups for donepezil or vehicle administration. A total of 77 animals (Tg2576 = 38, WT = 39, with equal numbers of each gender) were used for this study. Animals were treated with 1 of 3 dosages of donepezil (1mg, 2mg and 4mg/kg) or vehicle for a 6-month period. 42 animals were sacrificed and half of their brain tissues were used for A β measurement and the other halves were used for synaptophysin, A β immunohistochemical and thioflavin S staining [1mg/kg (Tg2576 = 4, WT = 5); 2mg/kg (Tg2576 = 6, WT = 6); 4mg/kg (Tg2576 = 5, WT = 6); vehicle (Tg2576 = 5, WT = 5)]. An additional 35 animals were sacrificed and their brain tissues were used for measurement of synapse density under electron microscopy [2 mg/kg (Tg2576 = 6, WT = 6); 4 mg/kg (Tg2576 = 6, WT = 6); vehicle (Tg2576 = 6, WT = 5)].

4.2. Drug treatment

Approximately equal groups of Tg2576 and WT mice received one of three doses of donepezil or vehicle for 6 months beginning at 3 months of age. Drinking water was prepared fresh weekly containing one of three doses of donepezil (equivalent to 1, 2, and 4 mg/kg/day). No other means were required to dissolve the drug in the water. The vehicle group received regular water for drinking that did not contain any drug. The amount of water consumed by each mouse was measured weekly.

4.3. Soluble A β analysis

The hippocampus and cortex from one brain hemisphere of each mouse was homogenized in PBS and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and protein quantification was performed using the bicinchoninic acid (BCA) assay (Bio-Rad Laboratories, Hercules, CA). Samples were analyzed for soluble A β using a sandwich ELISA specific for human A β ₁₋₄₀ or A β ₁₋₄₂ (BioSource International, Inc., Camarillo, CA) according to the manufacturer's instructions. Briefly, duplicate standards (A β ₁₋₄₀ or A β ₁₋₄₂, from 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.25pg/ml, 15.65pg/ml, 0pg/ml) and tissue samples (120 μ g), containing protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany) were incubated overnight in antibody-coated plates at 4 $^{\circ}$ C. After washing, the plates were incubated in rabbit anti-human A β ₁₋₄₀ or A β ₁₋₄₂ for 2 h at room temperature, and then with horseradish peroxidase-conjugated anti-rabbit IgG for another 2 h. Stabilized chromogen (100 μ l) was added to each well and incubated for 30 min in the dark.

The absorbance in each well was read with a microplate reader (SPECTRAmax Plus, Molecular Devices Corporation, Sunnyvale, CA). The intra-assay coefficient of variation was 2.9%.

4.4. Tissue preparation for Light and electron microscopy

For light microscopy studies, animals were deeply anesthetized and brains were dissected and one brain hemisphere of each mouse was fixed with 4% paraformaldehyde at 4°C. The brains were cut into 35µm thick serial sections in the coronal plane using a cryostat (Leica CM1850 UV, Leica Microsystems, Nussloch, Germany). Of the 6 series of sections (15-20 sections each), we used one series for synaptophysin immunohistochemical staining to assess synaptophysin expression, one series for Aβ immunohistochemical staining and another series for thioflavin S staining to assess compact (fibrillar) Aβ plaques and plaque burden.

For studies of synapse density, animals were deeply anesthetized and perfused transcardially with 0.01M PBS containing heparin sodium for 2 min, followed by a 30 min perfusion with 2% paraformaldehyde, 2% glutaraldehyde and 4% sucrose in 0.1M PBS. 250µm thick sections were cut in the coronal plane using a vibratome. Fifteen sections encompassing the whole hippocampus were selected from each brain and were rinsed in cold 0.1M PBS, treated with 2% OsO₄ in 0.1M PBS for 90 min at 4°C, and rinsed again in 0.1M PBS at room temperature. The sections were then dehydrated in a graded series of ethanol solutions, followed by propylene oxide, and left overnight in a 1:1 mixture of propylene oxide-Polybed 812 (Electron Microscopy Sciences, Hatfield, PA). Finally, the sections were flat embedded in Polybed 812 in an oven at 60°C for 48-72 hours. From the 15 embedded sections, three were selected for semi-thin and ultra-thin sectioning. Representative sections included the dorsal, medial and ventral hippocampus and underlying cortex.

