Analysis of the long-term actions of gabapentin and pregabalin in dorsal root ganglia and substantia gelatinosa

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Biggs JE, Boakye PA, Ganesan N, Stemkowski PL, Lantero A, Ballanyi K, Smith PA. Analysis of the long-term actions of gabapentin and pregabalin in dorsal root ganglia and substantia gelatinosa. J Neurophysiol 112: 2398–2412, 2014. First published August 13, 2014; doi:10.1152/jn.00168.2014.—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 embryonic day 13 rat embryos and maintained in organotypic culture for >5 wk (neuronal age equivalent to young adult rats). Exposure of similarly aged DRG neurons (dissociated and cultured from postnatal day 19 rats) to GBP or PGB for 5–6 days attenuated high-voltage-activated calcium channel currents (HVA $I_{\text{Ca}}$). Strong effects were seen in medium-sized and in small isoleucin B$_2$-negative (IB$_1$-) DRG neurons, whereas large neurons and small neurons that bound isoleucin B$_2$ (IB$_2$+) were hardly affected. GBP (100 μM) or PGB (10 μM) were less effective than 20 μM Mn$^{2+}$ in suppression of HVA $I_{\text{Ca}}$ in small DRG neurons. By contrast, 5–6 days of exposure to these α2δ-ligands was more effective than 20 μM Mn$^{2+}$ in reducing spontaneous excitatory postsynaptic currents at synapses in substantia gelatinosa. Spinal actions of gabapentinoids cannot therefore be ascribed to decreased expression of HVA Ca$^{2+}$ channels in primary afferent nerve terminals. In substantia gelatinosa, 5–6 days of exposure to PGB was more effective in inhibiting excitatory synaptic drive to putative excitatory neurons than to putative inhibitory neurons. Although spontaneous inhibitory postsynaptic currents were also attenuated, the overall long-term effect of α2δ-ligands was to decrease network excitability as monitored by confocal Ca$^{2+}$ imaging. We suggest that selective actions of α2δ-ligands on populations of DRG neurons may predict their selective attenuation of excitatory transmission onto excitatory vs. inhibitory neurons in substantia gelatinosa.

neuropathic pain; nerve injury; patch clamp; organotypic cultures; α2δ; Ca$^{2+}$ channel

NEUROPATHIC PAIN IS A MALADAPTIVE consequence of injury to, or disease of, the somatosensory system (Costigan et al. 2009; Sandkühler 2009; Treede et al. 2008). Because neuropathic pain 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 Ca$_{\text{V}2.2}$ 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 action of gabapentinoids suggest these agents 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 (<30 min; Sutton et al. 2002) and slowly developing effects (>40 h; Hendrich et al. 2012) can be observed in vitro. Interaction of gabapentinoids with α2δ-1 subunits also appears to interrupt interactions between high-voltage-activated (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 (100 μM) has little or no effect on stimulus-evoked excitatory postsynaptic currents (EPSCs) in nociceptive neurons in lamina II of the spinal dorsal horn (Moore et al. 2002). Frequency and amplitude of spontaneous EPSCs (sEPSCs) are similarly unaffected. Acute application of GBP (100 μM) also failed to affect sEPSCs 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 GBP for 40–48 h strongly reduced sEPSC frequency. Because chronic, but not acute, exposure to high (1 mM) concentrations of GBP inhibits Ca$_{\text{V}2.1}$ and Ca$_{\text{V}2.2}$ currents expressed in tsA-201 cells (Hendrich et al. 2012), 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 Ca$^{2+}$ channel current ($I_{\text{Ca}}$) seen in DRG neurons following 5–6 days of exposure to gabapentinoids with that produced by a relatively low concentration of Mn$^{2+}$ (20 μM). We then examined the effects of gabapentinoids and 20 μM Mn$^{2+}$ on sEPSCs 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 at both 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 in neurotransmitter release from all primary afferent terminals, they would produce an anesthetic rather than an antiallodynic effect. Gabapentinoids might therefore be expected to produce different effects on HVA $I_{\text{Ca}}$ in subsets of DRG neurons, and this might 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 $I_{\text{Ca}}$ in small, medium, and large DRG neurons in defined-medium culture. Small neurons were further subcategorized into those that bound the plant lectin isolectin B4 (IB4) and those that did not (IB4−) (von Banchet and Schaible 1999). Intense IB4 binding was originally reported to distinguish small C-fiber nociceptors (Fang et al. 2006).

Finally, within the dorsal horn, the substantia gelatinosa contains both excitatory and inhibitory neurons, and both receive monosynaptic excitatory input from nociceptive primary afferent fibers (Braz et al. 2014; Lu and Perl 2003, 2005; Todd 2010). Inhibitory neurons also receive low-threshold excitatory input (Daniele and MacDermott 2009). In view of their antiallodynic 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 Ca$^{2+}$ 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 previously (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 (Stiemkowski and Smith 2012a). Male Sprague-Dawley rats (18–20 days old) were euthanized with a high dose (1.5 g/kg ip) of ethyl carbamate (urethane; Sigma, St. Louis, MO). DRGs (14–21 per animal) were aseptically dissected from lower thoracic (T6–T12) and lumbar (L1–L6) spinal segments and collected in DMEM supplemented with 10% heat-inactivated horse serum (DMEMHS; both from GIBCO) and with low-resistance patch electrodes (2–5 MΩ) in two 50-mm culture dishes (Nunclon surface; VWR International, Mississauga, ON, Canada) precoated with 3 μg/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% CO$_2$. Defined medium was exchanged on days 1 and 3 and cultures maintained for 4 days.

