

# Memantine ameliorates motor impairments and pathologies in a mouse model of neuromyelitis optica spectrum disorders.

**Leung-Wah Yick**

University of Hong Kong Li Ka Shing Faculty of Medicine

**Chi-Ho Tang**

University of Hong Kong Li Ka Shing Faculty of Medicine

**Oscar Ka-Fai Ma**

University of Hong Kong Li Ka Shing Faculty of Medicine

**Jason Shing-Cheong Kwan**

University of Hong Kong Li Ka Shing Faculty of Medicine

**Koon Ho CHAN** (✉ [koonho@hku.hk](mailto:koonho@hku.hk))

University of Hong Kong <https://orcid.org/0000-0003-4665-2576>

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## Research

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# Abstract

**Background:** Neuromyelitis optica spectrum disorders (NMOSD) are central nervous system (CNS) autoimmune inflammatory demyelinating diseases characterized by recurrent episodes of acute optic neuritis and transverse myelitis. Aquaporin-4 immunoglobulin G (AQP4-IgG) autoantibodies, which target the water channel aquaporin-4 (AQP4) on astrocytic membrane, are pathogenic in NMOSD. Glutamate excitotoxicity, which is triggered by internalization of AQP4-glutamate transporter complex after AQP4-IgG binding to astrocytes, is involved in early NMOSD pathophysiology. We studied the effects of memantine, a N-methyl-D-aspartate (NMDA) receptor antagonist, on motor impairments and spinal cord pathologies in mice which received human AQP4-IgG.

**Methods:** Purified IgG from AQP4-IgG-seropositive NMOSD patients were passively transferred to adult C57BL/6 mice with disrupted blood-brain barrier. Memantine was administered by oral gavage. Motor impairments of the mice were assessed by beam walking test. Spinal cords of the mice were assessed by immunofluorescence and ELISA.

**Results:** Oral administration of memantine ameliorated the motor impairments induced by AQP4-IgG, no matter the treatment was initiated before (preventive) or after (therapeutic) disease flare. Memantine profoundly reduced AQP4 and astrocyte loss, and attenuated demyelination and axonal loss in the spinal cord of mice which had received AQP4-IgG. The protective effects of memantine were associated with inhibition of apoptosis and suppression of neuroinflammation, with decrease in microglia activation and neutrophil infiltration and reduction of increase in levels of proinflammatory cytokines including interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). In addition, memantine elevated growth factors including brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) in the spinal cord.

**Conclusions:** Our findings support that glutamate excitotoxicity and neuroinflammation plays important roles in complement-independent pathophysiology during early development of NMOSD lesions, and highlight the potential of oral memantine as a therapeutic agent in NMOSD acute attacks.

## Introduction

Neuromyelitis optica spectrum disorders (NMOSD) are inflammatory demyelinating disorders of the central nervous system (CNS) clinically characterized by recurrent attacks of acute optic neuritis, transverse myelitis and less frequently encephalitis. Patients with severe NMOSD can develop blindness, paraplegia, and even mortality [1]. The majority of NMOSD patients are seropositive for aquaporin-4 immunoglobulin G autoantibodies (AQP4-IgG), which target the water channel aquaporin-4 (AQP4) [2, 3] that is highly expressed on the membrane of astrocytic foot processes [4]. NMOSD seropositive for AQP4-IgG are considered as an autoimmune astrocytopathy [5, 6].

Diverse lesion pathologies have been observed in the CNS of NMOSD patients, suggesting that multiple pathophysiology are involved in acute attacks of NMOSD including both complement-dependent and -

independent mechanisms [7]. Notably, the initiator of complement cascade, C1q, is absent in the quiescent CNS. Complement-independent events likely contribute to the early development of NMOSD lesions. These events include AQP4 loss from internalization and degradation of the antigen-antibody complex [8], glutamate excitotoxicity from reduction of the glutamate transporter excitatory amino acid transporter 2 (EAAT2) [9, 10], neuroinflammation due to release of proinflammatory cytokines and chemokines from astrocytes [11], and antibody-dependent cell-mediated cytotoxicity (ADCC) which involves microglia/macrophages and granulocytes [12].

Our recent study showed that mice received IgG from AQP4-IgG-seropositive NMOSD patients developed complement-independent spinal cord pathologies, including AQP4 and glial fibrillary acidic protein (GFAP) loss, EAAT2 decrease, microglia activation, neutrophil infiltration, demyelination and axonal loss, which were associated with motor impairments [13]. These findings support that AQP4-IgG binding to astrocytes triggers glutamate excitotoxicity secondary to EAAT2 decrease, and neuroinflammation and astrocyte cytotoxicity through ADCC by activated microglia and infiltrated granulocytes. Targeting glutamate excitotoxicity caused by AQP4-IgG binding to astrocytes may be a potential therapeutic intervention in acute attacks of NMOSD.

Memantine (1-amino-3,5-dimethyladamantane hydrochloride) is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist. It is an approved drug for the treatment of dementia in Alzheimer's disease and Parkinson's disease [14, 15]. Memantine has been shown to be neuroprotective in different animal models of CNS damages. In an experimental autoimmune encephalomyelitis model with optic neuritis, memantine reduced optic nerve demyelination, and protected axons and retinal ganglion cells [16]. In experimental glaucomatous neurodegeneration, memantine prevented astrocytic dysfunction of the optic nerves by enhancing mitochondrial fission, increasing mitochondrial volume density and length, and reducing auto-phagosome formation [17]. Following subarachnoid hemorrhage, memantine suppressed apoptotic cascade via reducing neuronal nitric oxide synthase expression, peroxynitrite formation, and subsequent oxidative stress [18]. In this study, we investigated if memantine exerts neuroprotective effects in mice which received passive transfer of IgG from AQP4-IgG-seropositive NMOSD patients.

## Materials And Methods

### Patient samples and IgG purification

Sera/plasma were obtained from 13 NMOSD patients who were AQP4-IgG-seropositive as detected by cell-based indirect immunofluorescence assay [19] and 3 healthy subjects. IgG from sera or plasma was isolated using HiTrap Protein G Sepharose columns (GE Healthcare Bio-sciences, USA). Samples were further purified with Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, USA) and concentrated with Amicon Ultra-15 centrifugal filters (Merck Millipore, Germany). Protein concentration was measured by Bradford assay (Bio-Rad, USA). Pooled IgG isolated from AQP4-IgG-seropositive NMOSD patients was termed IgG(AQP4+). Pooled IgG isolated from healthy subjects was termed IgG(con).

## Mice

Female wild-type C57BL/6 mice of age 6-8 weeks were used. Mice were housed in the animal facilities at the Laboratory Animal Unit of The University of Hong Kong. They were maintained in groups of five per cage under a 12 h dark/light cycle and provided with free access of water and chow.

