Experimental Neurology 236 (2012) 79–87

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

# Huperzine A ameliorates experimental autoimmune encephalomyelitis via the suppression of T cell-mediated neuronal inflammation in mice

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ARTICLE INFO

*Article history:*
Received 6 December 2011
Revised 27 March 2012
Accepted 29 March 2012
Available online 15 April 2012

*Keywords:*
Huperzine A
Spinal cord
Multiple sclerosis
Experimental autoimmune encephalomyelitis
Inflammation
Demyelination

ABSTRACT

Huperzine A (HupA), a sesquiterpene alkaloid and a potent and reversible inhibitor of acetylcholinesterase, possesses potential anti-inflammatory properties and is used for the treatment of certain neurodegenerative diseases such as Alzheimer's disease. However, it is still unknown whether this chemical is beneficial in the treatment of multiple sclerosis, a progressive inflammatory disease of the central nervous system. In this study, we examined the immunomodulatory properties of HupA in experimental autoimmune encephalomyelitis (EAE), a T-cell mediated murine model of multiple sclerosis. The following results were obtained: (1) intraperitoneal injections of HupA significantly attenuate the neurological severity of EAE in mice. (2) HupA decreases the accumulation of inflammatory cells, autoimmune-related demyelination and axonal injury in the spinal cords of EAE mice. (3) HupA down-regulates mRNA levels of the pro-inflammatory cytokines (IFN-γ and IL-17) and chemokines (MCP-1, RANTES, and TWEAK) while enhancing levels of anti-inflammatory cytokines (IL-4 and IL-10) in the spinal cords of EAE mice. (4) HupA inhibits $MOG_{35–55}$ stimulation-induced T-cell proliferation and IFN-γ and IL-17 secretion in cultured splenocytes. (5) HupA inhibition of T-cell proliferation is reversed by the nicotinic acetylcholinergic receptor antagonist mecamylamine. We conclude that HupA can ameliorate EAE by suppressing autoimmune responses, inflammatory reactions, subsequent demyelination and axonal injury in the spinal cord. Therefore, HupA may have a potential therapeutic value for the treatment of multiple sclerosis and as a neuroimmunomodulatory drug to control human CNS pathology.



## Introduction

Multiple sclerosis (MS) is a progressive inflammatory disease of the central nervous system (CNS) that affects more than 2.5 million people worldwide. Inflammation, demyelination and axonal injury are the key pathological events of MS (Compston and Coles, 2008; Sospedra and Martin, 2005). In MS, myelin auto-reactive peripheral T cells and macrophages migrate into the CNS via a broken blood–brain barrier. The inflammatory cascade stimulated by these cells ultimately leads to neuroinflammatory injury and myelin sheath destruction. Although a number of immunosuppressive and immunomodulatory agents have been applied to MS treatment, more well-defined therapeutic strategies are required.

Acetylcholinesterase (AChE) inhibitor has been shown to have in vitro anti-inflammatory properties against macrophages (Ezoulin et al., 2007). Huperzine A (HupA), a sesquiterpene alkaloid extracted from club moss (*Huperzia serrata*), is a highly potent and reversible inhibitor of AChE (Wang et al., 1986; Zangara, 2003). This chemical has been used in traditional Chinese medicine for centuries for the treatment of swelling, fever, blood disorders and other diseases. Recent research has shown that HupA is beneficial in the prevention of the neuronal injury that occurs with various pathological conditions such as cerebral ischemia (Wang et al., 2006b, 2008), epilepsy (Coleman et al., 2008; Schneider et al., 2009) and Alzheimer's disease (AD) (Desilets et al., 2009; Fu and Li, 2009; Ha et al., 2011). A recent double-blind, placebo-controlled clinical trial in patients with AD indicated that HupA can significantly improve both cognitive functions and the quality of life (Wang et al., 2009).

HupA has been shown to possess potential anti-inflammatory properties in various animal models, including transient focal cerebral ischemia (Wang et al., 2008), chronic cerebral hypo-perfusion (Wang et al., 2010), and endotoxemia (Pavlov et al., 2009). Evidence has indicated that a pseudo-irreversible AChE inhibitor, rivastigmine, attenuates motor symptoms and inhibits T-cell proliferation in an animal model of EAE (Nizri et al., 2008). However, it is unclear whether HupA is as

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doi:10.1016/j.expneurol.2012.03.024

effective of an anti-inflammatory agent as rivastigmine and whether it can decrease the T cell-induced demyelination and axonal injury in the CNS that occurs in MS. Compared with currently available AChE inhibitors, HupA is more specific and more potent. In addition, HupA administered orally has a long duration of action and high bioavailability, and it is highly selective for brain AChE (Bai et al., 2000). Therefore, it is likely that HupA could be a therapeutic option for MS patients by decreasing T cell-induced demyelination and axonal injury in the CNS.

Due to its similarity to MS, experimental autoimmune encephalomyelitis (EAE) has been used as an animal model for proof of concept studies of MS therapy (Constantinescu et al., 2011; Hart et al., 2011). In this work, we used the EAE model to investigate the effects of HupA on the neurobehaviors of mice and to explore its underlying mechanisms of action by the assessment of inflammatory responses and demyelination in the spinal cord.

## Materials and methods

### *Animals*

Adult female C57BL/6 mice (Experimental Animal Center, Shanghai Medical College of Fudan University, China) aged 8–10 weeks with body weights of 18–20 g, were housed in a temperature- and light-controlled (22 ± 2 °C, 12:12 h light–dark cycle) room with food and water available ad lib. Prior to experimental manipulation, the mice were allowed to acclimate to the housing facilities for at least one week. All of the experimental protocols and animal handling procedures were approved by the Animal Care and Use Committee of Fudan University and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23).

### *Induction and neurological evaluation of EAE in C57BL/6 mice*

The mice were randomly divided into different groups. EAE was induced by subcutaneous immunization on day 0 with 300 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK, HD Biosciences Co., Ltd., Shanghai, China) and 500 μg mycobacterium tuberculosis (National vaccine and serum institute, Beijing, China). $MOG_{35–55}$ was emulsified in complete Freud's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). The 0.2-ml emulsion was injected subcutaneously into the flank. Pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA, 500 ng in 200 μl PBS) was injected intraperitoneally on the day of immunization and repeated 48 h later. The mice were weighed daily. The neurobehaviors of the mice were monitored, and their neurological scores were analyzed. All scoring was performed by two independent groups who were blind to the treatments. The criteria for the neurological scores were as follows: 0 = no clinical signs; 1 = partial loss of tail tonicity; 2 = completely limp tail and abnormal gait; 3 = complete hind limb paralysis; 4 = paraplegia with forelimb weakness or paralysis; and 5 = moribund or dead. The results are reported as the mean ± S.E.M. of the EAE score of each mouse.

