Analysis of the Long-Term Actions of Gabapentin and Pregabalin in Dorsal Root Ganglia and Substantia Gelatinosa.

James E Biggs¹², Paul A. Boakye¹, Naren Ganesan¹⁴, Patrick L Stemkowski¹⁴, Aquilino Lantero Garcia⁵, Klaus Ballanyi¹³ and Peter A Smith¹²

Neuroscience and Mental Health Institute¹ and Department of Pharmacology² or Physiology³ Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Canada T6G 2H7,

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Corresponding author:

P.A. Smith,
Department of Pharmacology, 9-75 Medical Sciences Building,
University of Alberta, Edmonton, AB, Canada T6G 2H7
E-mail: peter.a.smith@ualberta.ca; Phone; 780 492 2643; FAX;780 492 4325

⁴ Present Address: Department of Physiology and Biophysics, Faculty of Medicine, Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada, H2N 2N1

⁵ Present Address: Institute of Pharmacy University of Innsbruck, CCB - Center for Chemistry and Biomedicine, Innrain 80-82, A-6020, Innsbruck, Austria
The α2δ-ligands, pregabalin (PGB) and gabapentin (GBP) are used to treat neuropathic pain. We used whole-cell recording to study their long-term effects on substantia gelatinosa and dorsal root ganglion (DRG) neurons. Spinal cord slices were prepared from E13 rat embryos and maintained in organotypic culture for > 5 weeks (neuronal age equivalent to young adult rats). Exposure of similarly-aged DRG neurons (dissociated and cultured from P19 rats) to GBP or PGB for 5-6d attenuated high-voltage activated calcium channel currents (HVA ICa). Strong effects were seen in medium-sized and in small, isolectin B4 negative (IB4-) DRG neurons whereas large neurons and small neurons that bound isolectin B4 (IB4+) were hardly affected. 100μM GBP or 10μM PGB were less effective than 20μM Mn2+ in suppression of HVA ICa in small DRG neurons. By contrast, 5-6d exposure to these α2δ-ligands was more effective than 20μM Mn2+ in reducing spontaneous EPSCs at synapses in substantia gelatinosa. Spinal actions of gabapentinoids cannot therefore be ascribed to decreased expression of HVA Ca2+ channels in primary afferent nerve terminals.

In substantia gelatinosa, 5-6d exposure to PGB was more effective in inhibiting excitatory synaptic drive to putative excitatory neurons than to putative inhibitory neurons. Although sIPSC’s were also attenuated, the overall long-term effect of α2δ–ligands was to decrease network excitability as monitored by confocal Ca2+ imaging. We suggest that selective actions of α2δ-ligands on populations of DRG neurons may predict their selective attenuation of excitatory transmission onto excitatory versus inhibitory neurons in substantia gelatinosa.

**Keywords:** Neuropathic Pain; Nerve Injury; Patch-clamp; Organotypic Cultures; Alpha 2 delta; Ca2+ channel.
INTRODUCTION

Neuropathic pain is a maladaptive consequence of injury to, or disease of the somatosensory system (Costigan et al., 2009; Sandkuhler, 2009; Treede et al., 2008). Because it is characteristically resistant to the actions of conventional analgesics, treatment often involves the use of ‘anti-allodynic’ agents such as pregabalin (PGB) or gabapentin (GBP) (Moulin et al., 2007). In the clinic, these drugs take at least 3 days to exert an effect (Cheshire, 2002; Sharma et al., 2010).

Gabapentinoids are transported into neurons via the neutral amino acid transporter (Su et al., 1995) where they interact with the α2δ−1 accessory subunits of voltage-gated calcium channels (Bauer et al., 2009; Davies et al., 2007; Field et al., 2006; Gee et al., 1996; Hendrich et al., 2008; Patel et al., 2013). α2δ−1 subunits are involved in the trafficking of Cav2.2 channels to the cell surface (Cassidy et al., 2014; Hoppa et al., 2012) and signs of neuropathic pain induced by nerve injury have been associated with their upregulation in primary afferent terminals in the dorsal horn (Bauer et al., 2009; Luo et al., 2001; Zhou and Luo, 2014). Current hypotheses for the mechanism of gabapentinoid action suggest they interact with multiple α2δ-mediated trafficking processes (Cassidy et al., 2014; Hendrich et al., 2008; Hoppa et al., 2012; Patel et al., 2013; Tran-Van-Minh and Dolphin, 2010) and that both rapid (<30min) (Sutton et al., 2002) and slowly developing effects (> 40 hours) (Hendrich et al., 2012) can be observed in vitro. Interaction of gabapentinoids with α2δ−1 subunits also appears to interrupt interactions between HVA-Ca\(^{2+}\) channels and the vesicular release machinery (Hoppa et al., 2012; Matsuzawa et al., 2014). This results in decreased release of neurotransmitters from primary afferent terminals and presumably at other synapses in the spinal dorsal horn.
Acutely applied GBP (100μM) has little or no effect on stimulus-evoked EPSC’s in nociceptive neurons in lamina II of the spinal dorsal horn (Moore et al., 2002). Frequency and amplitude of spontaneous EPSCs (sEPSC) and IPSCs (sIPSC) are similarly unaffected. Acute application of PGB (100μM) also failed to affect sEPSC’s activated by capsaicin in co-cultures of dorsal horn and dorsal root ganglion (DRG) neurons (Hendrich et al., 2012). By contrast, exposure of these co-cultures to PGB for 40-48h strongly reduced sEPSC frequency. Because chronic, but not acute, exposure to high (1mM) concentrations of GBP inhibits CaV2.1 and CaV2.2 currents expressed in TSA 201 cells (Hendrich et al., 2008), it is tempting to speculate that decreased voltage-gated Ca\(^{2+}\) influx in primary afferent terminals is causally related to decreased neurotransmitter release (Bauer et al., 2009; Hendrich et al., 2012). If gabapentinoids do work in this way, Ca\(^{2+}\) channel blockade with a divalent cation such as Mn\(^{2+}\) should mimic their long-term effects on synaptic transmission in the dorsal horn. We therefore compared the reduction in HVA \(I_{Ca}\) seen in DRG neurons following 5-6d exposure to gabapentinoids to that produced by a relatively low concentration of Mn\(^{2+}\)(20μM). We then examined the effects of gabapentinoids and 20μM Mn\(^{2+}\) on spontaneous EPSC’s (sEPSC) in neurons in spinal cord slices maintained in defined medium organotypic culture (DMOTC; Biggs et al., 2012; Lu et al., 2006).

The current understanding of long-term gabapentinoid action (Bauer et al., 2009; Hendrich et al., 2008; Patel et al., 2013) has also yet to be integrated with what is known about nociceptive processing both at the spinal and peripheral level (Braz et al., 2014; Prescott et al., 2014; Todd, 2010; Zeilhofer et al., 2012). If gabapentinoids were to produce similar decreases neurotransmitter release from all primary afferent terminals, they would produce an anaesthetic rather than an anti-allodynic effect. Gabapentinoids might therefore be expected to produce different effects on HVA \(I_{Ca}\) in subsets of DRG neurons and this may predict their actions at different types of primary
afferent terminals in the dorsal horn. We therefore compared the long-term actions of
gabapentinoids on HVA I$_{Ca}$ in small, medium and large DRG neuron in defined-medium culture.
Small neurons were further subcategorized into those that bound the plant lectin, IB4 (IB4$^+$) and
those that did not (IB4$^-$) (von Banchet and Schaible, 1999). Intense IB4 binding was originally
reported to distinguish small C-fibre nociceptors (Fang et al., 2006).
Lastly, within the dorsal horn, the **substantia gelatinosa** contains both excitatory and
inhibitory neurons and both receive monosynaptic excitatory input from nociceptive primary
afferent fibres (Braz et al., 2014; Lu and Perl, 2003; Lu and Perl, 2005; Todd, 2010). Inhibitory
neurons also receive low threshold excitatory input (Daniele and MacDermott, 2009). In view of
their anti-allodynic action, gabapentinoids may exert preferential effects at excitatory synapses and
excitatory neurons as opposed to inhibitory synapses and inhibitory neurons. To test these
possibilities, we examined long-term actions of gabapentinoids on overall dorsal horn excitability
using confocal calcium imaging (Biggs et al., 2012) as well as whole-cell recordings from putative
excitatory and inhibitory **substantia gelatinosa** neurons in spinal cord slices maintained in
DMOTC.
A preliminary report of some of these findings has appeared (Biggs et al., 2008)

**METHODS.**
All procedures were carried out in compliance with the guidelines of the Canadian Council
for Animal Care and with the approval of the University of Alberta Health Sciences Laboratory
Animal Services Welfare Committee.

**DRG Neuronal Cultures, Drug Treatment and Electrophysiology**
Experimental procedures for establishing long-term defined medium cultures of DRG neurons were similar to those previously described.

http://www3.interscience.wiley.com/login.ezproxy.library.ualberta.ca/cgi-bin/fulltext/119027693/main.html,ftx_abs - b8 (Stemkowski and Smith, 2012a). Male Sprague-Dawley rats (18–20 days old) were euthanized with a high dose of 1.5 g/kg ethyl carbamate (urethane; IP; Sigma, St. Louis, MO, USA). DRGs (14-21/animal) were aseptically dissected from lower thoracic (T6-12) and lumbar (L1-6) spinal segments and collected in DMEM supplemented with 10% heat-inactivated horse serum (DMEMHS; both from Gibco, Grand Island, NY, USA).

