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Reduction by gabapentin of K^+-evoked release of [^{3}H]-glutamate from the caudal trigeminal nucleus of the streptozotocin-treated rat

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Recently, we showed that gabapentin can inhibit a facilitatory effect of substance P (SP) on K^+-evoked glutamate release in rat trigeminal slices (Maneuf *et al*., 2001), and we have now examined the effect of gabapentin on glutamate release in the trigeminal slice from the streptozotocin (STZ)-treated rat.

1 At 4 weeks following STZ treatment (50 mg kg^{-1} i.p.), blood glucose was increased in the majority of cases, compared to the control level. All the treated animals showed a significant degree ($P<0.001$) of tactile allodynia (assessed using von Frey filaments) that did not appear to correlate with blood glucose levels.

2 In this study, we demonstrated that, after STZ treatment, 30 μM gabapentin reduced K^+-evoked release of [^{3}H]-glutamate in either normal (11 mM) or high (30 mM) glucose conditions by 24 and 22%, respectively. In the normal rat, gabapentin (up to 100 μM) is ordinarily unable to affect release of glutamate from the trigeminal slice.

3 The uptake of glutamate in Sp5C punches from streptozotocin-treated rats was reduced under normal glucose conditions (41.7% of control), whereas high glucose restored uptake to normal (84.7% of control).

4 The addition of 1 μM substance P potentiated the evoked release of glutamate in both normal (40% increase) and high glucose (28%), and this was blocked by gabapentin (30 μM) in both conditions. It is interesting to speculate that this ability of gabapentin to reduce the release of glutamate in the trigeminal nucleus after streptozotocin treatment may be of relevance to the antihyperalgesic-allodynic actions of the drug.
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Abbreviations: CGRP, calcitonin gene-related peptide; NKA, neurokinin A; SP, substance P; Sp5C, caudal sensory subnucleus of the spinal trigeminal nucleus; STZ, streptozotocin

Introduction

Diabetes is frequently associated with painful polyneuropathies, with patients often reporting a complex picture of pain symptoms, numbness and paresthesias (Nabarro, 1991). The factors responsible for the diabetes-induced sensory neuropathy are multiple, and although nerve fibre demyelination accompanied by a characteristic marked loss of nerve-conductance velocity have been proposed as major determinants (Gilliatt, 1965; Thomas & Tomlinson, 1993), the aetiology of the condition remains unclear.

Gabapentin (Neurontin®), which was originally designed as an antiepileptic drug, and was approved for this use in 1993, has more recently been shown to attenuate the painful symptoms of diabetic neuropathy in the clinic (Backonja *et al*., 1998). In the animal, it was demonstrated that gabapentin blocked static and dynamic allodynia in streptozotocin (STZ)-treated diabetic rats (Field *et al*., 1999). Such findings have created much interest in the pharmacology of gabapentin, but, although recent advances have linked gabapentin with a binding site on the alpha-2-delta subunit of voltage-gated calcium channels (Gee *et al*., 1996), little is known about how its antihyperalgesic-antiallodynic properties are mediated. In spite of its 'gabamimetic' properties and action as an antiepileptic, gabapentin has been claimed not to interfere (at least directly) with GABAergic transmission within the brain (Timmerman *et al*., 2000). Indeed, in spite of recent findings claiming agonist actions at a $GABA_B$ receptor subtype (Ng *et al*., 2001), the possibility of an interaction between $GABA_B$ receptors and gabapentin remains highly contested (Jensen *et al*., 2002).

The modulatory action of gabapentin on other transmitter systems in the brain has been reported. Recently, evidence was given that gabapentin can reduce noradrenaline release from cortical slices (Dooley *et al*., 2000a). Furthermore, in the same cortical slice preparation, as well as in the hippocampus, a moderate reduction by gabapentin of K^+-evoked release of glutamate has been demonstrated (Dooley *et al*., 2000b). Release of glutamate from primary afferents plays a key role in pain transmission (Dickenson *et al*., 1997; Millan, 1999), and a

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reduction by gabapentin of the release of excitatory amino acids from presynaptic terminals in the dorsal horn from the normal animal has been reported (Shimoyama *et al*., 2000). However, in another study, this effect of gabapentin could only be found in the spinal cord from rats made 'hyperalgesic' after treatment with STZ (Patel *et al*., 2000).

