J. Cell. Mol. Med. Vol 13, No 2, 2009 pp. 341-351

Improved outcome of EAN, an animal model of GBS, through amelioration of peripheral and central inflammation by minocycline

Zhi-Yuan Zhang [#], Zhiren Zhang [#, *], Uwe Fauser, Hermann J. Schluesener

Institute of Brain Research, University of Tuebingen, Tuebingen, Germany

Received: February 11, 2008; Accepted: March 31, 2008

Abstract

Experimental autoimmune neuritis (EAN) is a widely used animal model of the human acute inflammatory demyelinating polyradiculoneuropathy, which is the most common subtype of Guillain-Barré Syndrome. EAN is pathologically characterized by breakdown of the blood-nerve barrier, infiltration of reactive immune cells, local inflammation, demyelination in the peripheral nervous system and mechanical allodynia. Minocycline is known to have neuroprotective and anti-inflammatory effects. Furthermore, relieve of neuropathic pain following minocycline administration was observed in a variety of animal models. Here, we investigated the effects of minocycline on rat EAN. Suppressive treatment with minocycline (50 mg/kg body weight daily immediately after immunization) significantly attenuated the severity and duration of EAN. Macrophage and T-cell infiltration and demyelination in sciatic nerves of EAN rats treated with minocycline were significantly reduced compared to phosphate-buffered saline (PBS)-treated EAN rats. mRNA expressions of matrix metallopeptidase-9, inducible nitric oxide synthase and pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α in EAN sciatic nerves were greatly decreased by administration of minocycline as well. Furthermore, minocycline attenuated mechanical allodynia in EAN rats and greatly suppressed spinal microglial activation. All together, our data showed that minocycline could effectively suppress the peripheral and spinal inflammation (immune activation) to improve outcome in EAN rats, which suggests that minocycline may be considered as a potential candidate of pharmacological treatment for autoimmune-mediated neuropathies.

Keywords: minocycline • EAN • neuropathic pain • inflammation • sciatic nerves

Introduction

Guillain–Barre Syndrome (GBS) is the world's leading cause of acute autoimmune neuromuscular paralysis and caused by an autoimmune attack on the peripheral nervous system [1]. Experimental autoimmune neuritis (EAN) is an autoantigen-specific T-cell-mediated inflammatory peripheral nervous system (PNS) demyelinating animal model and shares many clinical, electrophysiological and immunological features of the human acute inflammatory demyelinating polyradiculoneuropathy (AIDP), which is the most common subtype of the GBS [2]. GBS is characterized by motor disorders such as weakness or paralysis, as well as variable sensory disturbances [1]. Neuropathic pain, caused by lesion or inflammation of the nervous system, is a common symptom of GBS, occurring in 55–85% of cases [3]. Treatments of GBS include plasma exchange, intravenous immunoglobulin or supportive management such as intensive care and respiratory assistance. But all these treatments remain unsatisfying [2], and new therapeutic options are needed.

EAN can be actively induced by immunization with autoantigen (purified myelin, P0 or P2 peptide) and is pathologically characterized by breakdown of the blood-nerve barrier (BNB), infiltration of activated immune cells, local inflammation and demyelination in the PNS.

Rat EAN is a monophasic disease, with weight loss, ascending paraparesis/paralysis and spontaneous recovery [4]. EAN has been widely used as an animal model to study disease mechanism and therapy of AIDP. Recently, pain hypersensitivity has been successfully observed in a modified EAN model, which facilitates the investigation of the mechanisms underlying autoimmune neuropathies [5, 6].

Minocycline is a second-generation semi-synthetic tetracycline. Besides its broad-spectrum antibiotic activity, minocycline

[#]Both authors contributed equally to this work.
*Correspondence to: Zhiren ZHANG,
Institute of Brain Research, University of Tuebingen,
Calwer Str. 3, D-72076 Tuebingen, Germany.
Tel.: +49-7071-2984882
Fax: +49-7071-294846
E-mail: zhangzhiren@yahoo.com

doi:10.1111/j.1582-4934.2008.00333.x

has been shown to display neuroprotective and anti-inflammatory properties in a number of neurologic diseases or their animal models, including traumatic brain injury, spinal cord injury, ischaemia, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) [7]. Protective effects of minocycline in these disorders are considered due to inhibitory effects on immune cell activation, matrix metalloproteinase activity, nitric oxide production and cell apoptosis. Furthermore, minocycline has been shown to attenuate neuropathic pain in a variety of animal models through inhibiting spinal microglia activation [8, 9].

Minocycline is commonly used as an antibiotic for the treatment of acne and its toxicity has been well tested [10]. The high lipophilicity of minocycline allows it to diffuse into the central and peripheral nervous system (CNS and PNS) at therapeutically effective levels [7]. Therefore, the proven reliability and safety of minocycline's use as an antibiotic suggest its potential prospect as an effective treatment of various neurologic conditions and in the present investigation, we analysed a possible protective effect of minocycline on rat EAN.

Materials and methods

Animal experiments

Male Lewis rats (8–10 weeks old, 200–250 g, Charles River, Sulzfeld, Germany) were housed with equal daily periods of light and dark and free access to food and water. All procedures were performed in accordance with the published International Health Guidelines under a protocol approved by the local Administration District Official Committee. All efforts were made to minimize the number of animals and their suffering.

The standard EAN model was induced by subcutaneous injection into both hindpaws, with 100 μl of an inoculum containing 100 μg of synthetic neuritogenic P2 peptide of peripheral myelin-amino acids 53-78 (TESPFKNTEISFKLGQEFEETTADNR), which were synthesized by Gene Script Corporation, Scotch Plains, NJ, USA, under ether anaesthesia. The peptide was dissolved in phosphate-buffered saline (PBS) (2 mg/ml) and then emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 2 mg/ml mycobacterium tuberculosis to get a final concentration of 1 mg/ml [11]. For analysis of mechanical allodynia, EAN was induced by subcutaneous injection at the basal part of tails with reduced amount of P2 peptide (80 μg) as described [6], which was referred to as EAN pain model in this study.

The severity of EAN was scored daily as follows: 0—normal, 1—reduced tonus of tail, 2—limp tail, impaired righting, 3—absent righting, 4—gait ataxia, 5—mild paresis of the hind limbs, 6—moderate paraparesis, 7—severe paraparesis or paraplegia of the hind limbs, 8—tetraparesis, 9—moribund, 10—death.

For suppressive treatment, minocycline (Sigma, St. Louis, MO, USA; 50 mg/kg body weight in 1 ml PBS) was intraperitoneally injected once daily after immunization until the end of experiments, Day 17 or Day 30. As control, half of all EAN rats received intraperitoneal injection of the same volume of PBS (PBS control groups).

Tissue preparation

Five rats of each group (treated with minocycline or PBS, standard and pain model) were killed 17 days after immunization for the histological analysis of sciatic nerves and spinal cords. Rats were deeply anaesthetized with ether and perfused intracardially with 4°C, 4% paraformaldehyde in PBS. Sciatic nerves and spinal cords were quickly removed and post-fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Sciatic nerves were cut to two equal long segments and spinal cords were cut, divided into 8 mm segments. All these segments were embedded in paraffin, sectioned serially (3 μm) and mounted on silan-covered slides.

