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Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats

Rachel Wodarski, Anna K Clark, John Grist, Fabien Marchand, Marzia Malcangio *

Neurorestoration Group, Wolfson Centre for Age Related Diseases, King's College London, Hodgkin Building, London SE1 1UL, UK

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ABSTRACT

Diabetes mellitus is the leading cause of peripheral neuropathy worldwide. Despite this high level of incidence, underlying mechanisms of the development and maintenance of neuropathic pain are still poorly understood. Evidence supports a prominent role of glial cells in neuropathic pain states. Gabapentin is used clinically and shows some efficacy in the treatment of neuropathic pain. Here we investigate the distribution and activation of spinal microglia and astrocytes in streptozotocin (STZ)-diabetic rats and the effect of the gold standard analgesic, Gabapentin, on these cells. Mechanical allodynia was observed in four week-diabetic rats. Oral administration of Gabapentin significantly attenuated mechanical allodynia. Quantification of cell markers Iba-1 for microglia and GFAP for astrocytes revealed extensive activation of microglia in the dorsal horn of diabetic rats, whereas a reduction in the number of astrocytes could be observed. In addition, an attenuation of microglial activation correlated with reduced allodynia following Gabapentin treatment, while Gabapentin had no effect on the number of astrocytes. Here we show a role of microglia in STZ-induced mechanical allodynia and furthermore, that the anti-allodynic effect of Gabapentin may be linked to a reduction of spinal microglial activation. Astrocytic activation in this model appears to be limited and is unaffected by Gabapentin treatment. Consequently, spinal microglial activation is a key mechanism underlying diabetic neuropathy. Furthermore, we suggest that Gabapentin may exert its anti-allodynic actions partially through alterations of microglial cell function.

1. Introduction

Diabetes mellitus is the most common cause of peripheral neuropathy worldwide, with many patients experiencing chronic pain. The mechanisms that underlie the development and/or maintenance of diabetic neuropathy are not well understood and consequently the treatment of associated pain is inadequate. However, the antiepileptic drug Gabapentin has shown some efficacy in the treatment of diabetic neuropathy in multiple clinical trials (Vinik, 2005). In pre-clinical studies, the streptozotocin (STZ) model of diabetes in rats has been shown to be associated with sensory changes including allodynia and hyperalgesia which develop two weeks following STZ administration and continue over at least six weeks (Malcangio and Tomlinson, 1998; Calcutt and Chaplan, 1997). Similarly to the clinical scenario, Gabapentin is able to attenuate pain behaviours in STZ-induced diabetic neuropathy suggesting a good predictability of the animal model for the human disease (Luo et al., 2002).

Although the mechanisms of action of Gabapentin have yet to be ascertained, evidence implies that Gabapentin may act at the $\alpha_2\delta_1$ subunit of voltage-gated calcium channels, which is up-regulated in dorsal root ganglia and spinal cord in STZ-induced diabetic neuropathy (Luo et al., 2002; Yusaf et al., 2001). Gabapentin is thought to decrease neuronal activity through binding to $\alpha_2\delta_1$ and inhibition of calcium currents thereby preventing extracellular calcium entry essential for subsequent vesicular exocytosis (Stefani et al., 1998). However, recent evidence show that Gabapentin is an inhibitor of voltage-dependent calcium channels (VDCCs) trafficking via the inhibition of intracellular $\alpha_2\delta$ subunits (Hendrich et al., 2008). Central sensitization is thought to involve excessive calcium influx through VDCCs, resulting in elevated intracellular Ca^{2+} levels (Coderre et al., 1993), and consequently increases in neurotransmitter release. This is consistent with the efficacy of many specific VDCC blockers in neuropathic animals (Calcutt and Chaplan, 1997; Chaplan et al., 1994).

During the last decade growing evidence supports the prominent role of glial cells in the development and maintenance of neuropathic pain. Microglia and astrocyte activation in the spinal cord is observed following PNS or CNS injury (Scholz and Woolf, 2007; Watkins and Maier, 2003). Glial activation is also shown in inflammatory models (Qin et al., 2006; Raghavendra et al., 2004; Sweitzer et al., 1999) as well as in demyelinating disorders such as experimental autoimmune neuritis (EAN) (Beiter et al., 2005; Luongo

* Corresponding author. Tel.: +44 20 7848 6092; fax: +44 20 7848 6165.
E-mail address: Marzia.malcangio@kcl.ac.uk (M. Malcangio).

