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ORIGINAL ARTICLE

Activation of K_v7 channels with the anticonvulsant retigabine alleviates neuropathic pain behaviour in the streptozotocin rat model of diabetic neuropathy

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ABSTRACT

Diabetic peripheral neuropathy (DPN) is the most incapacitating complication of diabetes mellitus. Up to 50% of patients with DPN develop peripheral neuropathic pain (PNP). The underlying ionic and molecular mechanisms of diabetic PNP (DPNP) are poorly understood. However, voltage gated potassium (K_v7) channels which have been implicated in the pathogenesis of other types of PNP are likely to be involved. Here we examined, in the streptozotocin (STZ) rat model of DPNP, whether activating the Kv7 channels with a potent activator retigabine (ezogabine) would reverse/attenuate behavioural signs of DPNP. STZ rats exhibited behavioural indices of mechanical and heat hypersensitivity, but not cold hypersensitivity or spontaneous pain, 35 days after STZ injection. Retigabine given at a dose of 15 mg/kg (but not at 7.5 mg/kg, i.p.) significantly attenuated mechanical, but not heat hypersensitivity in DPNP rats, and was as effective as the positive control gabapentin. This analgesic effect of retigabine was completely reversed by the K_v7/M channel blocker XE991 (3 mg/kg, i.p.) indicating that the anti-allodynic effects of retigabine were mediated by K_v7 channels. In conclusion, the findings suggest that Kv7 channels are involved in DPNP pathogenesis, and that strategies that target their activation may prove to be effective in treating DPNP.

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Diabetic neuropathy; chronic pain; pain hypersensitivity; behaviour; neuropathic pain

Significance

This study demonstrates that streptozotocin (STZ) rats exhibit, 5 weeks after STZ injection, behavioural signs of mechanical and heat hypersensitivity, but not cold hypersensitivity or spontaneous pain. The study also shows that activation of K_v7 channels with retigabine dose dependently attenuated STZ-induced mechanical, but not heat hypersensitivity. The findings that these anti-allodynic effects of retigabine were reversed by the Kv7/M channel blocker XE991 indicate that they were mediated by Kv7 channels. The findings suggest that strategies that target activation of Kv7 channels may be effective in treating diabetic peripheral neuropathic pain.

Introduction

Peripheral neuropathic pain (PNP), pain arising as a direct consequence of a lesion or disease affecting the peripheral nervous system, is associated with many types of injury/disease, including diabetes mellitus. Diabetic peripheral neuropathy (DPN) is the most persistent and incapacitating complication of both type 1 and type 2 diabetes mellitus [1], and is the most common cause of PNP, with 34–50% of people with DPN having some degree of PNP [1–3]. Patients suffering from DPN can experience a large spectrum of sensory symptoms that are often concurrent with loss of sensation in the extremities which frequently leads to foot ulcers that may progress to limb amputations [4,5]. PNP induced by diabetes (DPNP) is characterised by unpleasant positive symptoms including spontaneous pain, mechanical hypersensitivity/allodynia (painful sensation caused by light touch), paresthesias (tingling), as well as negative symptoms notably heat hypoalgesia [6].

Despite its high prevalence and clinical importance, successful therapy for DPNP remains a challenge because the use of currently available drugs is limited by marginal efficacy and significant adverse side effects. Several mechanisms have been proposed for the pathogenesis of PNP including increased excitability of nociceptive dorsal root ganglion (DRG) and CNS neurons [7]. There is accumulating experimental evidence to support involvement of some of these mechanisms in DPNP pathogenesis [1]. Indeed preclinical studies suggest that, like other types of PNP, DPNP is due at least partly to abnormal hyperexcitability of DRG neurons [8,9]. This is based on the findings of studies using rodent models of DPNP showing that both C- and A-fibre DRG neurons exhibit aberrant spontaneous activity, the key characteristic of neuronal hyperexcitability [10–13].

The underlying ionic and molecular mechanisms of the aberrant hyperexcitability of DRG neurons associated with DPNP are not fully understood, but changes in expression and/or activation properties of voltage-gated K_v7 potassium channels, which produce an outward current (known as I_M) that normally exerts a powerful stabilising influence on neuronal excitability [14,15], are likely to be involved. This is because acute inhibition of K_v7 channels with a specific blocker XE991 increases excitability of DRG neurons [16,17], enhances Aδ-fibre discharges induced by mechanical or thermal stimuli [18] and causes nocifensive behaviour in

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rats [19,20]. Conversely, activators of K_v7 channels, such as retigabine and flupiritine, have been shown to block spontaneous activity in axotomised sensory fibres [21], attenuate C- and Aδ-fibre discharges induced by heat stimuli [18], reduce excitability of C-fibres in human sural nerves [22] and have an analgesic action in animal models of pain including surgically induced PNP [23]. In the present study, we examined whether activating Kv7 channels with retigabine would also attenuate pain hypersensitivity in the streptozotocin rat model of DPNP. Our results show that retigabine dose dependently attenuated mechanical, but not heat hypersensitivity in DPNP rats.