Immunohistochemistry and LFB staining

Immunohistochemistry (IHC) was performed on 3 μm paraffin-embedded sections using antibodies serially to evaluate local inflammation, cellular infiltration and demyelination in sciatic nerves: ED-1 (1:100; Serotec, Oxford, UK) to detect activated microglia/macrophages, W3/13 (1:50; Serotec) to identify T-lymphocytes, OX22 (1:100; Serotec, Oxford, UK) for B cells, GFAP (glial fibrillary acidic protein; 1:500; Chemicon International, Temecula, CA, USA) to detect astrocytes, P2X4R (P2X4 receptor; 1:200; Alomone Laboratories, Jerusalem, Israel) and MMP-9 (matrix metallopeptidase-9; 1:500; Neuromics, Edina, MN, USA). After dewaxing, sections were boiled (in an 850 W microwave oven) for 15 min. in citrate buffer (2.1 g citric acid monohydrate/L, pH 6) (Carl Roth, Karlsruhe, Germany). Endogenous peroxidase was inhibited by 1% H_2O_2 in pure methanol (Merck, Darmstadt, Germany) for 15 min. Sections were incubated with 10% normal pig serum (Biochrom, Berlin, Germany) to block non-specific binding of immunoglobulins and then with the primary antibodies overnight at 4°C. Antibodies binding to tissue sections were visualized with secondary biotinylated antibodies (rabbit anti-mouse or rabbit anti-goat) (1:400; DAKO, Hamburg, Germany). Subsequently, sections were incubated with a horseradish peroxidase-conjugated Streptavidin complex for 30 min. (1:100; DAKO, Hamburg, Germany), followed by development with diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany). Finally, sections were counterstained with hemalum. As negative controls, the primary antibodies were omitted.

After immunostaining, sections from minocycline and PBS control groups were examined by light microscopy and the numbers of ED-1$^+$, W3/13$^+$, OX22$^+$, P2X4R$^+$ and GFAP$^+$ cells were counted. Positively stained cell counting based on IHC results has been well developed to semi-quantify protein expression [12]. Positively stained cells were counted by independent observers. To evaluate positive cell numbers in sciatic nerves, four cross-sections for each rat were evaluated. Microphotos of the whole sciatic nerve cross-sections were taken under 100$\times$ magnification using Nikon Coolscope (Nikon, Düsseldorf, Germany) and only positive cells with the nucleus at the focal plane were counted. Areas of sciatic nerve cross-sections were measured on the same pictures using software MetaMorph Offline 7.1 (Molecular Devices, Toronto, Canada).

To evaluate positive cell numbers in dorsal horns of lumbar spinal cord, sections were first examined under dark field microscopy to determine the lumbar segmental level according to the method of Molander *et al.* [13]. Microphotos were taken for lumbar dorsal horns selected. Positive cells were counted and areas of dorsal horns were measured as described above. For each rat, three coronal lumbar spinal cord sections were analysed. Both left and right dorsal horns were counted for each of the section. Results were calculated as arithmetic means of positive cells per square millimetre and standard errors of means (SEM).

In addition, the routine Luxol fast blue (LFB) staining was applied to show myelin, particularly the situation of demyelination in sciatic nerves. For LFB staining, histological changes between minocycline and PBS-treated EAN rats were compared by an established semi-quantitative method. Briefly, four cross-sections from sciatic nerves of both sides of EAN rats were analysed. All perivascular areas present in cross-section were evaluated by two observers unaware of treatment, and the degree of pathological alteration was graded semi-quantitatively by the following scale: 0 = normal perivascular area; 1 = mild cellular infiltration adjacent to the vessel; 2 = cellular infiltration plus demyelination in immediate proximity to the vessel; 3 = cellular infiltration and demyelination throughout the section. Results were given as means of histological scores [14].

Flow cytometric analysis of peripheral T cells, B cells and monocytes

For fluorescent-activated cell sorting (FACS) analysis, three minocycline or PBS treated EAN rats were killed at Day 17 after immunization and blood was drawn intracardiacly with anti-clotting agent ethylenediaminetetraacetic acid (EDTA), under anaesthesia. 100 μl of blood samples were incubated at room temperature for 30 min. with 10 μl of following mouse anti-rat R-Phycoerythrin (RPE)-conjugated monoclonal antibodies: ED-9 for monocytes (Serotec, Oxford, UK), W3/13 for T cells (clone IF4, BD Pharmingen, Heidelberg, Germany) and OX33 for B cells (Serotec, Oxford, UK). Isotype control was used at the same concentration as antibodies mentioned above. Thereafter, erythrocytes were lysed with ERYTHROLYSE (red blood cell lysing buffer; Serotec, Oxford, UK) according to the manufacturer's instruction. Following two washing procedures, cells were analysed with a FACScan (Secton Dicinson, Ueberlingen, Germany). The mononuclear cells were gated by forward and sideward scatter.

RT–PCR

EAN rats receiving suppressive treatment with minocycline (and PBS controls), were killed at Day 17. Total RNA was isolated from the sciatic nerves using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA equivalent to 20 ng of total RNA was subjected to subsequent PCR analysis using primers specific for interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) or the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as described previously [15]. In preliminary experiments, optimal cycling conditions were established allowing amplification of each cDNA in the linear range. PCR products were separated on 1.5% agarose gels containing 10 μg/ml ethidium bromide, photographed using the UVsolo system (Whatman Biometra, Goettingen, Germany) and densitometric analysis was performed with the software BioDocAnalyze (Whatman Biometra, Goettingen, Germany). Results were calculated as levels of target mRNAs relative to those of the house-keeping gene GAPDH (Only three samples from each group were analysed with PCR).

Mechanical allodynia

Mechanical allodynia was assessed by measuring rat hind-paw withdrawal threshold (HWT) using an automatic von Frey system, namely a mechanical plantar test apparatus (Ugo Basile, Milan, Italy). HWT was tested between 9:00 and 12:00, on every second day after immunization. Eight days before the start of measurement, rats received training sessions every day. Rats were allowed to habituate to the environment of the test room for at least 10 min. first and then placed into a Perspex enclosure over a mesh floor and allowed acclimating for another 10 min. The mechanical force, which went from 0–50 g over a period of 15 sec., was then exerted onto the middle of the hind-paws using a fine metal filament. The force triggering the withdraw reflex was recorded automatically. Left and right hind-paws were measured eight times each, with a minimum 2 min. interval between stimuli and the mean values were calculated. As no significant difference was observed between the right and left hind-paw of each rat, results from both hind-paws were combined. Rats were re-grouped (stratified) before the immunization according the measurement results during the training sessions. EAN pain model rats were treated with minocycline or PBS, respectively (six rats per group).

Statistical analysis

The unpaired t-tests were performed to compare differences between minocycline and PBS treated EAN rats for single time points and two-way ANOVA tests were performed to compare differences between both groups over time (Graph Pad Prism 4.0 software). For all statistical analyses, significance levels were set at $P < 0.05$.

Results

Effects of minocycline on pathological scores and body weight of EAN rats

EAN was induced by subcutaneous injection of P2 peptide for experimental suppressive treatment. Minocycline or PBS (PBS control group) were injected once daily, from Day 0 to Day 30 after immunization. PBS-treated rats developed the first neurological signs of EAN (reduced tonus of tail) 9 days after immunization (mean clinical score: 0.33 ± 0.33). Severity of neurological signs was maximal at Day 15 (mean clinical score: 6.67 ± 0.33), but rats recovered fast from EAN by Day 20. However, in minocycline-treated EAN rats, the clinical signs were first seen at Day 14 (mean clinical score: 0.33 ± 0.18), reached the maximal level at Day 16 (mean clinical score: 1.22 ± 0.54) and disappeared after Day 18 (mean clinical score: 0.74 ± 0.25) (Fig. 1A). Comparative analysis revealed that suppressive treatment of minocycline not only delayed the onset and duration of EAN, but also significantly decreased clinical severity of EAN at every single day.

