Gabapentin attenuates neuropathic pain and improves nerve myelination after chronic sciatic constriction in rats

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HIGHLIGHTS

• Gabapentin improves myelin basic protein expression in the injured sciatic nerve.
• Gabapentin ameliorates neuropathic pain behaviors.
• Gabapentin has a dual role in improving neuropathic pain and nerve myelination following sciatic nerve injury.

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ABSTRACT

Gabapentin (GBP) is an anti-convulsive drug often used as analgesic to control neuropathic pain. This study aimed at evaluating oral GBP treatment (30, 60, 120 mg/kg, 60 min prior to chronic constriction of the sciatic nerve (CCSN) along 15-day treatment post-injury, 12 h(12 h) by monitoring spontaneous and induced-pain behaviors in Wistar rats on 5th and 15th days post-injury during early neuropathic events. CCSN animals receiving saline were used as controls. Another aim of this study was to evaluate GBP effects on myelin basic protein (MBP) on the 5th and 15th days post-injury and nerve morphology by transmission electron microscopy to address nerve regeneration. On the 5th and 15th days, GBP (60 mg/kg) reduced neuropathic pain behaviors (scratching and biting) in the ipsilateral paw and alleviated mechanical allodynia in comparison with the neuropathic saline group. GBP significantly increased climbing and rearing behaviors in CCSN and CCSN-free animals suggesting increased motor activity rather than sedation. We found three-fold significant increase in MBP expression by western blots on the 15th day when compared to controls. In addition, GBP (80 mg/kg) improved nerve axonal, fiber and myelin area 15 days post-surgery. In conclusion, GBP alleviated mechanical and thermal allodynia and spontaneous pain-related behaviors and improved later nerve morphology. Our findings suggest that GBP improve nerve remyelination after chronic constriction of the sciatic nerve.

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1. Introduction

Peripheral nerve injury (PNI) is an escalating problem worldwide mostly due to a growing prevalence of societal violence, occupational and traffic accidents, especially in more densely-populated urban areas. PNI may lead to a chronic condition associated with neuropathic pain and nerve function loss, with a tremendous impact in hospital costs and incapacity [1]. Although peripheral nerve regeneration is possible, current treatment is still unsatisfactory, especially for the elderly. Novel therapies to accelerate nerve regeneration are needed in association with benefits in reducing neuropathic pain. Mechanisms underlying pain relief with the use of anticonvulsants are a promis-

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ing target for therapy improvements [2], however to date no studies have addressed their potential dual benefit in improving pain and nerve regeneration.

The anticonvulsant gabapentin (GBP) (1-(aminomethyl) cyclohexaneacetic acid), one structural derivative of the gamma-amino butyric acid (GABA), is now further considered as an effective therapy for some forms of neuropathic and post-surgical pain [3]. GBP-specific inhibition of calcium–voltage dependent α2-δ subunits [4] or activation of protein kinase G-K+ channels [5] has been implicated in reducing allodynia by altering neurotransmitter release.

Our previous study has documented the benefit effect of oral GBP (60 and 120 mg/kg) treatment in improving heat-induced hyperalgesia with nerve pro-inflammatory effects in a model of sciatic nerve constriction in Wistar rats [6]. However, the potential nerve regenerative effect of GBP has been poorly explored.

In this study we explored whether prolonged GBP treatment improves sciatic nerve myelination by assessing nerve myelin basic protein (MBP) (a constitutive myelin protein) [7], spontaneous motor-related activity, and fine nerve myelin morphology, following sciatic nerve constriction in Wistar rats. In addition, we discuss whether better nerve remyelination and reduced myelin protein debris could improve neuropathic pain following GBP treatment.

2. Materials and methods

Protocols from this study were in accordance with the Brazilian College for Animal Experimentation (COBEA) and the International Association for the Study of Pain (IASP) and were approved by the Animal Care and Use Committee of Department of Physiology and Pharmacology, Federal University of Ceará.

2.1. Animal studies

160 male Wistar rats weighing between 250 and 300 g from the Department of Physiology and Pharmacology vivarium at the Federal University of Ceará were used in this study. Rats were housed in a temperature-controlled room (26 ± 2 °C) with free access to water and chow diet in a 12h/12h light/dark cycles. All surgical procedures were performed in the laboratory of Experimental Neurology in the Department of Physiology and Pharmacology at the Federal University of Ceará.