Materials and methods

Experimental animals

In the present study, 64 male Sprague Dawley rats (250–300 g) were used; they were housed four rats per cage in a room in the animal house of Liverpool University, UK. The room was maintained at room temperature between 21 and 24 °C under standard laboratory conditions with 12-h light/dark cycles and free access to food and water. The experimental protocols were approved by the University of Liverpool Ethical review committee, and complied throughout with the UK Animals (Scientific Procedures) Act 1986.

The animal model of diabetic peripheral neuropathic pain (DPNP)

A number of well-characterised animal models of DPNP have been developed to investigate its pathophysiology including the widely used STZ (streptozotocin) rat model. STZ (an alkylating chemotherapeutic agent that is used in the treatment of pancreatic β cell carcinoma) is the most prominent diabetogenic chemical agent in diabetes research. It causes hypoinsulinemia and hyperglycaemia as a result of its cytotoxicity to pancreatic β cells. The STZ model (model of type 1 diabetes) is more commonly used than other models of DPNP because of its low cost, rapid induction, greater stability and relative lack of toxicity to other organs [1,24,25]. Therefore we used this model which involves a single injection of STZ (60 mg/kg, i.p.) after an overnight fast to reduce competition between glucose and STZ for uptake into pancreatic β-cells. Experimental rats were observed daily.

Pain behavioural testing

Pain behavioural testing was conducted on 64 rats by an experimenter who was blind to the various treatment groups of the animals. As we have reported previously [26–28], testing was performed in plastic chambers after the rats were acclimatised for 3 consecutive days to the procedure room, the testing chambers and the devices used (see below). On the testing days, the rats were also habituated to the chambers by placing each rat in the plastic chamber for at least 15 min before testing. After the rats had habituated to the chambers (after the exploratory and grooming behaviour had ceased), paw withdrawal threshold (PWT), paw withdrawal latency (PWL), cold escape/nocifensive behaviour, and spontaneous foot lifting (SFL), if any, were assessed on the left hind paw of each control/vehicle and experimental rat (see below).

Behavioural testing for mechanical hypersensitivity (allodynia)

Behavioural testing for mechanical hypersensitivity/allodynia was performed using an automated von Frey type system known as a dynamic plantar aesthesiometer touch stimulator (Ugo Basile, Italy) as described previously [28]. Briefly, rats were placed in plexiglass boxes ($15 \times 15 \times 20$ cm) on a wire mesh, and the mid-plantar surface of the left hind paw of each rat was stimulated with a blunt metal probe (0.5 mm in diameter) three times with a minimum of 5-min interval between each trial. An abrupt withdrawal lifting or flicking of the hind paw in response to the pressure (mechanical force) applied with the metal probe was defined as a positive response. The average force of the three trials (in grams) that resulted in paw withdrawal was calculated and considered as PWT. To determine the baseline values, the test was conducted one day before STZ injection on experimental rats. The test was repeated 35 d post STZ to determine whether the rats developed mechanical hypersensitivity. As reported previously [27–29] mechanical hypersensitivity was indicated by a significant decrease in the mean PWT. To determine effects of drugs on mechanical hypersensitivity, the test was also conducted at different time points after treatment with various drugs or their vehicles.

Behavioural testing for heat hypersensitivity (heat hyperalgesia)

Behavioural testing for heat hypersensitivity/hyperalgesia was performed using Hargreaves analgesiometer (Ugo Basile, Comerio, Italy) as described previously [27–29]. Heat hypersensitivity was indicated by a significant decrease in the mean paw withdrawal latency (PWL) to a noxious heat stimulus applied to the mid-plantar surface of the hind paw (L4 dermatome) of each rat. The procedure of this test is similar to that used for mechanical hypersensitivity (see above) except that, in this test, the rats were placed on a 2-mm-thick glass floor under which the laser radiant heat source was positioned. The onset of the heat stimulus, activated a timer that stopped automatically when paw withdrawal was detected by a photocell of the analgesiometer. The average response latency (in seconds) of three trials was calculated and considered as PWL.

Behavioural testing for cold hypersensitivity

Behavioural testing for cold hypersensitivity was performed using the hot/cold plate analgesiometer (Ugo Basile, Milan, Italy) which can be cooled to −3 °C (at an ambient temperature between 20 and 25 °C). Before conducting the cold sensitivity test, rats were allowed to acclimate to the testing apparatus for ∼1 h, then the test was conducted by placing each rat on a metal surface of the cold plate maintained at 5 °C within a transparent plexiglass chamber ($16 \times 16 \times 27$ cm). As described previously by various investigators [28,30], the duration (in seconds) of the escape/nocifensive behaviour (licking, lifting, guarding, shaking or biting of the hind paw or jumping) per a 2 min period was measured, using a stop watch, in control (vehicle treated) and STZ-treated rats. To determine the baseline values of cold sensitivity, rats were tested one day before STZ injection. The average of two separate trials with at least 2 h separating each trial was used. The cold sensitivity test did not result in any signs of tissue injury.

Behavioural testing for spontaneous pain

Spontaneous foot lifting (SFL) duration has been used by numerous previous studies as a measure of spontaneous/ongoing pain in various animal models of pain (see [26,31] and references

therein). Therefore, in this study, we examined whether STZ treated rats exhibit this spontaneous pain behaviour. This test was performed by placing each rat in one of the plexiglass boxes ($n = 3$) used for the Hargreaves test and observing SFL (spontaneous lifting of the left hind foot off the glass floor) as described previously [26,31]. Any foot lifting associated with locomotion, body repositioning, or grooming was excluded [26]. This test was conducted only 35 d post-STZ because normal (untreated) rats do not exhibit SFL.