### *Treatment with acetylcholinesterase inhibitors: HupA*

The mice were injected intraperitoneal (i.p.) with HupA (0.05 or 0.2 mg/kg in a volume of 0.2 ml/mouse, Fuxing Drug Company, Shanghai, China) daily beginning either from the day of induction of EAE or from day 10 post-immunization. In some experiments, mecamylamine (Sigma-Aldrich, St. Louis, MO, USA, 10 mg/kg, i.p.), a nicotinic acetylcholine receptor (nAChR) antagonist, was administered 30 min prior to HupA treatment to evaluate the role of the nAChR in the effects of HupA. The control mice received the same volume of sterile PBS (0.01 M).

### *Histological analyses*

At the end of the study (day 32), the mice were anesthetized with chloral hydrate (300 mg/kg, i.p.) and then perfused via the left ventricle with 30 ml PBS (0.1 M) followed by 4% paraformaldehyde for 10 min. The spinal cords were resected and fixed in 4% paraformaldehyde at 4 °C. Paraffin sections (6 μm) from paraformaldehyde-fixed spinal cord tissues were stained with hematoxylin and eosin to assess inflammatory histological alteration. The frozen sections (25 μm) were prepared for luxol fast blue (LFB) to assess axon demyelination. In each group, at least 10 sections per mouse, obtained from the whole length of the spinal cord, were examined histologically. Inflammatory histological alterations were quantified with pathological scores, as previously described (Fagone et al., 2011). The criteria for the pathological scores were as follows: 0 = no inflammatory cells; 1 = scattered inflammatory cells; 2 = moderate, perivascular cuffing; and 3 = dense inflammatory cell infiltrates and parenchymal necrosis.

### *Transmission electron microscopy (TEM) imaging*

To further evaluate the demyelination and axonal injury, the mice were anesthetized and perfused transcardially with fixation solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4) at the end of the study (day 32). The spinal cords were dissected. After fixation in the same fixation solution, ultrathin sections were prepared with an ultra-microtome. The ultra-structures were analyzed and photographed with a Philips CM120 electron microscope in the Electron Microscope Laboratory at Shanghai Medical College of Fudan University.

### *Quantitative RT-PCR (qRT-PCR)*

To evaluate the mRNA changes of inflammatory cytokines/chemokines, the spinal cords were removed 14 d after $MOG_{35–55}$ immunization, and total RNA was isolated with Trizol reagent (Invitrogen, USA). The relative abundance of target mRNAs was quantified with SYBR Green qRT-PCR detection (iCycler iQ® real-time PCR detection system, Bio-Rad, CA, USA). The primer sequences for each target mRNA are as follows: mIL-17-Forward: 5′-ACCTCAACCGTTCCACGTCA-3′, mIL-17-Reverse: 5′-CAGGGTCTTCATTGCGGTG-3′; mIFN-γ-Forward: 5′-CTGCCACGGCACAGTCATTG-3′, mIFN-γ-Reverse: 5′-TGCATCCTTTTTCGCCTTGC-3′; mIL-4-Forward: 5′-CGAGGTCACAGGAGAAGGGA-3′, mIL-4-Reverse: 5′-AAGCCCTACAGACGAGCTCACT-3′; mIL-10-Forward: 5′-GGTTGCCAAGCCTTATCGGA-3′, mIL-10-Reverse: 5′-ACCTGCTCCACTGCCTTGCT-3′; mMCP-1-Forward: 5′-TTAACGCCCCACTCACCTGCTG-3′, mMCP-1-Reverse: 5′-GCTTCTTTGGGACACCTGCTGC-3′; mRANTES-Forward: 5′-ACACCACTCCCTGCTGCTTT-3′, mRANTES-Reverse: 5′-GACTGCAAGATTGGAGCACTTG-3′; mTWEAK-Forward: 5′-CGAGCTATTGCAGCCCATTA-3′, mTWEAK-Reverse: 5′-CCTGCTTGTGCTCCATCCT-3′; mFn14-Forward: 5′-GACCTCGACAAGTGCATGGA-3′, mFn14-Reverse: 5′-CGCATCCCAGGCAGAAGT-3′; and mHPRT-Forward: 5′-GCTGACCTGCTGGATTACATTAA-3′, mHPRT-Reverse: 5′-GATCATTACAGTAGCTCTTCAGTCT-3′. The housekeeping gene, HPRT, was used as an internal reference for standardization of the assay. Relative quantification was performed by determination of the n-fold differential expression with the $2^{-\Delta\Delta Ct}$ method and is expressed as % of HPRT. Melting curves were used to establish the purity of the amplified band. The PCR products were sequenced to confirm identity.

### *$MOG_{35–55}$-specific T-cell proliferation assay*

To assay T-cell proliferation, mouse spleens were dissected on day 14 after immunization with $MOG_{35–55,}$ and single-cell suspensions were prepared. The cells were then seeded on 96-well flat-bottom culture plates ($2\times10^5$ cells/well) and cultured in RPMI 1640 medium (Gibco, NY, USA) supplemented with $5\times10^{-5}$ M β-mercaptoethanol,

2 mM L-glutamine, 100 U/ml penicillin and streptomycin and 10% fetal bovine serum at 37 °C in a humidified incubator with 5% $CO_2$. $MOG_{35-55}$ peptide (1, 10, or 50 μg/ml) was added to stimulate cell proliferation. To evaluate the effect of HupA on $MOG_{35-55}$-stimulated T-cell proliferation ex vivo, HupA was added 30 min before $MOG_{35-55}$ stimulation. In some experiments, mecamylamine was also applied 30 min before HupA administration to check the role of nAChR in the HupA effect. Cell proliferation was measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, USA). As indicated in the manufacturer's protocol, 20 μl tetrazolium compound ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS®]) solution was added to each well, and the absorbance was read at a wavelength of 490 nm with a microplate reader (EL800,Bio-Tek Instruments, Inc., Winooski, VT). The results are expressed as the Stimulation Index (SI). The SI is equal to the mean OD value of the stimulated cells divided by the mean OD values of the unstimulated cells.

*Ex vivo assay for cytokine measurements*

As described above, cell suspensions of spleens ($2\times10^5$ cells/well) were prepared and re-stimulated with 50 μg/ml MOG for 48 h at 37 °C with 5% $CO_2$ in a humidified atmosphere. The supernatants were harvested and stored at 70 °C for cytokine assays. IL-17 and IFN-γ were measured with enzyme-linked immunosorbent assay (ELISA) kits as described by the manufacturer's instructions (Rapid-Bio Lab, CA, USA).

*Statistical analysis*

The data were expressed as the mean ± S.E.M. For the maximum neurological score of each mouse, the significance between the groups was examined using the Kruskall–Walis test followed by a Dunn post-hoc test. Other analyses were performed by a one-way analysis of variance followed by the Newman–Keul test. Changes were identified as significant if $p<0.05$.