Progressive weight loss during onset of EAN is another characteristic feature of this disease, which correlates with the severity of EAN. After a temporal weight loss at Day 1, which was probably due to the immunization, a progressive weight loss was observed during the onset of EAN from Day 9 to Day 22 in PBS treated EAN rats. Meanwhile, no delayed weight loss was apparent in minocycline-treated rats, but weight of these rats did not increase as in normal rats (Fig. 1B). And significant differences of

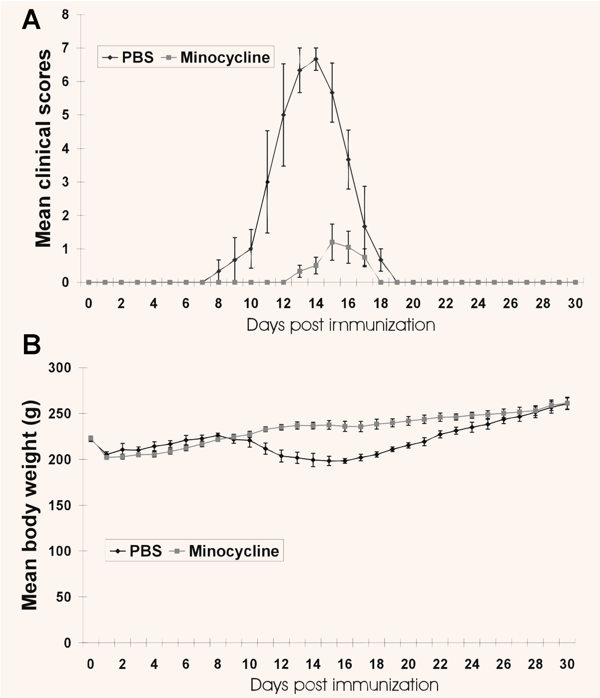

Fig. 1 Minocycline reduced pathological scores (**A**) and body weight loss (**B**) Experimental autoimmune neuritis (EAN) was induced by subcutaneous injection of synthetic neuritogenic P2 peptide (100 μg) and complete Freund's adjuvant (CFA) in both hind-paws of rats. Minocycline or PBS (five rats each, 50 mg/kg body weight in 1 ml PBS) were injected once daily starting immediately after immunization. Clinical scores and body weight were taken every second day after immunization. (**A**) Minocycline treatment significantly delayed onset, decreased neurologic severity and shortened duration of EAN compared to PBS controls. (**B**) Minocycline treatment also significantly attenuated body weight loss induced by EAN. Two-way ANOVA tests proved significant differences in pathological scores and body weight between both PBS and minocycline groups over time ($P < 0.05$).

pathological scores and body weight between both PBS- and minocycline-treated EAN rats were proved by Two-way ANOVA tests ($P < 0.05$). Taken together, these results indicated a very much reduced disease severity in minocycline-treated EAN.

Effects of minocycline on cellular infiltration and demyelination in EAN sciatic nerves

Sciatic nerves were taken from minocycline-treated and PBS control EAN rats ($n = 5$) at Day 17 and were analysed histologically. LFB staining demonstrated a significant decreased degree of perivascular demyelination and inflammatory cell infiltration in EAN rats treated with minocycline (mean histological score 0.55 ± 0.20) (Fig. 2A and C) as compared to the PBS control group (mean histological score 2.23 ± 0.42) (Figure 2B and C).

Distinct infiltration of different types of inflammatory cells into sciatic nerves was further analysed by IHC. Infiltration of macrophages (ED-1$^+$) (Fig. 3A), T cells (W3/13$^+$) (Fig. 3B) and B cells (OX22$^+$) (Fig. 3C) were seen in sciatic nerves of rats from the PBS control at Day 17 and the most dominant cell population were macrophages, with a mean density of about 592.7 ± 48.97 ED-1$^+$ cells per mm^2 (Fig. 3G). Minocycline treatment significantly decreased inflammatory infiltrations of all the three cell types ($P < 0.01$, compared to PBS control, respectively) (Fig. 3D–I).

Effects of minocycline on circulating monocytes and lymphocytes in EAN rats

In order to study the general suppressive effect of minocycline on immune cells, numbers of circulating monocytes (ED-9$^+$) (Fig. 3j), T cells (W3/13$^+$) (Fig. 3K) and B cells (OX33$^+$) (Fig. 3L) were analysed at Day 17. We had shown previously a significant increase of monocytes and T cells in EAN rats [16]. Following minocycline treatment, percentages of monocytes (Fig. 3J) and T cells (Fig. 3K) were significantly reduced, but the percentage of B cells (OX33$^+$) was increased (Fig. 3L) in comparison to the PBS control group.

Effects of minocycline on expressions of MMP9 in EAN sciatic nerves

MMPs, particularly MMP-9, are known to facilitate the passage of leucocyte across matrix barriers and are important for the pathology of autoimmune disorders [17]. Previous studies have shown an increased expression of MMP-9 in inflamed peripheral nerves in EAN [18]. In our study, minocycline significantly attenuated MMP-9 protein accumulation in sciatic nerves of EAN rats (Fig. 4A and B).

It was also reported that maximal levels of MMP-9 mRNA in sciatic nerves of EAN rats was concurrent with maximal disease severity [18]. So we further analysed MMP-9 mRNA expression in EAN sciatic nerves following minocycline treatment. Total mRNA was isolated from sciatic nerves of 17-days EAN rats treated with minocycline or PBS. As shown in Fig. 4C and D, mRNA expression of MMP-9 in EAN sciatic nerves was significantly reduced in the minocycline treated.

Effects of minocycline on expressions of inflammatory molecules in EAN sciatic nerves

iNOS and inflammatory cytokines IL-1β and TNF-α are up-regulated in EAN and known to play important roles in inflammatory progression of disease [19, 20]. Therefore, effects of minocycline on m-RNA levels of these mediators in EAN were investigated. Minocycline or PBS was given immediately following immunization until Day 17. As shown in Fig. 5, mRNA expressions of IL-1β, TNF-α and iNOS was significantly reduced by minocycline in sciatic nerves of EAN rats as compared to PBS controls.

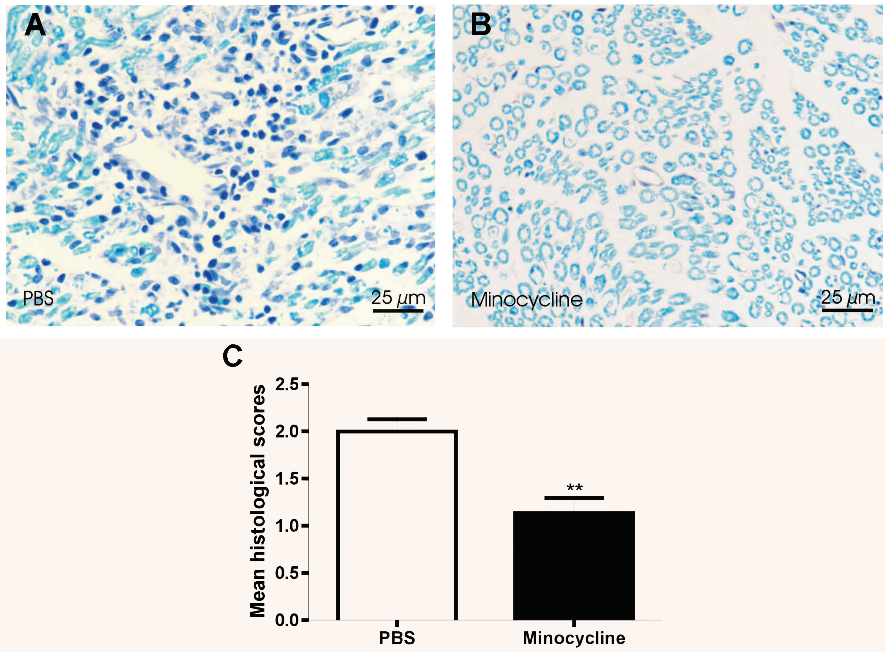

Fig. 2 Minocycline suppressed histopathological alterations in sciatic nerves of EAN rats. Seventeen days after immunization, sciatic nerves of minocyline treated and PBS control rats were taken and used for Luxol fast blue (LFB) staining, followed by haematoxylin counterstaining. Representative micrographs for PBS or minocycline treated EAN rats are shown in (**A**) and (**B**) respectively. (**C**) Means of histological scores were calculated as described in Experimental procedures. Bar figure shows that minocycline treatment significantly reduced mean histological scores compared to PBS controls. The unpaired t-test was performed (Graph Pad Prism 4.0 for windows). $^{**}P < 0.01$ compared to the PBS control.