Ganglia were treated for 1.5 h at 34 °C with 0.125 % type IV collagenase (Worthington, Lakewood, NJ, USA), washed twice in Ca2+ free PBS, treated with 0.25 % trypsin from bovine pancreas (Sigma) in PBS for 30 min, washed 3 times in DMEMHS and finally taken up in 2 ml of DMEMHS containing 80 μg/ml type IV DNAse (Sigma) and 100 μg/ml soybean trypsin inhibitor (Worthington). A single cell suspension was obtained by trituration of the enzymatically softened ganglia by 6-8 passages through the tip of a 1 ml Eppendorf pipette.

Neuronal cell enrichment was achieved by differential cell adhesion and treatment with anti-mitotic agents, followed by differential sedimentation procedures. Thus, dissociated neurons were plated in DMEMHS supplemented with the anti-mitotic combination, cytosine 3-D-arabinofuranoside (Ara-C), uridine and 5-fluoro-2’-deoxyuridine (all from Sigma and all at 10 μM) in two 50mm culture dishes (preplates; Corning, NY, USA) previously coated with 3 μg/ml polyornithine (Sigma). After 15-20 h, the non-neuronal cells become firmly attached to the dish, while most of the neurons were only weakly adherent to the dish or to flattened non-neuronal cells. Then, by carefully removing the culture medium, most dead cells and axonal / myelin debris were discarded prior to selectively dislodging the attached neurons with a gentle stream of serum free defined
medium (DMEM supplemented with 1/100 N-2 supplement and 1/100 penicillin /streptomycin/
amphotericin B; all from Gibco) delivered from a 1 ml Eppendorf pipette. The neurons from the
two preplates were collected in a total of 12 ml of defined medium in a conical test tube. Further
neuronal enrichment was achieved by centrifugation of the cell suspension at 500 rpm for 5 min,
whereupon viable neurons were lightly pelleted, leaving myelin debris, dead cells and small non-
neuronal cells in suspension. The supernatant was discarded and the cells were then re-suspended
in 1 ml of defined medium. At 100μl volumes, the cells were plated into 35 mm tissue culture
dishes ("Nunclon surface," VWR International, Mississauga, Ontario Canada) pre-coated with
3μg/ml polyornithine (Sigma) and 2 μg/ml laminin (Sigma). All dishes were then filled with a
neurotrophin- and serum-free defined medium at ~ 2 ml/dish. Cells were maintained at 36.5 °C,
95 % air, 5% CO₂. Defined medium was exchanged on days 1 and 3 and cultures maintained for 4
days.

For chronic treatment with GBP, a 10mM stock solution was prepared in saline and 10μl
added to 10 ml of neurotrophin- and serum-free defined medium to obtain final concentration of
100μM. Similarly, a final concentration of 10μM PGB was prepared from a 1mM stock solution.
Concentrations of 70 -120μM GBP and 6-60μM PGB in vitro correspond to those found in the
serum of patients using these drugs (Bockbrader et al., 2010; Johannessen et al., 2003).

Gabapentinoid containing medium was added on days 1 and 3 of culture (Fig 1). Control
cultures received 10μl of filtered saline which was added to the medium instead of the drug.

Figure 1 near here
Whole-cell recordings (at room temperature, 22°C) were made using an Axoclamp 2A amplifier in discontinuous single-electrode voltage-clamp mode as described previously (Abdulla and Smith, 1997). Patch pipettes were pulled from thin-walled borosilicate glass (WPI, Sarasota, FL, USA) and with low-resistance patch electrodes (2-5 MΩ), it was possible to use high switching frequencies >30 kHz with clamp gains as high as 30 mV/nA. DRG neurons were superfused at a flow rate of 2 ml/min with an extracellular solution containing (mM): 127 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 1.3 MgSO₄, 26 NaHCO₃, 25 D-Glucose, 2.5 CaCl₂, pH 7.2. The total volume of fluid in the recording dishes was about 1 ml. In order to record HVA I_{Ca} using Ba^{2+} as the change carrier, the superfusate was switched to an external solution which contained (in mM): 160 TEA-Cl, 10 HEPES, 2 BaCl₂, 10 glucose, adjusted to pH 7.4 with TEA-OH. Internal (pipette) solution contained (in mM): 120 CsCl, 5 Mg-ATP, 0.4 Na-GTP, 10 EGTA, 20 HEPES-CsOH, pH 7.2. DRG neurons were classified according to soma diameter as ‘small’ (< 30 μm), ‘medium’ (30-40 μm) or large (> 40 μM) as measured with a calibrated micrometer on the eye piece of a Nikon TE300 inverted fluorescence microscope (Nikon, Toronto, ON, Canada). Further sub-classification of small neurons into isolectin B4 (IB₄) positive and negative subtypes was achieved by adding IB₄-Alexa Fluor® 488 conjugate (Invitrogen, Eugene, OR, USA) to selected DRG cell cultures on day 1 (preplates for 30 min before removal) (Fjell et al., 1999). Neurons were observed under fluorescence microscopy. To limit misclassification, only the most intensely stained small DRG neurons were considered IB₄-positive.

Data were acquired using Pclamp 10.1 (Axon Instruments, Foster City, CA, USA), and final data records were produced using Origin 9.1 (Microcal, Northampton, MA, USA). Current densities were expressed as pA/pF. Input capacitance (C_{in}) was calculated by integrating the
capacitive current transient produced by a 10mV voltage step ($\Delta V$) to yield charge ($Q$) and using the equation $C_{in} = Q/\Delta V$.

Spinal Cord Organotypic Slice Culture Preparation, Drug Treatment and Electrophysiology.

 Defined medium organotypic cultures (DMOTC) of rat spinal cord slices with attached dorsal root ganglia (DRG) were prepared as previously described (Biggs et al., 2012; Lu et al., 2006). Briefly, embryonic day 13 (E13) rat fetuses were delivered by caesearean section from timed-pregnant female Sprague-Dawley rats (Charles River, Saint-Constant, QC, Canada) under 5% isoflurane anaesthesia. The dam was subsequently euthanized by cervical dislocation. The entire embryonic sac was placed in chilled Hank’s buffered salt solution containing (mM): 138 NaCl, 5.33 KCl, 0.44 KH$_2$PO$_4$, 0.5 MgCl$_2$-6H$_2$O, 0.41 MgSO$_4$-7H$_2$O, 4 NaHCO$_3$, 0.3 Na$_2$HPO$_4$, 5.6 d-Glucose and 1.26 CaCl$_2$. Individual rat fetuses were removed from their embryonic sac and rapidly decapitated. The spinal cord from each fetus was isolated in the above solution and sliced into 275-325μM transverse slices using a tissue chopper (McIlwain, St Louis, MO, USA). Only lumbar spinal cord slices with an intact spinal cord and two attached DRG were chosen and trimmed of excess ventral tissue and allowed to recover for 1 hour at 4°C. Each embryonic spinal cord slice was plated on a single glass coverslip (Karl Hecht, Sondheim, Germany) and attached with a clot of reconstituted chicken plasma (lyophilized; Sigma, St Louis, MO, USA) and thrombin (200 units ml$^{-1}$; Sigma, St Louis, MO, USA). Coverslips were then inserted into flat-bottomed tissue culture tubes (Nunc-Nalgene International, Mississauga, ON, Canada) filled with 1ml of medium, and then placed into a roller drum rotating at 120 rotations per hour in a dry heat incubator at 36°C. The medium in the tubes was composed of 82% Dulbecco’s modified Eagle’s
medium (DMEM), 10% fetal bovine serum and 8% sterile water (all from Gibco, Grand Island, NY, USA). The medium was supplemented with 20 ng ml\(^{-1}\) NGF (Alomone Laboratories, Jerusalem, Israel) for the first 4 days, and omitted thereafter. Antibiotic and antimycotic drugs (5 units ml\(^{-1}\) penicillin G, 5 units ml\(^{-1}\) streptomycin and 12.5 ng ml\(^{-1}\) amphotericin B, Gibco) were also included in the media during the first four days of culture. After 4 days in culture, DMOTC slices were treated with an antimitotic drug cocktail consisting of uridine, cytosine-b-D-arabino-furanoside (AraC), and 5-fluorodeoxyuridine (all at 10\(\mu\)M) for 24 hours to prevent the overgrowth of glial cells. During antimitotic/antibiotic treatment, the serum medium was progressively switched (first diluted 50:50 after 4 days, then completely exchanged after 5 days) to a defined, neurotrophin- and serum-free medium consisting of Neurobasal medium with N-2 supplement and 5 mM Glutamax-1 (all from Gibco). The medium within these tubes was exchanged regularly with freshly prepared medium every 3-4 days.

