

# Gabapentin inhibits high-threshold calcium channel currents in cultured rat dorsal root ganglion neurones

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**1** This study examined the action of gabapentin (gabapentin, 1-(aminomethyl) cyclohexane acetic acid (Neurontin<sup>®</sup>)) on voltage-gated calcium (Ca<sup>2+</sup>) channel influx recorded in cultured rat dorsal root ganglion (DRG) neurones.

**2** Voltage-gated Ca<sup>2+</sup> influx was monitored using both fura-2 based fluorescence Ca<sup>2+</sup> imaging and the whole-cell patch clamp technique.

**3** Imaging of intracellular Ca<sup>2+</sup> transients revealed that gabapentin inhibited KCl (30 mM)-evoked voltage-dependent Ca<sup>2+</sup> influx. Both the duration for 50% of the maximum response (W50) and total Ca<sup>2+</sup> influx were significantly reduced by ~25–30% in the presence of gabapentin (25 μM).

**4** Gabapentin potently inhibited the peak whole-cell Ca<sup>2+</sup> channel current (I<sub>Ba</sub>) in a dose-dependent manner with an estimated IC<sub>50</sub> value of 167 nM. Block was incomplete and saturated at a maximal concentration of 25 μM.

**5** Inhibition was significantly decreased in the presence of the neutral amino acid L-isoleucine (25 μM) but unaffected by application of the GABA<sub>B</sub> antagonist, saclofen (200 μM), suggesting a direct action on the α<sub>2δ</sub> subunit of the Ca<sup>2+</sup> channel.

**6** Gabapentin inhibition was voltage-dependent, producing an ~7 mV hyperpolarizing shift in current voltage properties and reducing a non-inactivating component of whole-cell current activated at relatively depolarized potentials.

**7** The use of specific Ca<sup>2+</sup> channel antagonists revealed a mixed pharmacology of the gabapentin-sensitive current (N-, L- and P/Q-type), which is dominated by N-type current.

**8** The present study is the first to demonstrate that gabapentin directly mediates inhibition of voltage-gated Ca<sup>2+</sup> influx in DRG neurones, providing a potential means for gabapentin to effectively mediate spinal anti-nociception.

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**Abbreviations:** Ca<sup>2+</sup>, Calcium; (DRG), dorsal root ganglia; (GBP), gabapentin, 1-(aminomethyl) cyclohexane acetic acid (Neurontin<sup>®</sup>); (HVA), high voltage-activated; I<sub>Ba</sub>, peak whole-cell calcium channel current carried by barium; (W50), duration for 50% of the maximum response

## Introduction

The anti-convulsant drug Gabapentin, 1-(aminomethyl) cyclohexane acetic acid (Neurontin<sup>®</sup>) has been shown to exhibit clinically effective anti-hyperalgesic activity via an unknown mechanism. Gabapentin was originally developed for the treatment of spasticity and partial epilepsy and has proved effective in a number of different animal seizure models (Taylor *et al.*, 1998; Cesena & Calcutt, 1999; Field *et al.*, 1999; Cheng *et al.*, 2000; Nicholson, 2000). Numerous open label case studies and three large double-blind trials now also provide supporting evidence that gabapentin is successful at treating a wide-ranging number of neuropathic pain conditions, including diabetic neuropathy (Backonja *et al.*, 1998), postherpetic neuralgia (Rowbotham *et al.*, 1998), trigeminal neuralgia, migraine and pain associated with

cancer and multiple sclerosis (Di Trapini *et al.*, 2000; Caraceni *et al.*, 1999; Houtchens *et al.*, 1997; see also Magnus, 1999; Laird & Gidal, 2000; Nicholson, 2000). Although gabapentin has now proved an effective treatment for a number of neuropathic pain conditions, numerous studies have investigated its possible mechanisms of action and yet no firm conclusions have been drawn (Taylor *et al.*, 1998).

Originally designed as a GABA mimetic, until very recently, there was no evidence for the ability of gabapentin to influence the functioning of either GABA receptors or GABA re-uptake mechanisms either directly or by inappropriate metabolic substitution (Taylor *et al.*, 1998). However, gabapentin has since been shown to mediate post synaptic membrane excitability through a subtype-specific interaction with a particular GABA<sub>B</sub> receptor heterodimer (Ng *et al.*, 2001). Gabapentin has also been demonstrated to substitute as a substrate for the system L amino acid transporter (Stewart *et al.*, 1993; Su *et al.*, 1995), providing

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an effective route of entry for gabapentin into the intracellular environment. Nevertheless, the only specific molecular target for gabapentin identified to date is the  $\alpha_2\delta$  subunit of voltage-dependent Ca<sup>2+</sup> channels (Gee *et al.*, 1996). Interaction with this high affinity binding site may provide a means for gabapentin to mediate voltage-dependent Ca<sup>2+</sup> influx, by indirectly influencing the functional interaction between the accessory  $\alpha_2\delta$  and the pore-forming  $\alpha_1$  subunit of Ca<sup>2+</sup> channels (reviewed in Walker & De Waard, 1998).

Previous studies have reported a mixed susceptibility of different Ca<sup>2+</sup> channel preparations to the action of gabapentin. Stefani *et al.* (1998) were the first group to demonstrate that gabapentin inhibited voltage-dependent Ca<sup>2+</sup> channel currents recorded from cortical neurons, however the gabapentin-mediated reduction in current varied between different cell types. Nevertheless, despite the inconsistent nature of the gabapentin-sensitive Ca<sup>2+</sup> current, a comparative survey of work undertaken by a number of independent groups suggests that, given the right conditions, gabapentin can produce a reduction in Ca<sup>2+</sup> channel influx. Gabapentin has been shown to reduce P/Q-type current into synaptosomes and inhibit release of excitatory amino acids from neocortical and trigeminal nucleus slices in a localized and stimulus-dependent manner (Dooley *et al.*, 2000; Fink *et al.*, 2000; Meder & Dooley, 2000; Maneuf & McKnight, 2000).