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et al., 2008), viral diseases like Borna Disease Virus (Stahl et al., 2006) and following intrathecal HIV-1 envelope glycoprotein injection (Milligan et al., 2001). Relevant to this study, recent reports demonstrate spinal microglial activation in STZ-diabetic rats (Daulhac et al., 2006; Tsuda et al., 2008) and the analgesic effect of inhibitors of phosphorylation of the Mitogen Activated Protein Kinases (MAPKs) Extracellular Signal-regulated Protein Kinase (ERK) (Tsuda et al., 2008) and p38 (Daulhac et al., 2006; Sweitzer et al., 2004), a marker for activated microglia which is essential for cytokine synthesis and release by microglia.

The aims of this study were first to investigate whether microglia and astrocytes were activated in diabetic rats showing pronounced allodynia. Then we examined whether the anti-allodynic effect of Gabapentin in diabetic rats was associated with changes in spinal glial activation states.

2. Materials and methods

2.1. Animals

All experiments were carried out using male Wistar rats (Harlan, Bicester, Oxon, UK), weighing 200–250 g, in accordance with United Kingdom Home Office regulations. Experimental study groups were randomised and behavioural studies were performed by an experimenter who was unaware of treatment groups.

2.2. Induction of diabetes

Diabetes was induced with an intraperitoneal (i.p.) injection of 55 mg/kg STZ (Sigma, UK) as described previously (Malcangio and Tomlinson, 1998). The tail vein blood glucose levels were measured one week after STZ injection to confirm diabetes. Animals with less than 14 mM blood glucose were excluded from the study.

2.3. Behavioural testing

2.3.1. Mechanical sensitivity

Mechanical withdrawal thresholds were tested using a Dynamic Plantar Aesthesiometer (Ugo-Basile, Milan). In brief, animals were placed in clear acrylic boxes (22 × 16.5 × 14 cm) with a metal grid floor in a temperature controlled room (~22 °C) and acclimatized for 15 min before testing. The stimulus was applied via a metal filament (∅ 0.5 mm) which applied a linearly increasing force ramp (2.5 g/s) to the plantar surface of the hind paw. A cut-off of 50 g was imposed to prevent any tissue damage. The force necessary to elicit a paw withdrawal was recorded. The paw withdrawal threshold (PWT) was calculated as the average of three consecutive tests with at least 5 min between each test. Mechanical allodynia was defined as reduced threshold after induction of diabetes compared to a baseline PWT before STZ injection. During Gabapentin treatment rats were tested 3 h after each morning Gabapentin administration.

2.3.2. Drug administration

Four weeks after STZ injection, Gabapentin (50 mg/kg; 5 ml/kg) or vehicle (0.9% saline) were delivered orally twice a day for 5 days.

2.4. Immunohistochemistry

Tissue was collected immediately following behavioural testing on the fifth day of treatment with Gabapentin/saline. Animals were anaesthetised with pentobarbital and transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Lumbar spinal cords (L3–L5) were excised, post-fixed over night in the paraformaldehyde solution, cryoprotected for 72 h in 20% sucrose in 0.1 M phosphate buffer and embedded in optimal cutting temperature embedding compound (O.C.T.). Tissue was then cryostat cut (20 μm) and thaw-mounted onto glass slides. Slides were incubated overnight with rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1; 1:1000; Wako Chemicals, Germany) or rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000; DakoCytomation, Denmark), followed by goat anti-rabbit IgG conjugated Alexa Fluor™ 488 or goat anti-rabbit IgG conjugated Alexa Fluor™ 546 (Molecular Probes, Oregon, USA), respectively. For double immunohistochemistry rabbit anti-phosphorylated p38 Mitogen Activated Protein Kinase (p-p38 MAPK; 1:100; Cell Signalling Technology, USA) was visualised with extra avidin-FITC (1:500, Sigma) following signal amplification with ABC (Vector Laboratories, CA, USA) and biotinyl tyramide (NEN life science products, UK). Sections were then incubated with primary antibody for a Iba-1 followed by secondary antibody solution. Slides were cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA) and visualised under a Zeiss ImagerZ1 (Zeiss, UK) fluorescence microscope. Iba-1, p-p38 and GFAP-positive cells were determined by counting the number of profiles (cell bodies). A box measuring 10^4 μm^2 was placed onto areas of the lateral, central and medial dorsal horn (laminae 1–3). These measurement protocols were carried out on three L5 spinal sections from each animal. Furthermore, microglia activation was assessed using a previously described qualitative scale: – baseline staining, + mild response, ++ moderate response (Colburn et al., 1997). For this, two sections per animal where $n = 3$ (naïve) and $n = 5$ (diabetic groups) were analysed. The experimenter was blind during image capture and cell counting.