Effects of minocycline on mechanical allodynia and spinal glia activation in EAN rats

Minocycline is known to suppress neuropathic pain in a variety of animal models [8, 9]. Therefore, we studied the influence of minocycline on neuropathic pain, which was induced with a reduced amount of P2 peptide and base tail immunization to avoid severe motor deficit and hind-paw inflammation that impairs the assessment of mechanical allodynia [6]. Minocycline greatly suppressed mechanical allodynia in EAN (Fig. 6A), which was proved by two-way ANOVA test ($P < 0.05$). In PBS-treated control EAN rats, mechanical allodynia, indicated by significant reduction of HWT compared to pre-immunization, was observed at Day 10 and reached the maximal level around Day 17 (Fig. 6A). However, in minocycline-treated EAN rats, pain sensitivity shown by HWT remained comparable before and after immunization, indicating the absence of mechanical allodynia (Fig. 6A). Therefore, minocycline could completely suppress the development of mechanical allodynia in EAN rats.

It is known that neuropathic pain can be due to central and/or peripheral sensitization [21]. So we further studied the effects of minocycline on peripheral and central inflammation in EAN pain model. Similar to the standard EAN model described above, in sciatic nerves of EAN pain model, the infiltration of T cells, B cells and macrophages were greatly reduced following minocycline treatment and the accumulation of IL-1β, TNF-α, iNOS mRNA and MMP-9 protein were significantly reduced (data not shown). So in EAN pain model, minocycline greatly inhibited peripheral inflammation.

We next examined the effects of minocycline on spinal glia activation, which has been recently recognized as an important factor for initiation and persistence of neuropathic pain. In the EAN pain model, spinal microglial activation was detected by ED-1 immunostaining at Day 17 EAN rats treated with minocycline or PBS. In PBS-treated EAN rats, emergence of $ED1^+$ cells were seen, mainly detected in grey matter, particularly in the superficial layers of dorsal horns (Fig. 6B). But in the minocycline treated EAN rats, spinal $ED1^+$ cells were rarely seen (Fig. 6D). Thus, a significant reduction of $ED1^+$ cells in lumbar dorsal horns of Day 17 EAN rats was detected after minocycline treatment (Fig. 6F). Spinal astrocyte activation in Day 17 EAN rats following minocycline was analysed as well. But no significant changes were seen (data not shown).

P2X4R is an adenosine-5′-triphosphate (ATP)-gated ion channel and its spinal up-regulation has been found to be crucial to the development of neuropathic pain following peripheral nerve injury [22]. Our previous data have shown an increased P2X4R expression in spinal microglia that was negatively correlated with mean HWT values in EAN rats [6]. As shown in Fig. 6C and E, appearance of $P2X4R^+$ cells was seen in PBS but not in minocycline treated EAN rats. Further, a significant reduction of $P2X4R^+$ cells in lumbar dorsal horns of Day 17 EAN rats was detected following minocycline treatment (Fig. 6G).

Discussion

Here we have studied the suppressive effects of minocycline on EAN. Suppressive treatment with minocycline greatly reduced neurologic severity of EAN through reducing local demyelination, suppression of local inflammatory cell infiltration and inhibition of

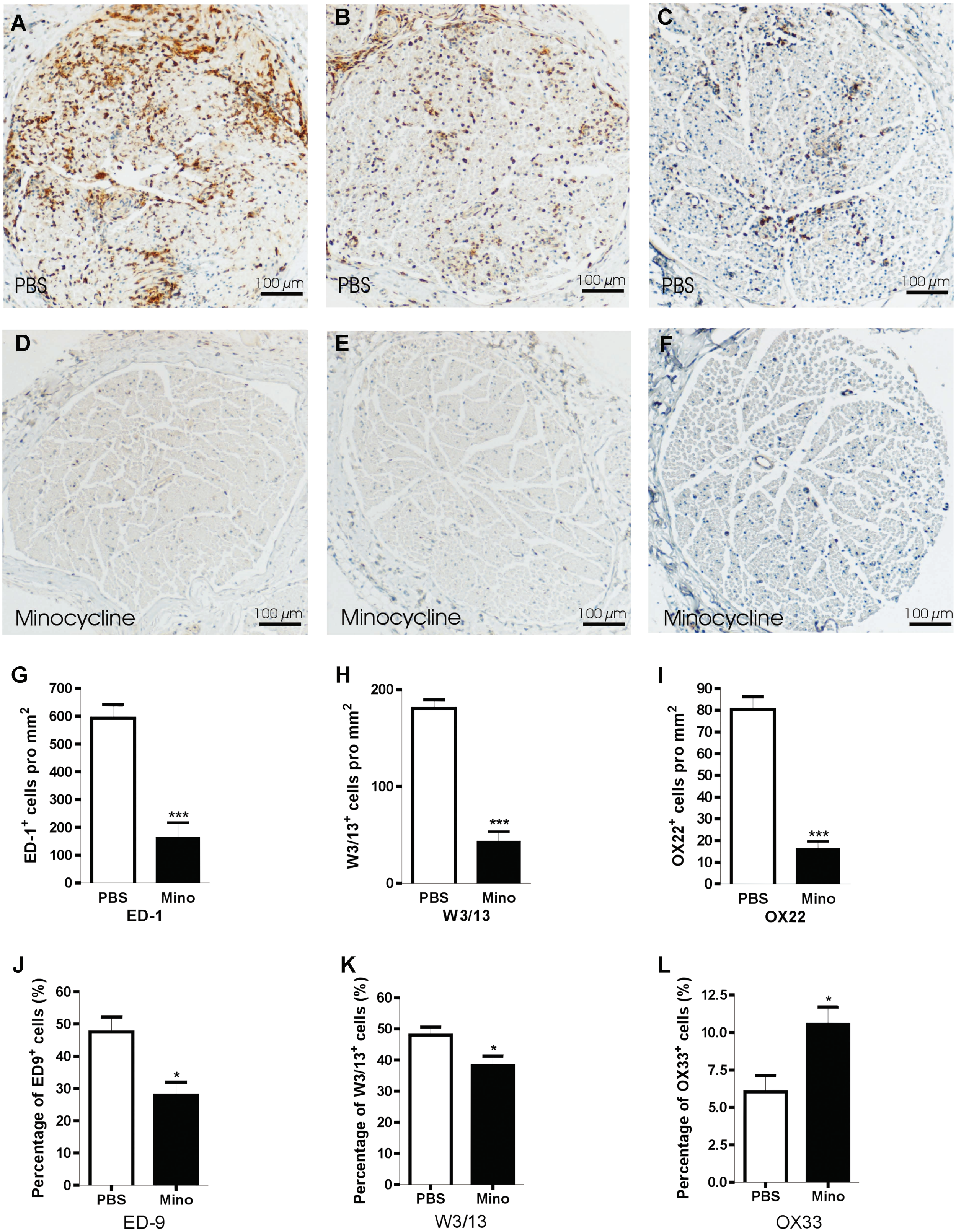
A
PBS
100 μm
B
PBS
100 μm
C
PBS
100 μm
D
Minocycline
100 μm
E
Minocycline
100 μm
F
Minocycline
100 μm
G
ED-1+ cells pro mm2
PBS
Mino
ED-1
H
W3/13+ cells pro mm2
PBS
Mino
W3/13
I
OX22+ cells pro mm2
PBS
Mino
OX22
J
Percentage of ED9+ cells (%)
PBS
Mino
ED-9
K
Percentage of W3/13+ cells (%)
PBS
Mino
W3/13
L
Percentage of OX33+ cells (%)
PBS
Mino
OX33