In view of these findings, the effects of gabapentin were therefore tested on voltage-dependent Ca<sup>2+</sup> influx recorded in cultured rat dorsal root ganglia (DRG) neurones. These neurones are thought to play a crucial role in pain processing (Reichling & Levine, 1999) and have been shown to express a variety of high voltage-activated (HVA) Ca<sup>2+</sup> channel  $\alpha_1$  subunits (N-, L-, P/Q- and R-type; Mintz *et al.*, 1992; Rusin & Moises, 1995) – all of whose properties have been shown to be affected by co-association with accessory  $\alpha_2\delta$  subunits (Gurnett *et al.*, 1996; Klugbauer *et al.*, 1999; Gao *et al.*, 2000; reviewed in Walker & De Waard, 1998; Felix, 1999). In the present study, the effect of gabapentin on HVA Ca<sup>2+</sup> influx was assessed using both Ca<sup>2+</sup> imaging and the whole-cell patch clamp technique. Some of the data in this manuscript has previously been published in abstract form (Martin *et al.*, 2000; McClelland *et al.*, 2000; Sutton *et al.*, 2000).

## Methods

### Cell culture

Briefly, dorsal root ganglia were dissected from 1- to 3-day-old Wistar rats that had been killed by decapitation. Ganglia were then incubated in Collagenase/Dispase (1 mg ml<sup>-1</sup>, Boehringer) at 37°C for 45 min in Ca/Mg-free PBS before being transferred to DMEM-based media (Gibco + 10% FBS, 2 mM L-glutamine, penicillin (5000 IU ml<sup>-1</sup>), streptomycin (5000 mg ml<sup>-1</sup>)) and mechanically dissociated by trituration through a 23G hyperdermic needle. The cell suspension was strained to remove debris (<40  $\mu$ m, Falcon), spun and resuspended in the above culture media containing 100 ng ml<sup>-1</sup> 7s-NGF (Promega) before being plated onto poly-L-lysine/laminin-coated coverslips (~5 × 10<sup>3</sup>/coverslip, 10 mm diameter). Cells were treated with cytosine arabinoside (2.5  $\mu$ M)

for the first 48 h in culture (37°C in air containing 5% CO<sub>2</sub>) after which they were transferred to F14-based culture media (Imperial Laboratories + 10% HS (Gibco), penicillin (5000 IU ml<sup>-1</sup>), streptomycin (5000 mg ml<sup>-1</sup>), 2 mM L-glutamine, 14 mM NaHCO<sub>3</sub>, 10 ng ml<sup>-1</sup> 2.5S NGF (Sigma)). Cells used for Ca<sup>2+</sup> imaging experiments were only cultured in F14-based media (20 ng ml<sup>-1</sup> NGF), and were not treated with cytosine arabinoside. Cells were used for experiments after an additional 48 h and re-fed with fresh culture media every 2–3 days.

### Calcium imaging

Cultured DRG neurones were incubated for 1 h in NaCl-based extracellular solution (mM): NaCl, 130; KCl, 3.0; MgCl<sub>2</sub>, 0.6; CaCl<sub>2</sub>, 2.0; NaHCO<sub>3</sub>, 1.0; HEPES, 10.0; glucose, 5.0 and 0.01, fura-2AM (Sigma, 1 mM stock in dimethylformamide), pH 7.4 (NaOH), osmolarity 310–320 mOsm (sucrose). The cells were then washed for 10–20 min with NaCl-based extracellular solution to remove the extracellular fura-2AM and to allow cytoplasmic de-esterification of the Ca<sup>2+</sup> sensitive fluorescent dye. The cells were constantly perfused with NaCl-based extracellular solution (1–2 ml/min) and viewed under an inverted Olympus BX50WI microscope with a KAI-1001 S/N 5B7890-4201 Olympus camera attached. The fluorescence ratiometric images from data obtained at excitation wavelengths of 340 nm and 380 nm were viewed and analysed using OraCal pro, Merlin morphometry temporal mode (Life Sciences resources, version 1.20). The neurones were stimulated with NaCl-based extracellular solution containing high K<sup>+</sup> (30 mM), which produced depolarization, activation of voltage-gated Ca<sup>2+</sup> channels and large transient increases in intracellular Ca<sup>2+</sup>. Three consistent transient increases of intracellular Ca<sup>2+</sup> could be obtained in a single experiment. The actions of gabapentin (25  $\mu$ M) were investigated on the response to the second stimulus. The actions of gabapentin on the Ca<sup>2+</sup> transient amplitude, duration at 50% peak amplitude (W50) and total Ca<sup>2+</sup> flux were measured.

### Measurement of membrane potentials

The effects of changing extracellular [K<sup>+</sup>] on membrane potential were evaluated using the whole-cell variant of the patch clamp technique. Culture DRG neurones were bathed in NaCl-based extracellular solution and low resistance patch pipettes were filled with a patch pipette solution containing (mM), KCl 140, EGTA 5, CaCl<sub>2</sub> 0.1, MgCl<sub>2</sub> 2, HEPES 10, ATP 2, pH 7.2 (TRIS), 310–315 mOsmol/l (sucrose). After entering the whole-cell recording configuration, neurones were allowed to equilibrate for approximately 5 min before stable membrane potentials were made. Extracellular solutions containing 10, 20, 30 or 40 mM K<sup>+</sup> were applied by low pressure ejection *via* a blunt micropipette (tip diameter about 10  $\mu$ m) positioned approximately 100  $\mu$ m from the neurone and the new stable membrane potentials recorded.

### Electrophysiology and solutions

The sensitivity of HVA calcium channels to gabapentin was assessed utilising the whole-cell mode of the patch clamp technique (2–5 mM Ba). Extracellular recording saline