DMOTC slices were treated after a 15-21 days \textit{in vitro} for a period of 5-6 days with either GBP (1-100\(\mu\)M) or PGB (1-100\(\mu\)M) with or without BCH (300\(\mu\)M). Thus, the age of the neurons in the spinal DMOTC corresponds to that of DRG neurons described above. The gabapentinoid containing medium was exchanged on the third treatment day and both spinal DMOTC and DRG neuron cultures received similar exposures to gabapentinoids (Fig 1). Age-matched untreated DMOTC slices served as controls. In some experiments, cultures were exposed to brain derived neurotrophic factor (BDNF, 200 ng ml\(^{-1}\), Alomone Labs, Jerusalem Israel) for 4-5 d (Lu et al., 2007; Lu et al., 2009a). Other cultures were exposed acutely (for 90s) to ATP (100\(\mu\)M), \textit{Trans} ACPD (\(\pm\)-1-aminocyclopentane-\textit{trans}-1,3-dicarboxylic acid, 25\(\mu\)M, Tocris), or Epinephrine (25\(\mu\)M).
Whole-cell patch-clamp recordings were obtained from neurons in DMOTC immediately after 5-6 days of gabapentinoid treatment. The use of infrared differential interference contrast optics allowed neurons to be selected under visual control. Neurons selected for recording were located ~250-800 μm from the dorsal edge of the cultures in an area presumed to reflect the substantia gelatinosa and up to a depth of 100 μm from the surface. Recordings were obtained using an NPI SEC-05L amplifier (npi Electronic Gmbh, Tamm, Germany) in discontinuous current- or voltage-clamp mode. For recording, slices were superfused at room temperature (~22°C) with 95% O₂-5% CO₂ saturated aCSF which contained (in mM): 127 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 26 NaHCO₃, 1.3 MgSO₄, 2.5 CaCl₂, 25 D-glucose, pH 7.4. Patch pipettes were pulled from thin-walled borosilicate glass (1.5/1.12 mm OD/ID, WPI, Sarasota, FL, USA) to 5-10 MΩ resistances when filled with an internal solution containing (in mM): 140 CsCl or 140 KCl, 5 HEPES, 10 EGTA, 2 CaCl₂, 2 Mg-ATP, 0.3 Na-GTP, pH 7.2, (290-300 mOsm). In some experiments, the use of Cs⁺ within the patch pipette precluded identification of neuronal types on the basis of their firing pattern in response to depolarizing current commands (Lu et al., 2007; Lu et al., 2006; Yasaka et al., 2010). In view of this, neurons were defined as high rheobase (>70pA) or low rheobase (<30pA). This afforded a method for distinguishing tonic firing inhibitory interneurons from higher rheobase, putative excitatory, delay firing neurons (Lu et al., 2007; Punnakkal et al., 2013; Yasaka et al., 2010).

Spontaneous excitatory postsynaptic currents (sEPSC) were recorded for 3 minutes with the neuron clamped at -70mV and spontaneous inhibitory synaptic currents (sIPSC) were recorded at 0mV (Lu et al., 2009b; Lu et al., 2012). Data were acquired using Axon Instruments pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA, USA). Synaptic events were analyzed using Mini Analysis™ software (Synaptosoft, Decatur, GA, USA). Peaks of events were first
automatically detected by the software according to a set of threshold criteria. All detected events were then visually re-examined and accepted only if they displayed a monophasic rise time to peak <25ms, a smooth offset and an amplitude > three times the background noise. Organotypically cultured spinal cord cultures derived from the embryos of each dam were used over a two week period. 5-8 slices. On the day of each experiment, studies were carried out on both control slices and those treated with PGB

Confocal Ca\(^{2+}\) imaging.

Ca\(^{2+}\) imaging experiments on DMOTC were carried out immediately after 5-6 days of gabapentinoid treatment. A single DMOTC slice was incubated for 1h prior to imaging with the membrane-permeant acetoxymethyl form of the fluorescent Ca\(^{2+}\)-indicator dye Fluo-4 (5mM Invitrogen, Canada). The conditions for incubating the dye were standardised across different slices to avoid uneven dye loading. After dye loading, the DMOTC slice was transferred to a recording chamber and perfused with external solution containing (mM): 131 NaCl, 2.5 KCl, 1.2 NaH\(_2\)PO\(_4\), 1.3 MgSO\(_4\), 26 NaHCO\(_3\), 25 D-glucose, and 2.5 CaCl\(_2\) (20°C, flow rate 4 ml min\(^{-1}\)). Changes in Ca\(^{2+}\)-fluorescence intensity were measured in dorsal horn neurons with a confocal microscope equipped with an argon (488nM) laser and filters (20x XLUMPlanF1-NA-0.95 objective; (Olympus FV300, Markham, Ontario, Canada). Changes were evoked by one of two methods; 1). Application of high K\(^+\) solution (20 or 35 mM) for 90 seconds or 2). electrical stimulation of the dorsal root entry zone (DRZ) (Biggs et al., 2012). Tungsten bipolar electrodes were used (WPI, Sarasota, FL, USA), the stimulating parameters were; frequency, 50Hz; pulse duration 100μs; number of pulses, 250. The stimulating electrode was repositioned until a reliable synaptic input to the region of interest was found. The threshold voltage to evoke a response was
determined and twice this voltage used to examine the excitability of the dorsal horn neurons. Typical stimulus intensities ranged between 10 and 60V. A total of 8 stimulus trains (S1-S8), were delivered at 5min intervals. Since the most stable response amplitudes were seen with the 5th and 6th stimuli (S5 and S6), responses to the 5th set of stimuli were used for all data comparisons (Biggs et al., 2012). Full frame images (512 x 512 pixels) in a fixed xy plane were acquired at a scanning time of 1.08 s per frame and stored as video files for off-line analysis (Ruangkittisakul et al., 2006). Videos were replayed and selected regions of interest drawn around distinct cell bodies and fluorescence intensity traces were generated with FluoView v 4.3 (Olympus).

To maximize objective sampling of cells for analysis, organotypically cultured spinal cord slices derived from the embryos of each dam, were used over a two week period. 5-8 slices, each previously subject to a different experimental protocol were studied on each experimental day. (i.e. each day slices were selected from the cohort of controls, or those treated with GBP, PGB, capsaicin, BCH or BDNF) and data were collected from at least 5 cells in each slice.

**Immunohistochemistry.**

DAB (3, 3'-diaminobenzidine) immunohistochemistry was adapted from previously published methods used in organotypic cultures (Avossa et al., 2003). Spinal cord DMOTC were maintained in vitro for 2 weeks and then fixed with 4% paraformaldehyde in PBS overnight at 4°C. The following day, cultures were rinsed three times in 1% PBS for 10 minutes each time at room temperature with gentle shaking. They were then incubated in 3% H₂O₂ in 1% PBS for 10 min at room temperature with no shaking. Following the H₂O₂, they were again rinsed 3 times with 1% PBS and incubated in 10% normal goat serum, 0.2% Triton X-100 in PBS (blocking solution) at room temperature for 1 hour. Immediately following the block, cultures were incubated with
either mouse Neuronal Nuclei (NeuN-Millipore) or rabbit Glial Fibrillary Acidic Protein (GFAP-
DAKO- kindly provided by Dr. Bradley Kerr) in 2% normal goat serum, 0.2% Triton X-100, and
100mg/ml bovine serum albumin at a 1:500 dilution. The NeuN antibody specifically recognizes
the DNA-binding, neuron-specific protein NeuN, which is specific to neuronal nuclei. The GFAP
antibody labels the intermediate filament protein expressed by numerous central nervous system
cell types including astrocytes and ependymal cells. Incubation with both antibodies was
overnight, approximately 18 hours, at room temperature in a humid chamber. Following
incubation, the cultures were washed 3 times with 1% PBS at room temperature while gently
rocking. They were then incubated with the corresponding mouse or rabbit biotinylated secondary
antibodies (Vector Laboratories) at a 1:200 dilution in the same 2% normal goat serum, 0.2%
Triton X-100, and 100mg/ml bovine serum albumin solution as the primary antibodies for 1.5hrs.
The cultures were then washed 3 times in 1% PBS for 10 minutes each time at room temperature
while gently rocking and then incubated with the Vectastain Elite ABC kit (Vector Laboratories)
dilated at 1:100 in PBS for 2 hours. Cultures were then washed again 3 times for 10 minutes each
time in 1% PBS, and then incubated for approximately 45 seconds in the developing
diaminobenzidine kit (Vector). The reaction was stopped by washing 3 times for 10 min each time
in 1% PBS. The cultures were dehydrated by subsequent 1minute treatments in 50, 70, 90%
ethanol, 50/50% ethanol and xylene, and 100% xylene and mounted with Permount mounting
medium. Images were captured using a Zeiss Axiocam MRm camera (Carl Zeiss, Oberkochen,
Germany) using a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen,
Germany).

Drugs and Chemicals.
Unless otherwise stated, all chemicals were from Sigma (St Louis, MO, USA). GBP was from TCI America, Portland OR, USA and PGB was received as a gift from Pfizer Global, Groton CT, USA.

Statistical Analysis

Statistical analysis was carried out using Student’s two tailed unpaired t-test or one way ANOVA with Tukey-Kramer Multiple comparisons test as appropriate. Cumulative probability plots for sEPSC’s and sIPSC’s were analyzed using Kolmogorov-Smirnoff (KS) statistics.