2.2. Sciatic nerve chronic constriction

In order to induce the experimental neuropathy, we used the chronic constriction of the sciatic nerve (CCSN) model, described by Bennett and Xie [8] and modified by Sommer et al. [9]. Animals were anesthetized with intraperitoneal injection of tribromoethilene (25 mg/kg), following trichotomy and anti-sepsis of the surgery field. A 15-mm longitudinal incision of the right thigh, at the level of the femoral trocar of the posterior limb, was used to access and expose the sciatic nerve after gluteus and femoral biceps dissection. We used three 4–0 cat-gut loose ligatures on the right paw sciatic nerve, 1-mm away from the sciatic trifurcation inducing a slight nerve ischemia. In the left thigh, the sciatic nerve was exposed, but remained untouched and surgery closed afterwards. Skin and muscular layers were sutured with a 5–0 mononylon thread.

2.3. Drugs and treatment regimens

Gabapentin (GBP) (1-(aminomethyl) cyclohexaneacetic acid, C9H17NO2) (Neurontin®, Pfizer) capsules were dissolved in 0.9% saline solution and then given orally by gavage every 12 h during either a 5 or 15-day treatment course. GBP doses of 30, 60 and 120 mg/kg were used based on previous studies with good clinical response [10]. As the maximum GBP effect occurs 60 min after oral administration [11], the first dose was given 60 min prior to the nerve surgery and the last dose was given 60 min before behavior tests.

2.4. Neuropathic pain assessment

In order to assess whether GBP treatment could ameliorate neuropathic pain following sciatic nerve injury, cohort animals were evaluated in spontaneous and induced behaviors.

2.4.1. Spontaneous pain behaviors

Rats were kept in a wooden cage (100 × 50 × 50 cm) for a 5-min acclimation time and testing. The observations were conducted as described elsewhere [6]. Each experimental and control rat was observed during a time of 30 min. Positioned in front of the cage, the observer could identify each behavioral component and record it using a computer software (Comporta®) designed by Prof. Marcus Vale (Federal University of Ceará, Brazil). Experimenters were unaware of the identity of the experimental groups.

The first observation was performed before the surgery (baseline) and then on the 5th and 15th post-surgery. A delta mean behavior value was derived from baseline. For measurements (in seconds), we considered the following pain-related spontaneous behaviors: (1) scratching: time spent raising the hind left or right paw to scratch parts of the body with rapid movements of the paw and claws; (2) biting: time spent piercing the skin with the mouth and teeth on the left or right side of the body.

2.4.2. Induced pain behaviors

2.4.2.1. Mechanical allodynia. In order to assess the GBP chronic effect on CCSN mechanical allodynia, the von Frey assessment was used according to Azevedo and colleagues [12]. Briefly, the testing consisted of poking the hind paw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. Paw stimulation was repeated until the animal presented three similar measurements (the difference between the highest and the lowest measurement should be less than 10 g). Animals were tested on the 5th and 15th day post-CCSN. The results are reported as the withdrawal threshold (g).

2.4.2.2. Cold acetone allodynia. In order to assess the GBP chronic treatment effect on CCSN-induced cold allodynia, the method described by Bennett and Flatters [13] was used. Briefly, rats were placed in acrylic cages with a wire grid floor 15–30 min before the beginning of the tests in a quiet room. A drop (0.05 mL) of acetone was placed against the center of hind paw ventral side and a stopwatch was started. Responses to acetone were graded to the following 4-point scale: 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking of the paw; 3, repeated flicking of the paw with licking directed at the ventral side of the paw. Acetone was applied alternately three times to each paw and the responses scored categorically. Animals were tested on the 5th and 15th day post-CCSN.

2.5. Spontaneous motor-related behaviors

In order to differentiate GBP treatment analgesic effects from sedation, we observed the control and experimental rats, as described above (Section 2.4.1), measuring the rearing (time spent suspending the forefoot and supporting the body on the hind paws and climbing behaviors (time spent climbing a 2-cm high three step ladder).
2.6. Myelin nerve morphology and MBP expression

In order to investigate nerve remyelination following CCSN injury, we assess ultra-structural myelin features and nerve fine histology and MBP expression by western blotting and immunohistochemistry on day 15th post-challenge.

