The anti-inflammatory effect of donepezil on experimental autoimmune encephalomyelitis in C57 BL/6 mice

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\textbf{A B S T R A C T}

Donepezil is a potent and selective acetylcholinesterase inhibitor. It has been reported to restore cognitive performance in multiple sclerosis (MS) patients and experimental autoimmune encephalomyelitis (EAE) mice, an established model of MS. However, there are no reports about the anti-inflammatory effects of donepezil on EAE. In this study, the donepezil treatments on EAE mice were initiated at day 7 post immunization (7 p.i., subclinical periods, early donepezil treatment) and day 13 p.i. (clinical periods, late donepezil treatment) with the dosage of 1, 2 and 4 mg/kg/d respectively and the treatments persisted throughout the experiments. Blood-brain barrier (BBB) permeability was detected by Evan's blue content, the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9, Akt and phosphorylated Akt (p-Akt) as well as nerve growth factor (NGF) and its precursor form (proNGF) in the brains of EAE mice were detected by Western blot, and the levels of interferon-γ and interleukin-4 in the brains of EAE mice were detected by ELISA. The results showed that the 2 mg/kg/d late donepezil treatment was the optimal dosage and could ameliorate clinical and pathological parameters, improve magnetic resonance imaging outcomes, reduce the permeability of BBB, inhibit the production of MMP-2 and MMP-9, modulate the expression of NGF and proNGF, increase Th2 bias and the phosphorylation of Akt in the brains of EAE mice. Our data suggested that the anti-inflammatory effects of donepezil may be a novel mechanism on treating EAE and provided further insights to understand the donepezil’s neuroprotective activities in MS.

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1. Introduction

Multiple sclerosis (MS) is the most common chronic inflammatory demyelinating disease of central nervous system (CNS), which is prevalent among young adults and usually leads to chronic disability (Geurts and Barkhof, 2008). The etiology of MS is not well understood, but inflammation is thought to play a central role in demyelination and axonal degeneration (El Behi et al., 2005; Schmidt et al., 2004). Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model for the study of the underlying pathogenesis of MS and also widely used to develop new therapies for MS (Stromnes and Goverman, 2006). Insights from EAE suggest that dysfunction of the blood–brain barrier (BBB) and migration of T lymphocytes into the CNS are the hallmark in the pathogenesis of EAE, which result in the elevated levels of cytokines, matrix metalloproteases (MMPs), and free radical species, then lead to demyelination and hamper nerve conduction (Feinstein et al., 2002; Geurts and Barkhof, 2008; Keegan and Noseworthy, 2002).

Donepezil is a potent and selective acetylcholinesterase inhibitor used for the treatment of Alzheimer’s disease (AD) and has been shown to provide neuroprotection by anti-inflammatory effects. It has been reported that donepezil could exert its anti-inflammatory effects through inhibiting the production of interleukin-1β (IL-1β), IL-6, IL-18 and monocyte chemoattractant protein 1 (MCP-1), and suppressing microglial activation which was independent of acetylcholine and its receptor (Hwang et al., 2010; Yoshiyama et al., 2010). Donepezil also could increase the IL-4 level and expression...
and reverse the AD-related down-regulation of the IL-4/MCP-1 axis (Gambi et al., 2004; Reale et al., 2006). Moreover, the P38/Akt pathway is involved in neuroprotection by donepezil in glutamate-induced neurotoxicity or lipopolysaccharide (LPS)-induced neuroinflammation via alpha7 nicotinic acetylcholine receptor (α7-nAChR) stimulation (Takatori et al., 2006; Tyagi et al., 2010). Although donepezil has already been used for treatment on cognitive dysfunction of MS patients and EAE mice (D’Intino et al., 2005; Krupp et al., 2011; Wiebenga et al., 2011), there are no reports about its anti-inflammatory effects on EAE or MS.

In this study, we examined the efficacy of donepezil on EAE model induced by myelin oligodendrocyte glycoprotein 35–55 amino acid peptide (MOG35–55) in C57Bl/6 mice. The permeability of BBB and the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) as well as nerve growth factor (NGF) and its precursor form (proNGF) in the brains of EAE mice were detected. We further evaluated the alterations of interleukin-17 (IFN-γ), IL-4, Akt and phosphorylated Akt (p-Akt) which were implicated in the pathogenesis of EAE. Our results demonstrated that donepezil could significantly attenuate the disease severity and neuropathology of EAE, improve outcomes of magnetic resonance imaging (MRI) and reduce the permeability of BBB. These effects were attributed to inhibition of the expression of MMP-2 and MMP-9, modulation of the expression of NGF and proNGF, alteration of the production/expression of IFN-γ, IL-4 and p-Akt in EAE mice. The results suggested that donepezil could be a promising drug for treatment on MS.