## Disruption of BBB and passive transfer of IgG from NMOSD patients

Animal procedures are summarized in Fig 1a. To disrupt the BBB, mice were anaesthetized with intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). They received subcutaneous injections of complete Freund's adjuvant (CFA, BD Biosciences, USA) containing heat-killed H37Ra *Mycobacterium tuberculosis* (Difco, USA) at 4 sites (50 µg in 50 µl CFA each site) on the hind flank on 7 days before IgG transfer. In addition, mice received i.p. injections of pertussis toxin (PTx, 200 ng in 0.2 ml PBS, List Biological Laboratories, USA) on 7 and 3 days prior to IgG transfer.

## Drug treatments

Memantine (Sigma-Aldrich, USA) was prepared by adding the compound to corn oil (Sigma-Aldrich, USA). The mixture was sonicated at room temperature for 20 min. It was freshly made immediately before each administration. Following BBB disruption, mice were randomly assigned to four sets of experiments. For preventive treatment, IgG(AQP4+) mice in set 1 received i.p. injection of 4 mg purified IgG from AQP4-IgG-seropositive NMOSD patients and oral gavage of vehicle or 60 mg/kg body weight memantine on the day of IgG passive transfer (day 0). Animals were sacrificed at 1 day post-injection (dpi). In set 2, beginning from day 0, IgG(AQP4+) mice received daily i.p. injection of purified IgG from AQP4-IgG-seropositive NMOSD patients and oral gavage of vehicle or memantine till 3 dpi. Animals were sacrificed at 4 dpi. In set 3, beginning from day 0, IgG(AQP4+) mice received daily i.p. injection of purified IgG from AQP4-IgG-seropositive NMOSD patients and oral gavage of vehicle or memantine till 7 dpi. Animals were sacrificed at 8 dpi. For therapeutic treatment in set 4, IgG(AQP4+) mice received daily i.p. injection of purified IgG from AQP4-IgG-seropositive NMOSD patients beginning from day 0 till 7 dpi. Oral gavage of vehicle or memantine was begun on the 4 dpi and continued once daily immediately after IgG injection till 7 dpi. Animals were sacrificed at 8 dpi. IgG(con) mice which received vehicle were used as sham controls in all sets of experiments. The dose of memantine was adopted from a previous study [16].

## Detection of motor impairments

Motor impairments were detected using beam walking test, in which examines the animal's ability to keep upright and walk across an elevated narrow beam to a platform [20]. The apparatus consisted of 80 cm

long beams with width of 1.2 or 0.6 cm, resting 50 cm above a table top on two stands. Prior to CFA and PTx treatment, mice were trained for 2 days, with 3 trials a day, with walking across each of the 12 and 6 mm-wide beams. One day after completion of IgG transfer and drug treatment, the time for the mice to cross each beam and the number of paw slips during the process were recorded.

## **Tissue preparation and immunofluorescence**

After beam walking test, mice were sacrificed by pentobarbital overdose and received intracardiac perfusion with ice-cold PBS and paraformaldehyde. Cervical spinal cords were harvested, and sectioned at 10- $\mu$ m thickness with a cryostat (Thermo Fisher Scientific, USA). Immunofluorescence was performed with standard procedure. The following primary antibodies were used: (1) rabbit anti-human IgG (1:600, Dako, USA), (2) rabbit anti-AQP4 (1:200, Sigma-Aldrich, USA), (3) mouse anti-glial fibrillary acidic protein (GFAP, 1:200, Santa Cruz Biotechnology, USA), (4) goat anti-myelin basic protein (MBP, 1:200, Dako, USA), (5) rabbit anti-neurofilament heavy polypeptide (NF-H, 1:400, Sigma-Aldrich, USA) (6) rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1, 1:200, Wako, Japan), and (7) rat anti-lymphocyte antigen 6 complex locus G6D (Ly6G, 1:400, Abcam, UK). Sections were then incubated with the appropriate Alexa-Fluor-conjugated secondary antibodies (Thermo Fisher Scientific, USA) at room temperature for 1 h. They were counterstained with DAPI and mounted with antifade reagent (Thermo Fisher Scientific, USA).

## **Image processing and quantification**

Measurement of immunofluorescent intensity was performed on eight rostral-to-caudal alternate cross sections of the ventrolateral white matter of the cervical spinal cord. All signals were captured with the same microscope (Nikon Eclipse Ni, Japan) and digitized with SPOT software 5.0 (Diagnostic Instruments, USA) in identical settings. Signal intensity was quantified using ImageJ software (Wayne Rasband, NIH, USA).

## **Histochemistry**

Luxol fast blue (LFB) staining was performed according to the manufacturer's instructions (Abcam, UK). Briefly, spinal cord sections were rehydrated and incubated in 0.1% Luxol fast blue solution at 40°C overnight. Excess stain was rinsed with 95% ethanol. Slides were washed in distilled water, immersed in 0.05% lithium carbonate solution for 30s, and then dehydrated in serial ethanols and mounted in Permount mounting medium (Sigma-Aldrich, USA). In addition, selected sections were stained with hematoxylin and eosin (H&E) using standard procedure.

## Detection of apoptotic cells

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using the In-situ Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instructions. Sections were mounted by counterstained with DAPI and mounted with antifade reagent (Thermo Fisher Scientific, USA). To quantify apoptotic cells, cells positive for TUNEL with condensed nuclei were counted on eight rostral-to-caudal alternate cross sections of the ventrolateral white matter of the cervical spinal cord.

## ELISA for cytokines and growth factors

Following transcardiac perfusion with ice-cold PBS, spinal cords of mice were harvested and homogenized using a lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor. Levels of proinflammatory cytokines including interleukin-1 $\beta$  (IL-1 $\beta$ ; RayBiotech, USA), interleukin-6 (IL-6; RayBiotech, USA) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; RayBiotech, USA), and growth factors including brain-derived neurotrophic factor (BDNF; MyBioSource, USA), glial cell line-derived neurotrophic factor (GDNF; MyBioSource, USA) and vascular endothelial growth factor (VEGF; MyBioSource, USA) in the homogenates were determined using mouse ELISA kits according to the manufacturer's instructions.

## Statistical analysis

Differences between groups were compared by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. Data are shown as mean  $\pm$  SEM. Levels of significance are indicated with \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . Calculation was performed using IBM SPSS Statistics Version 24 software.

# Results

## Memantine improved AQP4-IgG-induced motor impairments

Passive transfer of human AQP4-IgG to mice with disrupted BBB causes motor impairments [13]. To assess whether memantine improved motor performance in this animal model, beam walking test was performed in IgG(AQP4+) mice which had received either preventive or therapeutic treatment of memantine. We found that 60 mg/kg/day of memantine is effective on improving clinical severity, and therefore this dose was used in subsequent experiments (Additional file 1). On a 1.2 x 80 cm (width x length) beam, no significant differences on motor performance were observed between sham, vehicle-treated and memantine-treated mice at 1 dpi. Onset of motor impairments was found at 4 dpi, when vehicle-treated IgG(AQP4+) mice required significantly longer time to cross the beam and displayed significantly more paw slips during walking on the beam than sham mice. Preventive treatment with memantine at 60 mg/kg/day, when it was begun on the same day of IgG(AQP4+) passive transfer, completely prevented the development of motor impairments in terms of time to cross the beam and

number of paw slips during walking on the beam. Therapeutic treatment with memantine at 60 mg/kg/day, begun at 4 days after the first IgG(AQP4+) passive transfer, also prevented the development of motor impairments. The efficacy of therapeutic treatment was comparable to that of preventive treatment (Fig 1b and c). Similar findings were observed in walking test using a narrower 0.6 x 80 cm (width x length) beam that allowed detection of more subtle motor impairments. Onset of motor impairments was observed at 4 dpi. Both preventive and therapeutic treatment with memantine significantly reduced the time to cross the beam and the number of paw slips during walking on the beam (Fig 1d and e). These findings indicated that memantine markedly ameliorated motor impairments triggered by human AQP4-IgG in mice.