Animal groups and drug administration

A total of 64 rats were used in the present study. Of these, 52 rats received a single injection of a STZ (60 mg/kg, i.p.). The remaining 12 rats were naïve rats (not injected with STZ) and were used for examining the effects of the Kv7 agonist retigabine ($n = 6$ rats) and antagonist EX991 ($n = 6$ rats) on baseline pain sensitivity. The STZ rats ($n = 52$) were divided randomly into five groups: (1) group 1 (retigabine group A, $n = 10$ rats); each rat in this group received a single dose of 15 mg/kg retigabine; (2) group 2 (retigabine group B, $n = 10$ rats); each rat in this group received a single dose of 7.5 mg/kg retigabine; (3) group 3 (vehicle control group for groups 1 and 2, $n = 10$ rats); (4) group 4 (EX991 + retigabine group, $n = 10$ rats) received a single dose of the Kv7 antagonist EX991 (3 mg/kg) plus a single dose of 15 mg/kg retigabine and (5) group 5 (gabapentin, positive control group, $n = 12$). All the drugs and their vehicles were administered intraperitoneally. None of the STZ-treated rats showed any gross neurological abnormalities or any sign of self-mutilating behaviour or autotomy.

Retigabine (*N*-(-2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester), the k_v7 channels opener kindly donated by Dr Blackburn-Munro (NeuroSearch, Ballerup, Denmark), XE-991 (10,10-bis(4-pyridinylmethyl)-9(10H)-antracenone), the k_v7 channel blocker and STZ (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulphoxide (DMSO)and stored in aliquots at −20 °C. All compounds were diluted in sterile physiological saline immediately prior to their use to prevent degradation of the compound. Rats ($n = 52$) were randomised to five groups (see Results). The five group of rats were injected with these drugs or vehicle (at volume of 1 ml/kg) 35 d after STZ injection i.e. after the behavioural signs DPNP were fully established. The behavioural tests were performed before (pre-drug) drug treatment (35 d after STZ treatment) and 1–2 h, 2–3 h and 24 h after drug or vehicle (DMSO in physiological saline) treatment.

Statistics analysis

The data were found to be normality distributed (Shapiro–Wilk normality test) and are, therefore, presented as the mean ± SEM (and statistical analysis was made with *t*-test). One-way repeated-measures analysis of variance (ANOVA) with *post hoc* tests was used for comparisons between pre-drug and post-drug values at different time points. All of the statistical tests were performed using Graphpad Prism 5 (Graphpad Software, San Diego, CA). *p* Values of less than .05 were considered significant (statistical significance: $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$).

Results

Validation of the diabetogenic action of STZ in rats

As shown in Figure 1, prior to injection of STZ, there was no significant difference in the blood glucose concentrations between the rats designated as the control group ($n = 12$) and those designated as the experimental (STZ) group ($n = 18$). However, as shown in Figure 1(A), STZ rats exhibited significantly ($p < .001$, unpaired *t*-test) increased blood glucose concentrations 3 d post-STZ compared with the control group. STZ-treated rats remained hyperglycaemic thereafter. Indeed, as shown in Figure 1(A), the blood glucose of the STZ group remained significantly higher ($p < .001$, unpaired *t*-test) than that of controls 35 d post-STZ. Rats ($n = 5$) that did not exhibit hyperglycaemia after STZ injection were excluded from analysis.

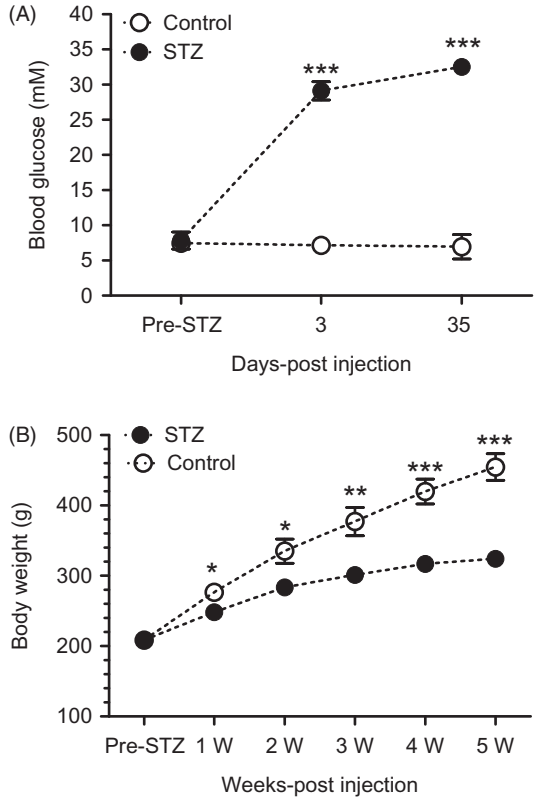

Figure 1. Hyperglycaemia and impairment of body weight gain in STZ rats. A single injection of STZ (60 mg/kg, i.p.) into rats caused a significant elevation in blood glucose as early as 3 days post-injection ($p < .001$) compared to pre-STZ baseline (control values) (A). STZ-treated rats remained hyperglycaemic thereafter as indicated by an even greater increase in blood glucose concentration as 35 d post-injection (A). This hyperglycaemia was accompanied by a gradual impairment in body weight gain from 1 to 5 weeks post-STZ (B). The mean body weight of STZ rats was compared with that of control rats at each time point using unpaired *t*-test. The level of significance is indicated with asterisks as follows: $^{***}p < .001$; $^{**}p < .01$ and $^{*}p < .05$.