comprised of (mM): Choline chloride 130, TTX 0.0025, TEA 25, KCl 3, BaCl<sub>2</sub> 2/5, MgCl<sub>2</sub> 0.6, NaHCO<sub>3</sub> 1, HEPES 10, Glucose 4, pH 7.4 NaOH, 320 mOsm (sucrose). Intracellular patch pipettes were filled with (mM): CsCl<sub>2</sub> 140, EGTA 10, CaCl<sub>2</sub> 0.1, MgCl<sub>2</sub> 2.0, HEPES 10, ATP, 2 pH 7.2. Tris, 310 mOsm (sucrose). GTP (1 mM) was included in the intracellular patch pipette solution for all experiments except the dose response data to increase stability of recordings over time. All experiments were performed at room temperature (20–22°C). Whole-cell patch-clamp recordings were performed using an Axopatch 200 amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.) linked to a personal computer equipped with pCLAMP Version 8.0. Patch pipettes (World Precision Instruments 1B120F-3) were pulled using a Sutter P-87 microelectrode puller and showed typical resistances of 2–4 MΩ. Series resistance had typical values of 8–12 MΩ and was electronically compensated by at least 90%. Recordings were low-pass filtered at 2 kHz using the built in Bessel filter of the amplifier and digitized at 5 kHz using a Digidata 1200 A/D converter (Axon Instruments). Peak inward currents (I<sub>Ba</sub>) were activated from a V<sub>h</sub> of –80 mV using 100 ms steps to V<sub>c</sub> of 0 mV every 15 s. To control for full equilibration with the internal patch solution, data was obtained from currents that had been allowed to stabilize for up to 10 min before any measurements were made. Drugs were applied by gravity-fed bath perfusion from an outlet placed close to the recorded cell. Data was analysed using Clampfit (Axon Instruments, Inc.). All curve fitting was carried out in GraphPad Prism Version 3.0. Unless stated otherwise, data are expressed as means ± s.e.mean, numbers in parentheses displayed on the figures reflect numbers of individual cells recorded, and *P* values given reflect unpaired 2-tailed Student's *t*-tests.

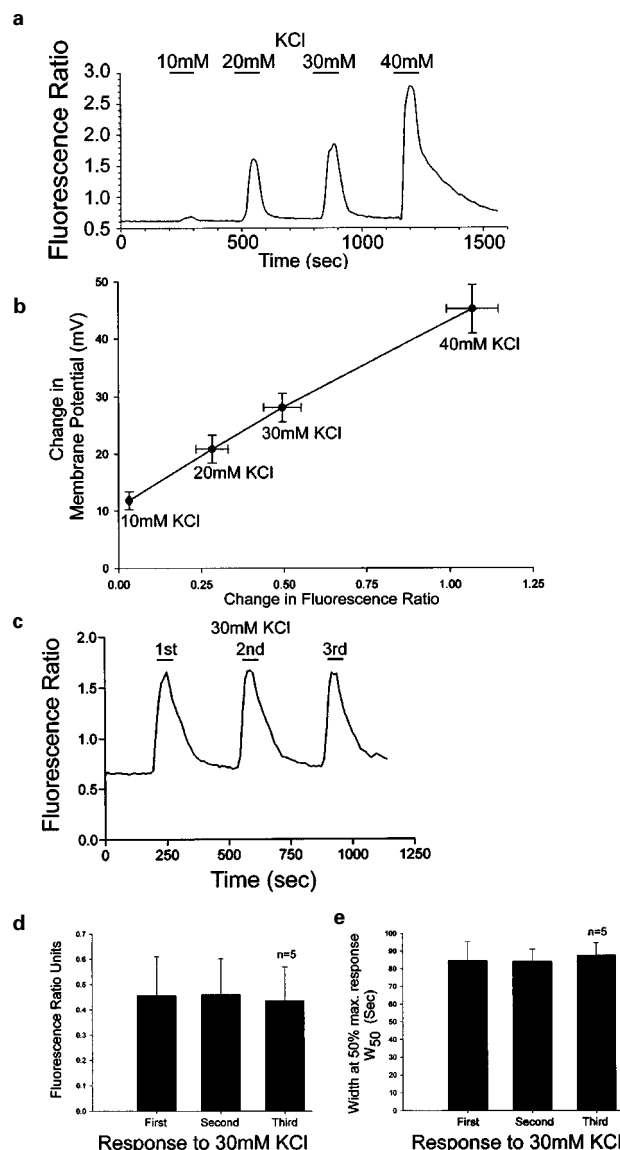
Stocks of gabapentin, L-isoleucine (Sigma), ω-Agatoxin IVA and ω-Conotoxin GVIA (Alamone) were prepared in water and stored at –20°C. Stocks of saclofen and nifedipine (Sigma) were prepared in DMSO and stored at –20°C.

## Results

### Gabapentin inhibits calcium influx into cultured DRG neurones

Experiments were initially carried out to optimize the recording conditions and to justify the protocols used in this study. Electrophysiological and fura-2-based fluorescence imaging data was obtained for neuronal responses over a range of stimulating KCl concentrations. Increases in intracellular Ca<sup>2+</sup> were shown to exhibit a graded linear correlation with changes to membrane potential over increasing concentrations of KCl (Figure 1a,b). Triplicate responses of consistent amplitude and duration which showed complete recovery from the stimulus were obtained using a maximal concentration of 30 mM KCl (Figure 1c–e). Mean values calculated for the duration (width) at 50% of the maximum response (W50) were 84.6 ± 10.8 s, 84.1 ± 7.0 s and 87.5 ± 7.3 s for three consecutive stimuli, respectively (Figure 1e).

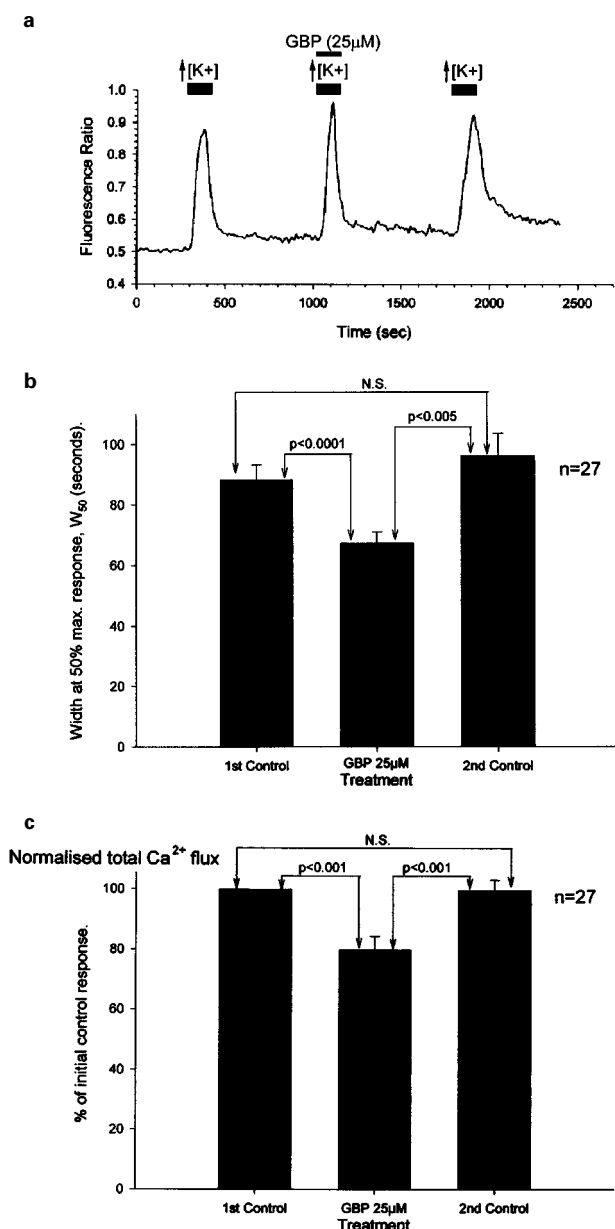
A 1–2 min pre-application of 25 μM gabapentin was not seen to alter the mean peak amplitude of the Ca<sup>2+</sup> transient evoked by 30 mM K<sup>+</sup>. Changes in fluorescence ratio were