Selected embedded sections (250µm thick) were trimmed and sectioned again using a Reichert Ultracut E Ultramicrotome (Vienna, Austria). Semi-thin (1µm) sections that included the hippocampus and underlying cortex were cut and stained with toluidine blue to study overall histopathological changes in the cortices and hippocampi. The semi-thin sections also served as reference sections for ultra-thin cutting. The sections were trimmed and ultra-cut. The thin (75-90nm) sections containing the outer molecular layer of the dentate gyrus (dorsal blade) were mounted on 400-mesh grids (each mesh grid is 62×62µm²; Electron Microscopy Sciences, Hatfield, PA). The sections were stained using 3% uranyl acetate for 20min followed by lead citrate for 5min and then were examined using a JEOL 100CX electron microscope (Japan).

4.5. Amyloid-β plaque staining and quantification

Selected sections were rinsed with 0.1M PBS (pH 7.4) and incubated in a blocking solution of 5% normal goat serum for 1 h. Sections were then incubated overnight in the primary antibody for Aβ at 4°C (rabbit polyclonal pan antibody raised against Aβ synthetic peptide). The antibody was purified using epitope-specific chromatography and was shown to recognize the sequence of Aβ in the region from amino acids 15-30 (1:1000, BioSource International, Inc., Camarillo, CA). After PBS washing, the sections were incubated in biotinylated anti-rabbit secondary antibody for 2 h at room temperature, then in an avidin-biotin complex for 1 h at room temperature (Vector Laboratories, Burlingame, CA). Aβ-like immunoreactivity was visualized using a DAB kit (Vector Laboratories, Burlingame, CA). To confirm that Aβ-immunoreactivity represented the presence of compact (fibrillar) Aβ plaques, selected sections were stained by using a 1% thioflavin S aqueous solution for 5 min, followed by a 70% alcohol wash for 3-5 min (Guntern et al., 1992).

Aβ-immunohistochemistry stained sections were used to measure both total plaque number and the total area occupied by amyloid plaque in each brain (i.e. total plaque burden) using the

CAST stereological program. A β plaques were counted on every sixth section throughout the entire brain for each structure, and the total number of plaques in the sections was calculated for statistical analysis.

4.6. Synaptophysin staining and quantification

For synaptophysin immunohistochemical staining, previously described methods were employed (Phinney et al., 1999). Briefly, selected sections were pre-incubated in Tris-buffered saline (TBS, 0.05 M) with 0.2% TritonX-100 (TBS-T, pH 7.4) containing 5% normal horse serum (Sigma, St. Louis, MO) at room temperature for 30 minutes. The sections were then incubated with a monoclonal antibody against synaptophysin, clone SVP38. This antibody was derived from BALB/c mice that were immunized by subcutaneous injections of crude fractions of presynaptic vesicles from bovine brain (Wiedenmann et al., 1985) (1:50, Monosan, Frontstraat, Netherlands) in TBS-T overnight at 4°C. After being washed with TBS, the sections were incubated with biotinylated secondary antibody (antimouse IgG, 1:200) for 2 hours at room temperature. The sections were rinsed again in TBS, and then incubated with an avidin-biotin complex for 1 hour at room temperature (Vector Laboratories, Burlingame, CA). Synaptophysin immunoreactivity was visualized using a DAB staining kit (Vector Laboratories). Developed sections were washed with PBS, mounted on gelatin-coated slides, dehydrated, cleared, and coverslipped using Permount. The C.A.S.T. Stereology System (Olympus, Albertslund, Denmark) was used for stereological counting. This system includes a light microscope equipped with a color video camera and high-resolution color video card with output to a color monitor and a motorized stage controlled by joystick to record movement in the x-y-z axes. The system also includes closed-loop feedback control to ensure accurate stage movement in the z-axis, and system software (CAST-GRID program), which overlays counting frames onto the video image. Synaptophysin-positive boutons were quantified using stereological methods previously described (Phinney et al., 1999; Rutten et al., 2005). Initial sampling was at low magnification in the outer molecular layer of the dentate gyrus. Boundaries were identified according to cellular structures. Synaptophysin-positive bouton counting was done using an optical fractionator method (West et al., 1991) under a 100 $\times$ objective. The density of synaptophysin-positive boutons (boutons per mm³) was calculated by dividing the total number of boutons counted by the volume sampled (Bonthuis et al., 1992; 2004). The sampled volume was determined as the number of disectors multiplied by the volume of one disector (312.5 μ m³ with 1.5 μ m guard height).