We have previously reported from work with the trigeminal nucleus slice, that in normal animals gabapentin does not affect the normal level of K^+-evoked release of [^{3}H]-glutamate, but blocks a facilitated component seen in the presence of substance P (Maneuf *et al*., 2001). We believe that this phenomenon may correlate with the efficacy of gabapentin in reversing hyperalgesia or allodynia, where the compound has no effect on acute pain, and points to a fundamental issue in understanding the mechanism of action of gabapentin: that there may be a prerequisite for modification to the system.

The experiments described here have again used the caudal sensory subnucleus of the spinal trigeminal nucleus in the brainstem (Sp5C), as an accessible source of sensory afferents in the mature rat. In the light of the whole-animal data showing an antiallodynic effect of gabapentin in the STZ model of diabetic neuropathy (Field *et al*., 1999), we have now evaluated the effect of gabapentin in punches from STZ-treated rats. Our finding that gabapentin is able to decrease K^+-evoked glutamate release in a significant manner here may be useful in explaining the action of gabapentin in reducing the pain in diabetic neuropathy.

Methods

Tissue preparation

Male Hooded Lister rats (250–350 g) were rapidly decapitated, the brain and upper spinal cord removed and coronal sections (400 μM) were cut from the brainstem using a McIllwain tissue. Consecutive sections containing Sp5C (running caudally from approximately 14 mm posterior to Bregma, according to Paxinos & Watson, 1986) were taken, and tissue punches were prepared using a 1-mm-diameter punch. Punches were then pooled from all the appropriate sections from all animals and incubated in aerated (95% O_2–5% CO_2) artificial cerebrospinal fluid (aCSF, composition mM: NaCl 118; KCl 4.8; $CaCl_2$ 1.3; $MgSO_4$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; ascorbic acid 0.6; glucose 11; peptidase inhibitors captopril, bestatin and phosphoramidon all 0.01) containing 0.1 μM [^{3}H]-glutamate for 30 min at room temperature. The peptidase inhibitors were present to minimise the breakdown of substance P. For experiments involving the use of high concentrations of glucose to mimic hyperglycaemia, the aCSF contained 30 mM glucose.

The tissue punches were transferred to individual chambers of a Brandel SF-20 superfusion system (two per chamber) and washed for 40 min with aCSF (flow rate 1 ml min^{-1}). Following the wash period, with the flow rate at 0.5 ml min^{-1}, aliquots were collected every 5 min for the duration of the experiment (60 min). At 30 min after starting collection, a single 5-min pulse of aCSF containing a 24 mM excess of K^+ (as KCl) was applied to evoke the release of [^{3}H]-glutamate (total concentration of K^+ ions was 30 mM). Drugs or vehicle were added 5 min before, and during the high potassium pulse in the presence of 0.4% BSA.

At the end of the experiment, the tissue punches were recovered and placed into vials for solubilisation using 0.5 ml dimethylsulphoxide in 4.5 ml of scintillation fluid (Ultima Gold MV, Packard Bioscience, Groeningen, The Netherlands). The amount of radioactivity present in both the perfusates (0.5 ml volume) and the solubilised tissue was counted overnight in a liquid scintillation counter. A measure of the amount of glutamate taken up by the punches was determined by the radioactivity present in the punches following solubilisation and expressed in DPM.

[^{3}H]-glutamate release was expressed as a fractional rate of release, with the radioactivity measured in a 5-min time bin divided by the total radioactivity present in the chamber (two punches chamber^{-1}) at the beginning of that period. The total radioactivity present in a chamber at the beginning of a period is equivalent to the radioactivity released after the beginning of this period plus the radioactivity present in the *chamber* at the end of the experiment. The amplitude of the peak of glutamate release in the presence of the test treatment was expressed as a percentage of that in the parallel control (vehicle-treated) fraction. Each mean data point is the result of 3–7 separate experiments, with four replicates per experiment.