2. Methods
2.1. Animals and regents

Six to eight-week-old female C57 Bl/6 mice weighting 16–18 g were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Bioethics Committee of Sun Yat-sen University (Approval ID: 2011-0029). Donepezil and complete Freund’s adjuvant (CFA) were purchased from Sigma–Aldrich (St. Louis, MO). MOG35–55 peptide (MEVQYWYSPFSRHYLRGK) was synthesized by CL BioScientific Co., LTD (Xi'an, China). Amino acid sequences were confirmed by amino acid analysis and mass spectrometry. The purity of the peptide was greater than 95%. Mycobacterium tuberculosis H37RA was purchased from Difco (Detroit, MI). Pertussis toxin (PTX) mass spectroscopy. The purity of the peptide was greater than 95%. Mycobacterium LTD (Xi and our preliminary dose-

2.2. Induction and assessment of EAE

EAE was induced in six to eight-week-old female C57 Bl/6 mice by the procedure which had been described previously (Chen et al., 2009). Briefly, mice received a subcutaneous injection in the flanks with 200 µg of MOG35–55 peptide per animal emulsified in CFA containing 500 µg of Mycobacterium tuberculosis H37RA. Immediately thereafter, and again 48 h later, the mice received an intraperitoneal (ip) injection of 300 ng of Pertussis in 100 µl of phosphate buffered saline (PBS). An additional injection of MOG35–55 peptide in CFA was delivered 7 days later. The animals were examined daily for disability. Clinical scores were defined as follows: 0, no signs; 1, loss of tail tonicity; 2, flaccid tail; 3, ataxia and/or paresis of hindlimbs; 4, complete paralysis of hindlimbs; 5, moribund or dead.

2.3. Dose-finding experiments and treatment of mice

Mice were randomly assigned to three groups: control mice, PBS-treated EAE mice and donepezil-treated EAE mice. The dose of donepezil was chosen on the basis of previous in vivo data (D’Intino et al., 2005; Janowsky et al., 2005; Scal et al., 2002) and our preliminary dose-finding experiment. In our dose-finding experiment, we initiated the therapies of donepezil at day 7 post immunization (7 p.i., subclinical periods, early donepezil treatment) and day 13 p.i. (clinical periods, late donepezil treatment) respectively because the apoptosis of neurons started around 1 week before clinical manifestation of EAE (Hobom et al., 2004). Donepezil was dissolved in PBS and used by intragastric administration at a dosage of 1, 2, 4 mg/kg/d respectively both in the subclinical and clinical periods in EAE mice and the treatments persisted throughout the study. PBS-treated EAE mice were used with intragastric administration of PBS only.

2.4. Histological evaluation

Histological evaluation was performed on paraffin-embedded sections of lumbar spinal cords of differently treated EAE mice (n = 6, respectively). Paraffin sections were stained with hematoxylin-eosin andSolochrome camassian impregnation for evaluating inflammatory infiltration and demyelination respectively. Histopathological examination was performed in a blinded fashion. The scale evaluated for inflammation was as follows (O’Neill et al., 2006; Racine et al., 1994): 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organization of inflammatory infiltrates around blood vessels; 3, extensive perivascular cuffing with extension into adjacent parenchyma, or parenchymal infiltration without obvious cuffing. Demyelination in the spinal cord was scored as previously described (Kuerten et al., 2007; Zappia et al., 2005): 1, traces of subpial demyelination; 2, marked subpial and perivascular demyelination; 3, confluent perivascular or subdural demyelination; 4, massive perivascular and subdural demyelination involving one half of the spinal cord with presence ofcellular infiltrates into CNS parenchyma; 5, extensive perivascular and subdural demyelination involving the whole cord section with presence of cellular infiltrates into CNS parenchyma.

2.5. MRI scans

All the MRI scans were conducted on a GE (Signa Twin Speed Excite II) 1.5 T scanner (using a 77 mT/m (150 mT/m ms) gradient system. Mice were anesthetized by intraperitoneal injection of 300 mg/kg body weight chloral hydrate at day 20 p.i. Animals were lying in prone position with their heads fixed in a small dual coil specially designed for brain MRI scans of the mice. The MRI scans included T1-weighted (TR 475 ms, TE 13 ms) sequences before and after administration of 0.5 mmol/kg body weight Gd-DTPA and a T2-weighted (TR 2500 ms, TE 80 ms) sequence in the coronal plane with a slice thickness of 1 mm.