**Figure 1** (a) Trace showing changes in fluorescence ratio produced by changes in KCl in imaging experiments. (b) Graph showing linear correlation between change in fluorescence ratio and membrane potential at varying concentrations of K<sup>+</sup>. The plot was produced from mean data ± s.e.mean obtained from imaging experiments (*n* = 19) and measuring membrane potential changes (*n* = 6–7); *R*<sup>2</sup> = 0.9977. (c) Trace from an individual DRG neurone showing that, three pulses of 30 mM KCl gave repeatable responses that fully recovered between stimulation. (d and e) Bar graphs show that consistent control Ca<sup>2+</sup> transients were obtained in response to repeated stimuli of 30 mM KCl applied to the same cells. Data plotted are the mean response as a fluorescence ratio or duration at 50% of the transient amplitude.

calculated to be  $0.3290 \pm 0.029$ ,  $0.3230 \pm 0.0240$  and  $0.3030 \pm 0.025$  for control, gabapentin and recovery responses, respectively (*n* = 27; Figure 2a). Nevertheless, gabapentin (25 μM) did significantly reduce both the duration of the Ca<sup>2+</sup> transients and the total Ca<sup>2+</sup> influx evoked by 30 mM KCl – measured as the area under the transient curve (Figure 2b,c). The duration for 50% of the maximum response (W50) was significantly and reversibly reduced from

control in the presence of gabapentin (calculated values were  $88.4 \pm 4.9$  s,  $67.4 \pm 3.8$  s and  $96.2 \pm 7.4$  s, for control, gabapentin and recovery, respectively,  $n = 27$ ; Figure 2b). Similar results were obtained when analysing the data for the gabapentin effect on total Ca<sup>2+</sup> flux normalized with respect to the first control response, where gabapentin reversibly reduced the influx to  $79.9 \pm 3.8\%$  with the recovery response calculated to be  $99.7 \pm 3.3\%$  ( $n = 27$ ; Figure 2c).



**Figure 2** Gabapentin inhibited Ca<sup>2+</sup> influx into cultured DRG as measured using fura-2 fluorescence imaging. (a) Trace from a single cultured DRG neurone showing a decrease in response duration and total Ca<sup>2+</sup> flux by gabapentin (25 μM), but no significant change in the amplitude of the peak Ca<sup>2+</sup> transient. (b and c) Bar charts showing the inhibitory actions of 25 μM gabapentin (GBP) on the duration of the Ca<sup>2+</sup> transients evoked by 30 mM KCl measured at 50% of the peak amplitude (W<sub>50</sub>) and the total Ca<sup>2+</sup> flux normalized with respect to the first control Ca<sup>2+</sup> transient.

### Gabapentin inhibits whole-cell voltage-gated calcium channel current in DRG neurones

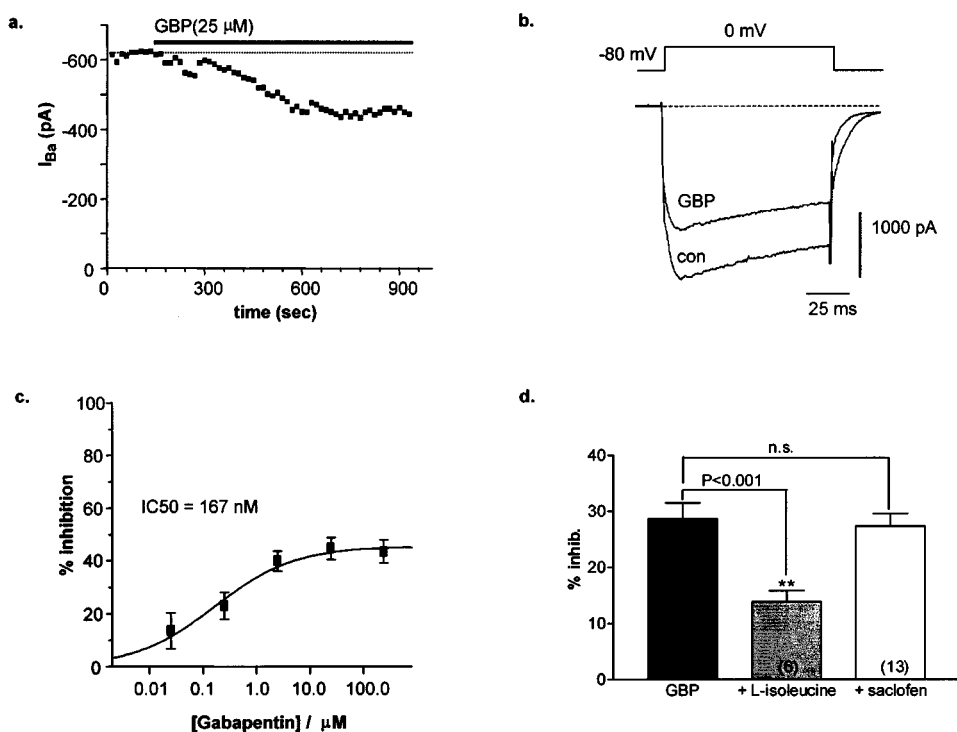
Gabapentin-mediated inhibition of HVA Ca<sup>2+</sup> channel current was initially assessed using the whole-cell patch clamp technique with barium as the charge carrier. From a holding potential of  $-80$  mV, peak inward currents were activated every 15 s using 100 ms step depolarizations to  $V_c = 0$  mV. Using these conditions the total peak recorded current is dominated by HVA channel influx. As shown in Figure 3a, sustained bath perfusion of gabapentin (25 μM) significantly and irreversibly blocked a component of peak HVA current ( $I_{Ba}$ ). Onset of block was slow to develop and completely saturated within 10 min of application.