2.6.1. Fine nerve histology and morphometry

Anesthetized rats were perfused intracardially with a solution containing 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After, a 5 mm segment (5 mm distal from the injury site) of the 15th day-post-injury sciatic nerve was removed and fixed with 2.5% glutaraldehyde in 0.1 M cacodilate buffer (pH 7.4) for 2 h and processed for transmission electron microscopy. After that, segments were post-fixed in 1% osmium tetroxide plus 0.8% potassium ferrocyanide and 5 mM CaCl₂ in 0.1 M cacodilate buffer (pH 7.4) during 1 h. Next, segments were dehydrated in increasing concentrations of acetone and finally embedded in Embed-812 resin (Electron Microscopy Sciences). Semithin (1 μm) and ultrathin (60 nm) sections were obtained in a RMC MT-6000 ultramicrotome. The semithin sections were stained with 1% toluidine blue solution and observed and photographed under a Zeiss Axioskop 2 Plus light microscope with 20× and 40× objectives. The ultrathin sections were collected on copper grids and stained 30 min in uranyl acetate followed by 10 min in lead citrate. Electron micrographs were acquired using Zeiss 900 transmission electron microscope operated at 120 kV.

2.6.2. Nerve morphometry

In order to identify myelinated fibers, five areas were systematically selected from the semi-thin cross sections of the nerve of each animal and were image-captured in high magnification by Axiovision Rel. 4.5 (Carl Zeiss Microimaging, Thornwood, NY, USA). The total of 30 areas from each group was analyzed. This quantification was performed using Image Java software (Bethesda, MD, USA).

2.6.3. MBP immunohistochemistry

On the 15th day post-CCSN and under deep anesthesia, rats were transcardially perfused with saline and then the distal sciatic nerve segment was harvested 5 mm-distal to the ligation, immediately immersed in buffered formaline (pH 7.4) for 24 h and sent to the histology core for immunohistochemistry processing. Immunohistochemistry for MBP (anti-MBP diluted 1:200, Santa Cruz, CA) was performed using the streptavidin-biotin-peroxidase method in formalin-fixed, paraffin-embedded tissue sections (4 μm thick) after citrate buffer antigen retrieval, according to manufacturer's instructions. Immunolabeling was visualized with the chromogen 3,3-diaminobenzidine (DAB). Negative control sections were processed simultaneously, as described above but with the primary antibody being replaced by 5% PBS-BSA. None of the negative controls showed immunoreactivity. Slides were counterstained with Harry's haematoxylin.

2.6.4. MBP immunoblotting

A 2.5 cm-long nerve tissue of the proximal sciatic nerve stump was harvested to assess the MPB on the 15th days post-surgery and immediately snap-frozen in liquid nitrogen and processed for western blotting, as described elsewhere [14]. MBP has been shown to be highly expressed in the proximal stump than the distal one 14 days-post-CCSN [15].

2.7. Statistical analyses

Normal distribution was assessed using Kolmogorov–Smirnov’s test. Effects amongst groups were assembled using one-way ANOVA with post-hoc Tukey for parametric data and Kruskal–Wallis with post-hoc Dunn’s for non-parametric data. In nerve morphometry, we used Mann–Whitney test. Values are shown as mean ± SEM.

3. Results

3.1. GBP effect on neuropathic pain behaviors

After 5-days of GBP treatment, all tested doses (30, 60 and 120 mg/kg) caused significant reductions in the scratching behavior of the right paw (93, 86.7, 94.4, and 83.6%, respectively) when compared to neuropathic saline group (p < 0.01). GBP (60 mg) lowered the biting behavior (131.8%), as opposed to the neuropathic saline group (p < 0.01). In CCSN-free rats, those behaviors were barely seen (N = 12). On the 15th day post-surgery, all neuropathic pain-related behaviors (scratching and biting) were worse (~2 and 10-fold more, respectively). GBP (60 mg) markedly improved the scratching and biting behaviors (76 and 73%, respectively) compared with the untreated challenged-group (Fig. 1A–D).

3.1.1. GBP effect on von Frey’s and cold acetone alldynia

On the 5th day post-constriction, all GBP doses caused significant increase in the right paw withdrawal threshold in relation to the neuropathic saline group. GBP at doses of 60 and 120 mg were responsible for the maximum effect, providing threshold catch-up of 45.9% and 57.7%, respectively, in relation to the neuropathic saline group, Fig. 1E (p < 0.00001). On the 5th day post-constriction, the score for cold allodynia was reduced (42.2%) by 120 mg of GBP in comparison with the neuropathic saline group. The 30 and 60 mg doses lead to reduction trends but neither reached a statistical difference (Fig. 1G). On the 15th day, GBP treatment (60 mg) improved mechanical and thermal alldynia compared with the untreated CCSN-group (p < 0.01) (Fig. 1F and H). GBP treatment did not induce significant differences in the contra-lateral CCSN-free paw (data not shown).