2.5. Data analysis

Two-way repeated measurement ANOVA followed by Tukey's test was used for behavioural studies. One-way ANOVA followed by Tukey's test was used for immunohistochemistry.

3. Results

STZ-induced allodynia can be reversed by the gold standard analgesic Gabapentin (Luo et al., 2002). Accordingly, in this study we first show that in four week diabetic rats the hind paw thresholds to mechanical stimulation were significantly reduced as compared to thresholds measured before the injection of STZ (from 37.5 ± 1.9 to 23.8 ± 1.2 g) (allodynia) (Fig. 1). Then we confirm that single oral administration of Gabapentin significantly reversed diabetes-induced allodynia as compared to saline administration (33.5 ± 2.5 and 24.8 ± 2.2 g, respectively) (Fig. 1). The anti-allodynic effect of Gabapentin remained significant for up to five days of daily treatment (Fig. 1).

In order to evaluate whether diabetes-induced allodynia and Gabapentin anti-allodynic effect altered the state of glial cell activation in the spinal cord, we performed immunohistochemical studies. Microglial cells were identified by immunoreactivity for Iba-1 and quantified throughout the lumbar spinal cord of naïve rats (Fig. 2A, D). In diabetic rats the number of microglial cells significantly increased in the dorsal horns (from 3.1 ± 0.5 to 5.4 ± 0.6 cells per 10^4 μm^2) (Fig. 2B, D). Furthermore, a change in morphology, as described in other studies (Tsuda et al., 2005, 2008), from "quiescent" to "activated" was observed. Microglial cells in naïve spinal cords showed a small soma with very thin and long processes whereas in diabetic spinal cords treated with Gabapentin/saline the size of the soma is increased and processes are thick and short. Importantly, in Gabapentin-treated diabetic rats the number of microglial cells in the dorsal horn returned to control values (3.2 ± 0.5 cells per 10^4 μm^2) (Fig. 2C, D). In addition, a

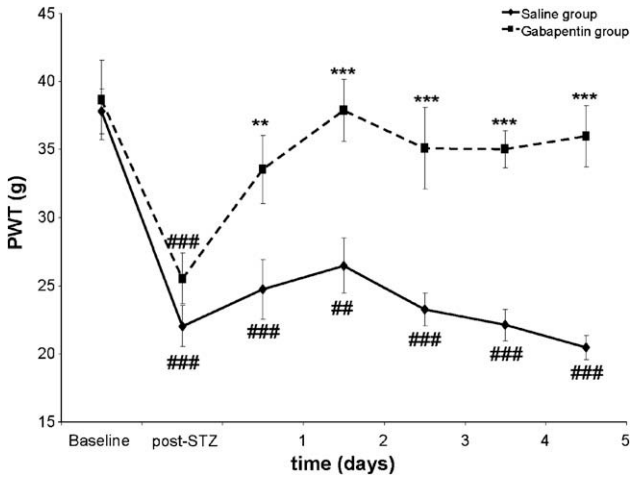

Fig. 1. Gabapentin reverses mechanical allodynia in STZ-diabetic rats. Rat hind paw thresholds to mechanical stimulation were assessed before STZ injection (baseline) and four weeks after STZ injection (post STZ), when allodynia was established. Vehicle (0.9% saline) or Gabapentin (50 mg/kg) were orally administered to diabetic rats and paw thresholds measured 3 h after the morning dose. Data shown as mean ± SEM, where $n = 7$ rats per group and data was analysed with a two-way repeated measures ANOVA (Tukey's post-hoc). ##/** $P < 0.01$, ###/*** $P < 0.001$; # = comparison within group to baseline, * = comparison between groups.

qualitative scale (Colburn et al., 1997) used to confirm the microglial activation state, shows resting morphology (−) in naïve sections, whereas sections from diabetic animals treated with saline show a mild to moderate activation (+/++). This response is decreased to only mild (+) responses in diabetic sections from Gabapentin treated diabetic animals.

We also examined levels of immunoreactivity for the phosphorylated form of p38 MAPK, a marker of rapid microglial cell activation. Low levels of immunoreactivity for p-p38 were observed. In diabetic rats the percentage of p-p38 positive cells within the population of Iba-1 positive cells was not altered compared to naïve (6.9 ± 1.6% and 6.8 ± 3.3% respectively) (Fig. 2D). Furthermore, Gabapentin treatment had no effect on p38 phosphorylation (5.4 ± 1.4) (Fig. 2D).