The action of gabapentin was tested over a broad range of concentrations (25 nM–250 μM). Inhibition of the HVA current was incomplete, with maximal block saturating at a concentration of 25 μM GBP ( $44.7 \pm 4.1\%$ ,  $n = 8$ ; Figure 3c). Block of the peak current was dose-dependent and fit of the mean dose-response data with the Hill equation yielded an estimated IC<sub>50</sub> value of 167 nM. This value is close to the K<sub>d</sub> value  $\sim 40$  nM obtained in binding studies (Suman-Chauhan *et al.*, 1993; Gee *et al.*, 1996; Brown & Gee, 1998) and below estimates of clinically effective plasma concentrations (10–100 μM; Goa & Sorkin, 1993). The lower potency of gabapentin *in vivo* may be due to endogenous levels of L-system substrates, such as L-leucine, which have been demonstrated to decrease the efficacy of gabapentin by competing for the  $\alpha_2\delta$  binding site (Gee *et al.*, 1996). Indeed, pre-application of L-isoleucine (25 μM) significantly reduced inhibition by gabapentin to just  $13.8 \pm 2.0\%$ ,  $n = 6$  ( $P < 0.001$ ; Figure 3d), sufficient to increase the estimated IC<sub>50</sub> value to a value greater than 10 μM, as observed previously for *in vivo* studies. This data therefore indicates that the Ca<sup>2+</sup> channel  $\alpha_2\delta$  subunit is the effective site of functional interaction for gabapentin in this preparation.

A recent report suggested that gabapentin may indirectly influence Ca<sup>2+</sup> channels by interacting with a specific splice variant of the GABA<sub>B</sub> receptor (Ng *et al.*, 2001). However, in agreement with previous observations (McClelland *et al.*, 2000), in this study inhibition by gabapentin (25 μM,  $28.6 \pm 7.1\%$ ,  $n = 10$ ) was unaffected by pre-application of the specific GABA<sub>B</sub> antagonist, saclofen (200 μM), reducing the calcium channel current by  $27.2 \pm 1.8\%$  ( $n = 13$ ;  $P = 0.66$ ; Figure 3d). This data, coupled with a reduced potency in the presence of neutral amino acids, therefore suggests that in DRG neurones gabapentin does indeed mediate its effect *via* a direct interaction with the Ca<sup>2+</sup> channel.

### Voltage-dependence of gabapentin inhibition

In DRG neurones, the voltage-dependence of the HVA whole-cell current represents the combined properties of a number of different channel subtypes (L-, N-, P/Q-, and R-type; Mintz *et al.*, 1992; Rusin & Moises, 1995), each differentially influenced by their associated accessory subunits (Walker & De Waard, 1998). Using 2 mM Ba<sup>2+</sup> as the charge carrier, maximal activation of the HVA current occurred within the voltage range of  $V_c = -5$  and 0 mV. Voltage-dependence of gabapentin inhibition was subsequently determined using a ramped step protocol (0.9 ms/mV,  $V_h = -80$  mV). Currents were activated under control



**Figure 3** (a) Time-course showing gabapentin inhibition of peak  $I_{Ba}$ . (b) Whole-cell current trace of peak HVA  $I_{Ba}$  activated under control conditions (con) and following a 10 min application of gabapentin (GBP). (c) Dose-response of GBP-mediated inhibition of peak HVA  $I_{Ba}$ . Data points represent mean  $\pm$  s.e.mean ( $n=3-8$  determinants) and were fitted with the following sigmoidal equation:  $Y = \max / 1 + (IC_{50} / [GBP])^n$  where  $n$  is the Hill slope factor = 0.55,  $\max$  is maximum inhibition = 46.8% and  $IC_{50} = 167$  nM. (d) Bar graph (mean  $\pm$  s.e.mean) showing that inhibition of peak HVA  $I_{Ba}$  by gabapentin (GBP) is significantly reduced in the presence of L-isoleucine (25  $\mu$ M) but is unaffected following treatment with saclofen (200  $\mu$ M,  $n=10$ , 6 and 13, respectively).

conditions or following a 10 min application of gabapentin. Boltzmann fit of the data showed that gabapentin inhibited a proportion of current activated at relatively depolarized potentials (Figure 4a). The decrease in HVA conductance was accompanied by an  $\sim 7$  mV hyperpolarizing shift in the voltage-dependence of current activation ( $V_{50act} = -8.6 \pm 1.5$ , and  $-15.2 \pm 0.8$  mV,  $n=3$  and 4, for control and gabapentin-treated cells respectively;  $P < 0.05$ ), with no corresponding change in the estimated reversal (null) potential determined from Boltzmann fit of the data ( $E_{rev} = 29.5 \pm 1.3$  and  $29.6 \pm 1.9$  mV).

The effects of gabapentin (2.5  $\mu$ M) were also investigated on voltage-gated currents using 2 mM Ca<sup>2+</sup> as the charge carrier. From a holding potential of  $-90$  mV, current/voltage (I/V) relationships were generated using 100 ms voltage step commands to potentials between  $-150$  to  $+30$  mV. No effects on the mean low threshold current ( $V_c = -50$  to  $-30$  mV) were detected. Nevertheless, as for Ba<sup>2+</sup>, a 3–5 min application of gabapentin produced a significant  $33 \pm 9\%$  inhibition of the peak current at  $V_c = -10$  mV, although no significant change in the mean voltage-dependence of channel activation was observed (Figure 4b;  $P < 0.03$ ,  $n=6$ ). However, in two of the six recorded neurones there was a similar tendency for the I/V relationship to shift to the left in the presence of gabapentin as for currents recorded in the presence of Ba<sup>2+</sup> (see inset traces, Figure 4c).