2.6. Evaluation of BBB disruption

The integrity of BBB was determined by evanescent measurement for Evan’s blue (EB) content at day 20 p.i. in six animals per group (Ma et al., 2010). Sterilized 2% EB (Sigma, USA) solution was administered intravenously at a dosage of 4 ml/kg per animal. 30 min after injection, mice were perfused with saline to remove intravascular EB dye.Brains were rapidly removed and each sample was weighed and then homogenized with 2.5 ml PBS and mixed with 2.5 ml 60% trichloroacetic acid to precipitate protein. The samples were centrifuged for 30 min at 1000 g and the supernatants were measured at 610 nm for absorption of EB by using a spectrophotometer (Genesis 10 uv; Thermo Electron Corporation, Madison, WI). EB was expressed as micromgrams per gram of brain tissue against a standard curve.

2.7. Western blot

To investigate the expression of NGF, proNGF, MMP-2, MMP-9, Akt and p-Akt in the brains of the control mice, PBS-treated and donepezil-treated EAE mice (n = 6, respectively), we performed western blot analysis. Samples of the brains from differently treated mice were loaded on 10% gradient sodium dodecyl sulfate-polyacrylamide gels (20 µg protein per lane). Proteins were transferred onto PVDF membrane (Bio-Rad). The membranes were blocked by 5% non-fat milk. Afterward, the membranes were incubated with monoclonal anti-proNGF (1:4000), anti-NGF (1:1000), polyclonal anti-MMP-2 (1:2000), polyclonal anti-MMP-9 (1:2000), anti-Akt (1:1000) and anti-p-Akt (1:1000) overnight respectively. After 3 times washes with TBST buffer, the membranes were incubated with anti-mouse-HRP and goat anti-rabbit-HRP for 30 min, respectively. The experiment was repeated in triplicate and β-actin was used as internal control. The bands were quantified with the Quantity one image analysis software.

2.8. ELISA assay

Spleens from differently treated mice were aseptically harvested. Spleenocytes (5 × 10^6 cells/well) from each group were incubated in 96-well flat-bottom plates in RPMI 1640 supplemented with 10% fetal calf serum, with the specific encephalitogenic peptide MOG 35–55 (20 µg/ml) used for the immunization. Culture supernatants were collected at day 4. The production of IFN-γ and IL-4 in the culture supernatants was determined by ELISA according to the manufacturer’s instructions. Cytokines concentrations were determined by interpolation from a standard curve by using the CurveExpert software (Chen et al., 2010).

Brain tissues were homogenized in 1 ml of ice-cold Tris buffer (pH 7.2, 4 ºC) containing 50 mM Tris, 1 mM EDTA, 6 mM MgCl2 and 5% (w/v) protease inhibitor cocktail. After homogenization, samples were sonicated for 10 s using an ultrasonic cocktail. After homogenization, all the extracts were centrifuged at 20,800 × g for 20 min at 4 ºC.
Supernatants were collected and cytokines (IFN-γ and IL-4) were estimated using ELISA kits. IFN-γ and IL-4 are expressed as pg/mg of total protein (Deak et al., 2003). The detection limits are 0.6–200 pg/ml for IL-4, 5–2000 pg/ml for IFN-γ in the cell supernatants. The detection limits are 1–200 pg/ml for IL-4, 10–2000 pg/ml for IFN-γ in the brain tissues.

2.9. Statistical analysis

Data were expressed as means ± SEM and analyzed by SPSS 13.0 software. Quantitative data were processed using Student’s t tests, Mann–Whitney U test, one-way analysis of variance (ANOVA), rank of one-way ANOVA, or pairwise comparison among groups with the least significant difference (LSD) test (level of test $\alpha = 0.05$). $p$ values less than 0.05 were considered statistically significant.