RESULTS

Gabapentinoids exert preferential long-term effects on HVA- \( I_{Ca} \) in medium-sized and small IB4 negative, DRG neurons.

To test whether long-term gabapentinoid exposure exerted differential effects on different types of primary afferent neuron, we compared their long-term actions on HVA- \( I_{Ca} \) using \( Ba^{2+} \) as a charge carrier (HVA-\( I_{Ba} \)) in small, medium and large DRG neurons. Small neurons were further subdivided into those that bound the plant lectin, IB4 (IB4+) and those that did not (IB4-)(von Banchet and Schaible, 1999). Maximum HVA-\( I_{Ba} \) density (at -10mV) was determined from current-voltage plots.

Exposure to 10 \( \mu M \) PGB for 5-6 d reduced maximal HVA-\( I_{Ba} \) density in small IB4- neurons to 67% of control amplitude (from 28.0±3.0 (n=10) to 18.8±2.5 pA/pF (n=12) \( p<0.05 \) Fig 2a). By contrast, no effect was seen on small IB4+ neurons. Peak current density in control small IB4+ neurons (22.2±2.0 pA/pF, n=14) did not differ from that seen in PGB treated neurons (22.5±3.8 pA/pF; \( p>0.9 \) Fig 2b).
The strongest effect of 10 μM PGB was seen in medium-sized neurons where it reduced HVA-IB$_{Ba}$ density to 40% of control amplitude. Thus maximal HVA-IB$_{Ba}$ density in medium neurons was reduced from 28.8±3.6 (n=11) to 11.6±2.4 pA/pF (n=22; p<0.0005; Fig 2c).

Large neurons (like small IB$_4^+$ neurons), were insensitive to the action of PGB. Maximal current density in control large neurons (30.0 ± 3.3 pA/pF, n=6) did not differ from that seen in PGB treated neurons (33.0± 8.7pA/pF; n =11; p > 0.8 Fig 2d).

Long-term actions of gabapentinoids on Ca$^{2+}$ channel currents depend on their entry into neuronal cytoplasm via the system L-amino acid transporter (Hendrich et al., 2008). In confirmation of this, we found that the effect of GBP on HVA-IB$_{Ba}$ in medium neurons was prevented in the presence of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH, 300μM); a specific blocker of this transporter (Hendrich et al., 2008). 100μM GBP reduced maximal HVA-IB$_{Ba}$ density in medium neurons to from 27.0±7.4 (n=13) to 6.8±3.9 pA/pF (n =13; p<0.02 Fig 2e). Maximal IB$_{Ba}$ density at -10mV in BCH-treated medium neurons was 41.0±2.4pA/pF (n=12) and this was unchanged in BCH plus 100μM GBP (39.1±3.8pA/pF; n=14; p>0.65; Fig 2f).

Fig 2g illustrates a typical family of HVA-IB$_{Ba}$ evoked in response to depolarizing voltage commands in a small IB$_4^-$ DRG neuron and Fig 2h illustrates similar currents recorded in another small IB$_4^-$ neuron following its exposure to 100μM GBP for 6 days. As noted by others (Hendrich et al., 2008), currents recorded in in the presence of gabapentinoids displayed less inactivation than controls. Fig 2i illustrates normalized and superimposed currents recorded at -10mV from Figs 2g and h. The difference in the rate of inactivation is clearly apparent. Inactivation was quantified by measuring the ratio of the peak to the end-of-pulse current as illustrated in Fig 2j. Changes in inactivation in response to 10μM PGB were highly significant for medium neurons, significant for IB$_4^+$ and IB$_4^-$ neurons but not for large neurons (Table 1).
Effects of Mn$^{2+}$ on HVA $I_{Ba}$ in DRG Neurons.

As illustrated in sample data records taken from a small DRG neuron (Fig 3a) Mn$^{2+}$ was highly effective in blocking HVA $I_{Ba}$ (ED$_{50}$ $\leq$ 10$\mu$M). Acute effects of various concentrations of Mn$^{2+}$ and chronic effects of 10$\mu$M pregabalin on small DRG neurons are compared in Fig 3b. Even the lowest concentration of Mn$^{2+}$ (20$\mu$M) was more effective than 10$\mu$M PGB in inhibiting HVA-$I_{Ba}$.

Long-term effects of gabapentinoids on excitatory synaptic transmission in substantia gelatinosa.

We next tested whether long-term gabapentinoid exposure reduced release of excitatory neurotransmitters in the substantia gelatinosa region of the spinal cord. We first examined whether there was any long-term effect of 10$\mu$M PGB on monosynaptic EPSC’s evoked by stimulation of the dorsal root entry zone. However, in view of the documented large variability in amplitude of such responses, (Lu and Perl, 2005), the difference in amplitude between the control and PGB treated group failed to attain statistical significance (data not shown).

In view of this, we examined effects of 10$\mu$M PGB on spontaneous excitatory synaptic currents (sEPSC). These responses were completely eliminated by 10$\mu$M CNQX (data not shown), Fig 4a illustrates typical recordings of sEPSC’s at -70mV. The main effect of 5-6d exposure to 10$\mu$M PGB was to reduce sEPSC amplitude in high threshold (putative excitatory) neurons by $\sim$25% ($p<0.0001$ KS test; Fig 4b and $p<0.0001$ by t-test on average amplitudes; Fig 4b inset).
All other effects of PGB were much smaller. Thus, the inter-event interval (IEI) of sEPSC’s in high threshold neurons was only slightly affected and was actually decreased by ~12% (Fig 4c and inset; \( p<0.0001 \) KS test and \( p<0.05 \) for average IEI, t-test). In putative inhibitory, low threshold neurons, PGB produced a very slight (4%) increase in sEPSC amplitude (\( P<0.0004 \) for KS test and \( p<0.05 \) for comparison of average amplitudes by t-test, Fig 4d and inset). IEI was also reduced, but by only ~10% (\( p<0.001 \) for KS test and \( p<0.01 \) for t-test on average amplitude, Fig 4e and inset).

*Figure 4 near here.*

*Effects of Mn\(^{2+}\) on excitatory synaptic transmission in dorsal horn.*

If PGB inhibition of excitatory neurotransmission in *substantia gelatinosa* can be ascribed to a decrease in presynaptic Ca\(^{2+}\) influx as a result of impaired Ca\(^{2+}\) channel function, similar or even larger effects should be seen with 20\( \mu \)M Mn\(^{2+}\) as this is more effective than 10\( \mu \)M PGB in reducing I\(_{Ba}\) in DRG cell bodies (Fig 3b). However, data pooled from 2 high threshold and 3 from low threshold neurons show that Mn\(^{2+}\) (20\( \mu \)M) had no significant effect on sEPSC amplitude or IEI (Fig 4f and g). This suggests that the ability of gabapentinoids to suppress excitatory synaptic transmission in *substantia gelatinosa* cannot be attributed to decreased Ca\(^{2+}\) channel expression in primary afferent terminals.

*Long-term effects of pregabalin and gabapentin on inhibitory synaptic transmission in substantia gelatinosa.*
We have shown previously that inhibitory synaptic events such as those illustrated in Fig 5a and recorded at 0mV are completely eliminated by a mixture of bicuculline and strychnine (Lu et al., 2012). They thus represent sIPSC’s with no contamination from sEPSC’s.

PGB was generally more effective in altering inhibitory synaptic transmission in low threshold (putative inhibitory) neurons than in high threshold (putative excitatory) neurons. 10μM PGB reduced the amplitude of sIPSC’s in low threshold neurons by ~39%. (p<0.0001 KS test Fig 5b). This change was highly significant when mean event amplitudes were compared (p<0.0001 T-test, Fig 5b inset). PGB also substantially increased IEI to 280% of control (decreased sIPSC frequency) of control in low threshold neurons (Fig 5c and inset, p<0.0001 for KS test and t-test on mean data values).

sIPSCs were quite infrequent in high threshold neurons. 10μM PGB altered the amplitude distribution of sIPSC’s with a tendency towards more small events (p<0.0001, KS test, Fig 5d). There was however no difference between mean sIPSC amplitude in control neurons and in those treated with PGB (Fig 5d, Inset). 10μM PGB also tended to increase the IEI of sIPSC in high threshold neurons (Fig 5e KS test p<0.02) but this was not reflected as a change in mean IEI (Fig 5e inset).

Effects of gabapentinoids on overall dorsal horn excitability.

Since gabapentinoids are effective anti-allodynic agents, they would be expected to produce an overall decrease in dorsal horn excitability. The observation that sEPSC amplitude in high threshold putative excitatory neurons is decreased (Fig 4a) is consistent with this possibility. However, gabapentinoids also inhibit sIPSC’s (Fig 5). In view of the complexity of dorsal horn
circuitry, the relative number of excitatory and inhibitory neurons and the nature and the number
and strength of synaptic connections between them it is difficult to predict the sum effect of such
changes. We therefore examined the overall effects of gabapentinoids on excitability of the dorsal
horn by confocal Ca\(^{2+}\) imaging.