## Results

*HupA ameliorates neurobehaviors and improves neurological scores of EAE mice*

To examine the effects of HupA on the neurological behaviors of mice with EAE, HupA was injected into EAE mice daily beginning either from the day of immunization or from day 10 post-immunization. The results showed that $MOG_{35-55}$-immunized mice developed the typical symptoms of MS between days 10 and 13 after immunization. These symptoms were manifested in all of the $MOG_{35-55}$-immunized mice (100% incidence) (n = 18). The EAE mice showed the following progression of symptoms: weakness of tail tonus, paralysis of the tail, and

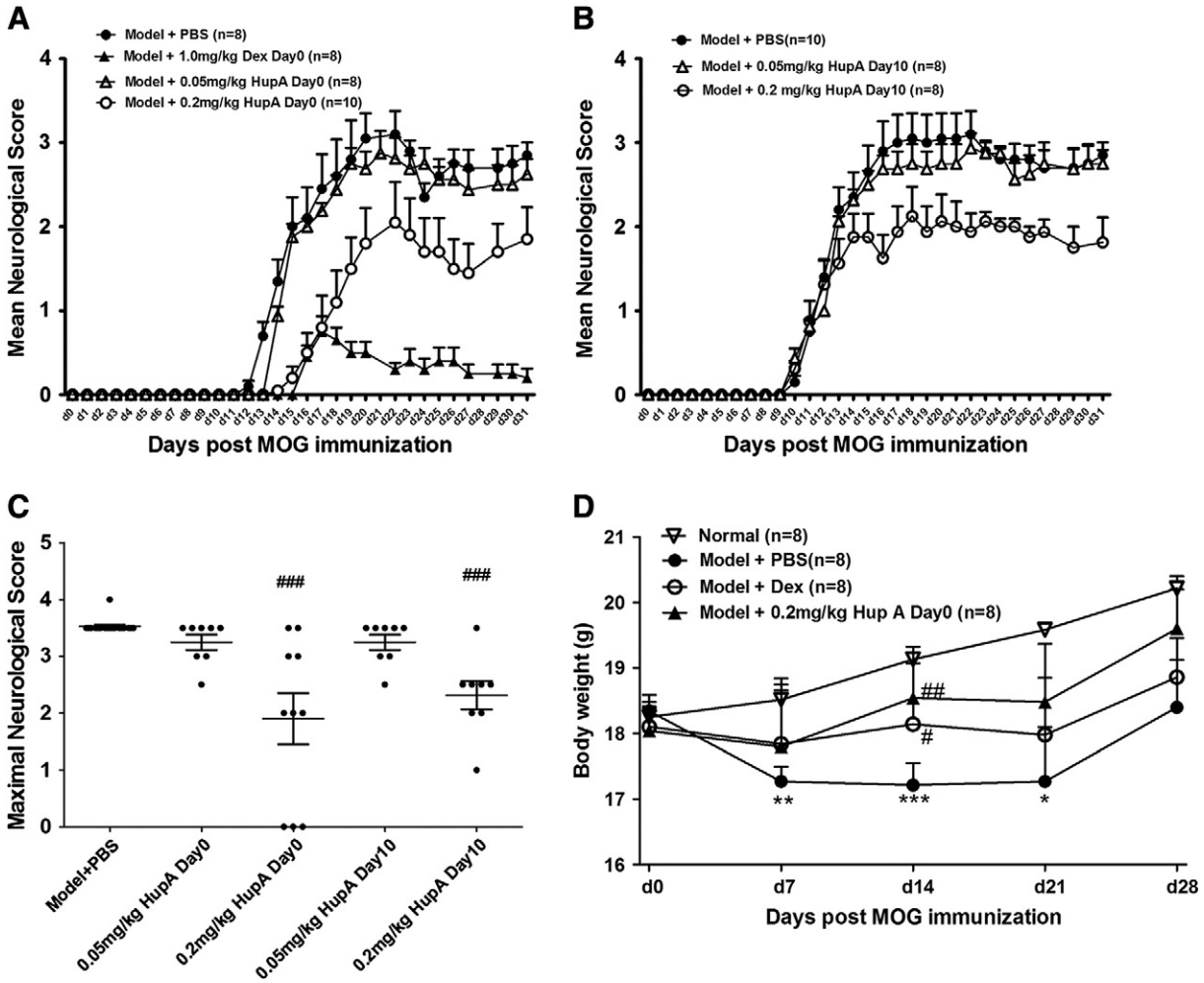


**Fig. 1.** Improved neurological behaviors in EAE mice with HupA treatment. Mice were intraperitoneally administered HupA (0.05 or 0.2 mg/kg) on a daily basis either from day 0 or from day 10 until day 31. Dexamethasone (Dex, 1 mg/kg) was used as a positive control for the treatment. The neurological scores (A: Prophylactic treatment and B: therapeutic treatment), the maximum EAE score of each mouse (C), and the mean body weight (D) are shown above (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with the normal group; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ compared with model + PBS group).

affliction of the hind limbs followed by the forelimbs. The mean maximal neurological score was as high as 3.53 in these mice (n = 18) (Fig. 1C). No abnormal neurological behaviors were observed in the control mice. High-dose glucocorticoid treatment is a widely adopted therapeutic strategy for the treatment of MS relapse. Dexamethasone (Dex) is a synthetic glucocorticoid and potent agonist for glucocorticoid receptors and is well known to alleviate the clinical symptoms of EAE, mainly through the suppression of inflammation via the inhibition of NF-κB-dependent gene expression (Eggert et al., 2008). Therefore, we used Dex as a positive control for the treatment in our experiments. In Dex-treated mice (n = 8), $MOG_{35-55}$ immunization induced only minor neurological signs. For example, these mice only showed paralysis of their tails. The mean neurological score in Dex-treated mice was only 0.625 (n = 8; $p<0.001$ vs. model group) (Fig. 1A). Similar to Dex, treatment with 0.2 mg/kg/d HupA significantly ameliorated EAE symptoms with considerably less severity when treatment was initiated at the day of disease induction (Figs. 1A and C). These mice showed only mild paralysis with a mean neurological score of 1.9 (n = 10; $p<0.001$ vs. model group) (Fig. 1C). Therapeutic intervention with HupA (0.2 mg/kg/d) at the onset of EAE symptoms (Fig. 1B) resulted in a significant reduction of EAE compared to vehicle-treated mice. Low-dose HupA treatment (0.05 mg/kg) had no impact on the EAE score (Figs. 1A and B). In addition to the development of neurological behaviors indicative of MS, the $MOG_{35-55}$-immunized mice showed a significant loss of body weight from day 7 to 21 after MOG injection (Fig. 1D). In contrast, the mice given prophylactic treatment with either HupA or Dex showed a gain in body weight on day 14 after $MOG_{35-55}$ injection (Fig. 1D). The results clearly indicate that HupA suppresses the development and progression of EAE.