Fig. 3 Minocycline suppressed macrophage, T cell and B-cell infiltration into sciatic nerves and suppressed circulating monocytes and lymphocytes in EAN rats. Seventeen days after immunization, sciatic nerves of both experimental groups were analysed by immunohistochemstry. ED-1 (**A** and **D**) immunostaining was used for macrophages, W3/13 (**B** and **E**) for T cells and OX22 (**C** and **F**) for B cells. Representative micrographs from minocycline treated and PBS control EAN rats are shown in (**A**–**C**) and (**B**–**D**), respectively. (**G**–**I**) Infiltration of macrophages, T cells and B cells into sciatic nerves was further semi-quantified as indicated in Experimental procedures and bar figures show quantified results. Minocycline treatment significantly reduced infiltration of macrophages, T cell and B cells into EAN sciatic nerves (mino: minocycline treatment). (**J**–**K**) Minocycline suppressed circulating monocytes and lymphocytes in EAN rats. Following the same treatment described above, blood was drawn intracardially and leucocyte populations analysed by FACS with monoclonal antibody ED-9 for monocytes (**J**), W3/13 for T cells (**K**) and OX33 for B cells (**L**). Bar figures show results of percentages of positive cells in blood. Minocycline significantly reduced the percentages of circulating monocytes and T cells. The unpaired t-test was performed to compare the differences (Graph Pad Prism 4.0 for windows). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to their respective control.

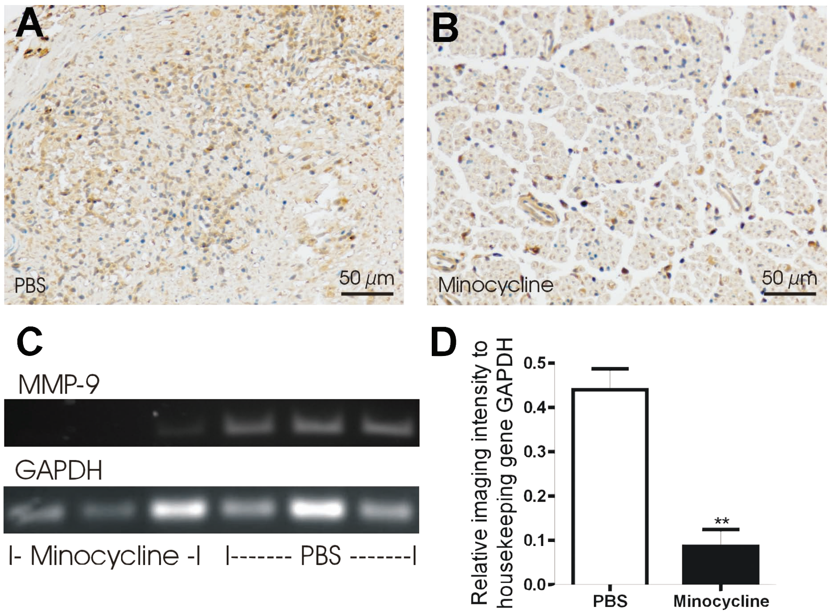

Fig. 4 Minocycline suppressed accumulation of matrix metalloproteases-9 (MMP-9) in sciatic nerves of EAN rats. Seventeen days after immunization, sciatic nerves of both groups were taken and used for immunohistochemical staining of MMP-9 (**A** and **B**). Representative immunohistochemical micrographs showed that minocycline significantly reduced the expression of MMP-9 in sciatic nerves. Another treatment group were used for analysis of mRNA level of MMP-9 (**C** and **D**). (**C**) Photos of gel electrophoresis with PCR products show obviously reduced mRNA levels of MMP-9 after treatment by minocycline. (**D**) Bar figures show semi-quantified results of imaging intensity relative to housekeeping gene GAPDH. The unpaired t-test was performed to compare the differences between minocycline treated and PBS control EAN rats (Graph Pad Prism 4.0 for Windows). $^{**}P < 0.01$ compared to the PBS control.

inflammatory molecule expression in sciatic nerves. Further, minocycline also significantly attenuated mechanical allodynia and inhibited microglia activation in spinal cords of EAN rats.

As a member of the tetracycline class of antibiotics, minocycline has been shown to possess anti-inflammatory properties. *In vitro* and *in vivo* data have suggested that minocycline inhibited inflammation by modulating cellular activation and subsequent release of cytokines, chemokines, lipid mediators of inflammation, matrix metalloproteases (MMPs), and nitric oxide [7].

In our EAN models, minocycline significantly suppressed infiltration of T cells, B cells and macrophages into peripheral nerves. Pathological development of EAN is characterized by the infiltration of reactive leucocyte into the PNS [23]. Activated autoreactive T cells, which can recognize peripheral nerve autoantigens on antigen presenting cells, are of importance for the initiation of EAN. Following activation, autoreactive T cells attach to the venular endothelium of the PNS, penetrate the BNB and generate an autoimmune reaction within the PNS that orchestrates the invasion of more lymphocytes and monocytes and local inflammation. Activated macrophages cause demyelination by direct phagocytic attack and secretion of inflammatory mediators. Depletion of macrophages and inhibition of their activity have been shown to suppress the development of EAN. Altogether, accumulation of reactive T cells and macrophages to the PNS are essential for EAN development [24]. Therefore, minocycline could inhibit local immune cell infiltration to favour EAN outcome.

How minocycline reduces immune cell infiltration in sciatic nerves of EAN rats was not clear but might be due to its effects on reducing circulating immune cells and inhibiting MMP expression in sciatic nerves. In our study, circulating monocytes and T cells in the EAN rat were reduced by minocycline. Kloppenburg *et al.* [25] reported that minocycline inhibited human T-cell proliferation. Minocycline also suppressed the proliferation of murine thymocytes induced by IL-1 [26]. So minocycline might inhibit lymphocyte proliferation to reduce circulating lymphocytes. This observation was in line with previous reports about the direct suppressive effect of minocycline on activation of immune cells [7] and suggests that generally reduced activated immune cells in circulation could also contribute to the suppressed cellular accumulation in areas of inflammatory lesions. However, we observed that