### **Memantine decreased AQP4-IgG-induced AQP4 and GFAP loss**

Our previous study has shown that passive transfer of human AQP4-IgG to mice with disrupted BBB does not cause complement activation in the spinal cord [13], consistent with the findings from others [21]. To evaluate AQP4-IgG-induced astrocytopathy in response to memantine, immunofluorescence analyses were performed. With pretreatment of CFA and PTx to disrupt the BBB, we found human IgG infiltration in the spinal cord parenchyma in all groups of mice (Additional file 2). Double immunofluorescence staining revealed colocalization of AQP4 and GFAP in the spinal cord white matter of the mice. At 1 dpi, no differences on AQP4 and GFAP immunofluorescences in the spinal cords were observed between sham, vehicle-treated and memantine-treated IgG(AQP4+) mice. Losses of AQP4 and GFAP immunofluorescences began at 4 dpi in vehicle-treated IgG(AQP4+) mice. Both preventive and therapeutic treatment with memantine decreased the losses of AQP4 and GFAP immunofluorescences at 4 and 8 dpi compared to vehicle-treated IgG(AQP4+) mice (Fig 2a). Evaluation of immunofluorescence intensity confirmed that the increases in AQP4 and GFAP levels in memantine-treated mice at 4 and 8 dpi were statistically significant compared to vehicle-treated IgG(AQP4+) mice (Fig 2b and c).

### **Memantine ameliorated demyelination and axonal loss**

We next examined whether memantine preserved myelin integrity and prevented axonal loss secondary to AQP4-IgG binding to astrocytes. MBP and NF-H immunofluorescence revealed, beginning from 4 dpi, prominent and patchy loss of myelin and axons in the spinal cord white matter of vehicle-treated IgG(AQP4+) mice compared to sham. Preventive and therapeutic treatment with memantine greatly increased MBP and NF-H signals at 4 and 8 dpi compared to vehicle-treated IgG(AQP4+) mice (Fig 3a). Evaluation of immunofluorescence intensity confirmed that the increase in MBP level in memantine-treated mice at 4 and 8 dpi were statistically significant compared to vehicle-treated IgG(AQP4+) mice (Fig 3b). Counting of NF-H-positive spots confirmed that at 4 and 8 dpi memantine-treated mice had more axons in the spinal cord than vehicle-treated IgG(AQP4+) mice (Fig 3c). Luxol fast blue staining further showed that memantine preserved myelin integrity in the spinal cord of IgG(AQP4+) mice (Additional file

3). These results indicated a protective effect of memantine against human AQP4-IgG-triggered demyelination and axonal loss in mouse spinal cord.

### **Memantine prevented apoptosis of astrocytes**

To investigate whether memantine prevented cell loss by apoptosis, we detected DNA fragmentation using the TUNEL assay. At 1 dpi, apoptotic cells in the spinal cord white matter of vehicle-treated IgG(AQP4+) mice were more than that in sham mice. Memantine treatment markedly reduced apoptotic cells (Fig 4a). Quantitative analysis revealed that the number of TUNEL-positive cells was significantly reduced in the memantine-treated mice, compared to the vehicle-treated IgG(AQP4+) mice (Fig 4b). At 4 and 8 dpi, no apoptotic cells were found in the spinal cord of mice in all groups (Data not shown). These results suggested that astrocytic apoptosis is an early event upon AQP4-IgG binding to astrocytes, and memantine treatment could prevent apoptotic cell death of astrocytes induced by human AQP4-IgG in mouse spinal cord.

### **Memantine attenuated microglia activation and neutrophil infiltration**

To examine whether memantine influenced neuroinflammation triggered by human AQP4-IgG, immunofluorescence analyzes on microglia activation and neutrophil infiltration were performed. At 1 dpi, no differences on microglia activation, as evaluated by Iba-1 immunofluorescence, and neutrophil infiltration, as evaluated by Ly6G immunofluorescence, were observed between the spinal cords of sham, vehicle-treated and memantine-treated IgG(AQP4+) mice. Profound increases in Iba-1 and Ly6G immunofluorescences were observed since 4 dpi in vehicle-treated IgG(AQP4+) mice. At 4 and 8 dpi, both preventive and therapeutic treatment with memantine decreased Iba-1 and Ly6G immunofluorescences compared to vehicle-treated IgG(AQP4+) mice (Fig 5a). Evaluation of immunofluorescence intensity confirmed that the decreases in Iba-1 and Ly6G levels in memantine-treated mice at 4 and 8 dpi were statistically significant compared to vehicle-treated IgG(AQP4+) mice (Fig 5b and c). H&E staining revealed tissue damage and inflammatory cell infiltration in the spinal cord parenchyma of vehicle-treated IgG(AQP4+) mice, while these were markedly reduced with memantine treatments (Additional file 3). These results indicated that memantine suppressed AQP4-IgG-induced microglia activation and neutrophil infiltration in the spinal cord.

### **Memantine reduced concentrations of proinflammatory cytokines in the spinal cord**

Activated microglia releases proinflammatory cytokines such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  [22]. AQP4-IgG can induce astrocytes to release a broad spectrum of proinflammatory mediators including IL-1 $\beta$  and IL-6 [11]. To investigate whether memantine treatment reduced neuroinflammation, ELISA of IL-1 $\beta$ , IL-6 and

TNF- $\alpha$  were performed in spinal cord homogenates of mice in different groups. Results revealed that concentrations of IL-1 $\beta$  and IL-6 in the spinal cords of vehicle-treated IgG(AQP4+) mice were markedly higher than that of sham mice at all the time points examined. Both preventive and therapeutic treatment with memantine significantly reduced IL-1 $\beta$  and IL-6 concentrations compared to vehicle-treated IgG(AQP4+) mice (Fig 6a and b). The concentration of TNF- $\alpha$  in the spinal cords of vehicle-treated IgG(AQP4+) mice was markedly higher than that of sham mice at 1 and 4 dpi, and then it decreased to that similar to sham. Preventive treatment with memantine significantly reduced TNF- $\alpha$  concentration at 1 and 4 dpi compared to vehicle-treated IgG(AQP4+) mice (Fig 6c).