In contrast, a decrease in GFAP positive astrocyte cell numbers was observed in diabetic animals compared to naïve animals (from 11 ± 0.8 to 6 ± 0.4 cell per 10^4 μm^2) (Fig. 3A, B, D). Furthermore, Gabapentin did not affect the number of astrocytes in diabetic animals (6 ± 0.4 cells per 10^4 μm^2) compared to saline treatment (6.7 ± 0.7 cells per 10^4 μm^2) (Fig. 3B–D).

4. Discussion

The present study demonstrates that in STZ-induced diabetic neuropathy mechanical allodynia was associated with increased spinal microglial activation. Gabapentin treatment attenuated established mechanical allodynia and this reversal of mechanical hypersensitivity was associated with reduced spinal microglial cell numbers in the dorsal horn of the spinal cord of diabetic rats.

Firstly, we observed that STZ-induced mechanical allodynia was effectively reversed following treatment with Gabapentin. These data support previous work demonstrating that following Gabapentin treatment, established mechanical allodynia in diabetic animals was reversed, and suggest that the STZ model offers good predictability for the disease in humans (Luo et al., 2002).

Activation of spinal microglia has been observed in a number of neuropathic pain models following peripheral nerve injury (Scholz and Woolf, 2007; Watkins and Maier, 2003). Here we show that diabetic rats exhibit enhanced microglial cell numbers in the dorsal horn of the spinal cord. We also observed morphological changes of Iba-1 positive microglia, suggesting a more active cell phenotype. Indeed, our results support recent work demonstrating spinal microglial activation, as observed by upregulation of Iba-1 and OX42, as well as morphological changes, in four week STZ-induced diabetic rats (Tsuda et al., 2008). In addition, increased phosphorylation of the MAPKs ERK and c-Jun N-terminal kinase (JNK) is observed in spinal microglia following induction of diabetes (Daulhac et al., 2006). However the role of p38 MAPK in diabetic neuropathy remains unclear. In this study we observed no change in the levels of p38 MAPK phosphorylation in the spinal cords of diabetic animals compared to naive animals. These data are supported