Statistical analysis was performed using analysis of variance (ANOVA) followed by a Tukey's multiple-comparison test when significance was attained for the ANOVA. Where only two groups were being compared, the Student's *t*-test was used.

Substance P (Sigma, Poole, U.K.) was dissolved at 10 mM in a 1 M acetic acid, 50% methanol solution containing 0.1% BSA, and aliquots stored at −20°C. Gabapentin was obtained under a powder form from Parke-Davis Neuroscience Research Centre, Cambridge, U.K., and was dissolved in distilled water. Streptozotocin (Sigma, Poole, U.K.) was dissolved in saline (50 mg ml^{-1}). [^{3}H]-glutamate was obtained from Amersham, Little Chalfont, U.K.

Induction of diabetes–STZ treatment

All experiments were conducted in agreement with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, and was reviewed by a local ethical committee. Male Hooded Lister rats (250–300 g initially) were housed in groups of three under a 12 : 12 h light–dark cycle (lights on at 07 : 00) with food and water available *ad libitum*. Animals were injected with STZ (50 mg kg^{-1} i.p.) or saline (i.p.) and after 4 weeks the presence of tactile allodynia was determined using the von Frey filament method. The animals were habituated to wire mesh-bottom cages for two sessions over 2 days prior to testing. Tactile allodynia was measured by application of von Frey filaments to the plantar area of the right hind paw starting with a filament strength of 3.63 g, which was taken as the threshold force indicative of the presence of allodynia (Chaplan *et al*., 1994). The filament was applied for up to 6 s to the paw or until a withdrawal response was observed within that 6-s time period. If a withdrawal was observed, the paw was retested 10 s later with the same filament, and then with lower filament strengths until no response was seen. If, however, no response was seen after application of 3.63 *g*, the process was repeated with higher strength filaments until a withdrawal response occurred. The lowest amount of force required to elicit withdrawal of the paw was recorded as withdrawal threshold in grams (force). The

animals were tested for the presence of tactile allodynia on the morning when the *in vitro* experiments were to be conducted, prior to sacrifice. The animals were killed by cervical dislocation. Samples of trunk blood were collected, and glucose levels measured using a Glucotrend blood glucose analyser (Boehringer Mannheim, Lewes, U.K.).

Statistical analysis was performed using a Mann–Whitney test.

Results

Effect of STZ treatment on blood glucose levels in the hooded Lister rat

At 4 weeks after the 50 mg kg^{-1} i.p. injection of STZ, a marked increase in blood glucose levels was seen in the majority of animals (Figure 1) when compared to the control group (6.08 ± 0.35 mM, $n=6$). In the STZ-treatment group ($n=33$), blood glucose was >15 mM in 20 animals, and the upper measurement limit of the Glucotrend instrument (33 mM) was reached in six. One animal showed a 'marginal' degree of hyperglycaemia (13.3 mM), but is six the level of blood glucose was in the control range of 5–10 mM. The low blood glucose levels were not taken as a rejection criterion for those animals, as tactile allodynia was obtained nevertheless (see below).

Induction of tactile allodynia following STZ treatment

At 4 weeks following the streptozotocin treatment, all the 33 animals showed the presence of tactile allodynia irrespective of the measured level of blood glucose in individual cases, producing a nocifensive withdrawal to filaments in the range 1.48–3. 63 g (Figure 2). By comparison, the animals in the control group responded only to the 8.50, 11.75 or 15.14 g hairs (median value 11.75, interquartile range 1.63). Statistical analysis using Mann–Whitney test showed a highly significant difference between the two groups ($P<0.001$).