Challenge with high potassium solution (20 or 35mM) produces robust increases in the
concentration of intracellular Ca\(^{2+}\) in dorsal horn neurons in DMOTC (Biggs et al., 2012; Lu et al.,
2009a). Typical recordings are illustrated in the *upper trace* of Fig 6a. K\(^+\) -evoked Ca\(^{2+}\) responses
were reduced in amplitude by 59.5±2.9% by 5μM CNQX (n=8) and almost completely eliminated
following Ca\(^{2+}\) channel blockade with a high concentration of Mn\(^{2+}\) (200μM, Fig 6a, *lower trace*).
This indicates that the responses to K\(^+\) result from glutamate release in addition to activation of
voltage-gated calcium channels. We therefore suggest that the amplitude and area under curve
(AUC) of K\(^+\) evoked Ca\(^{2+}\) responses provides an index of overall network excitability.

*Figures 6 and 7 and Table 2 near here*

As might be expected from published electrophysiological data (Hendrich et al., 2012;
Moore et al., 2002), acute (15 min) exposure of DMOTC slices to 10 or 100μM GBP had no
significant effect on the Ca\(^{2+}\) responses evoked with 35mM K\(^+\) (Table 2).

By contrast, when slices were exposed to GBP for 5-6 days, Ca\(^{2+}\) responses evoked by
35mM K\(^+\) were reduced in a concentration-dependent fashion (Fig 6b and c); whilst 1μM and
10μM GBP were without effect, pronounced reduction of the amplitude and area under the curve
(AUC) was seen with 100μM GBP (*p* < 0.01).
Similar effects were seen with PGB which depressed Ca\textsuperscript{2+} responses evoked by stimulation of the dorsal root entry zone (Fig 6d). PGB was more effective than GBP as clear depression of stimulation-evoked Ca\textsuperscript{2+} responses was seen with concentrations as low as 1\mu M (p < 0.0001 for 1, 10 or 100\mu M for both amplitude and AUC; Fig 6e and f).

Cellular origin of K\textsuperscript+-evoked Ca\textsuperscript{2+} signals.

Because glutamate is known to increase intracellular Ca\textsuperscript{2+} in cultured spinal astrocytes (Ahmed et al., 1990), there is a possibility that K\textsuperscript+-evoked Ca\textsuperscript{2+} signals in spinal DMOTC originate from astrocytes rather than neurons. This seems unlikely, as the cells generating Ca\textsuperscript{2+} signals in response to 35mM K\textsuperscript{+} (Fig 7a) are similar in size and morphology to NeuN positive cells (Fig 7b) but not to GFAP positive cells (Fig 7c). The difference in size and morphology of astrocytes relative to neurons is also clear from Fig 7d which illustrates a non-stimulated DMOTC in which both neurons and larger astrocytes showed a high level of resting Fluo 4 fluorescence.

Although most of the responding cells were similar in size to neurons, and the responses evoked therein were generally of large amplitude and decayed relatively rapidly (Fig 7e), we occasionally observed low amplitude, long duration Ca\textsuperscript{2+} responses (Fig 7f). The cell illustrated in Fig 7f responds to both norepinephrine (NE, 25\mu M) and ATP (100\mu M) whereas that illustrated in Fig 7e does not. Since it has been suggested that NE and ATP evoke Ca\textsuperscript{2+} signals selectively in astrocytes (Duffy and MacVicar, 1995; Fu et al., 2013; Huxtable et al., 2010), the recordings in Fig 7e may originate from a neuron and those in Fig 7f from an astrocyte.

Fig 7g illustrates the relationship between cell size and amplitude of response to 35mM K\textsuperscript{+}. No large, rapid changes in fluorescence were seen in largest cells (diameter > 80 arbitrary units).
As well as ATP and NE, astrocyte Ca\(^{2+}\) signalling may also be selectively evoked following activation of metabotropic glutamate receptors with \textit{trans}ACPD (25\(\mu\)M). Figures 7h-j compare the amplitude of responses to these three agonists to cell size. Although, in most cases ATP, NE and \textit{trans}ACPD are without effect on cells with diameter < 40 arbitrary units, they do not appear to distinguish reliably between small and large cells \textit{i.e.} between putative neurons and astrocytes) in spinal cord DMOTC.

\textit{Gabapentinoids effects on substantia gelatinosa neurons require entry via the system L-aminoacid transporter.}

Blockade of the long-term effects of gabapentinoids on HVA-I\(_{Ba}\) in DRG neurons by BCH (Fig 2e and f) confirm the observations of Hendrich et al (2008) and support their suggestion that the drugs must enter via the system L-amino acid transporter to exert their effect. Although the differences in the effects of Mn\(^{2+}\) and PGB illustrated in Fig 4 suggest that long-term spinal actions of gabapentinoids do not reflect decreased transmitter release as a result of decreased Ca\(^{2+}\) channel function, it remains to be determined whether their observed spinal actions are exerted intracellularly or as a result of an extracellular interaction with \(\alpha2\delta\) subunits (Eroglu et al., 2009).

Addition of 300\(\mu\)M BCH to spinal cord DMOTC together with 100\(\mu\)M GBP prevented its action. The first two columns of Figs 8a and b illustrate the extent of suppression of K\(^+\) evoked Ca\(^{2+}\) responses by 100\(\mu\)M GBP \((p < 0.01\) for amplitude and \(p < 0.001\) for AUC compared to control). By contrast, 100\(\mu\)M GBP failed to decrease K\(^+\)-evoked Ca\(^{2+}\) responses in the presence of 300\(\mu\)M BCH. In fact, responses evoked in BCH + GBP were larger than those evoked in BCH alone \((p < 0.05\) for amplitude and \(p < 0.001\) for AUC, right hand columns of Fig 8a and 8b).

Experiments using 10\(\mu\)M PGB and Ca\(^{2+}\) responses evoked by DRZ stimulation yielded similar but not identical data. PGB strongly depressed response amplitude and AUC \((p<0.001\) for...
both compared to control Fig 8c and d) but was ineffective in reducing response amplitude in BCH
treated slices (Fig 8c). BCH treatment also significantly reduced the amplitude and AUC of
responses (Fig 8c and d; \( p < 0.01 \) and < 0.001, respectively).

Gabapentinoid Effects on BDNF-Treated Slices.

Since gabapentinoids are primarily anti-allodynic agents rather than analgesics, it is
important to determine their actions in ‘neuropathic’ slices. Since brain derived neurotrophic factor
(BDNF) is strongly implicated in the central sensitization that contributes to the establishment of
neuropathic pain (Biggs et al., 2010; Coull et al., 2005; Liu and Sandkuhler, 1997; Lu et al., 2007;
Lu et al., 2009a; Merighi et al., 2008; Smith, 2014; Wang et al., 2009), we examined actions of
GBP on ‘neuropathic slices’ in which the extracellular environment of spinal neurons may
resemble that seen following peripheral nerve injury in vivo. This was done by culturing DMOTC
slices with 200ng/ml BDNF for 5-6 d (Lu et al., 2007; Lu et al., 2009a). This led to a significant
increase in the amplitude (\( p < 0.05 \)) but not the AUC of the \( \text{Ca}^{2+} \) response to 35mM \( \text{K}^{+} \) solution
(Fig 9a and b).

By contrast with the lack of effect of a low concentration of GBP (10\( \mu \text{M} \)) (Fig 6b and c) in
control cultures, this concentration was quite effective in attenuating \( \text{K}^{+} \)-evoked \( \text{Ca}^{2+} \) responses in
DMOTC slices cultured with BDNF. Thus, responses recorded in 10\( \mu \text{M} \) GBP + BDNF were of
significantly lower amplitude than those seen in BDNF alone (\( p < 0.001 \), Fig 9a) as was the AUC
\( (p<0.05 \text{ Fig 9b}). \) These data thus suggest that GBP is more efficacious in ‘neuropathic’ slices.
DISCUSSION.

These experiments addressed three different issues relating to the long-term action of gabapentinoids. First, we asked whether they have a preferential action on specific subsets of DRG neurons. This seems to be the case, as small, IB₄- negative and medium sized neurons were more sensitive to the actions of gabapentinoids than large and small IB₄+ neurons. Second, we asked whether spinal actions of gabapentinoids can be attributed to decreased functional expression of voltage gated Ca²⁺ channels in primary afferent terminals. Experiments in which we compared the actions of gabapentinoids with the Ca²⁺ channel blocker Mn²⁺ argued against this possibility. Thirdly, we asked whether gabapentinoids have preferential effects on excitatory neurons and processes in dorsal horn. In general, this seemed to be the case. We go on to suggest that the selectivity of action of α₂δ ligands on different subpopulations of DRG neurons may predict their differential actions at substantia gelatinosa synapses. Each of these issues is considered in additional detail below. In many parts of the study, we were able to compare the actions of PGB with those of GBP. No major differences in the actions of these two drugs were seen except that PGB was 10 times as effective as GBP.

Actions of Gabapentinoids on HVA I₈,a in DRG neurons.