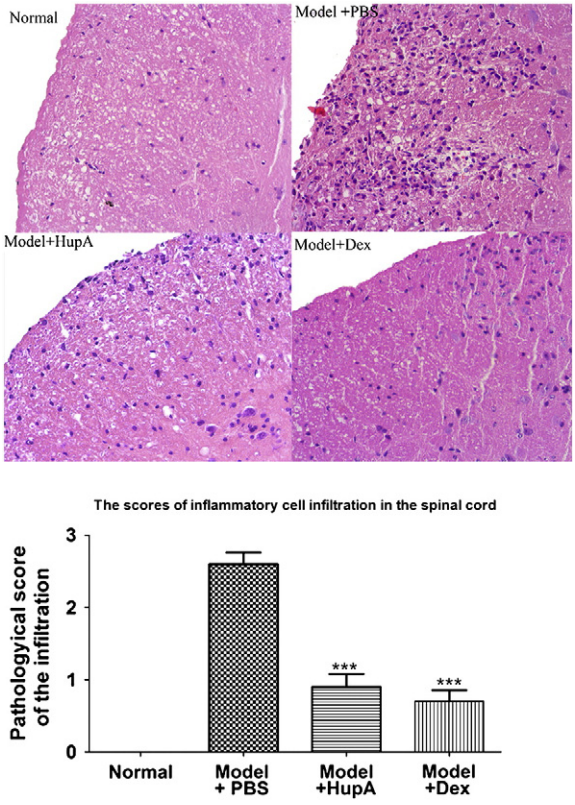


**Fig. 2.** Histological alterations in the spinal cord with HupA treatment in EAE mice. These histological assessments were performed in mice that lived until day 32 after immunization. Representative spinal cord sections (100× magnification; hematoxiline–eosin staining) from EAE mice showed intensive lymphocyte and macrophage infiltrations that were seldom observed in the sham group. These infiltrations of lymphocytes and macrophages could be largely attenuated with HupA and Dex (positive control) treatment (see the lower figure that shows the statistical results of the inflammation score; *** $p<0.001$ compared with model group).

*HupA reduces inflammatory infiltrates and demyelination in spinal cord tissue of EAE mice*

To examine whether the amelioration of neurological behaviors observed following prophylactic treatment with HupA resulted from a reduced infiltration of lymphocytes in the CNS, an inflammation assay was performed with histological staining of the spinal cord on day 32 after immunization. As shown in Fig. 2, there were no appreciable lymphocyte infiltrations in the perivascular area or white matter of the spinal cords of the control animals. However, in $MOG_{35-55}$-immunized mice, dense and widespread infiltrations of the spinal cord were observed. Prophylactic treatment with either HupA or Dex significantly attenuated the infiltrations; the mice in these two groups showed only minimal inflammation. Analysis of the inflammation score indicated a significant reduction with either HupA (n = 5; $p<0.001$, vs. model group) or Dex (n = 5; $p<0.001$ vs. model group) treatment (Fig. 2). These results suggest a protective effect of HupA in $MOG_{35-55}$-induced inflammation of the spinal cord. The LFB staining results revealed a normal myelin content of the spinal cord white matter (Fig. 3A). In non-treated EAE mice, the areas of demyelination were concentrated around the areas of inflammatory infiltration (Fig. 3B). Administration of either 0.2 mg/kg HupA or 0.2 mg/kg Dex preserved the myelin content and reduced the degradation of myelin in the spinal cord sections of the treated groups (Figs. 3C, D).

*HupA ameliorates demyelination and axonal injury in the spinal cord*

To further examine the effects of HupA on demyelination and axonal injury in the spinal cord of EAE mice, we performed an ultra-

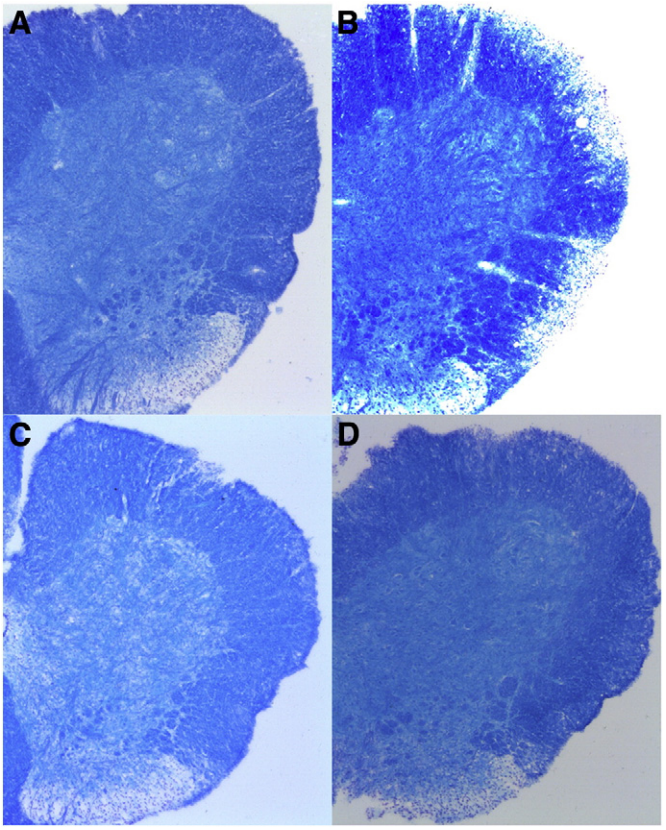


**Fig. 3.** HupA reduces the MOG-induced axonal damage in spinal cords of EAE mice. Mice were killed 32 days after EAE induction and sections of spinal cord were analyzed for damaged axons using LFB staining (100× magnification). (A) Normal mice; (B) MOG-induced EAE mice; (C) MOG-induced EAE mice treated with HupA (as described in Fig. 1); (D) MOG-induced EAE mice treated with Dex (positive control).

structural examination with transmission electron microscopy (TEM) on day 32 after $MOG_{35–55}$ immunization. At least two animals were used in each group. As shown in Fig. 4, in the spinal cords of $MOG_{35–55}$-immunized mice, severe axonal loss, extensive residual vacuoles and totally degenerated fibers were observed (Figs. 4A, B). Numerous hypo-myelinated fibers and naked axons were also observed, indicating progressive demyelination (Figs. 4A, B, arrow). In addition, macrophages were observed surrounding the myelinated axons in these mice (Figs. 4A, B), which has been regarded as an underlying mechanism of macrophage-associated myelin stripping and demyelination in MS (Dalcanto et al., 1975). The observed macrophages were contiguous to the myelin sheaths and caused them to degenerate (Fig. 4B, arrow). In contrast, the spinal cords of HupA-treated mice had significantly fewer pathological changes in their ultra-structures and predominantly intact myelin and normal axon appearances (Figs. 4C, D), suggesting that the HupA is protective against autoimmune-related demyelination and axonal injury.