### **Memantine elevated concentrations of growth factors in the spinal cord**

To further characterize the protective effects of memantine, we performed ELISA to evaluate the concentrations of BDNF, GDNF and VEGF in spinal cord homogenates of mice in different groups. Results revealed that BDNF concentration in the spinal cords of vehicle-treated IgG(AQP4+) mice was markedly lower than that of sham mice at all the time points examined. Both preventive and therapeutic treatment with memantine significantly increased BDNF concentration compared to vehicle-treated IgG(AQP4+) mice (Fig 7a). GDNF concentration in the spinal cord of vehicle-treated IgG(AQP4+) mice did not differ significantly compared to sham mice at all the time points examined. Both preventive and therapeutic treatment with memantine significantly increased GDNF concentration compared to sham and vehicle-treated IgG(AQP4+) mice at 4 and 8 dpi (Fig 7b). Similarly, VEGF concentration in the spinal cord of vehicle-treated IgG(AQP4+) mice did not differ significantly compared to sham mice. The memantine treatments significantly increased VEGF concentration compared to sham and vehicle-treated IgG(AQP4+) mice at all the time points examined (Fig 7c).

## **Discussion:**

We recently reported that passive transfer of human AQP4-IgG to mice with disrupted BBB led to complement-independent NMOSD-like spinal cord pathologies of AQP4 and astrocyte loss, EAAT2 loss, microglia activation, neutrophil infiltration, demyelination and axonal loss, which were associated with motor impairments [13]. Here we showed that memantine, when administered either simultaneously with or 4 days after passive transfer of human AQP4-IgG, ameliorated motor impairments in mice associated with marked alleviation of NMOSD-like pathologies, including decrease of AQP4 and GFAP loss, and reduction of demyelination and axonal loss. The mechanisms underlying the preventive and therapeutic effects of memantine involved prevention of apoptotic cell death, suppression of microglia activation and neutrophil infiltration, reduction of proinflammatory cytokines, and elevation of growth factors. These results indicate important pathological roles of glutamate excitotoxicity and neuroinflammation in NMOSD acute attacks, and highlight the potential of memantine as a therapeutic agent in NMOSD acute attacks.

Memantine is approved for the treatment of Alzheimer's disease. It is an open channel blocker of NMDA receptor with faster blocking / unblocking kinetics and stronger voltage dependency than other competitive and non-competitive NMDA antagonists [23]. These properties account for the better safety and tolerability of memantine compared to other NMDA receptor blockers, such as MK-801 and phencyclidine which may cause negative psychotropic effects [23, 24]. Memantine is an effective blocker of NMDA receptor only during pathological conditions, without affecting normal physiological functions. We found that both preventive and therapeutic treatment with memantine improved motor performance in mice which had received human AQP4-IgG. To our knowledge, this is the first study to show a clinical benefit of memantine in a NMOSD model.

Following AQP4-IgG binding to astrocytic AQP4, complement-independent pathophysiologies have been suggested to be important early processes in the development of NMOSD lesions [10, 25]. AQP4-IgG binding causes internalization of AQP4 together with the glutamate transporter EAAT2 from astrocytic membrane. This reduces the uptake of extracellular glutamate by astrocytes and disrupts glutamate homeostasis, leading to glutamate excitotoxicity against NMDA receptor-expressing glial cells and neurons [9, 26–29]. A recent study demonstrated that co-endocytosis of AQP4 and EAAT2 on astrocytic membrane upon AQP4-IgG binding required astrocytic Fcγ receptor, supporting a role of glutamate excitotoxicity in the early pathophysiologies of NMOSD acute attacks [10]. AQP4-IgG from NMOSD patients caused astrocyte injury with secondary damage to oligodendrocytes mediated by glutamate excitotoxicity, which may contribute to the demyelination observed in our model [29]. Overstimulation of NMDA receptors by glutamate triggers excessive Ca<sup>2+</sup> influx, mitochondrial dysfunction, oxidative stress and apoptotic cell death [30]. We found that memantine prevented astrocyte loss, demyelination and axonal loss in mice which had received human AQP4-IgG. We also detected significantly more apoptotic cells in the spinal cord white matter of vehicle-treated mice than that of memantine-treated mice early (1 day) after passive transfer of AQP4-IgG. Our previous study found that human AQP4-IgG did not cause significant loss of oligodendrocytes and neurons in this mouse model [13]. The apoptotic cells in the white matter observed in the current study were likely astrocytes. These findings suggested that memantine protected astrocytes from glutamate excitotoxicity and apoptosis that were triggered by AQP4-IgG binding. Indeed, loss of EAAT2 was observed in the spinal cord lesion of NMOSD patients, supporting a role of glutamate excitotoxicity in NMOSD pathophysiologies in human [5].

AQP4-IgG binding stimulates astrocytes to release proinflammatory cytokines, chemokines and other inflammatory mediators that can activate microglia, thus promote further production of inflammatory mediators in a vicious cycle [11]. This aggravates neuroinflammation which involves microglia activation and parenchymal infiltration of macrophages, granulocytes and natural killer cells from the peripheral circulation. These activated immune cells with Fc receptors can lead to astrocyte cytotoxicity via ADCC [12, 31, 32]. The protective effect of memantine on astrocytes likely attenuated the release of proinflammatory cytokines and chemokines from astrocytes triggered by AQP4-IgG binding, hence reduced microglia activation. In the EAE model of multiple sclerosis, memantine reduced neurological symptoms and decreased the expression of proinflammatory cytokines in the brain [33]. In the mouse

cuprizone model of demyelination, memantine inhibits the production of proinflammatory cytokines by astrocytes via modulation of NMDA receptor [34]. Moreover, the anti-inflammatory property of memantine was shown to be mediated through inhibition of microglia activation [35]. Consistent with these studies, we found that memantine suppressed human AQP4-IgG-triggered microglia activation and neutrophil infiltration, and reduced proinflammatory IL-1 $\beta$ , IL-6 and TNF- $\alpha$  levels in mouse spinal cord. These findings suggested that memantine could exert antiinflammatory effects through cellular mechanisms secondary to blocking NMDA receptors.

Neurotrophic factors play a critical role in the maintenance and survival of glial cells and neurons in the adult CNS. Astrocytes have been found to be a major source of neurotrophic factors [36]. Memantine markedly increased the levels of BDNF and its receptor trkB across the brain [37]. In dopaminergic neuron-glia cocultures, the neuroprotective effects of memantine was mediated by stimulating GDNF release from astrocytes [35]. In a streptozotocin-induced astrocytotoxicity model for Alzheimer's disease, memantine ameliorated BDNF and GDNF decline in astrocytes along with phosphorylation of IRS-1, Akt and GSK-3 $\alpha/\beta$  [38]. On the other hand, astrocytes were shown to produce VEGF upon brain injury, ischemia and neuroinflammation for regulating vascular remodeling and angiogenesis during the repair process [39, 40]. Memantine improved stroke outcomes via increasing BDNF, GDNF and VEGF levels, reducing reactive astrogliosis, and enhancing vascular density [41, 42]. In a rat model of chronic cerebral hypoperfusion, memantine enhanced neovascularization with increases in BDNF and VEGF expressions [43]. The precise mechanism of how memantine enhances the production of growth factors is not clear. Interestingly, in addition to its antagonistic effect on NMDA receptors, memantine is a highly lipophilic agent that can readily enter cells and act on intracellular processes [24]. We found that memantine prevented astrocyte loss and simultaneously increased BDNF, GDNF and VEGF levels in the spinal cord of mice which had received human AQP4-IgG. These results suggested that the elevated growth factor levels likely associated with the protective effect of memantine on astrocytes.