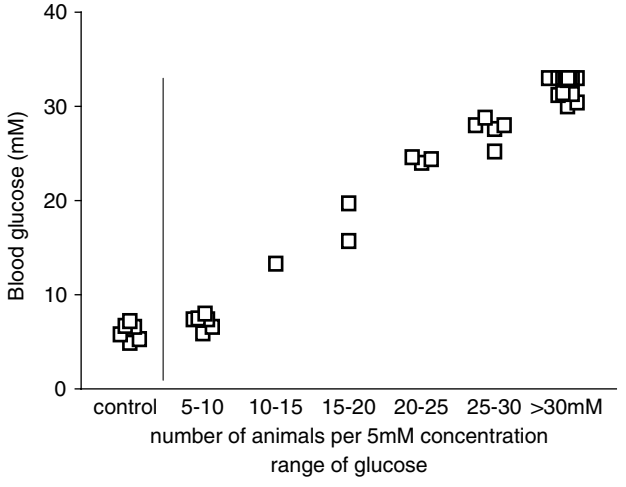

Figure 1 Range of blood glucose levels (mM) of control ($n=6$, left of line) and streptozotocin-treated ($n=33$, right of line) rats 4 weeks following injection of either vehicle or 50 mg kg^{-1} i.p. streptozotocin. Data from the streptozotocin-treated animals are grouped into 5 mM concentration-range bins to demonstrate the distribution of blood glucose levels throughout the treatment group. The highest value recorded at 33 mM, is the detection limit of the assay.

Glutamate uptake and release in aCSF containing 'normal' and or 'high' glucose

Punches from slices containing Sp5C from rats treated with STZ showed a significantly lower level of [^{3}H]-glutamate uptake (56% of control) when the experiment was performed in normal aCSF (11 mM glucose), compared to that for Sp5C punches harvested from animals in the control group ($P<0.001$, Figure 3). In high glucose (30 mM)-containing aCSF, the total slice content of [^{3}H]-glutamate was 90.5% of that of punches from control animals in normal aCSF. The

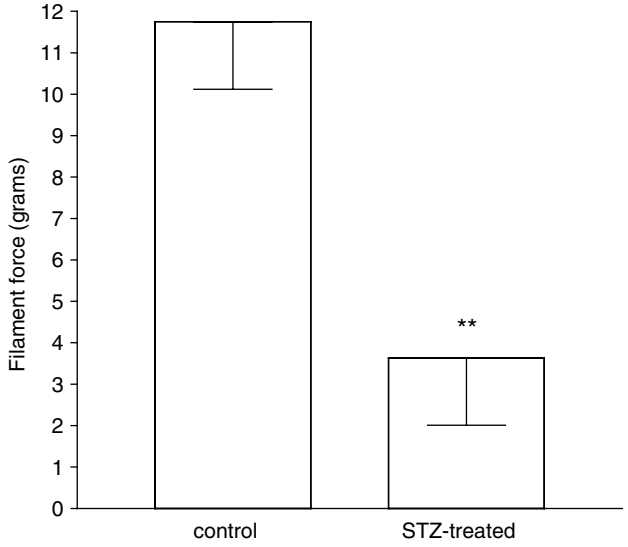

Figure 2 Median values and interquartile ranges of paw-withdrawal threshold (g) of control ($n=6$) and streptozotocin (STZ)-treated ($n=33$) animals. All the animals treated with STZ showed tactile allodynia. $^{**}P<0.01$ Mann–Whitney *U*-test, control *versus* STZ-treated rats.

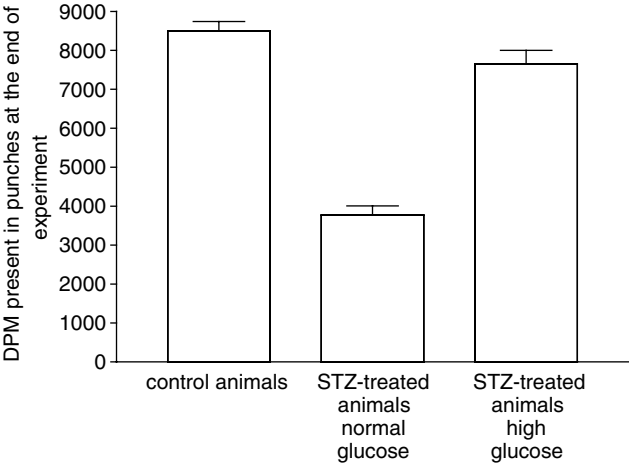

Figure 3 Content of [^{3}H]-glutamate in punches taken from control ($n=240$, normal glucose (11 mM)) and streptozotocin-(STZ) treated rats in normal (11 mM, $n=64$) and high (30 mM, $n=64$) glucose conditions. Histograms are mean values for content (dpm per chamber (two punches per chamber^{-1})), with one s.e.m.