*HupA down-regulates the expression of pro-inflammatory cytokines and chemokines in the spinal cords of EAE mice*

Similar to other inflammatory conditions, the release of cytokines and chemokines has been implicated in the pathogenesis of EAE (Alvarez et al., 2011). To further determine the effect of HupA on $MOG_{35–55}$-induced inflammation, we analyzed the expression of inflammatory cytokines and chemokines in the spinal cord with a qRT-PCR assay. We chose to measure cytokine and chemokine expression on day 14 after $MOG_{35–55}$ immunization because mice show obvious neurological symptoms (Fig. 1) and inflammation at this time point. As shown in Fig. 5, the pro-inflammatory cytokines IL-17 and IFN-γ were increased 19.08 ($n=6$; $p<0.001$) and 609.0 fold ($n=6$; $p<0.001$), respectively, in $MOG_{35–55}$-immunized mice compared to controls ($n=6$). In addition, chemokines that are proven to facilitate the inflammatory reaction in the CNS via the recruitment of macrophages and other leukocytes, such as MCP-1/CCL2, RANTES/CCL5 and TWEAK, were also up-regulated with $MOG_{35–55}$ immunization. For example, MOG immunization produced a 2134-, 463.4- and 5.1-fold increase in the mRNA levels of MCP-1/CCL2, RANTES/CCL5 and TWEAK, respectively (Fig. 5). Meanwhile, cytokines that are considered anti-inflammatory were also up-regulated or induced with $MOG_{35–55}$ immunization. For example, there was a 709.4-fold increase in gene expression of IL-10. In addition, IL-4 mRNA, which was undetectable in control mice, was significantly expressed in $MOG_{35–55}$-immunized mice suggesting an endogenous mechanism that limits the over-stimulated inflammatory response in EAE. In contrast, the enhanced expression of the pro-inflammatory cytokines IFN-γ and IL-17 was significantly decreased in HupA-treated mice to 99.65 fold ($n=6$; $p<0.001$, vs. model group) and 2.008 fold ($n=6$; $p<0.001$, vs. model group), respectively. The mRNA levels of the anti-inflammatory cytokines IL-10 and IL-4 were significantly increased ($n=6$; $p<0.05$ and $p<0.001$, respectively vs. model group) (Fig. 5). In addition to the changes in cytokine expression, chemokine expression was also suppressed by HupA treatment (Fig. 5). For example, there was a 95.13-fold decrease in MCP-1, a 232.0-fold decrease in RANTES, and a 3.985-fold decrease in TWEAK expression with HupA treatment. These results suggest that HupA depresses pro-inflammatory mediators while enhancing expression of anti-inflammatory cytokines in the spinal cords of EAE mice.

*HupA treatment inhibits the MOG-specific T-lymphocyte proliferation and Th1/Th17 response in the spleen*

Because T-lymphocytes play a key role in the pathology of MS, enhanced T-lymphocyte proliferation may aggravate an autoimmune reaction and neurological behaviors. We examined the effect of HupA on T-lymphocyte proliferation. We also tested the production of IFN-γ and IL-17, the hallmarks of Th1 and Th17 responses, because

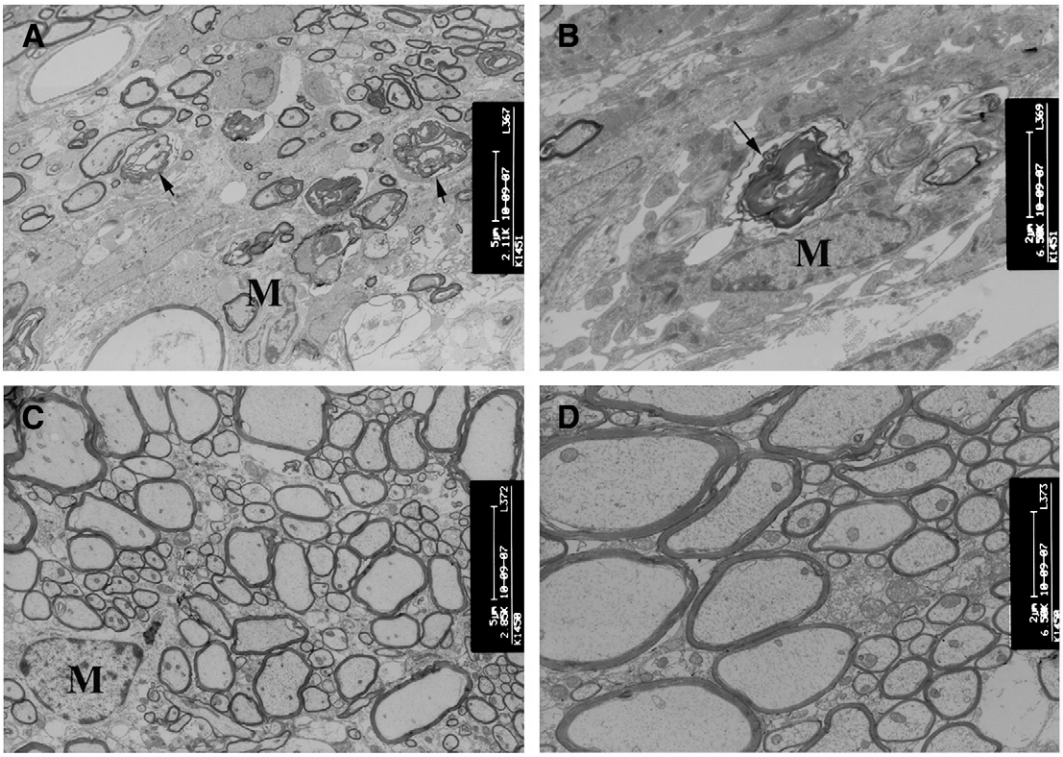


**Fig. 4.** Electron micrographs showing demyelination and axonal injury in the spinal cords of EAE mice. A and B are ultra-structures of the spinal cord of EAE mice with different scales (5 and 2 μm); C (5 μM) and D (2 μM) are that of HupA-treated mice. Note that demyelinated axons (arrow), along with infiltrating macrophage cells (M), are observed in the immunized mice with EAE (sacrificed at day 32) but are very sparse in the spinal cords of HupA treated mice. Most of the myelin sheaths and axonal structures are preserved after HupA treatment.