A limitation of our study is that complement-dependent pathophysiologies are absent in our model. In addition, the dose of memantine used in this study was higher than that in human patients with Alzheimer's disease (maintenance dose: 20 mg orally per day). The serum half-life of memantine in rodents is shorter than that in human [44]. Memantine will likely exert neuroprotective effects on patients with NMOSD at commonly used doses such as that for Alzheimer's disease.

## Conclusions

In summary, we found that memantine markedly ameliorated complement-independent motor impairments and spinal cord pathologies induced by human AQP4-IgG in mice. These findings suggested important roles of glutamate excitotoxicity and neuroinflammation in early pathophysiologies of NMOSD attacks. Memantine is a well-tolerated oral drug. Our results support perspectives for clinical studies on the use of memantine as preventive and therapeutic treatments of NMOSD acute attacks.

## Abbreviations

ADCC: Antibody-dependent cell-mediated cytotoxicity; ANOVA: Analysis of variance; AQP4: Aquaporin-4; AQP4-IgG: Aquaporin-4 immunoglobulin G ; BDNF: Brain-derived neurotrophic factor; CFA: Complete Freund's adjuvant; CNS: Central nervous system; EAAT2: Excitatory amino acid transporter 2; GDNF: Glial cell line-derived neurotrophic factor; GFAP: Glial fibrillary acidic protein; H&E: Hematoxylin and eosin; Iba-1: Ionized calcium-binding adapter molecule 1; IL-1 $\beta$ : Interleukin-1 $\beta$ ; IL-6: Interleukin-6; LFB: Luxol fast blue; Ly6G: Lymphocyte antigen 6 complex locus G6D; MBP: Myelin basic protein; NF-H: Neurofilament heavy polypeptide; NMDA: N-methyl-D-aspartate; NMOSD: Neuromyelitis optica spectrum disorders ; PTx: Pertussis toxin; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick-end labeling; VEGF: Vascular endothelial growth factor.

## **Declarations**

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Not applicable

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### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Authors' contributions**

LWY conceived, designed and performed all experiments and wrote the manuscript. CHT contributed to beam walking test and tissue preparation. OKFM contributed to beam walking test. JSCK contributed to IgG preparation and purification. KHC conceived the study, arranged patients for blood taking, and revised the manuscript.

### **Ethics approval and consent to participate**

Informed consent for using serum/plasma samples in the study was obtained from all study subjects. The experiments on C57BL/6 mice were approved by the Committee on the Use of Live Animals in

## Consent for publication

All authors read and approved the final manuscript.

## Competing interests

The authors declare no conflict of interest.

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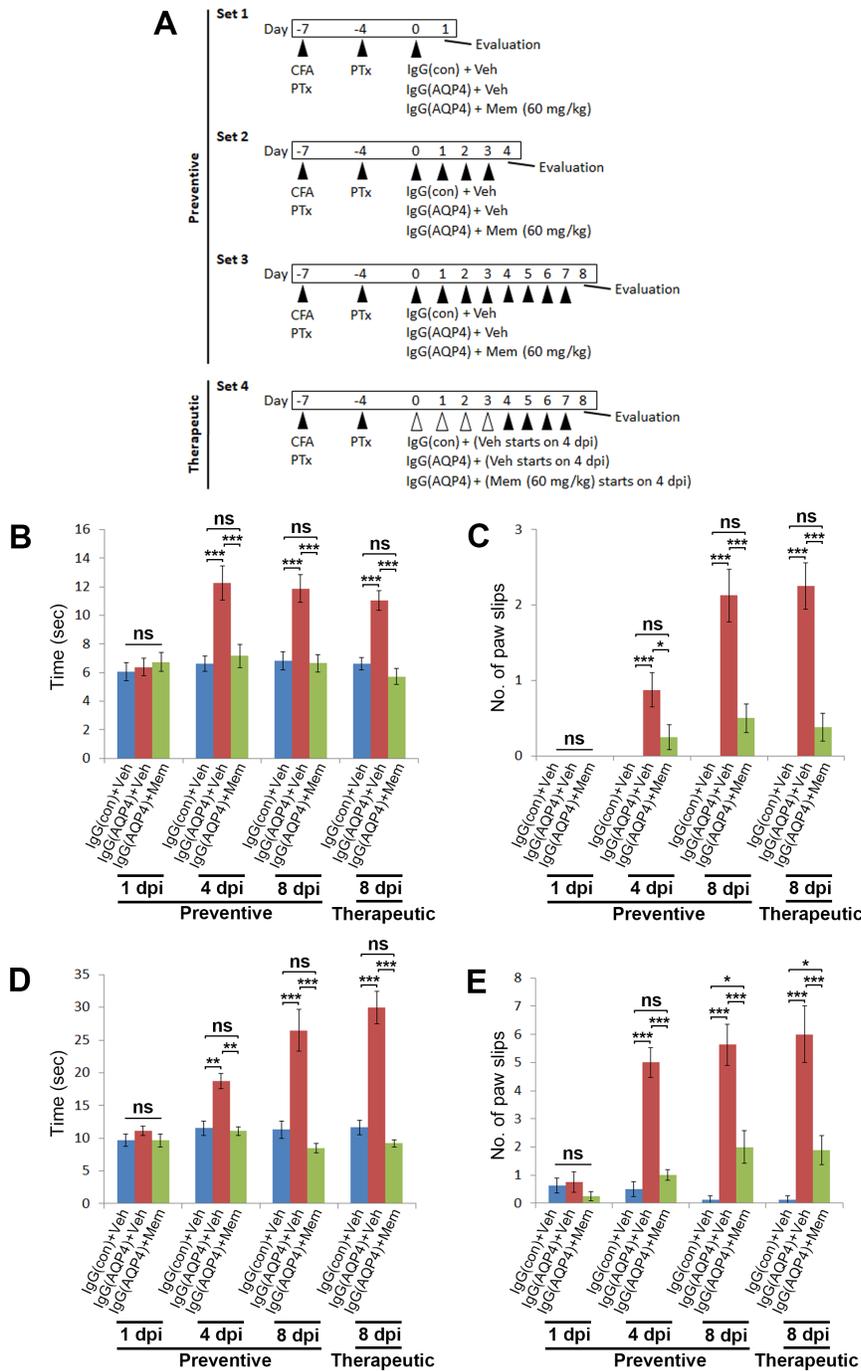
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## Figures