3.2. GBP effect on spontaneous motor-related behaviors

GBP (60 mg) raised the delta scores of rearing compared with the untreated CCSN-rats on day 5 post-nerve constriction. No differences were found for the rearing behavior on the 15th post-challenge. 60 and 120 mg of GBP-treated CCSN rats showed significant increase in climbing when compared with the neuropathic saline group, p < 0.05 on the day 5 post-surgery. All GBP tested doses improved climbing 15 days post-surgery compared with the untreated challenged-group (Supplementary Fig. 1).

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3.3. Nerve morphology

On the 15th day, qualitative observation of semi-thin cross sections of the sciatic nerve showed higher number of myelinated fibers within the challenged and GBP-treated group as opposed to the nerves of the neuropathic saline group. Ultra-thin nerve cross-sections revealed thicker and higher density of myelin sheaths. In addition, the nerves of the neuropathic saline group showed nerve fibers with signs of degeneration, including several myelin ovoids, among preserved and/or regenerated fibers and some fibers with thin myelin lamellae. Injured sciatic nerve treated with GBP showed many preserved and/or regenerated fibers among few myelin ovoids (Supplementary Figs. 2 and 3). Furthermore, oral GBP (60 mg/kg) improved by 63.3, 32.8 and 28.2% in sciatic nerve axonal and fiber areas and myelin area, respectively, in CCSN-challenged rats when compared with untreated nerve-injured controls (p < 0.05). In all nerve morphometric parameters untreated
Fig. 1. Effect of chronic gabapentin (GBP) treatment on scratching (A, B), biting (C, D) behaviors and on von Frey's pressure stimulus (E, F), and cold acetone (G, H) testing of the ipsilateral hind paw in CCSN-rats on either the 5° or 15th day. At least n = 8 animals per group were used. GBP 30, 60, 120 mg/kg was given by daily gavage (12/12 h). Behavioral data (mean ± SEM) are expressed in delta values. In pressure stimulus, data are expressed in threshold (g). In cold stimulus test, data are expressed in scores. *p < 0.05 vs saline group, by either ANOVA with Tukey's or by Kruskal–Wallis with Dunn's post-test when appropriate.
Prèssure stimulus

Cold stimulus (10°C)

Fig. 1. (Continued).
treated the online blockage and ipsilateral benefit from GBP (60 mg/kg) significantly increased (over three fold, \( p < 0.05 \)) MBP nerve expression, as compared with the neuropathic saline group. In addition, the MBP immunostaining was markedly seen in the GBP (60 mg/kg) treated rats after 15 days of treatment following sciatic nerve constriction (Supplementary Fig. 5). At the time point of the 5th day post-injury an increased in MBP nerve expression was seen in the GBP (60 mg/kg) treated group albeit not reaching statistically significance (data not shown).

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3.4. GBP effect on the sciatic nerve MBP expression

At the time point of the 15th day post-injury, GBP (60 mg/kg) significantly increased (over three fold, \( p < 0.05 \)) MBP nerve expression, as compared with the neuropathic saline group. In addition, the MBP immunostaining was markedly seen in the GBP (60 mg/kg) treated rats after 15 days of treatment following sciatic nerve constriction (Supplementary Fig. 5). At the time point of the 5th day post-injury an increased in MBP nerve expression was seen in the GBP (60 mg/kg) treated group albeit not reaching statistically significance (data not shown).

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4. Discussion

In this study, we addressed GBP effects on neuropathic pain behavior (5th and 15th day post-sciatic ligation) and on nerve myelination parameters following chronic sciatic nerve constriction in rats.

Our data have shown reduced biting and scratching behaviors in challenged rats, following GBP treatment on the 5th and 15th day post-nerve constriction. Early studies have demonstrated that ipsilateral scratching to the injured hind limb is a recognized hallmark feature of neuropathic pain [16] with a peak on the 14th day post-nerve constriction [17]. Biting likewise has been implicated in neuropathic distress but with a distinct evolution in models of chronic arthritis-associated neuropathy [18].