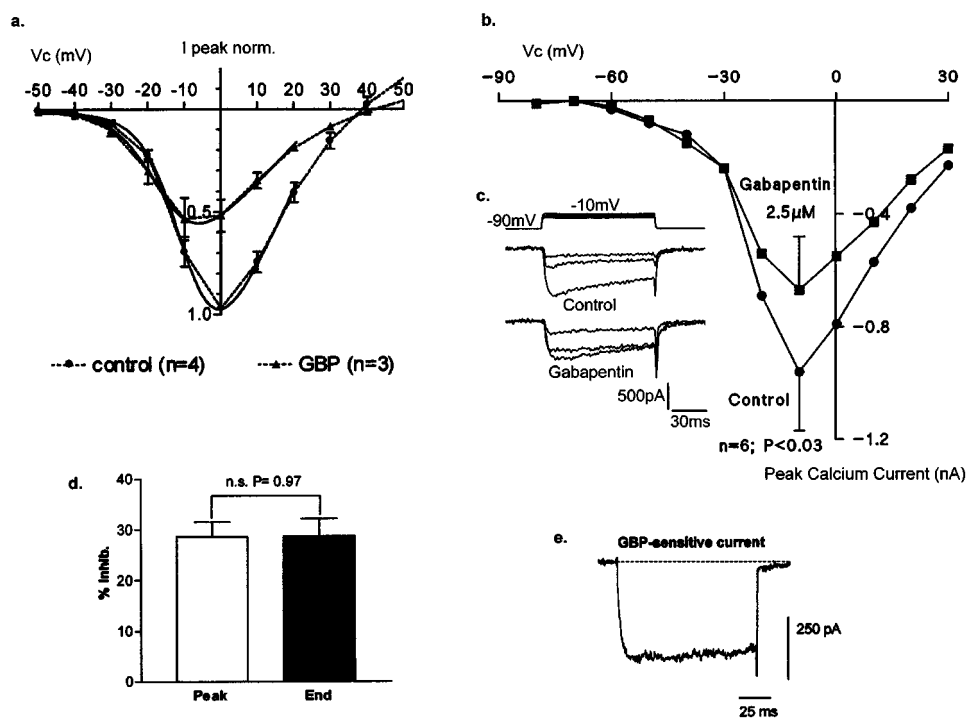
Examination of both the Ba<sup>2+</sup> and Ca<sup>2+</sup> current waveforms following inhibition by gabapentin (25  $\mu$ M) did not

reveal any changes in rate of current inactivation. Both peak and end Ba<sup>2+</sup> current amplitudes were equally inhibited by gabapentin ( $28.6 \pm 2.9\%$  vs  $28.8 \pm 3.4\%$  respectively;  $P = 0.97$ ,  $n=10$ ; Figure 4d). Current subtraction to obtain the gabapentin-sensitive difference Ba<sup>2+</sup> current revealed a square waveform, confirming its non-inactivating properties (Figure 4e).

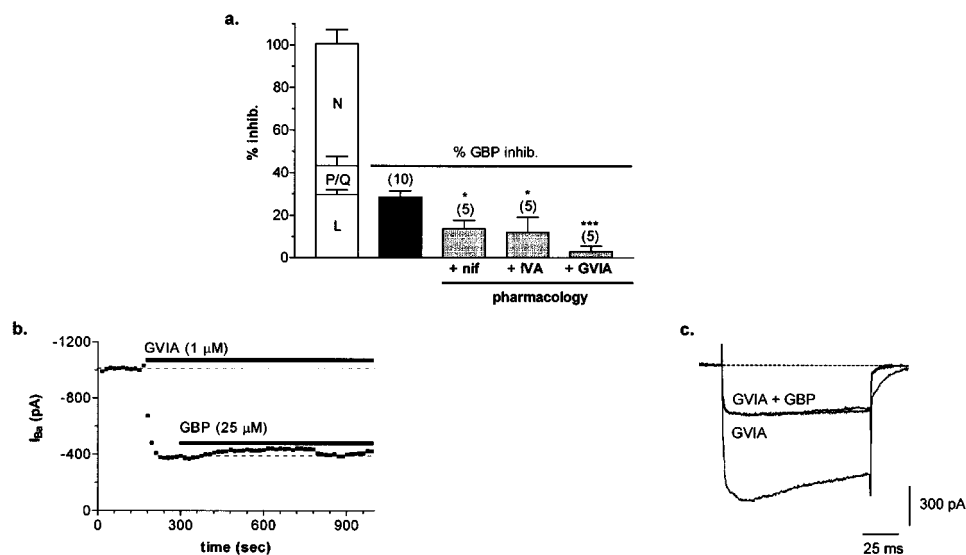
#### Pharmacological characterization of gabapentin-sensitive current

The pharmacological profile of the gabapentin-sensitive current was investigated using a variety of specific Ca<sup>2+</sup> channel antagonists. A number of the different HVA Ca<sup>2+</sup> channels expressed in DRG neurones can be blocked using selective inhibitors (N-, P/Q- and L-type; Mintz *et al.*, 1992; Rusin & Moises, 1995). Isolation of the components of HVA current were determined using 100 nM  $\omega$ -Agatoxin IVA to inhibit P/Q-type current ( $13.5 \pm 4.4\%$ ,  $n=8$ ), 1  $\mu$ M  $\omega$ -Conotoxin GVIA ( $\omega$ -CgTx GVIA) to inhibit N-type current ( $57.3 \pm 6.6\%$ ,  $n=7$ ) and the L-type blocker, nifedipine (5  $\mu$ M;  $29.8 \pm 2.2\%$ ,  $n=6$ ; Figure 5a).

As shown in Figure 5, pre-application of each Ca<sup>2+</sup> channel antagonist significantly reduced inhibition by gabapentin. Pre-block by  $\omega$ -CgTx GVIA almost completely eliminated the gabapentin-sensitive current, reducing it from  $28.6 \pm 2.9\%$  ( $n=10$ ) to just  $3.1 \pm 2.5\%$  ( $n=5$ ;  $P < 0.001$ ), suggesting that gabapentin inhibition is mediated mainly by N-type current



**Figure 4** (a) Mean  $\pm$  s.e. mean ramp-activated whole-cell currents (0.9 ms/mV) from  $-50$  mV under control conditions ( $n=4$ ) and following a 10 min application of gabapentin ( $25 \mu\text{M}$ ;  $n=3$ ). Solid line indicates Boltzmann fit to the data. (b) Mean ( $\pm$  s.e. mean;  $n=6$  determinants) I/V relationship for whole-cell voltage-gated Ca<sup>2+</sup> currents ( $2 \text{ mM Ca}^{2+}$ ) recorded under control conditions and following a 3–5 min application of gabapentin by low pressure ejection ( $2.5 \mu\text{M}$ ;  $P<0.03$ ). (c) Inset records showing Ca<sup>2+</sup> currents recorded in the absence or presence of gabapentin ( $V_c = -30, -20$  and  $10$  mV). (d) Bar graph of mean inhibition ( $\pm$  s.e. mean;  $n=10$  determinants) of HVA I<sub>Ba</sub> measured at the peak vs the end of the current step. (e) Gabapentin-sensitive difference current obtained by subtracting peak HVA current traces recorded under control conditions and following a 10 min application of gabapentin ( $25 \mu\text{M}$ ).