4.7. Synapse density quantification

At low magnification under the electron microscope, the boundaries of the outer molecular layer of the dentate gyrus were identified according to their characteristic cellular structures. Then, 8-15 photographs from each electron microscope section were taken systematically at 800 $\times$ magnification using alternate grid squares. Three sections from each animal, including the dorsal, medial, and ventral dentate gyrus, were assessed. A total of 922 photographs were taken for analysis. Synapses were identified under electron micrographs that were enlarged photographically to a final magnification of 29,000 $\times$. A magnification standard (grating replica) was used for each series of electron micrographs. Synapses in the molecular layer of the dentate gyrus were identified on photographs by the presence of synaptic vesicles and the postsynaptic densities. All asymmetrical and symmetrical synapses were counted. A stereological disector technique was used to measure the density of synapses (West and Gundersen, 1990; Geinisman et al., 2000; Dong et al., 2007, 2008). Each disector consisted of micrographs of two adjacent ultrathin sections, a reference section, and a look-up section immediately above it. Only synapses that appeared in the reference, but not in the look-up section were counted. The area of the unbiased counting frame was 247 μ m², the disector height was 0.085 μ m, and the disector volume was 20.99 μ m³. The latter value was used to

calculate the density of synapses (synapses per unit volume) as the quotient of the mean number of synapses counted per disector and the mean volume of dissectors.

4.8. Statistical analysis

All neuroanatomical measures were compared across groups using a two-way analysis of variance (ANOVA). Statistical significance was accepted for *p*-values less than 0.05. When significant genotype effects [i.e., Tg2576 mice versus WT controls], drug effects, or genotype × drug interactions were found, post-hoc analyses were performed using a Fisher's Protected Least Squares Design (PLSD) test.

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Figure 1.

Aβ₁₋₄₀ and Aβ₁₋₄₂ levels in brain tissue of 9-10 month old Tg2576 mice treated with 2 or 4mg/kg of donepezil or vehicle. The 4mg/kg dose of donepezil significantly decreased the Aβ₁₋₄₀ (A) and Aβ₁₋₄₂ (B) as compared with the vehicle and 2mg/kg groups.

Figure 2.

Representative A β -immunocytochemical stained sections in the brains of 9-10 month old Tg2576 mice treated with 1, 2 and 4 mg/kg of donepezil and vehicle. Donepezil (4mg/kg) administration for 6 months decreased the A β plaque deposition in the cortex and hippocampus [Tg2576 mice with 1 mg/kg (**Panel B**), 2mg/kg (**Panel C**), and 4mg/kg (**Panel D**)] as compared to vehicle control Tg2576 mouse (**Panel A**). Arrow heads indicate the plaques. Scale bar= 200 μ m in **Panel D** also applies to **A**, **B** and **C**.

Figure 3.

A β plaque number in the brain of Tg2576 mice treated with either 1, 2 or 4mg/kg of donepezil. The 4mg/kg of dose significantly decreased the β -amyloid plaque number (A) and burden (B) in the Tg2576 mouse brain.

Figure 4.

Synaptophysin-positive boutons in the hippocampus of Tg2576 mice after 6 months of treatment with donepezil (**Panel A**) and vehicle (**Panel B**). Arrows indicate synaptophysin-positive boutons. Scale bar = 10 μm. Stereological measurement indicates there was no significant difference in synaptophysin-positive boutons between the donepezil and vehicle treated groups (**Panel C**).

Figure 5.

Representative electron microscope photographs taken of the molecular layer of the dentate gyrus in Tg2576 mice at 9 months of age following 6 months of donepezil administration. Synapses were identified by the presence of synaptic vesicles and postsynaptic densities (see the **Panel A** high magnification insert). Asterisks indicate the synapses. **Panel A:** Tg2576 mice treated with vehicle. **Panel B and C:** Tg2576 mice given a 2mg/kg (**Panel B**) and 4mg/kg (**Panel C**) dose of donepezil. No remarkable degenerating axons were observed in this area. Scale bar=1 μ m in **C** applies to **A** and **B**.

Figure 6.

Synapse density in the molecular layer of the dentate gyrus in Tg2576 mice at 9 months of age following 6 months of donepezil administration. Statistical analysis indicates that the synapse density was significantly increased in the 4 mg/kg donepezil treated group as compared to the vehicle treated group ($p=0.037$). However, synapse density was not significantly changed in animals in the 2mg/kg treated group.