According to the classical literature, Aδ- and C-fibres, with medium and small-sized cell bodies respectively, are largely responsible for the transfer of nociceptive information whereas Aβ and Aα axons, which associate with large cell bodies, are responsible transfer of innocuous information. Over the last 10 years however, it has become apparent that situation is far more...
complex; some Aβ-fibres may carry nociceptive information whereas not all C-fibres are nociceptors (Daniele and MacDermott, 2009; Djouhri and Lawson, 2004; Fang et al., 2005; Light and Perl, 2003). Although the distinction is not absolute, small IB4+ DRG neurons tend to be non-peptidergic whereas IB4- neurons contain neuropeptides (Braz et al., 2014; Stemkowski and Smith, 2012c). Despite this, the assumption that IB4- neurons are always nociceptors has been challenged (Braz et al., 2014; Fang et al., 2006). Thus, when studying DRG neurons in culture, little can be said about the functional modality of each neuron type. Nevertheless, by adopting a frequently-used classification of DRG cell types (Abdulla and Smith, 1997; Scroggs and Fox, 1992; Stemkowski and Smith, 2012b), we are able to maintain consistency with previously published literature. Our finding that gabapentinoids have preferential actions on medium-sized and small IB4- neurons permit the straightforward conclusion that not all afferents are equally sensitive to gabapentinoids. This explains why they lack a simple anaesthetic effect. The antiallodynic as opposed to analgesic properties of gabapentinoids may also reflect their increased effectiveness in “neuropathic” BDNF-treated slices (Fig 9). As will be discussed below and as is illustrated in Figure 10, we suggest that the selective actions of gabapentinoids on specific populations of DRG neurons may be predictive of their selective actions in substantia gelatinosa.

Since the actions of gabapentinoids in DRG neurons are sensitive to BCH, and therefore depend on entry via the neutral aminoacid transporter, absence of this transporter in large and small IB4+ neurons may provide an explanation for their selective action on medium and small IB4- neurons. Another possibility relates to differences in the properties of α2δ subunits in the different neuron types. This is supported by the recent description of two splice variants of the α2δ–1 subunits in DRG neurons (Lana et al., 2013). One, designated α2δ–1 ΔA+B+C, is found in many brain regions whereas the other, α2δ–1 ΔA+BΔC, is upregulated in small DRG neurons.
following nerve injury. Interestingly the $\alpha_2\delta-1 \Delta A+B\Delta C$ variant expresses low affinity for gabapentinoids. Perhaps the relative abundance of $\alpha_2\delta-1 \Delta A+B\Delta C$ in different subpopulations of DRG neurons relates to their differential sensitivity to gabapentinoids.

Presynaptic $Ca^{2+}$ Channels and Gabapentinoid Action.

20$\mu$M Mn$^{2+}$ was more effective than 10$\mu$M PGB in blocking HVA I$_{Ba}$ in DRG neurons (Fig 3b). By contrast, this concentration of PGB was more effective than Mn$^{2+}$ in blocking synaptic transmission within the dorsal horn (Fig 4). The gabapentinoid-induced reduction in neurotransmitter release thus has little to do with altered function of $Ca^{2+}$ channels, and reduced voltage-gated $Ca^{2+}$ influx into in presynaptic nerve terminals. If this were the case, 20$\mu$M Mn$^{2+}$ would be more effective than 10$\mu$M PGB. Although, in expression systems and in dorsal root ganglion (DRG) cell bodies, interaction of gabapentinoids with $\alpha_2\delta$ impedes trafficking of pore-forming Ca$_{v}$.2.1 $\alpha$-subunits of voltage gated calcium channels to the cell surface (Cassidy et al., 2014; Hendrich et al., 2012; Hendrich et al., 2008; Tran-Van-Minh and Dolphin, 2010), they do not appear to prevent insertion of the $\alpha$-subunits of voltage-gated $Ca^{2+}$ channels into nerve terminals, at least in hippocampal neurons (Hoppa et al., 2012). It remains to be determined whether a similar situation applies to primary afferent terminals. Due to the co-localization of $Ca^{2+}$ channels and release sites (Cao and Tsien, 2010), the strength of synaptic transmission is normally saturated with regard to $Ca^{2+}$ channel expression. Thus, large decreases in $Ca^{2+}$ influx in nerve terminals, as would be seen in the presence of 20$\mu$M Mn$^{2+}$, would have only minor effects on
neurotransmitter release. Gabapentinoid reduction of neurotransmitter release must therefore engage a different mechanism. One possibility is that gabapentinoid binding to α2δ subunits interrupts the interaction between Ca\(^{2+}\) channel α subunits and neurotransmitter release sites (Hoppa et al., 2012). This may be consistent with the observation that long-term applications of gabapentinoids fail to affect Ca\(^{2+}\)-independent mEPSC’s in hippocampal pyramidal neurons (Cheng et al., 2006).

Effects of gabapentinoids in substantia gelatinosa.

The α2δ−1 subunit is expressed as a transmembrane protein with the α subunit of the mature Ca\(^{2+}\) channel (Cassidy et al., 2014; Dolphin, 2012). This and other data has led to the suggestion that it may function as a receptor for the neurotrophin, thrombospondin (Eroglu et al., 2009). Gabapentinoid binding to this site has been reported to block thrombospondin-induced neuronal sprouting. Since it has been suggested that sprouting of primary afferents contributes to central sensitization (Woolf et al., 1992), antagonism of the thrombospondin receptor has been implicated in the antiallodynic action of gabapentinoids (Eroglu et al., 2009). Our findings are at odds with this suggestion because the long-term actions of gabapentinoids in substantia gelatinosa are blocked by BCH (Fig 8), indicating its spinal actions are mediated intracellularly. There is also some doubt as to the role of primary afferent sprouting in the development of central sensitization (Bao et al., 2002; Hughes et al., 2003). In fact, some processes may retract (Bailey and Ribeiro-da-Silva, 2006).

Because we had used intracellular Cs\(^+\) to improve the signal to noise ratio of sEPSC’s, this precluded the use of firing pattern as a criterion for cell identification. Neurons studied with Cs\(^+\) in the recording pipette displayed very broad action potentials that presumably reflected blockade of outward K\(^+\) currents by Cs\(^+\). However as more studies emerge, it is becoming increasingly
apparent that most tonic firing, low threshold, neurons exhibit a GABAergic phenotype whereas delay firing neurons, which have a high threshold, are often glutamatergic (Yasaka et al., 2010). This idea is supported by a recent report on mouse dorsal horn where neurons expressing GFP-tagged vesicular glutamate transporter 2 (vGluT2-eGFP) had higher action potential thresholds than inhibitory neurons from mice that expressed GFP under the control of the glutamic acid decarboxylase promoter (Gad67-eGFP) (Punnakkal et al., 2013).

In view of this, it appears that the strongest actions of gabapentinoids involve a decrease in the amplitude of sEPSC’s in excitatory neurons (Fig 4b) with only a small and unexpected decrease in IEI (~12%, Fig 4c). Because the Ca$^{2+}$ imaging data (Figs 6,8 and 9) show that long-term exposure to gabapentinoids reduces overall neuronal activity, the small effects of PGB on sEPSCs in inhibitory neurons where amplitude increased by only 4% (Fig 4d) and IEI was decreased by 10% (Fig 4e), contribute little to the overall drug effect.

The inter-event interval of inhibitory events is up to 10’s of seconds (Figs 5c and e). This is far greater than that of excitatory events (Fig 4c and e, always < 1.5s) and this fits with the suggestion that the substantia gelatinosa is largely an excitatory network (Santos et al., 2007). Thus the action of gabapentinoids on sEPSCs may outweigh its effect on sIPSC’s and play a major role in its ability to promote an overall decrease in excitability. We cannot however rule out contributions from other mechanisms. For example, it has been shown that long-term exposure to gabapentinoids increases tonic GABA$_A$ mediated conductance in hippocampal neurons (Cheng et al., 2006). A similar conductance mediated by δ–subunit containing extrasynaptic GABA$_A$ receptors has also been described in substantia gelatinosa neurons (Bonin et al., 2011), but it remains to be determined whether this conductance is augmented by long-term gabapentinoid exposure.
Possible Determinants of Gabapentinoid Selectivity.

Because gabapentinoids affect both sEPSC’s (Fig 4) and sIPSC’s (Fig 5), they do not preferentially reflect the release of glutamate versus GABA. Their selectivity, which leads to an overall decrease in dorsal horn excitability may reflect their aforementioned preferential effect on sEPSC on excitatory (Fig 4b and c) versus inhibitory neurons (Fig 4d and e).

This selectivity of action of gabapentinoids towards excitatory terminals on excitatory substantia gelatinosa neurons may relate to their actions in the periphery (Fig 10). Since gabapentinoid sensitive, IB₄- neurons project primarily to excitatory vertical neurons in outer lamina II (Braz et al., 2014), this may explain the stronger effect of α2δ ligands at this synapse (Fig 4b). The ability of gabapentinoids to selectively affect the cell bodies of medium neurons may also play a role in their selectivity as Aδ fibres also appear to selectively project to excitatory dorsal horn neurons (Lu and Perl, 2005). By contrast, putative non-nociceptive, IB4+, C-fibre afferents appear to project to inhibitory low threshold dorsal horn neurons (Braz et al., 2014), Since these neurons are relatively insensitive to gabapentinoids (Fig 2b), this may explain their relatively weak effects at excitatory synapses onto inhibitory neurons (Fig 4d). Spontaneous synaptic activity in inhibitory neurons may also arise from low threshold Aβ-fibre input (Daniele and MacDermott, 2009) and given the lack of effect of gabapentinoids on large DRG neurons (Fig 2e), a weak drug effect at these synapses might also be expected.