We also examined whether there is a difference in body weight between hyperglycaemic (STZ) and control rats. We found that the STZ group had significantly lower body weights than controls throughout the study duration (Figure 1(B)). Indeed STZ rats weighed significantly less than control rats at each of the time points tested (1–5 weeks) post-STZ (Figure 1(B)). The difference in the mean body weight between STZ and control rats was more pronounced and more significant ($p < .0001$) 5 weeks post-STZ. Indeed, at this time point (5 weeks post-STZ), the mean weight of STZ rats (208.5 ± 5.4) was 1.6 times less than that (324.0 ± 9.5) of untreated (control) rats.

STZ rats exhibit behavioural indices of mechanical and heat, but not cold, hypersensitivity or spontaneous pain

Having established that the STZ rats displayed chronic hyperglycaemia, we sought to determine whether they exhibit behavioural

signs of PNP. Pain behaviours were assessed at 3 time points (21, 31 and 35 d after STZ treatment), and the values of pain behaviours at these time points were compared with the baseline (pre-STZ) values (Figure 2). These time points were based on pilot time-course experiments which showed mechanical hypersensitivity to be more significant at 35 d than at 31 d post-STZ, but not significant at 21 d. As shown in Figure 2(A), there was a significant decrease in the mean PWT at 31 ($p < .05$) and 35 ($p < .001$) days post-STZ (but not at 21 d) indicating that STZ-treated rats developed behavioural signs of mechanical hypersensitivity at later points than 3 weeks of STZ treatment. However, STZ rats developed heat hypersensitivity/hyperalgesia at all the time points tested (21, 31 and 35 d) as indicated by a highly significant decrease ($p < .001$) in the mean PWL at those time points (Figure 2(B)). In contrast, there was a slight but insignificant increase in the cold evoked responses at 35 d post-STZ (Figure 2(C)) indicating that STZ rats failed to develop cold hypersensitivity.

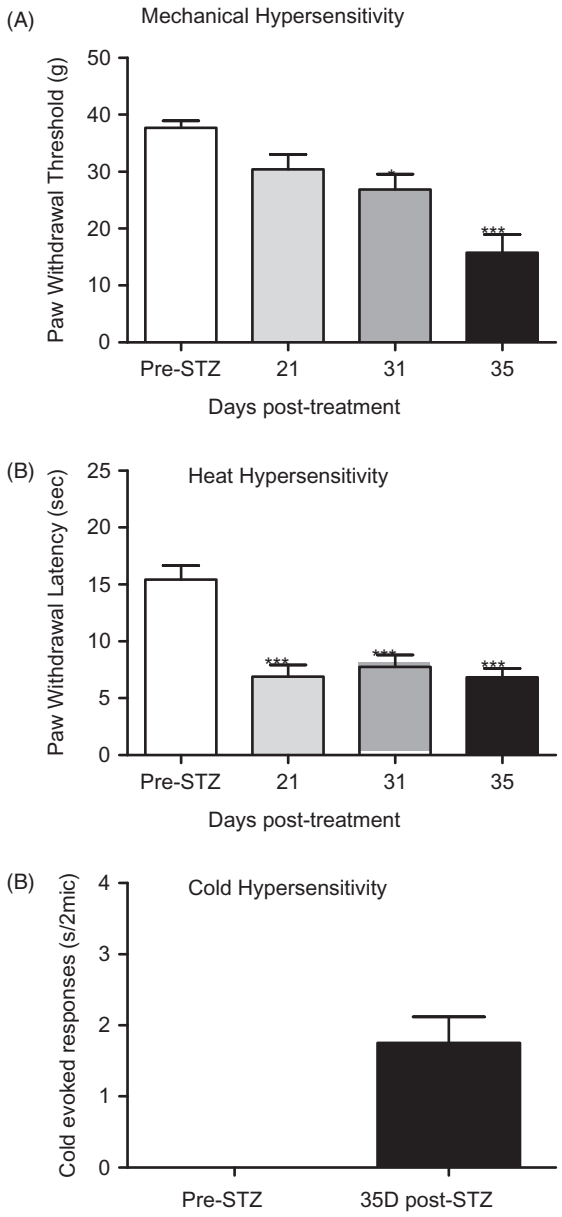

Figure 2. Behavioural indices of mechanical and heat hypersensitivity in STZ rats. The data presented in this figure and Figures 2–5 are presented as mean ± SEM. STZ treatment significantly decreased the mean PWT (A) and the mean PWL (B) at different time points after STZ treatment. The decrease in PWL was highly significant ($p < .001$) at all three time points tested (21, 31 and 35 d post-treatment) indicating development of heat hypersensitivity at these time points (B), whereas the decrease in the PWT was significant at 31 ($p < .05$) and 35 ($p < .001$) days, but not at 21 d post-STZ (A) indicting a delay in the development of mechanical hypersensitivity. There was no significant change in the mean duration of cold evoked responses (C) indicating that STZ rats do not exhibit cold hypersensitivity. Note that for each behavioural type, comparisons were between values before STZ treatment (pre-STZ, open bars) and 21 (light grey bars), 31 (dark grey bars) and 35 (black bars) days post-STZ. Statistical tests were made with one-way ANOVA with Tukey's multiple comparison test. For the level of significance, see legend to Figure 1.