3. Results

3.1. Donepezil treatment ameliorated clinical severity of EAE mice

In our experiments, the onset day of disease indicated the day when individual mouse showed the first symptom. The mean clinical score represented the average score of each mouse during the experiment. The maximal score indicated maximum severity of disease in individual mouse. In the 2 and 4 mg/kg/d early donepezil-treated EAE mice, the disease onset day was at day $15.4 \pm 0.2$ and $15.4 \pm 0.4$ respectively, while the disease onset day was at day $14.1 \pm 0.3$ and $14.6 \pm 0.2$ in the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice respectively ($p < 0.01$, both. Fig. 1A, B, C and Table 1). We found no significant difference between the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice in the disease onset day ($p > 0.05$). Fig. 1A, B, C and Table 1. The mean clinical scores of 2 and 4 mg/kg/d early donepezil-treated EAE mice were $1.0 \pm 0.1$ and $1.0 \pm 0.1$ respectively, while the mean clinical scores of the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice were $2.1 \pm 0.2$ and $1.8 \pm 0.2$ respectively ($p < 0.01$, both. Fig. 1A, B, C and Table 1). And there was no significant difference between the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice in the mean clinical scores ($p > 0.05$, Fig. 1A, B, C and Table 1). The maximal clinical score represented the average score of each mouse during the experiment. The maximal score indicated maximum severity of disease in individual mouse. In the 2 and 4 mg/kg/d early donepezil-treated EAE mice, the disease onset day was at day $15.4 \pm 0.2$ and $15.4 \pm 0.4$ respectively, while the disease onset day was at day $14.1 \pm 0.3$ and $14.6 \pm 0.2$ in the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice respectively ($p < 0.01$, both. Fig. 1A, B, C and Table 1). We found no significant difference between the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice in the disease onset day ($p > 0.05$). Fig. 1A, B, C and Table 1. The mean clinical scores of 2 and 4 mg/kg/d early donepezil-treated EAE mice were $1.0 \pm 0.1$ and $1.0 \pm 0.1$ respectively, while the mean clinical scores of the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice were $2.1 \pm 0.2$ and $1.8 \pm 0.2$ respectively ($p < 0.01$, both. Fig. 1A, B, C and Table 1). And there was no significant difference between the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice in the mean clinical scores ($p > 0.05$, Fig. 1A, B, C and Table 1). The maximal clinical score represented the average score of each mouse during the experiment. The maximal score indicated maximum severity of disease in individual mouse. In the 2 and 4 mg/kg/d early donepezil-treated EAE mice, the disease onset day was at day $15.4 \pm 0.2$ and $15.4 \pm 0.4$ respectively, while the disease onset day was at day $14.1 \pm 0.3$ and $14.6 \pm 0.2$ in the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice respectively ($p < 0.01$, both. Fig. 1A, B, C and Table 1). We found no significant difference between the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice in the disease onset day ($p > 0.05$). Fig. 1A, B, C and Table 1. The mean clinical scores of 2 and 4 mg/kg/d early donepezil-treated EAE mice were $1.0 \pm 0.1$ and $1.0 \pm 0.1$ respectively, while the mean clinical scores of the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice were $2.1 \pm 0.2$ and $1.8 \pm 0.2$ respectively ($p < 0.01$, both. Fig. 1A, B, C and Table 1). And there was no significant difference between the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice in the mean clinical scores ($p > 0.05$, Fig. 1A, B, C and Table 1). The maximal
scores of 2 and 4 mg/kg/d early donepezil-treated EAE mice were 2.4 ± 0.1 and 2.6 ± 0.2 respectively, while the maximal scores of the early PBS-treated and 1 mg/kg/d late donepezil-treated EAE mice were 3.7 ± 0.2 and 3.6 ± 0.2 respectively (p < 0.01, both, Fig. 1A, B, C and Table 1). And there was no significant difference between the early PBS-treated and 1 mg/kg/d early donepezil-treated EAE mice in the maximal scores (p > 0.05, Table 1 and Fig. 1A, B, C).

With the second section of experiments, we investigated whether donepezil would have beneficial effects on the disease course when the therapy was started at clinical period. We found there was no significant difference between the late PBS- and 1, 2, 4 mg/kg/d late donepezil-treated EAE mice in the onset day of disease (p > 0.05, Table 1). The mean clinical scores of 2 and 4 mg/kg/d late donepezil-treated EAE mice were 1.1 ± 0.1 and 1.1 ± 0.1 respectively, while the mean clinical scores of the late PBS-treated and 1 mg/kg/d late donepezil-treated EAE mice were 2.1 ± 0.2 and 1.7 ± 0.2 respectively (p < 0.01, both, Table 1 and Fig. 1D, E, F). And there was no significant difference between the late PBS-treated and 1 mg/kg/d late donepezil-treated EAE mice in the mean clinical scores (p > 0.05, Table 1 and Fig. 1D, E, F). The maximal scores of 2 and 4 mg/kg/d late donepezil-treated EAE mice were 2.5 ± 0.2 and 2.4 ± 0.1 respectively, while the maximal scores of the late PBS-treated and 1 mg/kg/d late donepezil-treated EAE mice were 3.7 ± 0.2 and 3.5 ± 0.2 respectively (p < 0.01, both, Table 1 and Fig. 1D, E, F). And there was no significant difference between the late PBS-treated and 1 mg/kg/d late donepezil-treated EAE mice in the mean clinical scores and maximal scores respectively (p > 0.05, Table 1) while only 2 and 4 mg/kg/d early donepezil treatment delayed the EAE disease onset when compared with the 2 and 4 mg/kg/d late donepezil-treated mice (p < 0.05, Table 1).