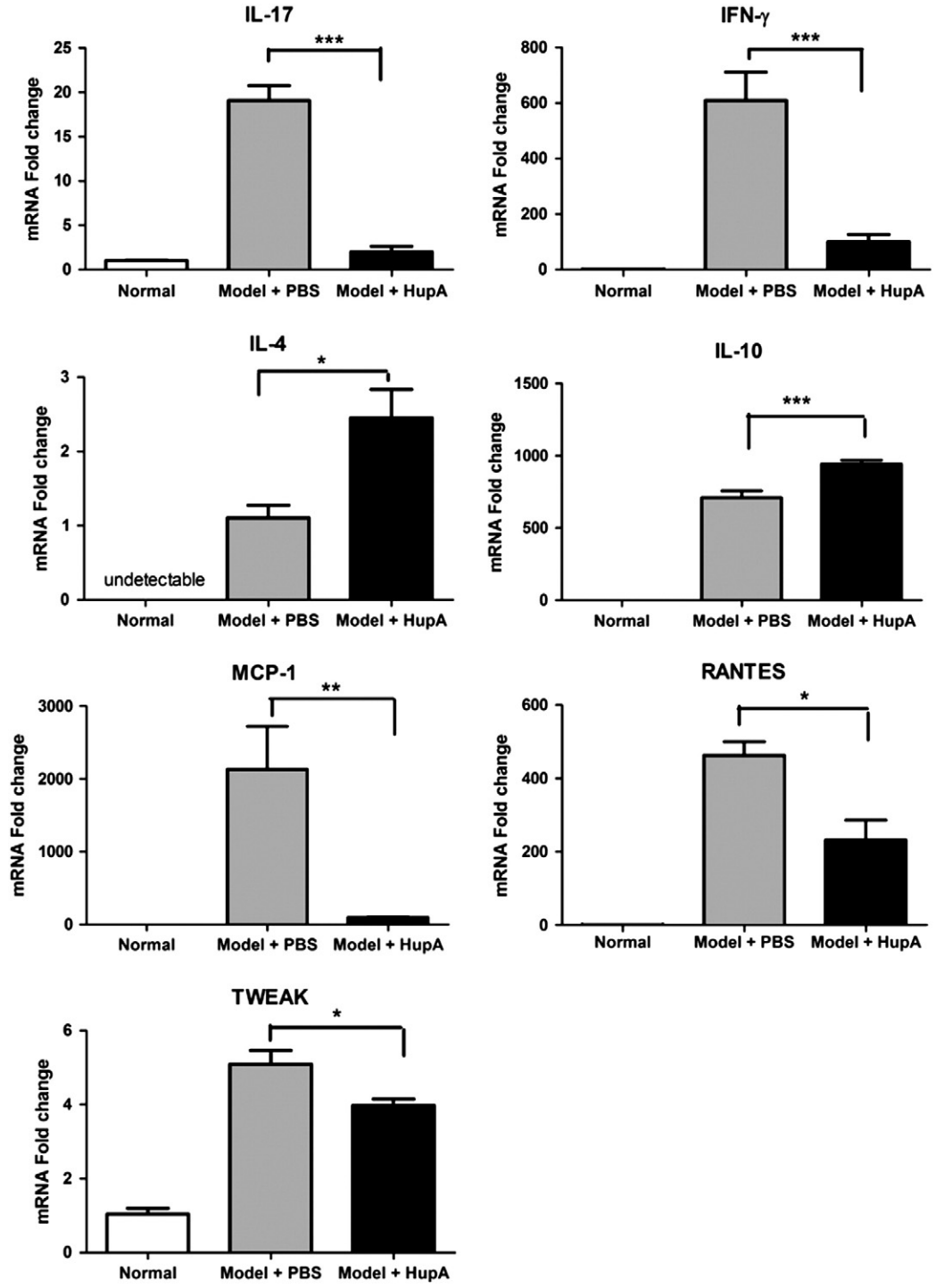


**Fig. 5.** Alterations in the mRNA expression of cytokines and chemokines in the spinal cords of $MOG_{35-55}$-immunized mice with HupA treatment. Quantitative mRNA expression was detected with qRT-PCR on day 14 post-immunization (n = 5/group). The data are presented as relative amount of transcript normalized to HPRT (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with the model). Note that $MOG_{35-55}$ immunization stimulates an increase in the expression of pro-inflammatory cytokines (IL-17, IFN-γ), chemokines (MCP-1, RANTES, and TWEAK) and anti-inflammatory cytokines (IL-4, IL-10). HupA treatment significantly down-regulates the increased expression of pro-inflammatory cytokines (IL-17, IFN-γ) and chemokines (MCP-1, RANTES, and TWEAK) but further enhances the expression of anti-inflammatory cytokines (IL-4, IL-10) in the spinal cord.

these pro-inflammatory cytokines are secreted by encephalitogenic T-cells to recognize the cognate antigens and therefore contribute to the pathogenesis of EAE. We found that $MOG_{35-55}$ dose-dependently stimulated the proliferation of T-cells in the spleen (n = 6; Fig. 6); the stimulation index increased to $1.833 \pm 0.16$, $2.64 \pm 0.30$ and $4.352 \pm 1.168$ after 1, 10, and 50 μg/ml MOG stimulation, respectively. The stimulation index dropped from $4.352 \pm 1.168$ to $2.955 \pm 0.422$ when stimulated with 50 μg/ml MOG and treated with HupA ($p<0.001$; Fig. 6). The release of IFN-γ and IL-17 from isolated splenocytes of EAE mice increased after 50 μg/ml MOG stimulation, from $65.42 \pm 32.88$ to $4623 \pm 1209$ pg/ml ($p<0.001$) and $16.5 \pm 4.68$ to $555.3 \pm 58.33$ pg/ml ($p<0.001$), respectively, in comparison to controls (Fig. 7). Moreover, HupA treatment also decreased the levels of IFN-γ and IL-17 to $2241 \pm 769.5$ pg/ml ($p<0.001$, vs. model) and $324.1 \pm 51.02$ pg/ml ($p<0.001$, vs. model), respectively (Fig. 7). These results suggest that T cells from HupA-treated EAE mice exhibit reduced proliferation and

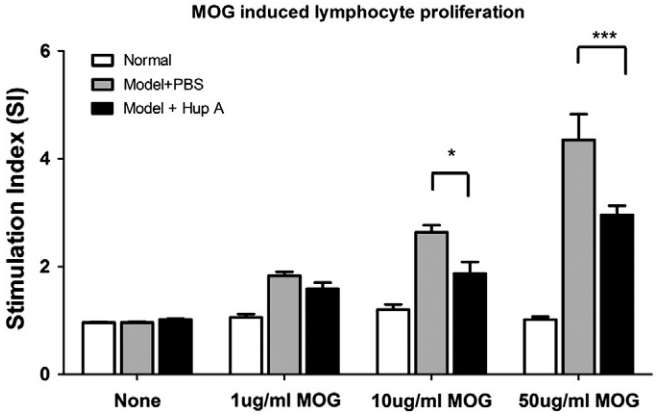


**Fig. 6.** HupA inhibition of $MOG_{35-55}$-specific T-cell proliferation in the spleen. Splenocytes were isolated on day 14 after $MOG_{35-55}$ immunization and cultured in the presence or absence of $MOG_{35-55}$ for 3 days. Proliferation was measured. The results are expressed as a Stimulation Index (SI). SI = mean OD value of the stimulated cells/mean OD values of the un-stimulated cells (* $p<0.05$, *** $p<0.001$, in comparison with the model group; $n=5$ mice per group). Note that HupA treatment strongly reduced $MOG_{35-55}$-stimulated T cell proliferation.

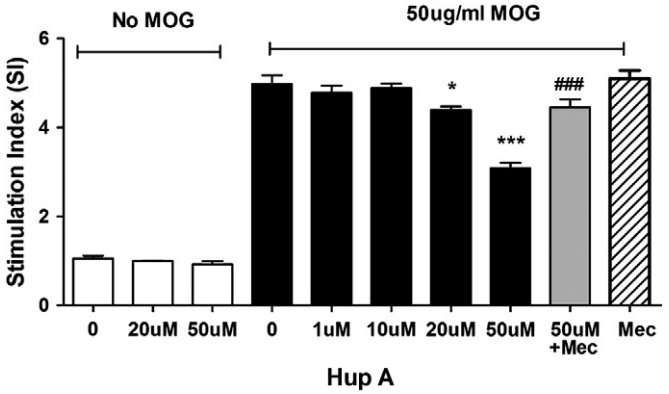


**Fig. 8.** HupA inhibition of $MOG_{35-55}$-induced T-cell proliferation ex vivo. Splenocytes isolated from EAE mice on day 14 post-immunization were exposed to 50 μg/ml $MOG_{35-55}$. HupA was added 30 min before $MOG_{35-55}$. The nicotinic receptor antagonist mecamylamine was applied 30 min before HupA treatment. The data shown here represent four independent experiments (* $p<0.05$, *** $p<0.001$ in comparison with the group of 0 μM HupA; ### $p<0.001$ in comparison with 50 μM HupA group). Note that HupA, at concentrations of 20 and 50 μM, significantly inhibits $MOG_{35-55}$-stimulated T cell proliferation, and this inhibition can be reversed by the nicotinic receptor antagonist mecamylamine.