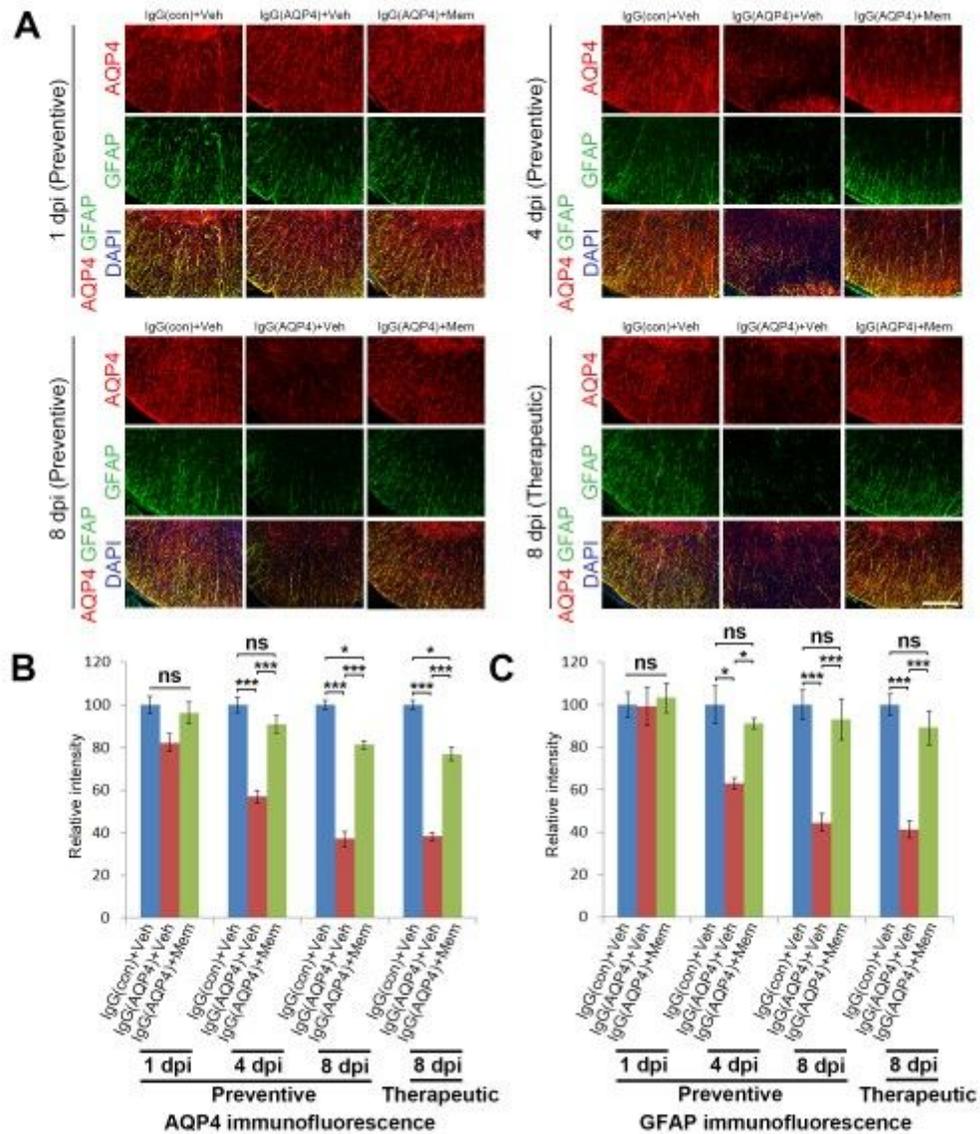


**Figure 1**

**Figure 1**

Oral administration of memantine ameliorates human AQP4-IgG-induced motor impairments in mice. a Animal groups and experimental procedures. b-c Time required b and number of paw slips c during walking across a 1.2 x 80 cm (width x length) beam in beam walking test on IgG(AQP4+) mice treated with preventive memantine or vehicle at 1, 4, 8 days post-injection (dpi); or with therapeutic memantine or vehicle at 8 dpi. IgG(con) mice treated with vehicle were used as a sham control. d-e Time required d and

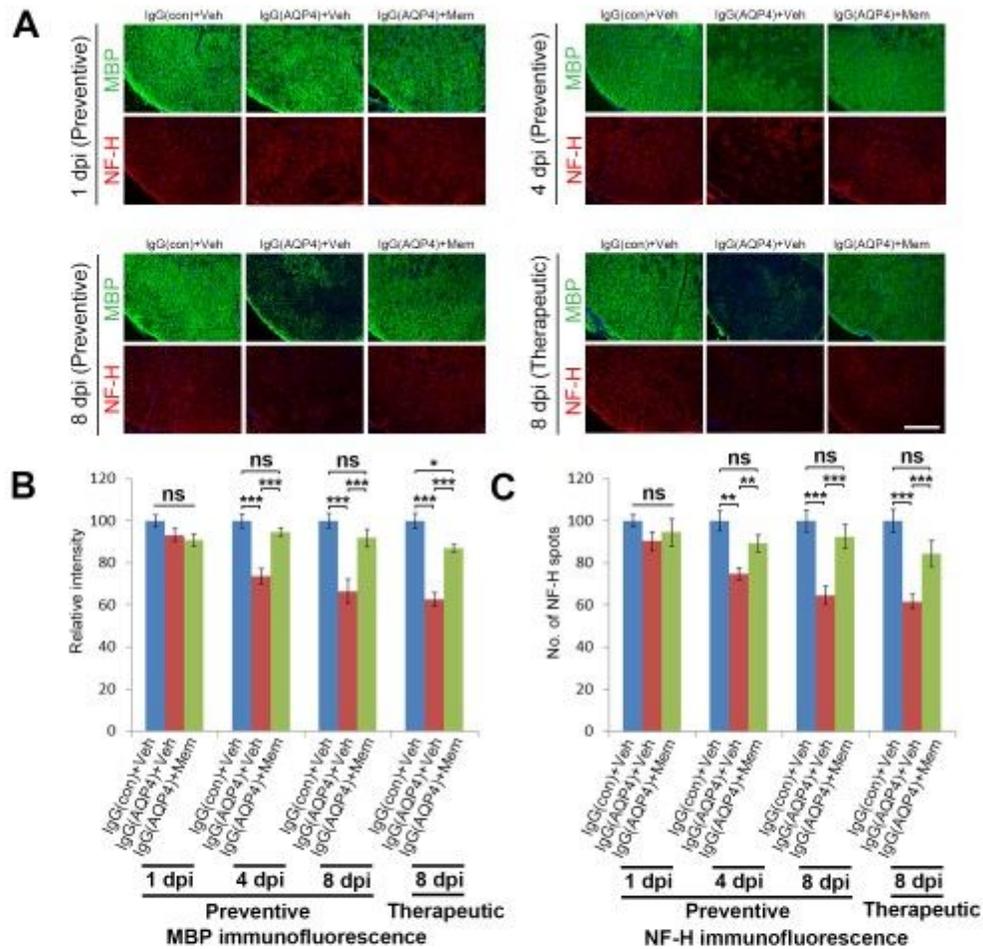
number of paw slips e during walking across a 0.6 x 80 cm (width x length) beam in beam walking test on mice in different groups. n = 8 per group. Data are mean  $\pm$  SEM. One-way ANOVA with Tukey-Kramer post hoc test. ns, not significant; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.