Oral GBP treatment (12/12 h) at doses of 30, 60, and 120 mg/kg induced significant reductions in the scratching time (in the right ipsilateral paw) spent by the neuropathic rats in comparison with the untreated group. However, this finding was not seen in a dose-dependent manner, in fact the best effect was seen with a GBP dose of 30 mg/kg. Interestingly, Kayser and Christensen found a benefit with the same GBP dose (30 mg/kg i.p.) on the vocalization thresholds (a supraspinal-derived behavior) following sciatic nerve constriction in rats [11]. On the other hand, our data show that GBP (60 mg/kg) was more effective in reducing the biting behavior.

Bagriyanik et al. have found deficits in motor-related behaviors (open field and rotarod test) 14 days post-nerve constriction in rats [19]. Diverging from Gustafsson et al. findings that show decreased locomotion and rearing behaviors after acute and cumulative doses of GBP (200 \( \mu \)mol/kg, ~40 mg/kg) [20], we found that prolonged GBP treatment increased motor-related behavior delta scores (rearing and climbing), as compared to controls therefore suggesting that oral GBP did not induce sedative effects in these animals. However, we cannot rule out an excitatory effect of GBP enhancing these behaviors. More studies are needed to dissect better these findings.

In accordance with our data, LaBuda and Little [10] used a spinal nerve (L5) ligation model and found increased paw withdrawn threshold (tactile von Frey) with GBP treatment (30, 60 and 120 mg/kg i.p.) as compared with the saline control after 1 h post-surgery. The anti-allodynic GBP effect could be explained by blockage of the \( \alpha_2\delta \) in the Cav2.1 calcium channel subunits [21]. In addition, GBP can suppress in vivo and in vitro ectopic discharges in the sciatic nerve afferent fibers from neuropathic animals but not from normal animals [22].

MBP and PLP constitute 85% of the protein found in the myelin sheath and help to stabilize the myelin structure scaffolding the lipid component [23]. Setton-Avruj et al. studied the temporal course of MBP nerve expression (3, 7 and 14 days), after sciatic nerve constriction in adult rats, distally to the ligature, and found that there was a maximum peak of MBP expression seven days post-constriction, reducing thereafter. In the proximal nerve stump, a maximum peak of MBP was found on the 14th day post-constriction [24]. Similarly to Setton-Avruj findings, a GBP redistribution was seen in the distal stump of injured nerves, suggesting an increased cellular phagocytosis and scattering of MBP debris. GBP redistribution may be ongoing during simultaneous processes of distal Wallerian degeneration and nerve regeneration. Noteworthy, axon-released MBP may be digested by metalloproteinase-9 to generate MBP84-104 and MBP68-86 fragment peptides that are strongly immunogenic, activating T-cells and causing T-cell-mediated mechanical allodynia [25]. Our previous [6] and current findings suggest that GBP-induced myelin debris removal from the injured nerve site and better regenerating axons could be beneficial to improve neuropathic pain by reducing GBP digested fragments of the nerve millieu.

Macrophages and Schwann cells both cooperate during myelin degeneration, removing myelin and axon debris that may facilitate nerve regeneration [26]. In addition, according to Hall [1978], rapid Schwann cell proliferation (3–4 days post-nerve injury) is key factor to promote axonal regeneration. Removal of myelin debris by macrophage-like activated cells is an important factor benefitting nerve recovery after sciatic nerve constriction, as myelin debris could be inhibitory to nerve regeneration, since several myelin-associated inhibitors of axon regeneration have been found in the peripheral myelin [27,28].

One study has found no nerve regenerative effect with chronic injected pregabalin, a GBP analogus, (10 mg/kg by daily subcutaneous injection) at the 21st day following sciatic nerve crush [29], this may be due to a different dose used. In light of our findings, Machado et al. have documented benefits of a high dose of oral GBP (300 mg/kg) in improving the area and density of myelinated fibers 30 days following stretch-induced nerve injury in Wistar rats [30].

More studies are warranted to appreciate in more detail the mechanisms, time-course and fine histology of myelin removal and remodeling during GBP pro-myelination effects in their association with the amelioration of neuropathic pain.

5. Conclusion

In summary, altogether our findings reinforce the analgesic effects of GBP and suggest a beneficial role of GBP on nerve morphology following sciatic nerve injury, through modulation of MBP expression and myelin remodeling.

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