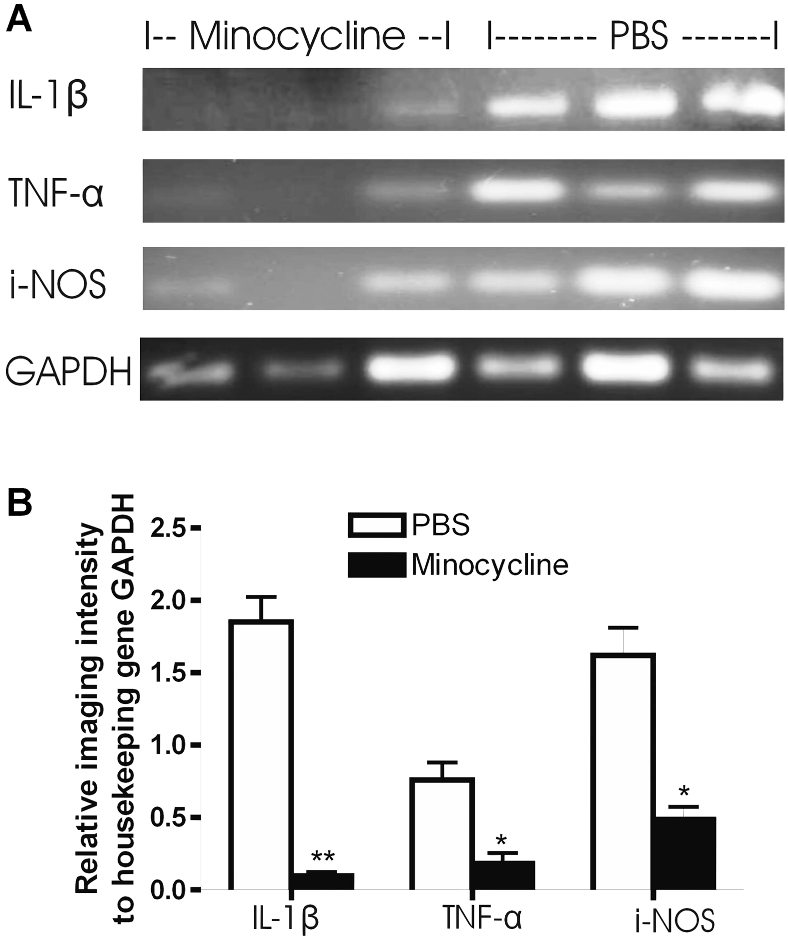

Fig. 5 Minocycline suppressed expression of interleukin (IL)-1β, tumour necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS) in sciatic nerves of EAN rats. Seventeen days after immunization, mRNA of sciatic nerves of both groups was isolated and relative expression levels analysed by RT-PCR. (**A**) Photos of gel electrophoresis with PCR products showed obviously reduced mRNA levels of IL-1β, TNF-α and iNOS after treatment by minocycline. (**B**) Bar graph show semi-quantified results of imaging intensities relative to the housekeeping gene GAPDH. The unpaired t-test was performed to compare the differences (Graph Pad Prism 4.0 for windows). $*P < 0.05$ and $**P < 0.01$ compared to their respective control.

percentages of B cells in blood increased following minocycline treatment, which was not in line with the observation of reduced B cells infiltration in sciatic nerves. Following the application of minocycline, while percentages of circulating T cells and monocytes were greatly reduced compared to the PBS control, percentages of circulating B cells relatively increased, which could be owe to the strong decrease of circulating T cells and monocytes. While percentages of circulating B cells increased following minocycline treatment in EAN rats, the infiltration of B cells into sciatic nerves decreased. This might be because minocycline has also effects on immune cell infiltration. Under normal condition, it is impossible or rare for immune cells to pass the BNB to access peripheral nerves. However, in EAN, auto-active T cells and B cells and reactive monocytes can penetrate BNB to reach lesion site. Minocycline is well known to inhibit MMP to decrease immune cell infiltration [7]. Therefore, following minocycline administration, infiltration of reactive immune cells to sciatic nerves was greatly reduced regardless of their percentages in blood.

MMPs are important for development of inflammation [27], like enhancing effects of pro-inflammatory cytokines, regulating chemokine activity and activating defensins. In EAN, MMPs could participate in the disruption of the BNB, breakdown of the myelin sheath, the release of TNF-α, and finally facilitate leucocyte invasion into the PNS [28]. In multiple sclerosis and its animal model EAE, MMPs are important for disease progression [29]. MMPs facilitate leucocyte infiltration into the CNS parenchyma by degrading the basement membrane that surrounds blood vessels, greatly impairing the integrity of the blood brain barrier (BBB) [29]. Further, serum MMP-9 levels closely correlated with disease activity demonstrated by gadolinium-enhanced MRI in MS patients [30]. In the nervous system, aberrant expression of MMPs may support disease activity by converting pro-forms of several inflammatory molecules, such as TNF-α, into their active forms, resulting in the propagation of inflammation [31]. In addition, MMPs were reported to induce the degradation of myelin or axonal injury after injection into the brain [32], and fragments of MMP-mediated digestion of myelin basic protein are encephalitogenic when injected into mice. Thereby, a cascade of demyelinating and pro-inflammatory events is generated in the nervous system as a result of aberrant MMP expression [33].

Minocycline not only inhibits the enzymatic activity of MMPs, but also reduces the expression of several MMP family members