Th1/Th17 pro-inflammatory responsiveness than T cells from untreated mice.

*HupA inhibits the MOG-induced proliferation of encephalitogenic T-cells ex vivo*

Splenocytes isolated from EAE mice were cultured in the medium with or without MOG stimulation. Different doses of HupA were added into the medium to evaluate the effect of HupA on MOG-induced proliferation. We found that the proliferation of cells stimulated with 50 μg/ml MOG was significantly down-regulated in the presence of 20–50 μM HupA. HupA significantly suppressed the 50 μg $MOG_{35-55}$-stimulated proliferation of T-cells in a concentration-dependent manner, with a maximal suppression at a concentration of 50 μM of HupA (Fig. 8). HupA inhibition of the $MOG_{35-55}$-stimulated proliferation of T-cells could be reversed by mecamylamine, a nicotinic receptor antagonist (Fig. 8). For example, administration of mecamylamine (50 μM) 30 min before HupA (50 μM) treatment could significantly increase the stimulation index, from $3.089 \pm 0.285$ to $4.453 \pm 0.430$ ($p<0.001$), suggesting that nicotinic receptors are involved in the anti-proliferative effect of HupA (Fig. 8).

*Mecamylamine abolished the therapeutic effect of HupA on EAE mice*

To evaluate the role of the nicotinic receptor in the therapeutic effect of HupA, mecamylamine, an antagonist of the nAChR, was administrated prior to HupA treatment. It was found that daily pretreatment with mecamylamine (10 mg/kg b.w.) markedly reduced the improvement of $MOG_{35-55}$-induced neurological behaviors and neurological scores observed following HupA treatment (Figs. 9A and B).

## Discussion

The goal of the present study was to determine whether HupA can improve the neurological behaviors of $MOG_{35-55}$-induced EAE mice and to explore the underlying mechanisms. The present findings suggest that HupA treatment can suppress the clinical and histopathological signs of EAE by suppressing autoimmune responses, inflammatory reactions and the subsequent demyelination and axonal injury in the spinal cord and that the effect of HupA involves nicotinic receptor-mediated events.

Cholinesterase inhibitors (ChEIs) have been widely used for the treatment of certain neurological diseases, such as myasthenia gravis and Alzheimer's disease (Barner and Gray, 1998; Maggi and Mantegazza, 2011). To date, four ChEIs, tacrine, donepezil, galanthamine and rivastigmine, have been approved by the US Food and Drug Administration for the treatment of AD. The efficacy of ChEI is somewhat limited by the gastrointestinal side effects caused by activation of peripheral cholinergic systems, which are often worsened by lack of compliance. Compared with the ChEIs currently in use, HupA has better penetration through the blood–brain barrier, higher oral

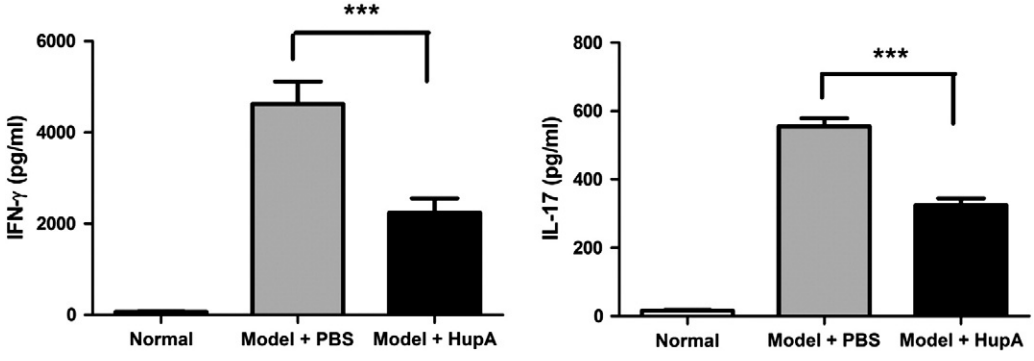


**Fig. 7.** Decreased Th1/Th17 cytokines release by HupA in splenocytes cultures of EAE mice. Splenocytes isolated from $MOG_{35-55}$-induced EAE mice (day 14) were stimulated ex vivo with $MOG_{35-55}$ peptide. Cytokines secreted into the culture supernatant were measured by ELISA 48 h after stimulation with $MOG_{35-55}$ (*** $p<0.001$, compared with model group).

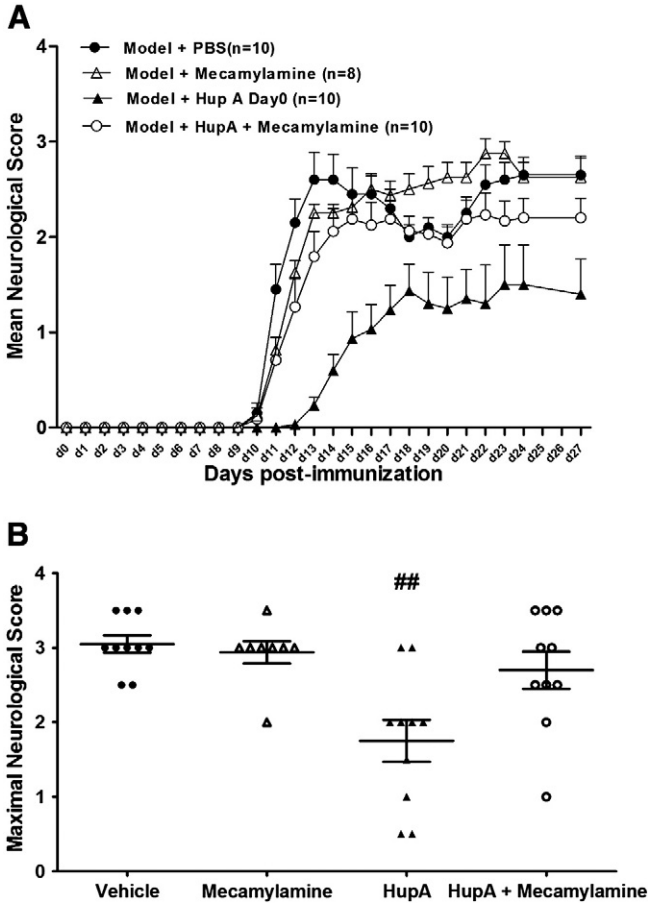


**Fig. 9.** Nicotinic receptor blocker mecamylamine antagonizes ameliorated neurological behaviors of HupA on EAE mice. Daily treatment with HupA, beginning on the day of EAE induction, reduces neurological scores of EAE mice, and this effect can be reversed by mecamylamine (10 mg/kg, ip; ## $p<0.01$ compared with vehicle group).

bioavailability, and a longer duration of AChE inhibitory action (Bai et al., 2000; Desilets et al., 2009; Wang et al., 2006a). HupA effectively reduces neurological behaviors and prevents the pathological alterations in the spinal cords of EAE mice. These findings suggest that HupA may be beneficial in the treatment of MS.