As noted in the Methods, numerous previous studies have used spontaneous foot lifting (SFL) as a measure of spontaneous/ongoing pain in various animal models of pain (see Djouhri et al., 2006, 2012 and references therein). Therefore, we sought to determine whether STZ-treated rats exhibit SFL. We found that unlike other models of PNP such as spinal nerve injury model [26,31] and chemotherapy-induced PNP [28] which show significant SFL, none of the STZ rats showed SFL indicating the STZ model does not exhibit spontaneous pain behaviour.

Retigabine attenuates STZ-induced behavioural signs of mechanical but not heat hypersensitivity

In the present study (which was conducted few years ago), we examined in STZ rats, whether pharmacological activation of Kv7 channels with retigabine could reverse or attenuate mechanical and heat hypersensitivity. As shown in Figure 3, a single injection of retigabine at a dose of 15 mg/kg, but not at 7.5 mg/kg, given 35 d after STZ treatment, resulted in a significant increase in the mean PWT at all time points tested (0–1 h, 1–2 h and 2–3 h post-drug) indicating that retigabine injection caused attenuation of the behavioural manifestations of mechanical hypersensitivity induced by STZ treatment. However, the increase in PWL caused by retigabine was statistically not significant at all of the time points tested (Figure 3(B)) indicating that retiagbine does not reverse/reduced heat hypersensitivity in STZ rats.

Blockade of Kv7 channels with XE991 reverses the analgesic effects of retigabine

To confirm that the analgesic effects of retigabine are mediated by activation of Kv7 channels, we used the Kv7 channel blocker XE991 to examine whether the effects of retigabine on STZ-induced mechanical and heat hypersensitivity could be reversed. As shown in Figure 4, the effects of retigabine (15 mg/kg) on PWT (Figure 4(A)) was antagonised by XE991 (3 mg/kg), compared with the vehicle, indicating that the effects of retigabine on mechanical hypersensitivity in the STZ rats can be reversed by this blocker. Interestingly, the antiallodynic effects of retigabine were similar to those of the positive control gabapentin (Figure 4(A)). As a control, we also tested both retigabine and XE991 for effect on PWT and PWL in naïve (normal) rats. The result showed that neither retigabine nor XE991 had any effect on the baseline PWT or PWL, compared with the vehicle group (Figure 5(A,B)) indicating that the effects of these drugs were mediated by Kv7 channels.

Discussion

The present study was aimed at investigating whether retigabine, a potent non-selective activator of neuronal Kv7 channels that has been approved for its use as an antiepileptic in humans, attenuates pain hypersensitivity in the STZ rat model of DPNP. The findings show that 35 d after STZ injection: (a) STZ-diabetic rats