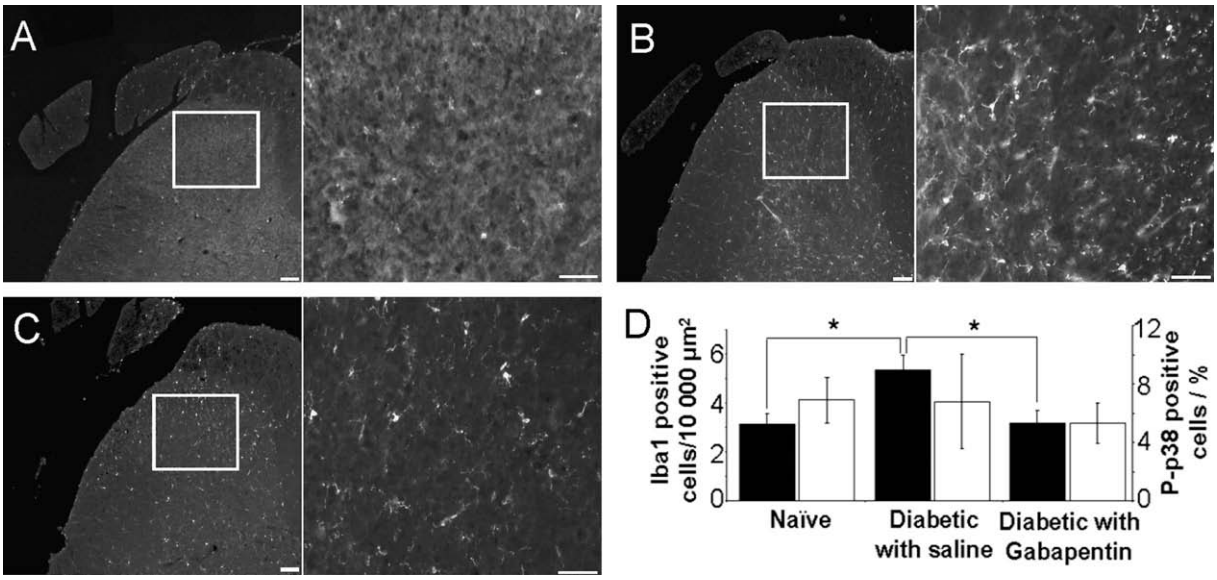

Fig. 2. Immunoreactivity (IR) for Iba1 in spinal cord sections. (A–C) Iba1 IR of naive (A) and diabetic animals treated with saline (B) or Gabapentin (C). Boxes indicate higher magnifications of the dorsal horns. Scale bars = 50 μm. (D) Cell counting for Iba1 positive cells in the dorsal horn (black columns) and percentage of p-p38 positive cells within the Iba-1 positive population (white columns). Data shown as mean ± SEM, where $n = 3$ (naive) and $n = 4$ (diabetic groups). Data were analysed with a one way ANOVA (Tukey's post-hoc). * $P < 0.05$.

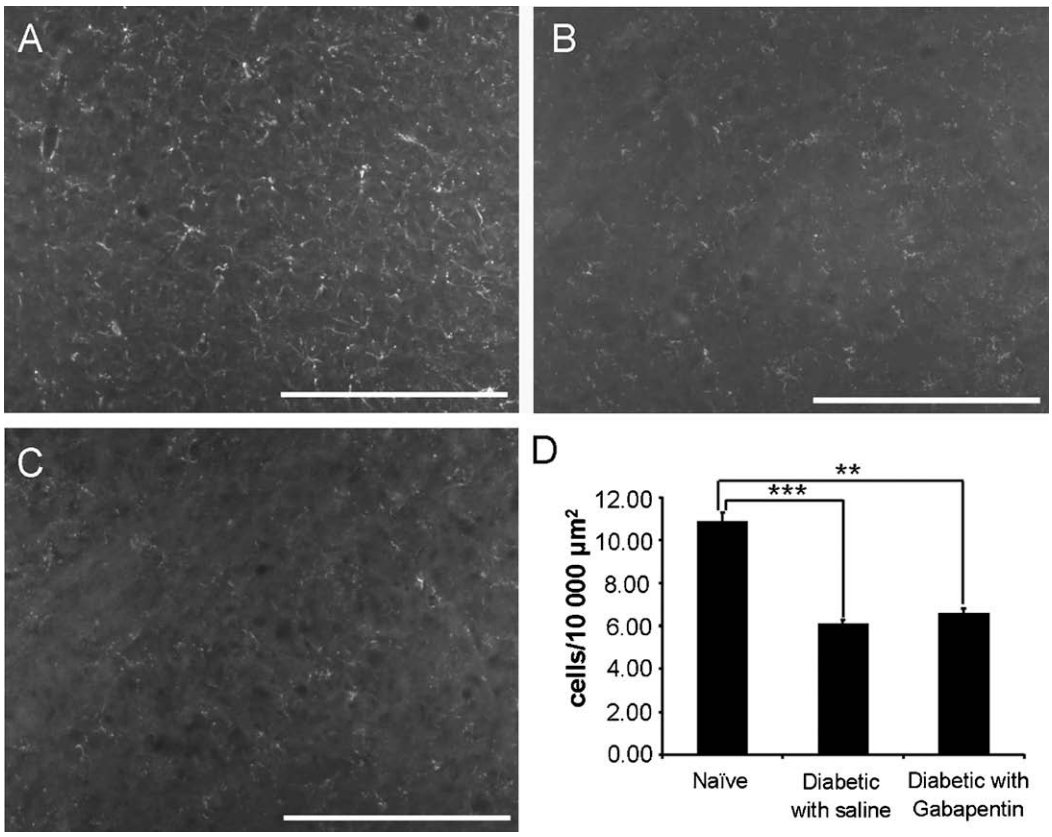

Fig. 3. Immunoreactivity (IR) for GFAP in spinal cord sections. (A–C) GFAP IR of naive (A) and diabetic animals treated with saline (B) or Gabapentin (C). Scale bars = 100 µm. (D) Cell counting for GFAP positive cells in the dorsal horn. Data shown as mean ± SEM where $n = 3$ (naive) and $n = 5$ (diabetic groups). Data were analysed with a one way ANOVA (Tukey's post-hoc). $^{**}P < 0.01$, $^{***}P < 0.001$.

by Tsuda and colleagues, who recently reported increases in ERK phosphorylation four weeks following STZ administration, but no change in the level of p38 MAPK phosphorylation (Tsuda et al., 2008). In contrast, a second study reported enhanced levels of p38 phosphorylation three weeks following STZ administration (Daulhac et al., 2006). This discrepancy may be explained by the early time point (three weeks) at which Daulhac et al. conducted their investigations. P38 phoshorylation is a rapid marker of microglial activation which declines over time, and consequently at three weeks following STZ, in the relatively early stages of the model significant p38 phosphorylation is present (Daulhac et al., 2006). However, with progression of the disease enhanced p38 phosphorylation may be lost and therefore be absent by 4–5 weeks post-STZ as observed here as well as by others (Tsuda et al., 2008).