effect on uptake had no net effect on the fractional rate of [^{3}H]-glutamate release, either basal or K^+-evoked, since the actual amount released per time bin was proportionately lower for punches from STZ-treated rats in normal aCSF (data not shown). The baseline fractional rate of [^{3}H]-glutamate release for both conditions was similar, and the K^+-evoked peak of [^{3}H]-glutamate release was also similar in amplitude for both conditions.

Effect of gabapentin on K^+-evoked release of [^{3}H]-glutamate

In punches of slices from Sp5C from untreated animals, gabapentin (up to 100 μM) did not affect the K^+-evoked release of [^{3}H]-glutamate (Figure 4). In the punches from STZ-treated animals, however, gabapentin was able to reduce both the baseline level of K^+-evoked release of [^{3}H]-glutamate, and the SP-facilitated K^+-evoked release. A measure of 30 μM of gabapentin inhibited [^{3}H]-glutamate release by 24% in normal glucose aCSF, and 22% in high glucose (see Figure 5a and b; $F(3,9) = 31.29$ normal glucose and $F(3,21) = 23.94$ high glucose). This concentration had been found to be maximal in inhibiting the substance P-facilitated K^+-evoked release of [^{3}H]-glutamate from trigeminal slices (Maneuf *et al*., 2001). The addition of 1 μM SP caused an increase in the evoked release of glutamate in both normal (40%, $P<0.05$) and high glucose (28.3%, $P<0.05$). This increase was similar to that observed in tissue from untreated animals (Maneuf *et al*., 2001). This effect was blocked by gabapentin (30 μM) in both normal and high glucose conditions. This inhibition was similar to that of gabapentin against K^+-evoked release.

Discussion

Following a noxious stimulus in their peripheral territory, the activation of small-diameter sensory fibres results in the release of excitatory amino-acid transmitters (glutamate and aspartate) together with neuropeptides (tachykinins and calcitonin gene-related peptide, CGRP) in the superficial laminae of the dorsal horn. The distribution of A and C fibres in the caudal trigeminal nucleus corresponds to that of the dorsal horn proper (Tracey, 1985), and on practical grounds we have used the caudal trigeminal nucleus slice in this study to investigate the potential role of the modulation of sensory glutamatergic transmission, in the utility of gabapentin in neuropathic pain.

The importance of the role of glutamate as the main excitatory sensory neurotransmitter in the dorsal horn is supported by a wealth of evidence (for a review, see Millan, 1999). The possible contribution of the neuromodulatory peptides SP, neurokinin A (NKA) and also CGRP in the regulation of neuronal hyperexcitability in chronic pain is also understood (for a review, see Millan, 1999). Tactile allodynia in the STZ-treated rat is reported to be blocked by spinal administration of antagonists of glutamate receptors (Calcutt & Chaplan, 1997), or of NK_1 and NK_2 receptors (Coudoré-