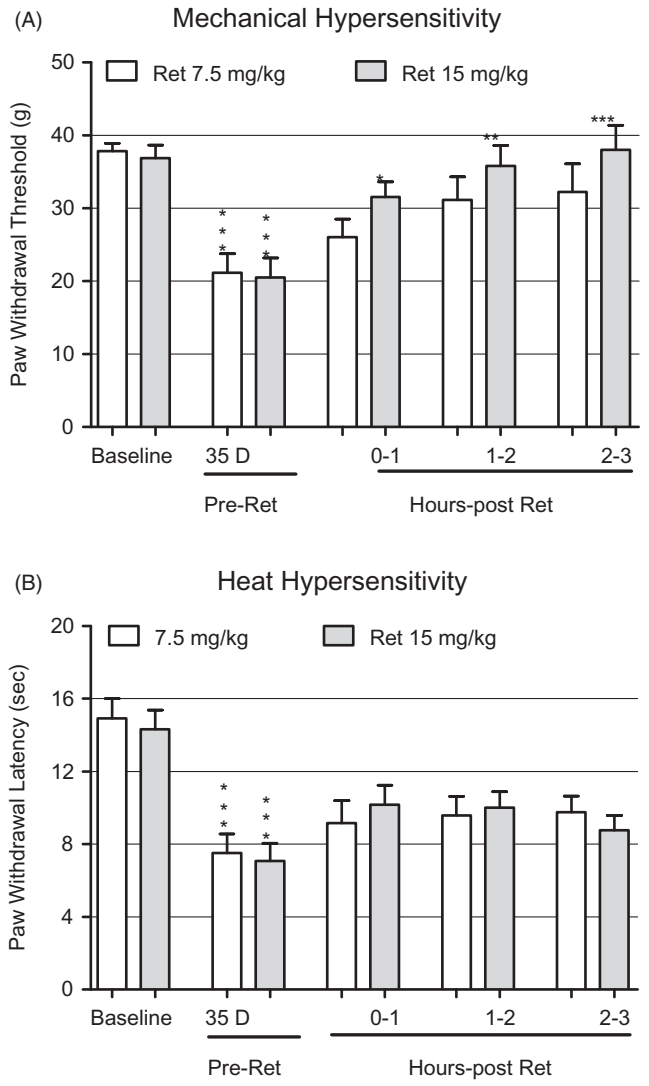

Figure 3. Effects of activating Kv7 channels with retigabine on mechanical and heat hypersensitivity in STZ rats. A single injection of retigabine at a dose of 15 mg/kg (grey bars), but not 7.5 mg/kg (open bars), significantly reversed the STZ-induced decrease in the mean PWT at all three time points tested (A) indicating that retigabine attenuated mechanical hypersensitivity induced by STZ. However, retigabine given at both doses had no significant effect on heat hypersensitivity induce by STZ (B). Comparisons were between the values at different time points after retigabine (post-Ret) and those at 35 d after STZ but just before retigabine injection (pre-Ret). Statistical tests were made with one-way ANOVA and Tukey's multiple comparison test. For the level of significance, see legend to Figure 1.

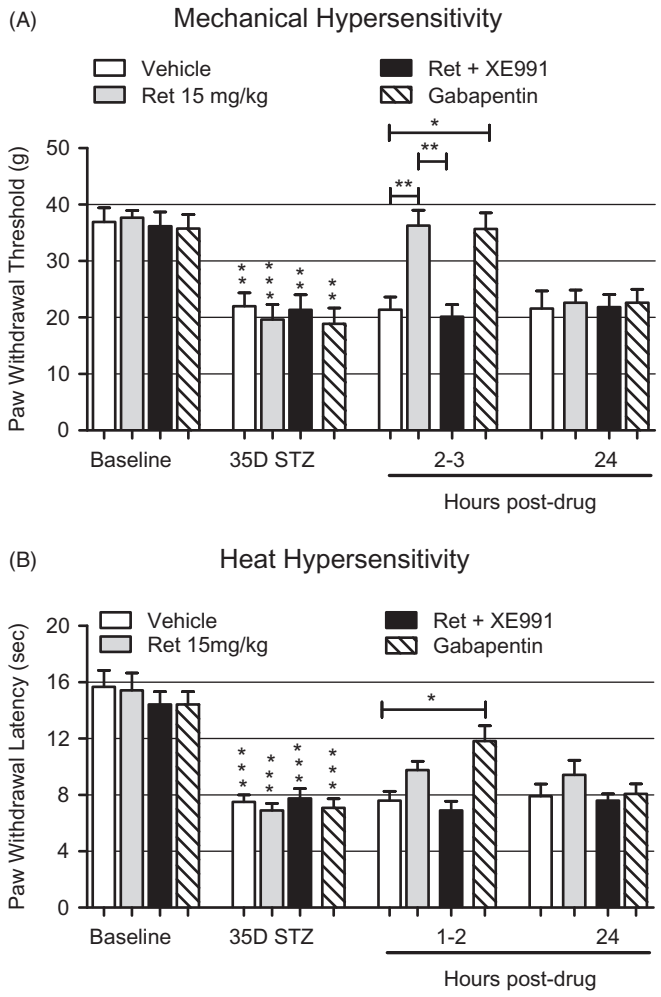

Figure 4. Effects of retigabine, XE991 and gabapentin on mechanical and heat hypersensitivity induced by STZ. STZ treatment significantly decreased mean paw withdrawal threshold (PWT) to a mechanical stimulus (A) and mean paw withdrawal latency (PWL) to a noxious heat stimulus (B) 35 d post-treatment in all four groups of rats (vehicle, retiagbine, retigabine plus XE991 and gabapentin) indicating that all rat groups developed mechanical (A) and heat (B) hypersensitivity. Compared with vehicle ($n = 10$ rats), intraperitoneal injections of retigabine (15 mg/kg, $n = 10$) and gabapentin (10 mg/kg, $n = 12$) significantly reduced mechanical hypersensitivity at 2–3 h post-drug treatment (B). However, gabapentin but not retigabine was also effective in alleviating heat hypersensitivity at 1–2 h post-drug. The effects of retigabine on mechanical hypersensitivity (A) was blocked by injection of retigabine with the Kv7 channel antagonist XE991 ($n = 10$) indicating that the retigabine effects were mediated by Kv7 channels. For statistical tests, see legend to Figure 3.

exhibited behavioural indices of mechanical and heat hypersensitivity, but not cold hypersensitivity or spontaneous/ongoing pain, (b) retigabine dose-dependently attenuated mechanical, but not heat hypersensitivity in STZ-diabetic rats, (c) the effects of retigabine were similar to those of the positive control gabapentin and (d) the anti-allodynic effects of retigabine were completely reversed by the K_v7/M channel blocker XE991 indicating that the effects of retigabine are mediated by Kv7 channels, and that these channels are involved in the pathogenesis of DPNP.