**Figure 5** (a) Pharmacology of gabapentin-sensitive peak HVA I<sub>Ba</sub> (mean  $\pm$  s.e. mean). Clear bars represent mean data for inhibition of peak HVA I<sub>Ba</sub> with Ca<sup>2+</sup> channel antagonists (L-, P/Q- or N-type;  $n=6, 8$  and  $7$ , respectively). The black bar represents mean total current inhibited by gabapentin. Grey bars represent mean gabapentin-sensitive current remaining in the presence of L- (+ nif), P/Q- (+ IVA) or N- (+ GVIA) type blockers. (b) Time-course showing gabapentin inhibition of peak I<sub>Ba</sub> is virtually eliminated following block of the N-type current with  $\omega$ -CgTx GVIA. (c) Whole-cell current trace of peak HVA I<sub>Ba</sub> activated under control conditions and following sequential applications of  $\omega$ -CgTx GVIA ( $1 \mu\text{M}$ , GVIA) followed by gabapentin ( $25 \mu\text{M}$ ) in the presence of GVIA (GVIA + GBP).

(Figure 5b,c). However, prior inhibition of either L- or P/Q-type current also significantly reduced the effectiveness of gabapentin, although to a lesser extent, as gabapentin inhibition was just  $13.7 \pm 3.9\%$  and  $12.0 \pm 7.2\%$ , in the presence of nifedipine and  $\omega$ -Agatoxin IVA, respectively ( $n=5$ ,  $5$ ;  $P<0.05$ ). The mixed pharmacology of the gabapentin-sensitive current suggests that inhibition may reflect a direct action of gabapentin on a number of different sub-types of calcium channel, dominated by the N-type current in this cell-type.

## Discussion

This study is the first to demonstrate a potent inhibition by gabapentin of the Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels in DRG neurones. Voltage-gated Ca<sup>2+</sup> channels are critical in mediating both the development and maintenance of the neuronal sensitization process associated with neuropathic pain, and provide attractive candidates for the development of antihyperalgesic drugs (reviewed in Vanegas & Schaible, 2000). DRG neurones are thought to play a crucial role in pain processing (Reichling & Levine, 1999) and have been shown to express a variety of HVA Ca<sup>2+</sup> channel  $\alpha_1$  subunits (Mintz *et al.*, 1992; Rusin & Moises, 1995). Recordings taken from dorsal horn neurons in spinal cord slices from adult hyperalgesic rats or neonatal control slices have confirmed a presynaptic (DRG) site of action for gabapentin (Patel *et al.*, 2000; Shimoyama *et al.*, 2000), and depression of presynaptic Ca<sup>2+</sup> currents participating in nociceptive synaptic transmission may well be expected to contribute to the antihyperalgesic action of gabapentin. Nevertheless, previous studies indicated that gabapentin had no effect on freshly dissociated DRG neurones, suggesting that inhibition of Ca<sup>2+</sup> channels in this cell-type did not contribute to its mechanism of action (Rock *et al.*, 1993). More recently however, a decrease in Ca<sup>2+</sup> channel current has been detected in DRG neurones by a number of different groups using both electrophysiological and Ca<sup>2+</sup> imaging techniques (Martin *et al.*, 2000; McClelland *et al.*, 2000; Sarantopoulos *et al.*, 2000; Sutton *et al.*, 2000). Under conditions used in the present study, gabapentin potently inhibited voltage-dependent Ca<sup>2+</sup> influx in DRG neurones, with effective reductions achieved using nM concentrations of drug.

The mixed pharmacology of the gabapentin-sensitive Ca<sup>2+</sup> current in DRG neurones suggests that inhibition may reflect a direct action of gabapentin on a number of different channel sub-types. Of particular interest was the unique sensitivity of the  $\omega$ -CgTx GVIA-sensitive, N-type current to inhibition by gabapentin. N-type Ca<sup>2+</sup> channels are believed to play an important role in mediating neuropathic pain (Vanegas & Schaible, 2000) and the potential for gabapentin-dependent modulation of presynaptic N-type channels in DRG neurones would prove an important means for mediating neuronal excitability and neurotransmitter release (Dunlap *et al.*, 1995). In this study, reduced sensitivity to gabapentin was also demonstrated following inhibition of other subtypes of HVA Ca<sup>2+</sup> channel (P/Q- and L-type). Not only did the gabapentin-responsive current exhibit mixed Ca<sup>2+</sup> channel pharmacology, there was also a degree of overlap in the sensitivity of gabapentin inhibition to various Ca<sup>2+</sup> channel blockers. These findings are supported by a recent paper by Stefani *et al.* (2001), who also noted an apparent overlap in the Ca<sup>2+</sup>-

dependent pharmacology of gabapentin action in isolated rat cortical neurones. In the current study, the reduced gabapentin sensitivity observed following the use of specific Ca<sup>2+</sup> channel antagonists might reflect an additional, more indirect influence of the blockers on the gabapentin-sensitive target. Activation of voltage-dependent Ca<sup>2+</sup> influx *per se* could participate in 'priming' the cell for gabapentin action, and the addition of Ca<sup>2+</sup> channel inhibitors could play a secondary role in altering any Ca<sup>2+</sup>-dependent modulation of the level of gabapentin responsiveness of the cell.