**Figure 2**

Memantine decreases AQP4 and GFAP loss in IgG(AQP4+) mice. Pictures are representative photomicrographs of cross sections showing the white matter in ventrolateral region of cervical spinal cord. a Double immunofluorescence staining of AQP4 and GFAP (astrocyte marker) in IgG(AQP4+) mice treated with preventive memantine or vehicle at 1, 4, 8 dpi; or with therapeutic memantine or vehicle at 8 dpi. IgG(con) mice treated with vehicle were used as a sham control. Sections were counterstained with DAPI. Scale bar = 100  $\mu$ m. b Relative intensity of AQP4 immunofluorescence in the spinal cord. c Relative

intensity of GFAP immunofluorescence in the spinal cord. n = 5 per group. Data are mean  $\pm$  SEM. One-way ANOVA with Tukey-Kramer post hoc test. ns, not significant; \*p < 0.05; \*\*\*p < 0.001.



**Figure 3**

**Figure 3**

Memantine reduces demyelination and axonal loss in IgG(AQP4+) mice. a Immunofluorescence staining of MBP (myelin marker) and NF-H (axon marker) in the spinal cords of IgG(AQP4+) mice treated with preventive memantine or vehicle at 1, 4, 8 dpi; or with therapeutic memantine or vehicle at 8 dpi. IgG(con) mice treated with vehicle were used as a sham control. Sections were counterstained with DAPI. Scale bar = 100  $\mu$ m. b Relative intensity of MBP immunofluorescence in the spinal cord. c Quantitative analysis of

the number of NF-H spots in the spinal cord normalized to sham. n = 5 per group. Data are mean  $\pm$  SEM. One-way ANOVA with Tukey-Kramer post hoc test. ns, not significant; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

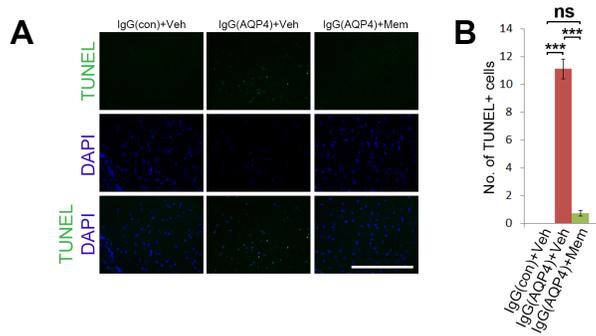
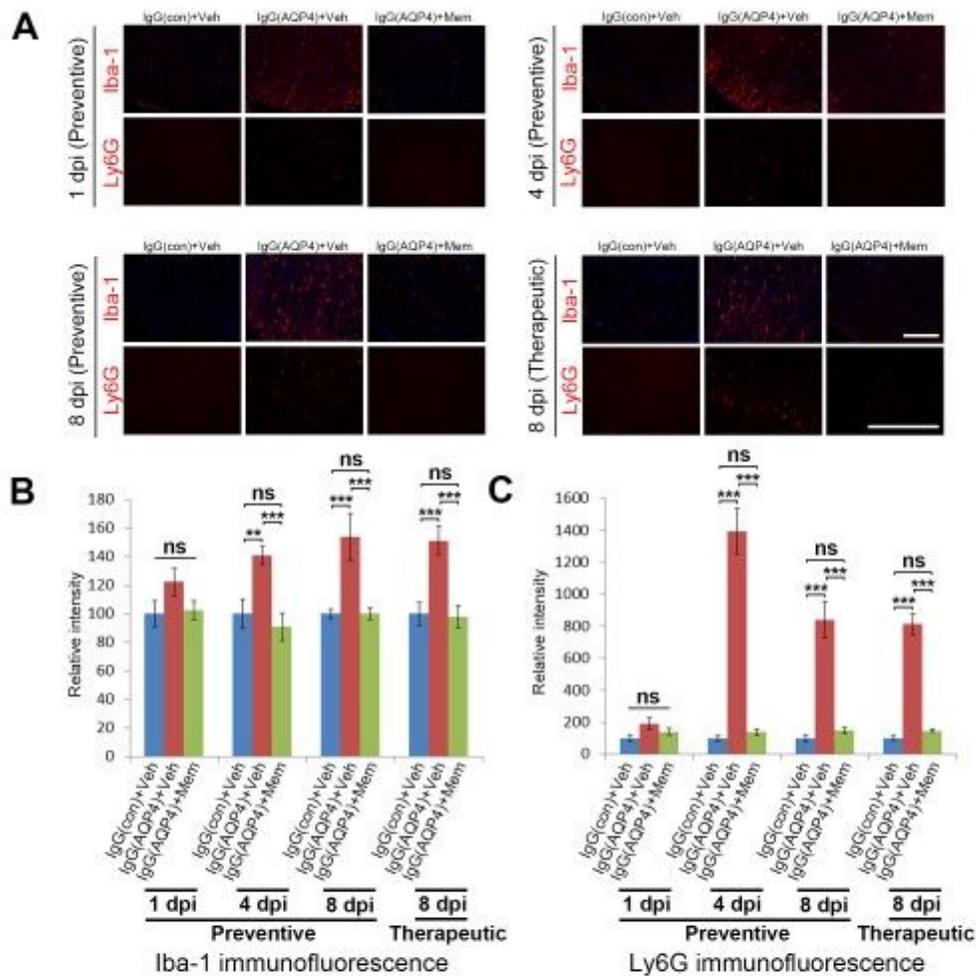


Figure 4

## Figure 4

Memantine inhibits apoptosis in IgG(AQP4+) mice. a TUNEL staining of apoptotic cells in the spinal cords of IgG(AQP4+) mice treated with memantine or vehicle at 1 dpi. IgG(con) mice treated with vehicle were used as a sham control. Sections were counterstained with DAPI. Scale bar = 100  $\mu$ m. b Quantitative

analysis of the number of TUNEL-positive cells in the spinal cord. n = 5 per group. Data are mean  $\pm$  SEM. One-way ANOVA with Tukey-Kramer post hoc test. ns, not significant; \*\*\*p < 0.001.