In the present study, we used the STZ model which has been shown by numerous preclinical studies to exhibit long-lasting behavioural signs of DPNP including mechanical and heat hypersensitivity [32–42]. Consistent with these studies, we found that STZ-diabetic rats developed significant behavioural signs of mechanical hypersensitivity (allodynia) 35 d post-STZ. This behaviour is believed to correspond to the cutaneous hypersensitivity in patients that leads to acute distress on contact with an external stimulus, such as clothing. Indeed, such mechanical hypersensitivity can be so painful in some patients preventing them from performing their daily activities (thereby impacting their employment and social life) and may lead to depression [43]. The anti-allodynic effects of retigabine observed in the present study are unlikely to be due to motor impairment because administration of retigabine (10 mg/kg) causes only a transient (15 min) impairment of motor performance in rats [44].

Although heat hypersensitivity (hyperalgesia) is infrequent in patients with PDN [7], animal studies using rodent models of diabetes including the present study and the aforementioned studies show that heat hypersensitivity develops within weeks of onset of diabetes in most models of type 1 and type 2 diabetes. This pain behaviour has been shown to persist in animals that retain

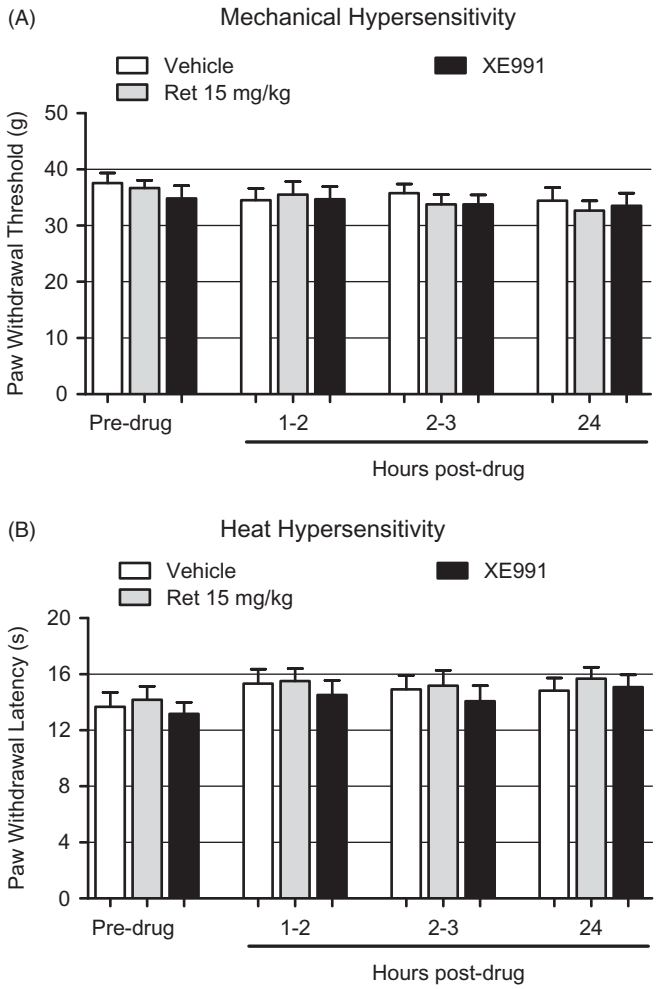

Figure 5. Effects of retigabine and XE991 on pain sensitivity in naïve rats. Compared to vehicle ($n = 10$), injections of an intraperitoneal dose of XE991 (3 mg/kg/$n = 6$) or retigabine (15 mg/kg, $n = 6$) into naive rats had no obvious effects on pain sensitivity on normal rats as indicated by no significant changes in the mean PWT (A) or PWL (B). Statistical tests were made with two-way repeated measures ANOVA, with Bonferroni post-hoc test.

residual endogenous insulin production or are supplemented by exogenous insulin [45]. However, heat hypersensitivity may progress to heat hypoalgesia/hyposensitivity in more insulinopenic animals [45]. Indeed, Fox et al. (1999) reported that STZ rats exhibited transient heat hyposensitivity lasting for about 4 weeks post-STZ. A recent study by McNaughton and his co-workers [46] has also shown that STZ-treated mice develop heat hyposensitivity 6–8 weeks post-STZ treatment. Heat hyposensitivity may be due to sensory dysfunction and/or depletion of sensory nerve terminals in the epidermis of patients as a result of diminished trophic support by insulin [47,48]. Regardless of the mechanism of heat hyposensitivity, it should be noted that the present and the aforementioned studies that used the STZ model have not reported heat hyposensitivity. The discrepancy between the studies may be due to differences in the animal strain and/or the time point at which the heat testing was conducted. However, development of heat hyposensitivity in animal models of DPNP is not surprising given that chronic hyperglycaemia is known to cause neuronal degeneration and subsequent impairment of thermal perception in human diabetic patients [49]. It is believed that the dual manifestation of mechanical hypersensitivity and heat hyposensitivity mirrors the clinical presentation of PDN in humans [46].