Figure 10 near here.

Unanswered questions
Since clinical benefit from gabapentinoids takes 3 or more days to develop in the clinic (Cheshire, 2002; Sharma et al., 2010), our studies were designed to replicate this situation in vitro. In confirmation of the results of Moore et al (2002), we found that gabapentinoids had little or no effect in spinal cord when applied acutely (Table 2). Despite this, gabapentinoids have been reported to act within 30 mins or less in animal models of neuropathic pain in vivo (Coderre et al., 2005; Kayser and Christensen, 2000; Kumar et al., 2013; Patel et al., 2013). The reasons for this discrepancy remain to be elucidated. In our own unpublished in vivo studies, we have also found that intra-peritoneal administration of GBP attenuates mechanical allodynia 3 h after administration. Interestingly, the amount of depression of HVA I_{Ca} we see with chronic application of 10μM PGB in medium and IB4- DRG neurons is comparable with the 40% depression seen with acute application of 100μM GBP or 10μM PGB to undefined types of cultured DRG neuron (McClelland et al., 2004; Sutton et al., 2002). This may imply that the rapidly developing actions of gabapentinoids seen in animal models in vivo (Kumar et al., 2013) relate more to acute peripheral actions of the drug (Carlton and Zhou, 1998), rather than spinal actions that take days to develop.

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DICLOSURES
None

AUTHOR CONTRIBUTIONS.
JEB carried out most of the experiments, contributed to study design and wrote the first draft of the manuscript. Other experiments were carried out by PAB, NG, PLS and ALG. KB provided Ca²⁺ imaging facilities, supervised experiments and reviewed various versions of the manuscript.
PAS conceived study, supervised experiments and wrote the final version of manuscript.
TABLE 1. Effect of PGB on ratio of peak to end-of-pulse HVA I\textsubscript{Ba} in DRG Neurons.

Decreased ratio = decreased inactivation refer to fig 1j

<table>
<thead>
<tr>
<th></th>
<th>Small IB4 -</th>
<th>Small IB4 +</th>
<th>Medium</th>
<th>Large</th>
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<tr>
<td>Control Ratio</td>
<td>2.94±0.68 (n=5)</td>
<td>1.94±0.32 (n=6)</td>
<td>1.57±0.17 (n=6)</td>
<td>2.35±0.30 (n=6)</td>
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<tr>
<td>Ratio in 10µM PGB</td>
<td>1.34±0.2 (n=7 (p&lt;0.03))</td>
<td>1.00±0.08 (n=5 (p&lt;0.01))</td>
<td>1.27±0.3 (n=12 (p&lt;0.0001))</td>
<td>1.92±0.39 (n=11 (p&gt;0.45))</td>
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TABLE 2. Lack of effect of acutely applied Gabapentin (GBP) on \(K^+\) evoked \(Ca^{2+}\) responses in dorsal horn neurons.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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<th>Control</th>
<th>100µM GBP</th>
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<td>n=19</td>
<td>n=13</td>
<td>n=19</td>
<td>n=13</td>
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<tr>
<td>Amplitude</td>
<td>863.7±166.8</td>
<td>649.8±145.7</td>
<td>1135.6 ±149.76</td>
<td>1041.8 ±109.8</td>
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<td>Arbitrary Units</td>
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<td>AUC</td>
<td>104734±13742</td>
<td>98676±19158</td>
<td>153760±21693</td>
<td>180241±20267</td>
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<tr>
<td>Arbitrary Units</td>
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<td>(p&gt;0.8)</td>
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Figure 1. Scheme to show time course for generation of spinal DMOTC cultures and dissociated DRG neuron cultures as well as periods of exposure to the α2δ ligands, PGB (10μM) and GBP (100μM). DRG neurons were exposed to drugs for 3-4d and DMOTC cultures were exposed to drugs for 5-6d.

Figure 2. Long-term effects of gabapentinoids on HVA-\(I_{Ba}\) in DRG neurons. a-d. HVA-\(I_{Ba}\) density - voltage plots for small IB\(_4\)-, small IB\(_4\)+, medium and large DRG neurons, in the presence or absence of 10μM PGB (applied for 3-4d). Note profound suppression of current in small IB\(_4\)- and medium sized neurons but not in small IB\(_4\)+ or in large neurons. e. HVA-\(I_{Ba}\) density - voltage plots for control medium neurons and neurons exposed to 100μM GBP for 3-4d. f. HVA-\(I_{Ba}\) density - voltage plots for control medium neurons and medium neurons exposed to 300μM BCH or 300μM plus 100μM GBP for 5-6d. Note lack of effect of GBP on BCH treated neurons. g. Family of HVA-\(I_{Ba}\) evoked in a control small IB\(_4\)- neuron in response to a series of voltage commands. Voltage recordings are shown in lower trace. h. Family of HVA-\(I_{Ba}\) evoked in another small, IB\(_4\)- neuron recorded after 6d in the presence of 100μM GBP. Note reduction in current amplitude and attenuation of inactivation. i. Current recordings obtained at -10mV normalized and superimposed from g and h, note decreased rate of inactivation in continued presence of GBP. j. Definition of peak and end-of-pulse current measurements used to quantify inactivation of HVA-\(I_{Ba}\). Error bars define SEM.

Figure 3 a. Recordings of HVA-\(I_{Ba}\) from a small DRG neuron prior to and after the addition of 200μM Mn\(^2+\). \(V_h = -100\)mV, voltage commands in 10mV increments up to +30mV, note strong
suppression of currents at all voltages by 200μM Mn\textsuperscript{2+}. b. Log concentration-effect plot for suppression of HVA I\textsubscript{Ba} by Mn\textsuperscript{2+} in small DRG neurons (open circles n=5). Error bars define SEM. Grey data point shows average effect of 5-6 d exposure of 10μM PGB on HVA I\textsubscript{Ba} in small IB4+ neurons.

**Figure 4 a.** Sample recording of spontaneous EPSC’s (sEPSC) from a high threshold neuron in the substantia gelatinosa region of a spinal DMOTC (V\textsubscript{h} = -70mV). b and c. cumulative probability plots of sEPSC amplitude and interevent interval (IEI) from high threshold neurons in the substantia gelatinosa region of a spinal DMOTC. Comparison of control neurons with those exposed to 10μM PGB for 5-6d. 3692 events from 6 neurons in control slices (black points), 9825 events from 10 neurons in PGB treated slices. (grey points p<0.0001 for amplitude and IEI, Kolgomorov-Smirnoff test). Insets display mean amplitudes and IEI’s from same the data. d. and e. Cumulative probability plots of sEPSC amplitude and IEI from low threshold neurons. Comparison of control neurons (black points), with those exposed to 10μM PGB for 5-6d (grey points), 11139 events from 14 neurons in control slices, 9680 events from 8 neurons in PGB treated slices (p<0.0004 for amplitude, p<0.0001 for IEI Kolgomorov-Smirnoff test). Insets display mean amplitudes and IEI’s from the same data (Black bars control, grey bars PGB). f and g. lack of effect of 20μM Mn\textsuperscript{2+} on amplitude and IEI of sEPSC’s data pooled from 3 low threshold and 2 high threshold neurons (black points control, grey points Mn\textsuperscript{2+}).

**Figure 5.** Sample recording of spontaneous IPSC’s (sIPSC) from a low threshold neuron in the substantia gelatinosa region of a spinal DMOTC (V\textsubscript{h} = 0mV). b and c. Cumulative probability plots of sIPSC amplitude and IEI from low threshold neurons. Comparison of control neurons
(black points), with those exposed to 10μM PGB for 5-6d (grey points). 1548 events from 9 neurons in control slices, 1538 events from 6 neurons in PGB treated slices. \( p<0.0001 \) for both amplitude and IEI; Kolgomorov-Smirnoff test). Insets display mean amplitudes and IEI’s from the same data (control data black bar, PGB data grey bar). d. and e. Cumulative probability plots of sIPSC amplitude and interevent interval (IEI) from high threshold neurons in the substantia gelatinosa region of a spinal DMOTC. Comparison of control neurons with those exposed to 10μM PGB for 5-6d. 62 events from 4 neurons in control slices (black points), 54 events from 6 neurons in PGB treated slices. (grey points; \( p<0.0001 \) for amplitude and \( <0.02 \) for IEI, Kolgomorov-Smirnoff test). Insets display mean amplitudes and IEI’s from same the data, differences are not significant by t-test (control data black bar, PGB data grey bar).