The improvement of neurological behaviors with HupA treatment is most likely attributable to the attenuated immune response/inflammatory reaction and decreased demyelination and axon damage in the spinal cords of EAE mice. In our study, inflammatory alterations in histology and ultra-structures (Figs. 2, 3 and 4) clearly indicate the attenuation of inflammatory reactions, demyelination and axonal injury in the spinal cords of EAE mice. In fact, it has been reported that rivastigmine, a pseudo-irreversible ChEI, attenuates motor symptoms in an animal model of EAE by the inhibition of T-cell proliferation (Nizri et al., 2008), which is similar to HupA. There are two possible mechanisms by which HupA may attenuate the immune response/inflammatory reaction: the inhibition of peripheral immune activation or a direct effect on the neurons or oligodendrocytes in the CNS involved in progression of the inflammatory process. HupA inhibition of T-cell proliferation and Th1/Th17 pro-inflammatory responses indicates that attenuated peripheral immune activation is one of the mechanisms underlying the improvement of neurological behaviors. Several studies have shown that HupA protects neuronal cells from insults such as hydrogen peroxide, beta-amyloid protein (or peptide), glutamate, ischemia and staurosporine-induced cytotoxicity and apoptosis (Hemendinger et al., 2008; Zhou and Tang, 2002). It is not clear whether the observed attenuated immune responsiveness and decreased neuronal damage in the CNS are caused by inhibiting inflammatory cell infiltration or by the neuroprotection of neurons by HupA. Whether HupA acts directly on neurons and oligodendrocytes in EAE mice requires further investigation.

IFN-γ and IL-17 are cytokines produced by Th1 and Th17 cells, respectively. These two cytokines have been shown to trigger Th1/Th17-type pro-inflammatory responses and induce EAE (El-behi et al., 2010; Harrington et al., 2005; Langrish et al., 2005). The down-regulation of IFN-γ and IL-17 and up-regulation of IL-4 and IL-10 by HupA indicates a shift of Th1/Th2 balance toward a Th2 type response. Namely, HupA not only suppresses pro-inflammatory responses but also increases anti-inflammatory responses, thus preventing inflammation-related demyelination and axonal injury and leading to the improvement of neurological behaviors in EAE. Similar observations regarding the anti-inflammatory properties of AChE inhibitors have been reported in AD, in which ChEIs induced the inflammatory response by shifting from Th1-type to Th2 (Reale et al., 2006, 2008). Nizri reported that rivastigmine suppresses neuroinflammation by inhibiting Th1/Th17 cytokine expression in EAE mice without any impact on Th2 responses (Nizri et al., 2008). Together, these findings suggest that ChEIs, including HupA, play their neuroprotective role by inhibiting inflammation in the CNS.

The observed inhibition of $MOG_{35-55}$-induced encephalitogenic T-cell proliferation and Th1/Th17 cytokine expression in the CNS suggests that the T-cells responsible for the production of these cytokines may be direct targets of HupA. Indeed, the AChE inhibitors galantamine and donepezil have been reported to modulate the functioning of the immune system by inhibiting both immuno-competent cell viability and pro-inflammatory cytokine secretion (Pavlov et al., 2009; Reale et al., 2005). Moreover, the AChEI edrophonium can inhibit phytohemagglutinin-induced proliferation of lymphocytes (Nizri et al., 2006). Because a high proliferation rate is a core response of immune cells following exposure to various stimuli, such as antigens, mitogens and cytokines, the potent anti-proliferative activity of HupA may contribute to its therapeutic effect and is consistent with our observation of diminished inflammatory cells in the spinal cords of EAE mice. Our findings also support the notion that AChE inhibitors, including HupA, have anti-proliferative and anti-inflammatory effects (Hao et al., 2011; Nizri et al., 2006; Pavlov et al., 2009; Wang et al., 2010).

Chemokines play a key role in the migration of peripheral immune cells to the CNS through the blood–brain barrier, which is normally impermeable to peripheral immune cells (Wilson et al., 2010). The chemokines MCP-1/CCL2 and RANTES/CCL5 have been found to be closely related to the pathophysiology of EAE (Mahad et al., 2006). Blockade of the interactions between chemokines and their receptors CCR2 and CCR5, which are expressed on many monocytes and T cells, leads to a reduction in inflammation in mouse models of immune-mediated demyelination (Brini et al., 2009; Glass et al., 2004; Mahad et al., 2006). Because MCP-1 attracts activated monocytes to the site of inflammation and RANTES attracts activated T-cells (Wilson et al., 2010), inhibition of the expression of MCP-1 and RANTES by HupA suggests a limited migration of monocytes and T cells into the CNS that may contribute to the diminished infiltration of inflammatory cells and the resultant injury in the spinal cord of HupA-treated mice.

Like other AChE inhibitors, HupA exerts its anti-proliferative effects by increasing both the level and duration of action of acetylcholine through the prevention of acetylcholine breakdown. HupA's effects can be blocked by the nAChR antagonist mecamylamine, which indicates that nAChR is involved. The recently discovered "cholinergic anti-inflammatory pathway" makes anti-inflammatory therapy more feasible (Tracey, 2007; Wang et al., 2003). Stimulation of the cholinergic anti-inflammatory pathway by electrical or pharmacological manipulations significantly inhibits the production of systemic pro-inflammatory cytokines (Pavlov et al., 2009). This inhibition

may contribute to the attenuated demyelination and axonal injury in the CNS we observed in this study. In fact, our results indicate that mecamylamine markedly reverses the HupA improvement of $MOG_{35-55}$-induced neurological behaviors in EAE mice.

In summary, we have shown that HupA treatment in $MOG_{35-55}$-immunized mice reduces the severity of the clinic signs of EAE by the inhibition of inflammation, demyelination, and axonal injury in the spinal cord as well as the proliferation of encephalomyelitic T-cells. These effects are mediated by nAChR. HupA is more potent than other currently prescribed AChE inhibitors, more selective for central AChE than peripheral AChE (Bai et al., 2000), and has already been approved for the treatment of AD (Wang et al., 2009). Therefore, we conclude that HupA may be of potential therapeutic value for the treatment of autoimmune or neurodegenerative diseases such as MS.

## Statement of interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

This work was sponsored by the Shanghai Scientific and Technological Innovation program (09dZ1974100) and the National Key Basic Research Program of China (2007CB512502). The authors are grateful to Dr. DongMan Chao (The University of Texas Medical School, Houston, USA) for his valuable suggestions, critical comments and English revision of the manuscript.

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