Our findings that STZ-diabetic rats did not show significant cold hypersensitivity are not in line with those of Courteix et al. (1993), who reported that over 70% of their STZ rats exhibited cold hypersensitivity. The reason for this apparent discrepancy is not clear, but it might be due to differences in rat strains, dose of the STZ and/or different methodologies used to assess cold hypersensitivity. Indeed, Courteix et al. (1993) used a tail immersion test to measure cold-evoked pain, as opposed to the cold plate used in the present study. Those co-workers also used a higher dose (75 mg/kg, i.p.) of STZ than the dose (60 mg/kg, i.p.) used in the present study. These factors may also account for the variability in the onset of DPNP behaviours between the different animal studies.

One of the interesting findings of the present study is that, STZ rats did not show any SFL, a behavioural index of spontaneous pain (see Djouhri et al., 2006, 2016 and references therein), in agreement with those of Biessels et al. (2014) [50] who reported that diabetic rodents do not show spontaneous pain behaviours such as limb guarding, audible or ultrasonic vocalizations, or autotomy. Taken together, these findings suggest that the STZ model differs from other models of chronic pain which we and others have previously shown to exhibit significant SFL including models of inflammatory pain [26] and PNP induced by traumatic nerve injury [26,27,31] or chemotherapy [28]. Unfortunately, however, none of the aforementioned animal studies reported whether or not STZ rats show spontaneous pain behaviour.

It is noteworthy that the validity of STZ-treated animals as a model of DPNP has been challenged by a number of investigators [34,37,39] who suggested that the apparent PNP behaviour seen in the STZ animals is independent of hyperglycaemia, and that it is due to the antineoplastic effects of STZ. This proposal is based on their findings that all STZ-treated rats, whether diabetic or not, displayed mechanical hypersensitivity. In contrast, there is substantial evidence arguing against the suggestion that STZ toxicity is directly responsible for the neuropathy phenotype including that: (a) reversing hyperglycaemia by intensive insulin therapy was found to alleviate pain hypersensitivity in STZ-diabetic rats [32] and (b) animals treated concurrently with both STZ and O-methyl-glucose, an agent that blocks the effects of STZ on the pancreas, show no evidence of neuropathy [51]. It should be pointed out that, in the present study, only diabetic STZ rats were assessed for DPNP behaviours. It is also worth mentioning that it was beyond the remit of the present study to determine whether the non-diabetic STZ rats develop pain behaviours.

Numerous previous studies have shown that activating Kv7 channels with retigabine is effective in attenuating pain behaviours in various experimental models of pain. Indeed, retigabine has been shown to display analgesic efficacy in multiple animal pain models including models of temporomandibular joint pain [52], visceral pain [53], inflammatory and nerve injury-induced PNP [16,44,54,55], spinal cord injury [56]; trigeminal neuropathy [57], bone cancer pain [58] and chemotherapy-induced PNP [59]. However, as far as we know, there has been only one study showing that retigabine attenuated both mechanical and heat hypersensitivity in STZ rats [60]. Our findings that retigabine reduced mechanical hypersensitivity in STZ-diabetic are in line with these findings. In contrast, however, our results show that retigabine given at higher doses (15 mg/kg and 7.5 mg/kg) than that (5 mg/kg) used by these investigators [60] had no effect on heat hypersensitivity in STZ-diabetic rats. The reason for this apparent discrepancy might be due to a difference in the rat strain (Sprague Dawley in the present study versus Wistar in their study). However, since separate nociceptor subsets mediate

mechanical and heat hypersensitivity, it is possible that the different effects of retigabine on mechanical and heat hypersensitivity are due to differential effects of the Kv7 channel activator on different nociceptor subpopulations (see [27] for discussion).

As noted in the Introduction, DPNP is likely to be due to increased excitability of both DRG and CNS neurons both of which express Kv7.2–7.5 voltage-gated K^+ channels that encode the M-current [16]. Although it is difficult to ascertain precisely the mode and site of retigabine action, it is possible that the anti-allodynic effects of retigabine are mediated by Kv7 channels in large myelinated A-fibre neurons. This is because (a) STZ-induced mechanical allodynia in mice was reversed by blockade of myelinated Aβ-afferent fibres [40], and (b) capsaicin-sensitive C-fibre nociceptors are not required for the development of STZ-induced mechanical allodynia in both rats [13] and mice [40]. It should be noted that large myelinated A-afferent fibres, in particular Aβ-fibre, have also been implicated in mechanical allodynia seen in other models of PNP [10,61,62]. This is based on the findings of these and other studies [6,63–65] that blockade or ablation of unmyelinated C-fibre nociceptors has limited effects on mechanical allodynia. These findings suggest that nerve injury-induced mechanical and heat hypersensitivities are mediated by separate nociceptor subsets [66,67]. Thus, it is possible that differential effects of retigabine on mechanical and heat hypersensitivity observed in the present study are due to different effects of the K_v7 channel opener on nociceptor subpopulations.

Conclusion

The present study provides direct evidence that, unlike other models of PNP, STZ-diabetic rats do not exhibit behavioural indices of cold allodynia or spontaneous pain, and that the analgesic effects of retigabine were similar to those of gabapentin. Our findings that the anti-allodynic effects of retigabine were completely reversed by the K_v7/M channel blocker XE991 implicate Kv7 in the DPNP pathogenesis. These findings suggest that strategies that target activation of Kv7 channels may be effective in treating DPNP.

Disclosure statement

The authors have no potential or actual conflicts of interest to declare.

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