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

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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

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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 (TESPKNTEIFKLGQFEETADNR), 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 after emulsification 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 intradermal 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 into 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 metalloproteinase-9; 1:500; Neuroemics, 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% H2O2 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 diamobenzidine (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× 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 cross-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).
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 intracardially 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-Phycocerythrin (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 decrease as in normal rats (Fig. 1B). And significant differences of
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 $0.55 \pm 0.20$) (Fig. 2A and C) as compared to the PBS control group (mean histological score $2.23 \pm 0.42$) (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 $592.7 \pm 48.97$ 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-1$\beta$ and TNF-$\alpha$ are up-regulated in EAN and known to play important roles in inflammatory progression of disease [19, 20]. Therefore, effects of minocycline on mRNA 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-1$\beta$, TNF-$\alpha$ and iNOS was significantly reduced by minocycline in sciatic nerves of EAN rats as compared to PBS controls.
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...
Activated macrophages cause demyelination by direct phagocytic sion of more lymphocytes and monocytes and local inflammation. An autoimmune reaction within the PNS that orchestrates the invasion of the endothelium of the PNS, penetrate the BNB and generate an inflammatory response. Following activation, autoreactive T cells attach to the venular presenting cells, are of importance for the initiation of EAN. T cells, which can recognize peripheral nerve autoantigens on antigen presenting cells, are of importance for the initiation of EAN. Thymocytes induced by IL-1 [26]. So minocycline might inhibit 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 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 leucocytes 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
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-\(\alpha\), 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-\(\alpha\), 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.
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,
Nerve injury or inflammation is often accompanied with release of nerve terminal, at the axon, or at the cell body of sensory neurons, primary sensory neurons to stimuli, which can occur at the distal spontaneous pain. Neuropathic pain can be due to peripheral sensitization of 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.

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