The interesting observation of the relatively non-inactivating nature of the gabapentin-sensitive current given its mixed pharmacology, can be explained accordingly. The binding site for gabapentin is located on the accessory  $\alpha_2\delta$  subunit and not the major  $\alpha_1$  pore-forming unit of the channel. Moreover, DRG neurones have been shown to express a mixed population of voltage-gated Ca<sup>2+</sup> channels consisting of a number of different subtypes of  $\alpha_1$  subunits. The  $\alpha_2\delta$  subunits (1–3) have all been shown to functionally associate with a variety of different  $\alpha_1$  subunits (Gurnett *et al.*, 1996; Dolphin *et al.*, 1999; Klugbauer *et al.*, 1999; Gao *et al.*, 2000; reviewed in Walker & De Waard, 1998; Felix, 1999). Through its interaction with  $\alpha_2\delta$ , gabapentin may therefore indirectly affect a heterogeneous mix of  $\alpha_1$  subunits as no preferential partnership between  $\alpha_1$  and  $\alpha_2\delta$  subtypes has yet been demonstrated. Co-expression experiments have shown that functional association with  $\alpha_2\delta$  affects comparable changes in current amplitude and voltage-dependent properties of a variety of different  $\alpha_1$  subunits (Walker & De Waard, 1998; Felix, 1999). Likewise in a similar manner, through its interaction with  $\alpha_2\delta$ , gabapentin might also be inferred to produce a similar overall trend of a decrease in current amplitude carried by a variety of different  $\alpha_1$  subunits, regardless of the underlying biophysical properties of the  $\alpha_1$  subunit. This data therefore suggests that in DRG neurones, gabapentin may modulate a mixture of different  $\alpha_1$  subunits producing a reduction of a non-inactivating high-voltage activated component of the whole-cell current. This finding is supported by previous work, whereby gabapentin has been shown to inhibit disparate components of L- vs P/Q- and N-type current in different preparations (Stefani *et al.*, 1998; Fink *et al.*, 2000).

In support of these findings, the mixed ability of gabapentin to inhibit Ca<sup>2+</sup> channels has been demonstrated previously for a number of different preparations. Stefani *et al.* (1998) recorded a differing sensitivity of rat brain neurones to gabapentin and no detectable effects of gabapentin were observed on Ca<sup>2+</sup> channel currents recorded from hippocampal neurones taken from patients with temporal lobe epilepsy (Schumacher *et al.*, 1998). Gabapentin inhibition of synaptosomal P/Q-type Ca<sup>2+</sup> current and effects on neurotransmitter release have also been shown to be both tissue-specific and dependent upon the stimulus protocol (Dooley *et al.*, 2000; Fink *et al.*, 2000; Meder & Dooley, 2000; Maneuf & Mcknight, 2000). The recent identification of three different subtypes of  $\alpha_2\delta$  provides a means for subunit-specific interactions with gabapentin, which could restrict its effects to more localized sites of action. (Klugbauer *et al.*, 1999; Su *et al.*, 2000). Moreover, sub-cellular distribution and tissue specificity of Ca<sup>2+</sup> channel subunit combinations have not yet been determined. Thus, gabapentin may require particular subunit variants ( $\alpha_1$ ,  $\alpha_2\delta$ , and  $\beta$ ), coupled with the presence of additional accessory proteins and/or activation of

second messenger cascades to be effective (Sutton *et al.*, 2000; Reichling & Levine, 1999). Consequently, the pre-requisite of distinct, permissive conditions may well act to limit the actions of gabapentin, in contrast to other established Ca<sup>2+</sup> channel ligands. Indeed, as a drug gabapentin is not effective in all patients (Laird & Gidal, 2000), a finding which could reflect a biovariability in its pharmacokinetic properties and/or the target binding interactions of gabapentin.

As the only ligand identified to-date that interacts with the accessory  $\alpha_2\delta$  subunit, gabapentin provides a unique, alternative candidate for mediating Ca<sup>2+</sup> influx to control the development and maintenance of chronic pain. In support of this theory, the differing  $\alpha_2\delta$  binding affinities of stereo-selective analogues of gabapentin correlate well with their structure-activity relationships in animal models of pain (Field *et al.*, 2000). Moreover,  $\alpha_2\delta$  subunits have been shown to be upregulated in dorsal horn tissue obtained from hyperalgesic rats (Philp *et al.*, 1999a,b; Luo *et al.*, 2000). Levels of mRNA, protein and [<sup>3</sup>H]-gabapentin binding sites were all shown to be increased and were accompanied by a corresponding increase in expression of the pore-forming  $\alpha_{1B}$  (N-type) subunit (Philp *et al.*, 2000). A finding mirrored in this study, by the observed sensitivity of the N-type current in DRG neurones to inhibition by gabapentin

Nevertheless, the affects of gabapentin are not restricted to a presynaptic  $\alpha_2\delta$  site of action. Postsynaptic affects on spinal synaptic transmission have also been demonstrated, whereby gabapentin has been shown to variably modulate either NMDA- or AMPA-mediated synaptic transmission in freshly dissociated DRG neurones, control slices and *in vivo* (Rock *et al.*, 1993; Chizh *et al.*, 2000; Shiyoma *et al.*, 2000). Most recently, gabapentin has also been shown to target a specific GABA<sub>B</sub> receptor heterodimer, promoting activation of a postsynaptic inwardly rectifying K<sup>+</sup> (Kir) conductance and

altering membrane excitability (Ng *et al.*, 2001). Nevertheless, experiments using saclofen undertaken in this study support previous findings that the actions of gabapentin on Ca<sup>2+</sup> channel currents in this preparation are not mediated *via* a corresponding interaction with the GABA<sub>B</sub> receptor (McClelland *et al.*, 2000). Gabapentin can also gain access to the intracellular cytoplasmic environment by virtue of its ability to substitute as a substrate for the system L amino acid transporter (Stewart *et al.*, 1993; Su *et al.*, 1995). As a consequence, accumulating concentrations of gabapentin could interact with any number of different cytoplasmic proteins and second messenger cascades, indirectly influencing Ca<sup>2+</sup> influx and/or other neuronal functions. Indeed, gabapentin has been shown to alter metabolic turnover and promote non-vesicular release of the inhibitory neurotransmitter, GABA (Taylor *et al.*, 1998; Honmou *et al.*, 1995a,b; Petroff *et al.*, 1996).

Thus, in summary, the overall mechanism of action for gabapentin may well reflect a combined action mediated by both extra and intracellular targets, which together provide a means for reducing overall neuronal excitability. However, the  $\alpha_2\delta$  subunit of the calcium channel is the only molecular target identified to date that interacts with gabapentin with high affinity. Moreover, as this study has demonstrated, in DRG neurones voltage-gated Ca<sup>2+</sup> channel current is sensitive to inhibition by gabapentin, which consequently provides a potential means for gabapentin to effectively mediate spinal anti-nociception.

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