From these results, we found that 1 mg/kg/d donepezil could hardly affect the disease course whether the treatment was initiated at the subclinical or clinical periods. However, 2 mg/kg/d or 4 mg/kg/d donepezil could suppress the severity of EAE, and the early donepezil treatment could markedly delay disease onset while no significant difference between 2 mg/kg/d and 4 mg/kg/d early donepezil-treated EAE mice in the mean clinical scores and maximal scores (Fig. 1 and Table 1). Therapy was chosen only after the disease onset, so we selected the 2 mg/kg/d late donepezil treatment in our following experiment, which was thought as the optimal dosage for therapy on EAE.

3.2. Donepezil treatment improved histopathological outcomes of EAE

To confirm whether donepezil treatment could provide protective effects on the pathological changes in the CNS, neuropathological analysis was conducted on lumbar spinal cords in the 2 mg/kg/d late donepezil- and PBS-treated EAE mice. Blinded analyses revealed that the inflammatory scores which indicated the diffuse infiltration of mixed macrophages, T and B lymphocytes into CNS white matter in the lumbar spinal cords of EAE mice were significantly reduced in donepezil-treated EAE mice when compared with PBS-treated EAE mice (1.50 ± 0.18 v.s. 2.83 ± 0.11, p < 0.01, Fig. 2). Moreover, a large plaque of demyelination was seen in the PBS-treated EAE mice at day 20 p.i., while the demyelination was markedly attenuated by donepezil treatment (1.92 ± 0.20 v.s. 2.67 ± 0.11, p < 0.01, Fig. 2). Representative sections of lumbar spinal cords showing the effects of PBS and donepezil treatment on inflammation and demyelination in EAE mice were presented in Fig. 2. These results indicated that donepezil treatment could attenuate the inflammation and demyelination in lumbar spinal cords of EAE mice.

3.3. Donepezil treatment reduced the MRI lesions in the brains of EAE mice

Previous observations demonstrated that the damage in MRI became more severe on day 18 p.i. and could last 1 week, regardless of the recovery phase of EAE (Xiao et al., 2004), so we did MRI scans of mice on day 20 p.i. MRI scans were performed on the PBS- and 2 mg/kg/d late donepezil-treated EAE mice (n = 6, respectively). Focal lesions were seen in the brains of PBS-treated EAE mice (10 lesions in 6 animals, Table 2). The lesions were located in the subcortex (Fig. 3A, B and C), pons (Fig. 3D, E and F), cerebellum and around the lateral ventricle, with isointense signal intensities on T1-weighted MRI which could not be unequivocally detected (Fig. 3A and D), slightly increased or isoointense signal intensities on T2-weighted MRI (Fig. 3B and E) and pronounced Gd-DTPA enhancement on T1-weighted MRI (Fig. 3C and F). The inflammatory active demyelinated lesion was seen as a hyperintense region on the T2-weighted images. Postcontrast T1-weighted image was acquired to evaluate BBB integrity (Noseworthy et al., 2000), which showed gadolinium enhancement at the lesion site. Gd-DTPA enhancement of meninges was also seen in six PBS-treated EAE mice (Fig. 3C and F). However, there were only Gd-DTPA enhancement in meninges but no unequivocal focal lesions in the brains of donepezil-treated EAE mice (Fig. 3G, H, I).

3.4. Donepezil treatment reduced BBB dysfunction in EAE mice

BBB destruction accompanying with large numbers of leucocytes infiltrating into CNS plays an important role in the development and progression of MS and EAE (De Vries et al., 1997). In this experiment, the integrity of BBB of differently treated mice was detected by quantitative measurement for EB content at day 20 p.i. (n = 6). 30 min after the intravenous injection of EB, animals were carefully perfused to remove excess dye and the content of EB in the brain tissue was measured at 610 nm using a spectrophotometer. The concentrations of extravasated EB (expressed as micrograms per gram of brain tissue) of different groups were shown in Fig. 4. In
donepezil treatment (D, EAE mice were 185.75 mg/kg/day late donepezil treatment in the lumbar spinal cords of EAE mice. Data were expressed as mean ± S.E.M., **p < 0.01 compared with PBS-treated EAE mice.