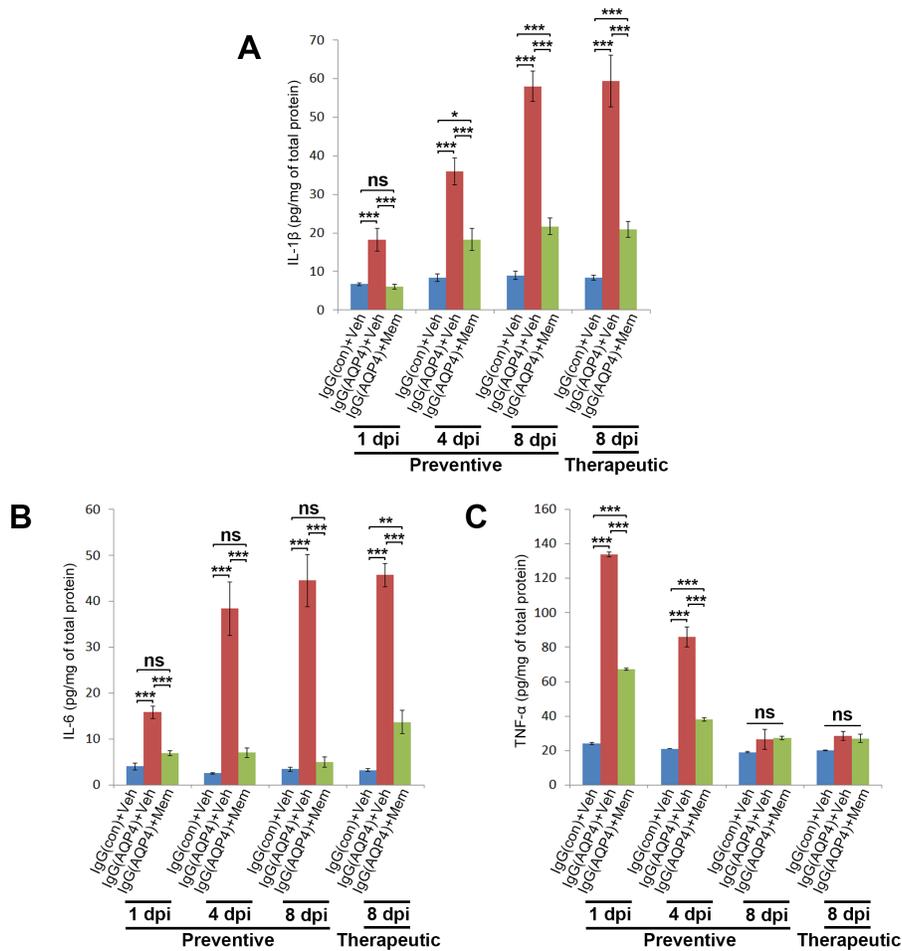


**Figure 5**

**Figure 5**

Memantine suppresses microglia activation and neutrophil infiltration. a Immunofluorescence staining of Iba-1 (microglia marker) and Ly6G (neutrophil marker) in the spinal cords of IgG(AQP4+) mice treated with preventive memantine or vehicle at 1, 4, 8 dpi; or with therapeutic memantine or vehicle at 8 dpi. IgG(con) mice treated with vehicle were used as a sham control. Sections were counterstained with DAPI. Scale bar = 100  $\mu$ m. b Relative intensity of Iba-1 immunofluorescence in the spinal cord. c Relative

intensity of Ly6G immunofluorescence in the spinal cord. n = 5 per group. Data are mean  $\pm$  SEM. One-way ANOVA with Tukey-Kramer post hoc test. ns, not significant; \*\*p < 0.01; \*\*\*p < 0.001.

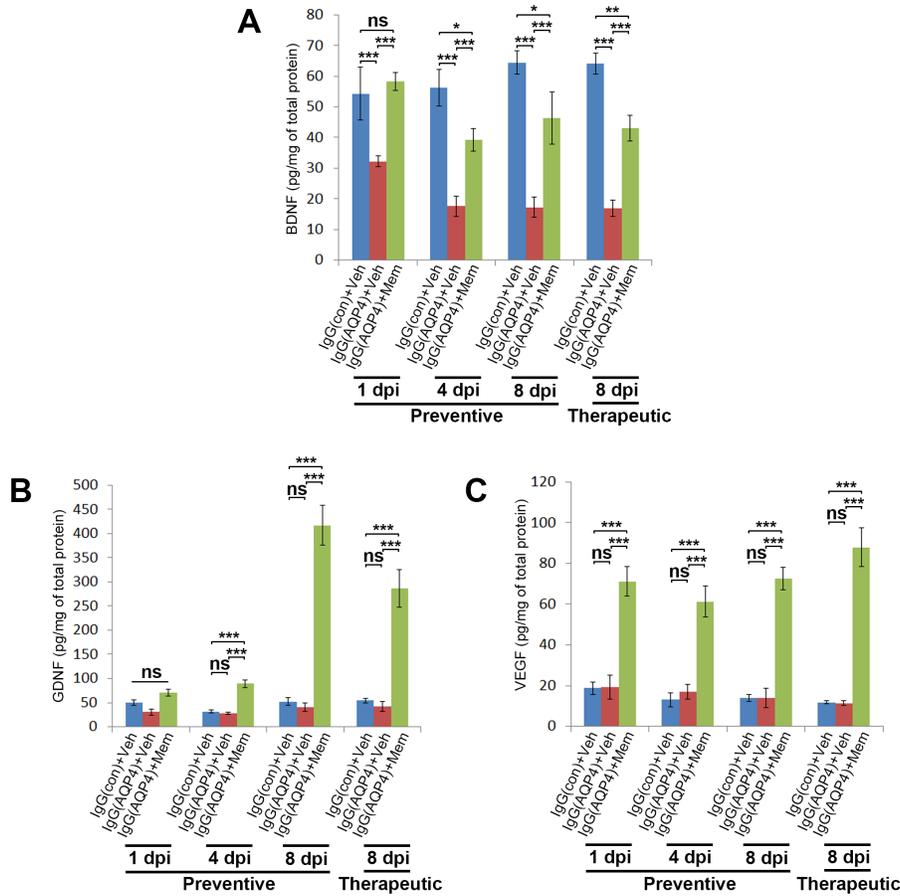


**Figure 6**

**Figure 6**

Reduction in proinflammatory cytokines in memantine-treated IgG(AQP4+) mice. a-c ELISA analyses of IL-1 $\beta$  a, IL-6 b and TNF- $\alpha$  c in the spinal cords of IgG(AQP4+) mice treated with preventive memantine or vehicle at 1, 4, 8 dpi; or with therapeutic memantine or vehicle at 8 dpi. IgG(con) mice treated with vehicle

were used as a sham control. n = 3 per group. Each ELISA was performed in duplicates. Data are mean  $\pm$  SEM. One-way ANOVA with Tukey-Kramer post hoc test. ns, not significant; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.



**Figure 7**

**Figure 7**

Elevation of growth factors in memantine-treated IgG(AQP4+) mice. a-c ELISA analyses of BDNF a, GDNF b and VEGF c in the spinal cords of IgG(AQP4+) mice treated with preventive memantine or vehicle at 1, 4,

8 dpi; or with therapeutic memantine or vehicle at 8 dpi. IgG(con) mice treated with vehicle were used as a sham control. n = 3 per group. Each ELISA was performed in duplicates. Data are mean  $\pm$  SEM. One-way ANOVA with Tukey-Kramer post hoc test. ns, not significant; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

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