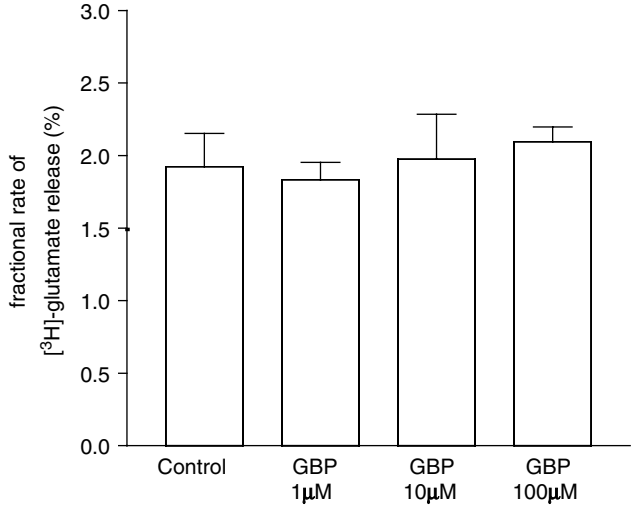

Figure 4 Percentage fractional rate of K^+-evoked [^{3}H]-glutamate release from caudal trigeminal punches from untreated rats in the absence or presence of gabapentin (GBP, 1–100 μM) Data are mean values, plus one s.e.m., from four separate experiments. (see Methods for experimental details). Increasing concentrations of gabapentin did not affect the K^+-evoked release of [^{3}H]-glutamate ($P>0.05$).

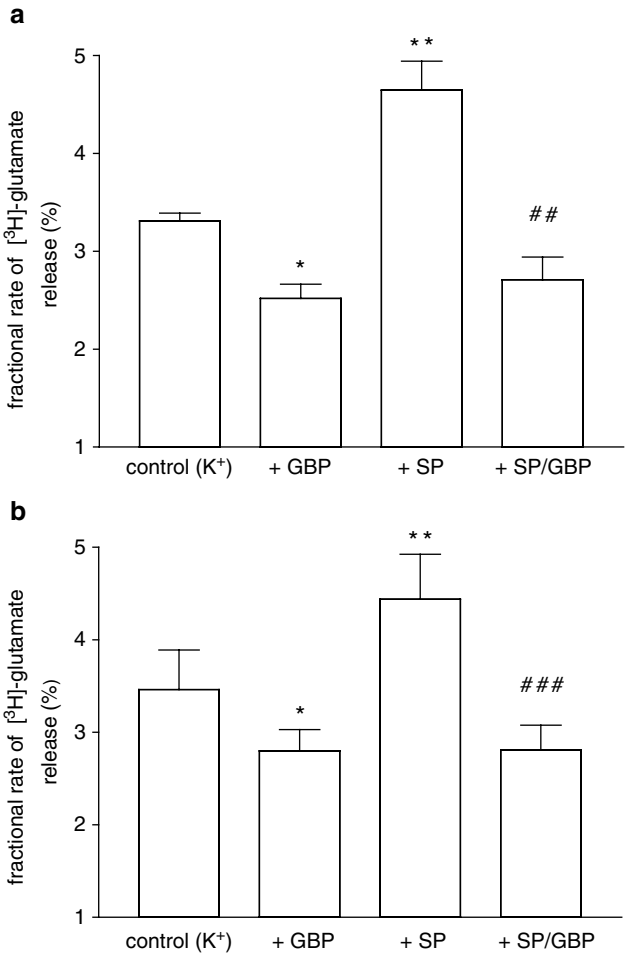

Figure 5 Effect of gabapentin (GBP, 30 μM) alone or in the presence of substance P (SP, 1iM) on K^+-evoked release of glutamate under normal (11 mM (**a**)) or high (30 mM; (**b**)) glucose concentrations in punches from trigeminal slices from STZ-treated animals. Data are mean values from eight and four separate experiments respectively, plus one s.e.m. Gabapentin inhibited both the K^+-evoked glutamate release, and the SP-facilitated K^+-evoked release of [^{3}H]-glutamate from punches from trigeminal slices. $\#\#P<0.01$: Tukey's post hoc test *versus* SP. $*P<0.05$ and $**P<0.01$: Tukey's post-hoc test *versus* control.

Civiale *et al*., 2000), supporting the involvement of both glutamate and tachykinins. It has also been established that SP and NKA (and CGRP) are released in the caudal trigeminal nucleus following stimulation of the trigeminal ganglion (Samsam *et al*., 2000). The cooperativity between glutamate and neurokinins in participating in the establishment of a neuropathic condition in the spinal cord has also been documented (see Urban *et al*. (1994) for review).