Table 2

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Focal lesion</th>
<th>Enhancement of meninges</th>
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<tr>
<td>1</td>
<td>Around lateral ventricle</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Subcortex</td>
<td>+</td>
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<tr>
<td>3</td>
<td>Subcortex</td>
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<td>4</td>
<td>Cerebellum</td>
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<tr>
<td>5</td>
<td>Around lateral ventricle</td>
<td>+</td>
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<tr>
<td>6</td>
<td>Subcortex</td>
<td>+</td>
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<td>7</td>
<td>Subcortex</td>
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<td>8</td>
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MRI was performed on PBS and late donepezil treatment (2 mg/kg/d) EAE mice at day 20. Mouse no., mouse number; +, detected; −, not detected; mouse no. 1–6 were PBS-treated EAE mice; and mouse no. 7–12 were 2 mg/kg/d late donepezil-treated EAE mice.

3.5. Donepezil treatment suppressed the expression of MMP-2 and MMP-9 in the brains of EAE mice

The content of EB in the brains of donepezil-treated EAE mice was not remarkably as that of the PBS-treated EAE mice (Fig. 5B, p < 0.01). These data suggested that donepezil treatment could block the reduced expression of NGF and down-regulate the expression of proNGF in the brains of EAE mice.

3.6. Donepezil treatment blocked the reduced expression of NGF and down-regulated the expression of proNGF in the brains of EAE mice

We further determined the expressions of NGF and proNGF by western blot in the brains of differently treated mice. The expression of NGF was significantly reduced in the brains of PBS-treated EAE mice when compared with the control mice (Fig. 5B, p < 0.05), and donepezil treatment markedly blocked the reduction of NGF expression in EAE mice (p < 0.05, Fig. 5B). And the expression of proNGF was significantly up-regulated in the PBS-treated EAE mice when compared with the control mice (p < 0.01, Fig. 5B), while donepezil treatment markedly down-regulated the expression of proNGF in the brains of EAE mice (p < 0.01, Fig. 5B). These data suggested that donepezil treatment could block the reduced expression of NGF and down-regulate the expression of proNGF in the brains of EAE mice.

3.7. Donepezil treatment modulated the levels of IFN-γ and IL-4 in the splenocytes culture supernatants and brains of EAE mice

IFN-γ and IL-4, which was the hallmark cytokines that direct Th1 and Th2 development in EAE (Oreja-Guevara et al., 2012), were detected by ELISA in the supernatants of splenocytes culture and brains from different groups. In splenocytes culture supernatants, the IFN-γ levels of PBS-treated EAE mice showed much higher than that of the control mice (447.18 ± 19.68 v.s. 104.46 ± 13.52 pg/ml, p < 0.01, Fig. 6). The treatment of donepezil could significantly reduce the production of IFN-γ in splenocytes culture supernatants when compared with PBS-treated EAE mice (309.88 ± 16.73 v.s. 447.18 ± 19.68 pg/ml, p < 0.01, Fig. 6). The levels of IL-4 in the PBS-treated EAE mice were significantly lower than that in the control mice (5.26 ± 0.54 v.s. 18.51 ± 0.67 pg/ml, p < 0.01, Fig. 6). And donepezil treatment could significantly prevent the reduction of IL-
4 secretion in splenocytes culture supernatants of EAE mice (10.67 ± 0.91 pg/ml, p < 0.01 for PBS-treatment, Fig. 6).

In the brains, we found IFN-γ level was significantly increased in the PBS-treated EAE mice than that in the control mice (75.21 ± 2.20 vs. 59.13 ± 1.72 pg/ml, p < 0.01, Fig. 6). The treatment of donepezil could suppress the production of IFN-γ in the brains of EAE mice (62.00 ± 1.68 pg/ml, p < 0.01 for PBS-treatment, Fig. 6). The IL-4 level was significantly lower in the brains of PBS-treated EAE mice when compared with the control mice (3.88 ± 0.14 vs. 4.85 ± 0.16 pg/ml, p < 0.01, Fig. 6). The treatment of donepezil could reduce secretion of IL-4 level in the brains of EAE mice (4.14 ± 0.12 pg/ml, p < 0.01 for PBS-treatment, Fig. 6).