**Figure 6 a.** Sample records of changes in Fluo-4 fluorescence intensity in dorsal horn neurons in DMOTC in response to superfusion of saline containing 35mM K+. Responses from three neurons (regions of interest) are superimposed. *Upper traces.* recorded under control conditions, *Lower traces* recorded in the presence of 200μM Mn\(^{2+}\). b. Effects of 5 days exposure to 1, 10 or 100μM GBP on amplitude of K\(^+\)-evoked Ca\(^{2+}\) responses. c. Effects of GBP on area under curve AUC of responses summarised in b. For untreated slices, data collected from 40 neurons in 6 slices, for 1μM GBP 18 neurons in 3 slices, for 10μM GBP 30 neurons in 4 slices, for 100μM GBP, 27 neurons in 4 slices. Note lack of effect of 1 and 10μM GBP and significant suppression of responses with 100μM \( F(\text{degrees of freedom between groups}=3; \text{degrees of freedom within groups}=111) = 3.031 \) for data in b, \( F(\text{degrees of freedom between groups}=3; \text{degrees of freedom within groups}=111) = 3.763 \) for data in c ANOVA / Tukey-Kramer Multiple comparisons test, untreated vs 100μM \( p<0.01 \). d. Sample records of changes in Fluo-4 fluorescence intensity in
response to dorsal root entry zone (DRZ) stimulation. 5th and 6th stimuli from a series of 8 stimuli are shown to illustrate the consistency of amplitude of these two responses (see methods). Responses from three neurons (regions of interest) in the dorsal horn region of a spinal cord slice in DMOTC. Responses are staggered for clarity of display. e. Effects of 5 days exposure to 1, 10 or 100μM pregabalin (PGB) on amplitude of DRZ-evoked Ca²⁺ responses. f. Effects of PGB on AUC of responses summarised in e. for untreated, 48 cells from 6 slices, for 1μM PGB, 181 cells from 9 slices, for 10μM PGB, 44 cells from 6 slices, for 100μM PGB, 40 cells from 5 slices. Note that all concentrations of PGB produce strong suppression of responses (F(degrees of freedom between groups=3; degrees of freedom within groups=309) = 19.32 for data in e, F(degrees of freedom between groups=3; degrees of freedom within groups=309) = 15.67 for data in f; ANOVA / Tukey-Kramer Multiple comparisons test untreated versus 1, 10 or 100μM p<0.0001).

Figure 7. a. Effect of 35mM K⁺ on Fluo 4 intensity in cells in a field from the dorsal horn region of a DMOTC slice. b. Field of NeuN stained neurons from another slice. c. Field of GFAP stained glial cells from a third slice. Note similarity in size and morphology of cells responding to 35mM K⁺ in a and neurons in b and differences to GFAP stained glia in c. d. Field of cells expressing high resting Fluo 4 florescence, note differences in size and shape of putative neurons and astrocytes. e and f. Examples of two different types of Ca²⁺ responses seen in dorsal horn cells. The response to K⁺ in the putative neuron illustrated in e is large and rapid and the cell is unaffected by ATP (100μM) or norepinephrine (NE, 25μM). The response to K⁺ in the putative astrocyte illustrated in f is relatively small and slow and the cell responds to both ATP and NE. Black bar represents time of 90s application of K⁺ or agonists. g-j Comparison of amplitude of Ca²⁺ responses evoked by 35mM K⁺, 100μM ATP, 25μM NE and trans ACPD (25μM) with cell
size in arbitrary units generated by analysis software. Note lack of response of some small cells in
h-j to ATP, NE and trans ACPD.

**Figure 8.** Reduction in amplitude of DRZ-evoked Ca$$^{2+}$$ responses following exposure to 100$$\mu$$M GBP and restoration of amplitude following inclusion of 300$$\mu$$M BCH in culture with GBP.

Control n = 33 neurons in 4 slices, GBP n = 27 neurons in 4 slices ($p<0.01$ compared to control)
BCH alone n= 57 neurons in 4 slices (not significantly different from control), BCH + GBP n = 37 neurons in 4 slices (not significantly different control, $p<0.001$ compared to GBP alone; $p<0.05$ compared to BCH alone; F *(degrees of freedom between groups=3; degrees of freedom within groups=151)*=7.45 for data in a, F(*(degrees of freedom between groups=3; degrees of freedom within groups=151)*) = 11.36 for data in b; ANOVA/Tukey-Kramer Multiple comparisons test). b.

Corresponding AUC data for results shown in a. For GBP versus control, $p < 0.001$; GBP/BCH not significantly different from control but different from GBP alone $p<0.001$ and from BCH alone $p<0.001$; responses recorded in BCH alone were significantly smaller than control ($p<0.01$; Tukey-Kramer Multiple comparisons test). c. Reduction in response amplitude of nerve stimulation evoked Ca$$^{2+}$$ responses following exposure to10$$\mu$$M PGB and restoration of responses following inclusion of 300$$\mu$$M BCH in culture with PGB. One way ANOVA revealed significant differences ($p < 0.0001$). Control n = 103 neurons from 17 slices, PGB n = 37 neurons from slices ($p < 0.001$ compared to control), BCH n = 23 neurons from 5 slices, BCH + PGB n = 46 neurons from 4 slices ($p < 0.001$ compared to PGB alone, not significant compared to control, $p<0.001$ compared to BCH alone; F *(degrees of freedom between groups=3; degrees of freedom within groups=205)*=13.39 for data in c; F(*(degrees of freedom between groups=3; degrees of freedom within groups=205)*)=11.36 for data in b; ANOVA/Tukey-Kramer Multiple comparisons test).
Corresponding AUC data for results shown in i. One way ANOVA revealed significant differences $(p < 0.001)$. PGB significantly different from control $(p < 0.001)$, PGB + BCH slightly smaller than control $(p < 0.05)$ but not different from BCH alone or PGB alone (Tukey-Kramer Multiple comparisons test). Figure 9. Increase in amplitude of $K^+$-evoked $Ca^{2+}$ responses following 5d exposure to 200ng/ml BDNF and suppression of responses by 5d exposure to a normally sub-effective concentration of GBP (10μM; compare with Fig 6b and c). a. ANOVA reveals significant differences $(p < 0.0001, F(degrees of freedom between groups=3; degrees of freedom within groups=83) =11.72)$. Control n = 33 neurons in 6 slices neurons, BDNF n = 36 neurons in 3 slices $(p < 0.01$ compared to control), BDNF 10μM GBP n= 17 neurons in 4 slices $p < 0.001$ compared to BDNF alone). b, Corresponding data for AUC, although the effect of BDNF is not significant, 10μM GBP produces significant reduction in AUC of responses evoked in BDNF-treated slices $(p < 0.05 F(degrees of freedom between groups=3; degrees of freedom within groups=83) =4.32$ ANOVA/Tukey-Kramer Multiple comparisons test).

Figure 10. Scheme to illustrate possible relationship between actions of gabapentinoids in DRG with their actions in spinal dorsal horn. Drugs exert strongest effects on the medium sized cell bodies associated with A-δ fibres and on small IB4- neurons which project to excitatory substantia gelatinosa neurons. The cell bodies of large DRG neurons which associate with Aβ fibres and small IB4+ neurons that may project to inhibitory substantia gelatinosa neurons are less sensitive to α2δ ligands.
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a) Small IB4- Neurons 10 μM PGB

b) Small IB4+ Neurons 10 μM PGB

c) Medium Neurons 10 μM PGB,

d) Large neurons 10 μM PGB

e) Medium Neurons 100 μM GBP

f) Medium Neurons 100 μM GBP + BCH

g) Control $I_{\text{Ba}}$

h) $I_{\text{Ba}}$ 100μM GBP

i) Inactivation

j) Inactivation
a) Control

-100mV

Mn²⁺

+ 40mV

b) Amplitude of DRG cell body $I_{Ba}$

% of control

$\text{[Mn}^{2+}\text{]}$ (µM)

0 10 20 30 40 50 60 70

1 10 100 1000

$\leq 10$µM PGB

-40mV
sEPSC at -70mV

High Threshold Neurons (Amplitude)

Cumulative Fraction

Amplitude (pA)

Control

PGB

P<0.0001

Low Threshold Neurons (Amplitude)

Cumulative Fraction

Amplitude (pA)

Control

PGB

P<0.0004

High Threshold Neurons (IEI)

Cumulative Fraction

IEI (ms)

Control

PGB

P<0.0001

Low Threshold Neurons (IEI)

Cumulative Fraction

IEI (ms)

Control

PGB

P<0.05

Control

10μM PGB

P<0.01

n.s.

Control

20μM Mn^{2+}

n.s.
a  sIPSC at 0mV

b  Low Threshold Neurons

c  Low Threshold Neurons

d  High Threshold Neurons (amplitude)

e  High Threshold Neurons (IEI)
a  Before 35mM K^+  

b  Neu N  

c  GFAP  

d  

35mM K^+  

ATP (100µM)  

NE (25µM)  

Trans ACPD (25µM)  

% Change in Flu o 4 Fluorescence  

% Change in Flu o 4 Fluorescence  

% Change in Flu o 4 Fluorescence  

Time (s)  

Cell Size (Arbitary Units)  

Cell Size (Arbitary Units)  

Cell Size (Arbitary Units)  

Fluo 4 Fluorescence  

Arbitary Units  

Fluo 4 Fluorescence  

Arbitary Units  

Fluo 4 Fluorescence  

Arbitary Units  

Time (s)  

Cell Size (Arbitary Units)  

Cell Size (Arbitary Units)  

Cell Size (Arbitary Units)
a) 
Peak Amplitude of Ca\(^{2+}\) Response
Arbitrary Units (au)

- Control
- BDNF
- BDNF 10\(\mu\)M GBP

p<0.01  p<0.001

b) 
Area Under Curve
Arbitrary Units (au)

- Control
- BDNF
- BDNF 10\(\mu\)M GBP

ns  p<0.05