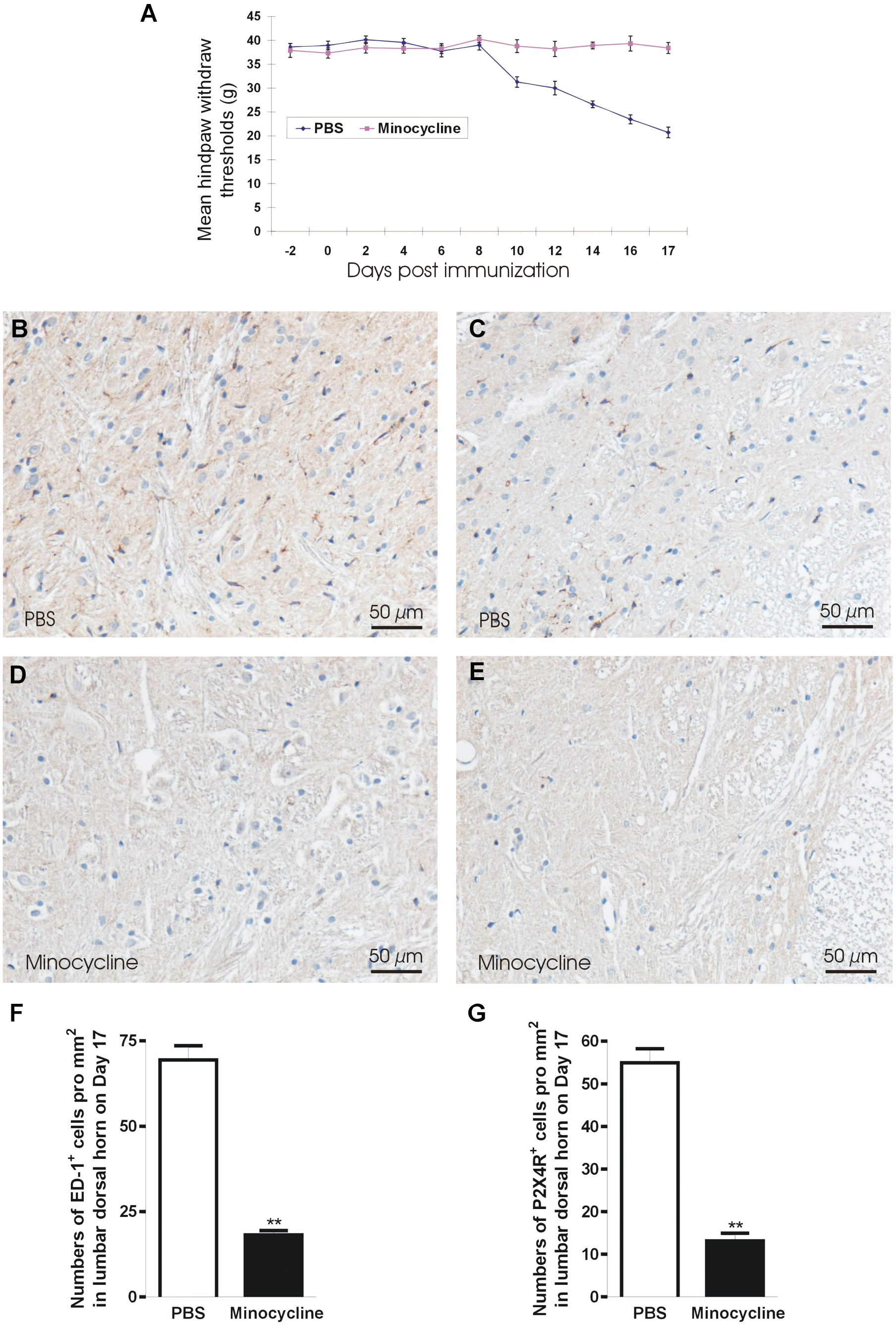

Fig. 6 Minocycline suppressed mechanical allodynia and spinal microglial activation in EAN rats. EAN was induced by subcutaneous injection on the basal part of tails with reduced amount of P2 peptide (80 μg) and CFA in rats. Mechanical allodynia was evaluated by measuring rat hind-paw withdrawal threshold (HWT) using an automatic von Frey system in EAN rats treated with minocycline or PBS controls (six rats per group). (**A**) Minocycline greatly suppressed mechanical allodynia in EAN, namely no significant changes of HWT were observed in the minocycline-treated group. In the PBS control group, mechanical allodynia was observed as early as Day 9 up to the end of the experiment (Day 17). Significant differences of HWT between both PBS and minocycline groups over time was proved by two-way ANOVA tests ($P < 0.05$). Representative micrographs show that ED-1$^+$ (**B**) and P2X4R$^+$ (**C**) cells were pronounced in dorsal horns of lumbar spinal cords in PBS control EAN rats at Day 17. Minocycline treatment significantly suppressed the accumulation of ED-1$^+$ (**D**) and P2X4R$^+$ (**E**) cells in dorsal horns of lumbar spinal cords of EAN rats at Day 17. (**F** and **G**): Numbers of ED-1$^+$ (**F**) and P2X4R$^+$ (**G**) cells in dorsal horns of lumbar spinal cords were counted as described in Experimental procedures. Bar graphs show semi-quantified results of arithmetic means of positive cells per square millimetre and standard errors of means. The unpaired t-test was performed to compare the differences between minocycline treated and PBS control EAN rats (Graph Pad Prism 4.0 for Windows). $^{**}P < 0.01$ compared to their respective control.

[34]. Here, our data showed that minocycline reduced MMP-9 level in sciatic nerves of EAN rats, which could not only inhibit leucocyte infiltration but also diminish their effects on demyelination in EAN.

Minocycline could also attenuate inflammatory cytokines in sciatic nerves, which may favour its use in EAN. Cytokines are produced and released by many cell types and regulate inflammation and immunity. Pro-inflammatory cytokines, such as IL-1β and TNF-α are produced by microglia, astrocytes and macrophages, and augment both inflammation and subsequent immune responses [35]. In EAN, IL-1β may participate in initiating the autoimmune response. Expression of IL-1β mRNA in EAN lymph node and sciatic nerves was reported and could be related to the presence of macrophages/monocytes, initiating a local immune response in lymph nodes and PNS. TNF-α activates macrophages, up-regulates iNOS-specific mRNA, regulates release of nitrite and also stimulates Schwann cells to further produce pro-inflammatory cytokines, like IL-6 [20]. TNF-α can greatly enhance BNB permeability, which contributes to EAN development [36], and in particular to central and peripheral demyelination [20]. Therefore, the suppression of these inflammatory cytokines in EAN by minocycline can improve EAN outcome.

Similar to inflammatory cytokines, iNOS is also important for the pathogenesis of EAN. iNOS functions to produce nitric oxide which possesses pro-inflammatory property including vasodilation, oedema, cytotoxicity and mediates cytokine-dependent processes that can result in tissue destruction [37]. In EAN, up-regulation of iNOS was reported and was particularly related to pathogenesis of PNS cell-mediated demyelination and even axonal damage [38]. In our study, reduced expression of iNOS mRNA was also observed under administration of minocycline, which may inhibit activation of immune cells, and be an important part of minocycline's anti-inflammatory effect in EAN. Interestingly, inhibition of iNOS expression is considered to be a major mechanism of minocycline [7]. While accumulated data proposed several potential mechanisms of inhibitory activity of minocycline to microglia and macrophages, the exact mechanisms are not clear yet. One of the potential mechanism is that minocycline could inhibit the expression of iNOS, resulting in reduced release of NO and NO-induced phosphorylation of p38 MAPK (mitogen-activated protein kinase) [34]. Our observation in this study also supports this idea.

An interesting finding in this study is that minocycline attenuates mechanical allodynia in EAN rats. Neuropathic pain is a common symptom of human autoimmune polyneuropathy, occurring in 55-85% of cases [39]. In EAN, mechanical allodynia was successfully observed recently [5, 6]. Neuropathic pain, which is caused by lesion or inflammation of the nervous system, is characterized by increased sensitivity to painful stimuli (hyperalgesia), the perception of innocuous stimuli as painful (allodynia) and spontaneous pain. Neuropathic pain can be due to peripheral sensitization or central sensitization.

Peripheral sensitization refers to the increased sensitivity of primary sensory neurons to stimuli, which can occur at the distal nerve terminal, at the axon, or at the cell body of sensory neurons. Nerve injury or inflammation is often accompanied with release of multiple inflammatory mediators, like IL-1β, TNF-α, from infiltrating leucocyte or damaged axons. The nerve terminal, axon and soma of primary sensory neurons express receptors for these inflammatory mediators. The binding of these inflammatory mediators to their receptors may result in an increase or suppress the activity of certain ion channels to cause excitability change of primary sensory neurons by post-translational and transcriptional regulation [40]. In EAN, systemic administration of minocycline significantly reduced immune cell infiltration to peripheral nerves and local inflammatory cytokines, like IL-1β, IL-6, which could partly contribute to the reduced pain hypersensitivity.

In the CNS, particularly in spinal cords, glia activation plays an essential role in the mediation of neuropathic pain. In spinal cord, microglia are activated in response to a variety of peripheral stimuli, resulting from degeneration of central terminals of dying sensory neurons or through the release of substances by incoming sensory afferents or pain-responsive neurons in the dorsal horn [41]. Activated microglia secretes pro-inflammatory cytokines, like IL-6, TNF-α and IL-1β, which contribute to central sensitisation of neuropathic pain [42].

In EAN spinal cords, glia activation is known and considered to be related to neuropathic pain [6, 43]. In this study, we observed an increase of reactive microglia (ED-1$^+$ or P2X4R$^+$ cells) pronounced in the dorsal horn, an area closely associated with nociceptive signalling. Following minocycline treatment, significant reduction of numbers of reactive spinal microglia was seen and accompanied by attenuated mechanical allodynia. Minocycline is well recognised as an inhibitor of microglia activation [44]. Being lipophilic, minocycline can diffuse into the CNS and PNS to inhibit microglia activation, thereby suppressing neuropathic pain in EAN. Certainly, greatly attenuated peripheral inflammation and decreased local release of multiple inflammatory cytokines, like IL-1β, TNF-α, which are known to play an important role in peripheral sensitization [40], may also greatly contribute to the absence of mechanical allodynia.

In conclusion, we have studied the effects of minocycline in EAN, an animal model of GBS. Our data showed that minocycline could effectively suppress peripheral and spinal inflammation to improve outcome in EAN rats, which suggests that minocycline should be considered a potential candidate for treatment of autoimmune neuropathies.

Acknowledgement

This work has been supported in part by a grant of the BMBF (03134298). The authors are solely responsible for the content of the article.

References

1. **Willison HJ.** The immunobiology of Guillain-Barré syndromes. *J Peripher Nerv Syst.* 2005; 10: 94–112.
2. **Hughes RA, Cornblath DR.** Guillain-Barré syndrome. *Lancet.* 2005; 366: 1653–66.
3. **Howarth AL.** Pain management for multiple sclerosis patients. *Prof Nurse.* 2000; 16: 824–6.
4. **Gold R, Hartung HP, Toyka KV.** Animal models for autoimmune demyelinating disorders of the nervous system. *Mol Med Today.* 2000; 6: 88–91.
5. **Moalem-Taylor G, Allbutt HN, Iordanova MD, Tracey DJ.** Pain hypersensitivity in rats with experimental autoimmune neuritis, an animal model of human inflammatory demyelinating neuropathy. *Brain Behav Immun.* 2007; 21: 699–710.
6. **Zhang Z, Zhang ZY, Fauser U, Schluesener HJ.** Mechanical allodynia and spinal up-regulation of P2X4 receptor in experimental autoimmune neuritis rats. *Neuroscience.* 2007; doi: 10.1016/j.neurosience.2007.12.042.