3.8. Effect of donepezil on phosphorylation of Akt in the brains of EAE mice

Previous studies have shown that the neuroprotective effect of donepezil is mediated by the PI3K-Akt pathway activated by a7-nAChRs (Takatori et al., 2006; Tyagi et al., 2010). We tested whether Akt, a downstream target of PI3-kinase, was involved in neuroprotection in donepezil-treated EAE mice. Our results showed that p-Akt in the brains of PBS-treated EAE mice significantly decreased when compared with the control mice. The donepezil treatment significantly increased p-Akt in the brains when compared with PBS-treated EAE mice (p < 0.01, Fig. 7).

4. Discussion

Donepezil was reported to restore cognitive performance in MS patients and EAE mice (D’Intino et al., 2005; Krupp et al., 2011; Wiebenga et al., 2011), while there are no reports about its anti-inflammatory effects on EAE or MS. In this report, our observations show that the disease severity of EAE mice is significantly reduced by donepezil treatment which is initiated at subclinical and clinical periods (early and late donepezil treatment) compared with PBS-treated EAE mice. And neuropathological investigations of lumbar spinal cords reveal that few signs of cell infiltration and
Demyelination could be seen in donepezil-treated EAE mice in contrast to PBS-treated EAE mice. Moreover, donepezil treatment could improve the outcomes of brain MRI examination of EAE mice. These results are not consistent with the previous results of D’Intino and his colleagues (D’Intino et al., 2005). In their report, the clinical severity of EAE was not ameliorated by either donepezil or rivastigmine which both were selective acetylcholinesterase inhibitors while they only could restore cognitive performance in recovery stage of the disease. The possible explanation of this discrepancy may be that donepezil could produce different effects in different EAE models. In our experiment, EAE is induced by MOG35–55 in C57BL/6 mice while guinea pig spinal cord tissue was used in Lewis rats in D’Intino and his colleagues’ experiment (D’Intino et al., 2005). Different EAE models may have different

![Fig. 5.](image)

Fig. 5. (A) The expression of MMP-2 and MMP-9 in the brains of differently treated mice (n = 6, respectively). The brains from differently treated mice were harvested for western blot at day 20 p.i. Values represented the mean ± S.E.M., **p < 0.01, *p < 0.05 PBS-treated EAE mice compared with control mice; ##p < 0.01, #p < 0.05 2 mg/kg/d late donepezil-treated EAE mice compared with PBS-treated EAE mice.

![Fig. 6.](image)

Fig. 6. Protein level of IFN-γ and IL-4 in the brains (pg/mg) and splenocytes culture supernatants (pg/ml) of differently treated mice (n = 6, respectively). Values represent the mean ± S.E.M., **p < 0.01 compared with control mice; ##p < 0.01 2 mg/kg/d late donepezil-treated EAE mice compared with PBS-treated EAE mice.

![Fig. 7.](image)

Fig. 7. The expression of Akt and p-Akt in the brains of differently treated mice (n = 6, respectively). The brains from differently treated mice were harvested for western blot at day 20 p.i. Values represent the mean ± S.E.M., **p < 0.01, *p < 0.05 compared with control mice; ##p < 0.01 2 mg/kg/d late donepezil-treated EAE mice compared with PBS-treated EAE mice.
pathological conditions (Hartung and Rieckmann, 1997; Pachner, 2011). In our model, we immunize the animals twice, 7 days apart, and we additionally use PTX on two days after the immunization. In Lewis rats (D’Intino et al., 2005), models were typically immunized just once, without the PTX application. Since both the additional immunization with MOG35–55 in CFA (containing mycobacteria) and the injection with PTX lead to a pronounced activation of macrophages (Hofstetter et al., 2003; Kassiotis et al., 2001), donepezil particularly dampens the activation of macrophages (Arikawa et al., 2011; Zhou et al., 2009). In the experiment of Nizri and his colleagues, rivastigmine also was reported to suppress the neuroinflammation and immunomodulation in EAE which was induced by MOG35–55 in C57 Bl/6 mice (Nizri et al., 2008). Different dosage used in the experiment may be another explanation. In our experiment, 2 mg/kg/d donepezil could significantly affect the disease course whether the treatment is initiated at the subclinical or clinical periods. And we also find that the early donepezil treatment could markedly delay disease onset when compared with the late donepezil treatment in EAE mice at this dosage.