Daulhac et al. also observed anti-hyperalgesic effects following p38 inhibition in the STZ model of diabetes (Daulhac et al., 2006), however treatment was administered beginning on day 14 following STZ during the very early stages of the disease. A second study utilised a selective inhibitor of the p38α isoform. In four week diabetic rats systemic administration of a p38α inhibitor attenuated mechanical and thermal pain behaviours (Sweitzer et al., 2004), however this treatment also significantly reduced blood glucose levels in diabetic animals suggesting that the analgesic effects of inhibition of p38α may be partially due to modification of the disease. In the spinal cord p38α is expressed by neurons, whereas the microglial expression is exclusively of the p38β isoform (Svensson et al., 2005), such that p38α inhibition alone will likely be ineffective against any enhanced phosphorylation of microglial p38β.

Interestingly, we observed a decrease in the numbers of GFAP-positive astrocytes in diabetic animals compared to naïve controls. The extent of this decrease was similar in both Gabapentin and saline treated groups, suggesting that Gabapentin treatment has little effect, if any, on spinal astrocytes. Similarly, a decrease in GFAP staining has been previously reported in the hypothalamus of rats four and eight weeks after STZ-injection (Lechuga-Sancho et al., 2006;Coleman et al., 2004). Coleman et al. suggest that this may be due to a direct effect of insulin on the morphology of astrocytes, as has been observed *in vitro* (Aizenman and de, 1987). However, a recent study has shown no difference in the cell number of GFAP-positive cells in the lumbar dorsal horn of the spinal cord between STZ-diabetic and control rats four weeks after STZ-injection (Tsuda et al., 2008).

Astrocytes have been shown to become activated after microglia following peripheral nerve injury and are thought to contribute to the maintenance of chronic pain (Scholz and Woolf, 2007; Watkins and Maier, 2003; Kawasaki et al., 2008). It may be that astrocyte activation can be observed in STZ rat spinal cord at a different time interval than the one we have examined.

Interestingly, we have observed a reduction in the numbers of Iba-1 positive microglia in the dorsal horn of diabetic rats treated with Gabapentin, compared to saline. The reduction of spinal microglia cell marker by Gabapentin may be due to a direct action on microglia, or an indirect effect following neuronal inhibition.

Recent evidence indicates that Gabapentin might act on the $\alpha_2\delta_1$ calcium channel subunit expressed by neurons (Maneuf et al., 2006). Importantly, expression of $\alpha_2\delta_1$ by microglial cells has yet to be demonstrated. Up-regulation of $\alpha_2\delta_1$ in DRGs and/or spinal cord has been reported in traumatic models of neuropathic pain (spinal nerve ligation, spinal nerve transection and chronic constriction injury) as well as in diabetes (Luo et al., 2002). This up-regulation correlates with both the development

and maintenance of mechanical allodynia. In addition, the behavioural hypersensitivity that occurs in all of these models is sensitive to Gabapentin with a clear reduction of allodynia following treatment (Luo et al., 2002). However, in another clinically relevant model of neuropathy, resulting from administration of the chemotherapy agent vincristine, no up-regulation of the $\alpha_2\delta_1$ subunit in DRGs and spinal cord dorsal horns could be observed and Gabapentin attenuated pain behaviour only after repeated treatment (Xiao et al., 2007). It has been suggested that the anti-allodynic effect of Gabapentin in models displaying variations in $\alpha_2\delta_1$ regulation may be due to additional mechanism(s) of action. It is possible that a combination of factors, both dependent and independent of $\alpha_2\delta_1$, may result in reduced microglial activation following Gabapentin.

In summary, these data show microglial activation in the dorsal horn of the spinal cord following STZ-induced diabetic neuropathy. A role for microglia in STZ-induced mechanical allodynia is suggested by the novel observation that the anti-allodynic effect of Gabapentin is associated with a reduction of spinal microglial activation. In contrast, the importance of astrocyte activation in this model appears to be limited. Consequently, microglia are an important player in spinal neuropathic pain mechanisms during diabetic neuropathy.

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