7. **Stirling DP, Koochesfahani KM, Steeves JD, Tetzlaff W.** Minocycline as a neuroprotective agent. *Neuroscientist.* 2005; 11: 308–22.
8. **Raghavendra V, Tanga F, DeLeo JA.** Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther.* 2003; 306: 624–30.
9. **Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, Watkins LR.** Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain.* 2005; 115: 71–83.
10. **Zouboulis CC, Piquero-Martin J.** Update and future of systemic acne treatment. *Dermatology.* 2003; 206: 37–53.
11. **Schluesener HJ, Seid K, Zhao Y, Meyermann R.** Localization of endothelial-monocyte-activating polypeptide II (EMAP II), a novel proinflammatory cytokine, to lesions of experimental autoimmune encephalomyelitis, neuritis and uveitis: expression by monocytes and activated microglial cells. *Glia.* 1997; 20: 365–72.
12. **Leifeld L, Fielenbach M, Dumoulin FL, Speidel N, Sauerbruch T, Spengler U.** Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) expression in fulminant hepatic failure. *J Hepatol.* 2002; 37: 613–9.
13. **Molander C, Xu Q, Grant G.** The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. *J Comp Neurol.* 1984; 230: 133–41.
14. **Hartung HP, Schäfer B, Heininger K, Stoll G, Toyka KV.** The role of macrophages and eicosanoids in the pathogenesis of experimental allergic neuritis. Serial clinical, electrophysiological, biochemical and morphological observations. *Brain.* 1988; 111: 1039–59.
15. **Pannu R, Barbosa E, Singh AK, Singh I.** Attenuation of acute inflammatory response by atorvastatin after spinal cord injury in rats. *J Neurosci Res.* 2005; 79: 340–50.
16. **Zhang Z, Zhang ZY, Fauser U, Schluesener HJ.** FTY 720 ameliorates experimental autoimmune neuritis by inhibition of lymphocyte and monocyte infiltration to peripheral nerves. *Exp Neurol.* 2008; 210: 681–90.
17. **Rosenberg GA.** Matrix metalloproteinases in neuroinflammation. *Glia.* 2002; 39: 279–91.
18. **Kieseier BC, Clements JM, Pischel HB, Wells GM, Miller K, Gearing AJ, Hartung HP.** Matrix metalloproteinases MMP-9 and MMP-7 are expressed in experimental autoimmune neuritis and the Guillain-Barré syndrome. *Ann Neurol.* 1998; 43: 427–34.
19. **Lee Y, Shin T.** Expression of constitutive endothelial and inducible nitric oxide synthase in the sciatic nerve of Lewis rats with experimental autoimmune neuritis. *J Neuroimmunol.* 2002; 126: 78–85.
20. **Zhu J, Mix E, Link H.** Cytokine production and the pathogenesis of experimental autoimmune neuritis and Guillain-Barré syndrome. *J Neuroimmunol.* 1998; 84: 40–52.
21. **Campbell JN, Meyer RA.** Mechanisms of neuropathic pain. *Neuron.* 2006; 52: 77–92.
22. **Nasu-Tada K, Koizumi S, Tsuda M, Kunifusa E, Inoue K.** Possible involvement of increase in spinal fibronectin following peripheral nerve injury in upregulation of microglial P2X4, a key molecule for mechanical allodynia. *Glia.* 2006; 53: 769–75.
23. **Schabet M, Whitaker JN, Schott K, Stevens A, Zürn A, Bühler R, Wiethölter H.** The use of protease inhibitors in experimental allergic neuritis. *J Neuroimmunol.* 1991; 31: 265–72.
24. **Mäurer M, Toyka KV, Gold R.** Cellular immunity in inflammatory autoimmune neuropathies. *Rev Neurol.* 2002; 158:S7–15.
25. **Kloppenburg M, Dijkmans BA, Verweij CL, Breedveld FC.** Inflammatory and immunological parameters of disease activity in rheumatoid arthritis patients treated with minocycline. *Immunopharmacology.* 1996; 31: 163–9.
26. **Ingham E.** Modulation of the proliferative response of murine thymocytes stimulated by IL-1, and enhancement of IL-1 beta secretion from mononuclear phagocytes by tetracyclines. *J Antimicrob Chemother.* 1990; 26: 61–70.
27. **Parks WC, Wilson CL, López-Boado YS.** Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol.* 2004; 4: 617–29.
28. **Hughes PM, Wells GM, Clements JM, Gearing AJ, Redford EJ, Davies M, Smith KJ, Hughes RA, Brown MC, Miller KM.** Matrix metalloproteinase expression during experimental autoimmune neuritis. *Brain.* 1998; 121: 481–94.
29. **Yong VW, Power C, Forsyth P, Edwards DR.** Metalloproteinases in biology and pathology of the nervous system. *Nat Rev Neurosci.* 2001; 2: 502–11.
30. **Lee MA, Palace J, Stabler G, Ford J, Gearing A, Miller K.** Serum gelatinase B, TIMP-1 and TIMP-2 levels in multiple sclerosis. A longitudinal clinical and MRI study. *Brain.* 1999; 122: 191–7.
31. **Brundula V, Rewcastle NB, Metz LM, Bernard CC, Yong VW.** Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain.* 2002; 125: 1297–308.
32. **Newman JP, Verity AN, Hawatmeh S, Fee WE Jr, Terris DJ.** Ciliary neurotrophic factors enhances peripheral nerve regeneration. *Arch Otolaryngol Head Neck Surg.* 1996; 122: 399–403.
33. **Opdenakker G, Van Damme J.** Cytokine-regulated proteases in autoimmune diseases. *Immunol Today.* 1994; 15: 103–7.
34. **Zemke D, Majid A.** The potential of minocycline for neuroprotection in human neurologic disease. Clin *Neuropharmacol.* 2004; 27: 293–8.
35. **Saliba E, Henrot A.** Inflammatory mediators and neonatal brain damage. *Biol Neonate.* 2001; 79: 224–7.
36. **Hartung T, Döcke WD, Gantner F, Krieger G, Sauer A, Stevens P, Volk HD, Wendel A.** Effect of granulocyte colony-stimulating factor treatment on *ex vivo* blood cytokine response in human volunteers. *Blood.* 1995; 85: 2482–9.
37. **Abramson SB, Amin AR, Clancy RM, Attur M.** The role of nitric oxide in tissue destruction. *Best Pract Res Clin Rheumatol.* 2001; 15: 831–45.
38. **Conti G, Rostami A, Scarpini E, Baron P, Galimberti D, Bresolin N, Contri M, Palumbo C, De Pol A.** Inducible nitric oxide synthase (iNOS) in immune-mediated demyelination and Wallerian degeneration of the rat peripheral nervous system. *Exp Neurol.* 2004; 187: 350–8.
39. **Moulin DE, Hagen N, Feasby TE, Amireh R, Hahn A.** Pain in Guillain-Barré syndrome. *Neurology.* 1997; 48: 328–31.
40. **Ji RR, Strichartz G.** Cell signaling and the genesis of neuropathic pain. *Sci STKE.* 2004; 252:reE14.
41. **Watkins LR, Maier SF.** Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. *Physiol Rev.* 2002; 82: 981–1011.
42. **Inoue K.** The function of microglia through purinergic receptors: neuropathic pain and cytokine release. *Pharmacol Ther.* 2006; 109: 210–26.
43. **Beiter T, Artelt MR, Trautmann K, Schluesener HJ.** Experimental autoimmune neuritis induces differential microglia activation in the rat spinal cord. *J Neuroimmunol.* 2005; 160: 25–31.
44. **Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J.** Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J Neurosci.* 2001; 21: 2580–8.