The underlying mechanisms by which donepezil exerts its beneficial effects may be multifactorial. In the development and progression of MS and EAE, enhanced BBB permeability accompanying with large numbers of leucocytes infiltrating into CNS plays an important role (De Vries et al., 1997). The gelatinases MMP-2 and MMP-9 seem to be involved in mechanisms of T cell migration into the CNS and the disruption of the BBB (Hartung and Kieseier, 2000; Yong et al., 2001). MMP-2 and MMP-9 can specifically degrade type IV collagen, a key structural component of the basement membrane that surrounds blood vessels which induces the destruction of BBB (Mun-Bryce and Rosenberg, 1998; Conant et al., 1999; Matrisian, 1990). Increased expressions of MMP-2 and MMP-9 have been demonstrated in autopsied MS brains (Anthony et al., 1997; Benesová et al., 2009; Cuzner et al., 1996). In EAE, the expressions of MMP-2 and MMP-9 peaked at the same time as the appearance of clinical signs (Dong et al., 2008). In this study, we find enhanced BBB permeability accompanies with up-regulation of MMP-2 and MMP-9 expressions in brains of EAE mice, which is consistent with our previous report (Ma et al., 2010), and donepezil treatment reduces the up-regulation of MMPs expressions and the permeability of BBB.

It has been shown that the precursor of NGF (proNGF) was cleaved extracellularly by plasmin to form mature NGF and that mature NGF was degraded by MMP-9 (Bruno and Cuello, 2006). The proNGF is a potent apoptotic ligand for the p75 neurotrophin receptor–sortilin complex and induces neuronal cell death (Nykaer et al., 2004). Bone marrow stromal cell therapy reduces proNGF and p75 expression and improves functional recovery in EAE mice (Zhang et al., 2009). The expression of NGF is down-regulated in the CNS (brain and spinal cord) and the peripheral (sera and splenocytes supernatants) during the acute stage in EAE (Chen et al., 2012; D’Intino et al., 2005; Yin et al., 2012). Our present study has shown the levels of NGF reduced while proNGF increased in the brains of PBS-treated EAE mice, which is consistent with our previous report (Chen et al., 2010). Donepezil treatment can suppress the production of IFN-γ and prevent the down-regulation of IL-4 in the splenocytes supernatants and brains of EAE mice in our study. Donepezil is known to increase Th2 bias (Reale et al., 2006), and these findings support that donepezil could regulate Th2 bias in EAE and provide further support of its anti-inflammatory effects in EAE.

In our result, donepezil treatment could increase IL-4, NGF and suppress IFN-γ production in EAE mice. NGF could induce a decrease of IFN-γ in the CNS of EAE rodents (Villoslada et al., 2000). IFN-γ could inhibit NGF secretion by mouse astrocytes (Awatsujii et al., 1995; Brodie, 1996). IL-4 has been shown to increase the synthesis of NGF by mouse astrocytes (Brodie et al., 1998), rat astrocytes (Awatsujii et al., 1993), lymphocytes (Arredondo et al., 2001) and C6 glial cells (Brodie and Goldreich, 1994). The production of NGF by IL-4 points to a possible role of this cytokine as a trophic factor during inflammation in the CNS. The alteration of NGF and IFN-γ, IL-4 in EAE mice might reflect the result of neuro-immune crosstalk in EAE development to some degree (Kerschensteiner et al., 2003, 2009) and provide neurotrophic support during anti-inflammation in the CNS by donepezil treatment.

PI3K signaling plays a major role in the development of EAE. PI3K inhibition prevents further development of EAE (Comerford et al., 2012; Haylock-Jacobs et al., 2011). Akt is a downstream target of PI3 kinase and its phosphorylation (p-Akt) contributes to the protection of mice against EAE (Tsiperson et al., 2013). In this study, we find donepezil increases the expression of p-Akt in the brains of EAE mice. It is well established that increased p-Akt expression, an effector of PI3K, is the prerequisite for the neuroprotective effects of some active ingredients in vitro and in vivo (Kaya et al., 2005). And recent researches showed that PI3K/Akt pathway was involved in neuroprotection by donepezil in glutamate-induced neurotoxicity or LPS-induced neuroinflammation via α7-nAChR stimulation (Takatori et al., 2006; Tyagi et al., 2010). From above, we suggest that donepezil could suppress neuroinflammation in EAE involving the PI3K-Akt signaling pathway.

In this report, we provide that donepezil is effective on the treatment of EAE according to clinical evaluation, neuropathological criteria and MRI examination. And donepezil can reduce the permeability of BBB, inhibit the expression of MMP-2 and MMP-9, block the reduction of NGF expression and down-regulate the expression of proNGF, increase Th2 bias and the phosphorylation of Akt in EAE mice. Our results show that the anti-inflammatory effects of donepezil may be a novel mechanism on treating EAE and provide further insights to understand the mechanisms of donepezil’s neuroprotective activities in MS.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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