We recently reported that gabapentin inhibited the facilitated component of K^+-evoked release of [^{3}H]-glutamate in the Sp5C slice in the presence of SP (Maneuf *et al*., 2001), while it had no effect on the normal level of evoked release. This lack of effect of gabapentin on its own was tentatively linked to the lack of efficacy in acute pain, whereas an antihyperalgesic-antiallodynic action of the drug in neuropathic pain has been clinically proven and could then relate to the changes in glutamatergic transmission under those conditions. It is noteworthy to mention that the active concentrations of gabapentin responsible for an action on glutamatergic transmission in this study (tens of micromolar of gabapentin) correlate with *in vivo* studies (Field *et al*., 1999) and the clinic in humans (Muscas *et al*., 2000). To further these findings, we extended our studies on the action of gabapentin in the Sp5C slice, by using tissue from animals in a validated model of neuropathic pain. The STZ-treated rat is used very commonly as a model of diabetic neuropathy, and reproduces many of the behavioural symptoms of diabetic neuropathy such as allodynia, although other aspects of the pathology do not seem to be present (Walker *et al*., 1999). With the Hooded Lister rat in the present study, we saw a 100% success rate with STZ treatment for the production of tactile allodynia, whereas the Sprague–Dawley rat is reported to show a lower incidence of allodynia (Lynch *et al*., 1999). Interestingly, the presence of allodynia did not always correlate with high levels of blood glucose, at least at the 4-week time point. Earlier studies reported that a significant increase in blood glucose levels was only seen after 16 weeks following STZ treatment (dose $\geqslant 45\,mg\,kg^{-1}$) (Tancrede *et al*., 1983). In our protocol, the animals were tested 4 weeks following STZ treatment ($50\,mg\,kg^{-1}$). It is possible that a proportion of animals did not yet show the hyperglycaemia expected at 16 weeks.

In the light of recent studies (Fox *et al*., 1999; Walker *et al*., 1999) criticising the STZ model, we would be cautious about adding to the controversy. We take our results to point less towards a failure of the STZ model, than to a limited rate of success in the establishment of a real diabetic neuropathy due to the time needed to establish the full hyperglycaemia and allodynia pathology.

STZ purportedly created a chronic diabetic state, which resulted in a decrease in the net glutamate content in the Sp5C slice from STZ-treated animals, compared to the normal animal. This effect was cancelled if the experiments were performed in high-glucose (30 mM) conditions. This finding can be related to the observations of Madl & Royer (1999) that hypoglycaemia (which we speculate to be equivalent to 'normal glucose' for the STZ-treated, diabetic rats in our study) induced a large loss of glutamate in the hippocampal slice, which was not due to hypoxia. Those authors suggested that an increased metabolic rate of glutamate *via* the aminotransferase is responsible for this decrease. Increasing the glucose levels resulted in normal glutamate content in our punches (equivalent to normal glucose conditions in normal animals).

In the STZ-treated rat, gabapentin was able to reduce the K^+-evoked release of [^{3}H]-glutamate on its own either in normal or high glucose-containing aCSF. A similar action of gabapentin was also described by Patel *et al*. (2000), who reported that excitatory transmission in the spinal cord was inhibited by gabapentin only in preparations from STZ-treated animals showing hyperalgesia, and not from normal animals. Substance P, which we reported was able to facilitate the evoked release of glutamate from Sp5C (Maneuf *et al*., 2001), again increased the K^+-evoked release of glutamate, and gabapentin inhibited this facilitated release. This component of release sensitive to gabapentin, which is not present under normal circumstances, might be either part of or a consequence of the changes occurring within the hyperalgesic animal (Yaksh & Chaplan, 1997).

Although a prejunctional site of action of gabapentin may seem likely from our findings, it would be difficult and imprudent to eliminate the possibility of a postjunctional component. It is likely that gabapentin-binding sites (purportedly alpha-2-delta subunits of voltage-gated calcium channels) are situated both pre- and postsynaptically, a localisation shared by NK_1 receptors (Hu *et al*., 1997; Liu *et al*., 1997). In conclusion, we would suggest that changes have occurred in the caudal trigeminal nucleus of the streptozotocin-treated rat, that allow gabapentin to reduce the release of glutamate, therefore contributing to the antihyperalgesic properties of this compound.

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