For chronic treatment with GBP, a 10 mM stock solution was prepared in saline and 10 μl were added to 10 ml of neurotrophin- and serum-free defined medium to obtain a final concentration of 100 μM. Similarly, a final concentration of 10 μM PGB was prepared from a 1 mM stock solution. Concentrations of 70–120 μM GBP and 6–60 μM 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 10 μl of filtered saline, which was added to the medium instead of the drug.

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), 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 (in mM) 127 NaCl, 2.5 KCl, 1.2 NaH$_2$PO$_4$, 1.3 MgSO$_4$, 26 NaHCO$_3$, 25 D-glucose, and 2.5 CaCl$_2$, pH 7.2. The total volume of fluid in the

Neuronal cell enrichment was achieved by differential cell adhesion and treatment with antimitotic agents, followed by differential sedimentation procedures. Thus dissociated neurons were plated in DMEMHS supplemented with the antimitotic combination cytotoxic 3-α-arabino-furanoside (Ara-C), uridine, and 5-fluoro-2’-deoxyuridine (all from Sigma and all at 10 μM) in two 50-mm culture dishes (preplates; Corning, NY) previously coated with 3 μg/ml polyornithine (Sigma). After 15–20 h, the nonneuronal cells became firmly attached to the dish, whereas most of the neurons were only weakly adherent to the dish or to flattened nonneuronal cells. Next, by carefully removing the culture medium, most dead cells and axonal/myelin debris were discarded before the attached neurons were selectively dislodged with a gentle stream of serum-free defined medium (DMEM supplemented with 1:100 N-2 supplement and 1:100 penicillin-streptomycin-ampicillin 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 nonneuronal cells in suspension. The supernatant was discarded, and the cells were then resuspended in 1 ml of defined medium. At 100-μl volumes, the cells were plated into 35-mm tissue culture dishes (Nunclon surface; VWR International, Mississauga, ON, Canada) precoated with 3 μg/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% CO$_2$. Defined medium was exchanged on days 1 and 3 and cultures maintained for 4 days.

**Fig. 1.** Scheme showing time course for generation of spinal defined-medium cultures, drug treatment, and electrophysiology. Experimental procedures for establishing long-term defined-medium cultures of DRG neurons were similar to those previously described (Stiemkowski and Smith 2012a). Male Sprague-Dawley rats (18–20 days old) were euthanized with a high dose (1.5 g/kg ip) of ethyl carbamate (urethane; Sigma, St. Louis, MO). DRGs (14–21 per animal) were aseptically dissected from lower thoracic (T6–T12) and lumbar (L1–L6) spinal segments and collected in DMEM supplemented with 10% heat-inactivated horse serum (DMEMHS; both from GIBCO, Grand Island, NY). Ganglia were treated for 1.5 h at 34°C with 0.125% type IV collagenase (Worthington, Lakewood, NJ), washed twice in Ca$^{2+}$-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 with 6–8 passages through the tip of a 1-ml Eppendorf pipette.

**Fertilization**

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α2δ ligands

α2δ ligands

| e1 | e11 | Birth | p10 | p20 | p30 | p40 |

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recording dishes was about 1 ml. To record HVA I_Ca using Ba^{2+} as the change carrier, the superfuse was switched to an external solution containing (in mM) 160 tetraethylammonium (TEA)-Cl, 10 HEPES, 2 BaCl_2, and 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, and 20 HEPES-CaCl_2, 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 eyepiece of a Nikon TE300 inverted fluorescence microscope (Nikon, Toronto, ON, Canada). Further subclassification of small neurons into IB_4-positive and -negative subtypes was achieved by adding IB_4-AlexaFluor 488 conjugate (Invitrogen, Eugene, OR) 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 IB_4 positive.

Data were acquired using pCLAMP 10.1 (Axon Instruments, Foster City, CA), and final data records were produced using Origin 9.1 (Microcal, Northampton, MA). Current densities were expressed as picoamperes per picofarad (pA/pF). Input capacitance (C_in) was calculated by integrating the capacitive current transient produced by a 10-nV voltage step (ΔV) to yield charge (Q) and using the equation C_in = Q/ΔV.

Spinal cord organotypic slice culture preparation, drug treatment, and electrophysiology. DMOTC rat spinal cord slices with attached DRG were prepared as previously described (Biggs et al. 2012; Lu et al. 2006). Briefly, embryonic day 13 (E13) rat fetuses were delivered by cesarean section from timed-pregnant female Sprague-Dawley rats (Charles River, Saint-Constant, QC, Canada) under 5% isoflurane anesthesia. The dam was subsequently euthanized by cervical dislocation. The entire embryonic sac was placed in chilled Hank’s buffered salt solution containing (in mM) 138 NaCl, 5.33 KCl, 0.44 KH_2PO_4, 0.5 MgCl_2·6H_2O, 0.41 MgSO_4·7H_2O, 2.5 NaHCO_3, 0.3 Na_2HPO_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- to 325-μm transverse slices using a tissue chopper (McIlwain, St. Louis, MO). Only lumbar spinal cord slices with an intact spinal cord and two attached DRG were chosen, trimmed of excess ventral tissue, and allowed to recover for 1 h at 4°C. Each embryonic spinal cord slice was plated on a single glass coverslip (Carl Hecht, Sondheim, Germany) and attached with a clot of reconstituted chicken plasma (Iyophilized; Sigma) and thrombin (200 units/ml; Sigma). Coverslips were then inserted into flat-bottom tissue culture tubes (Nunc-Nalgene International, Mississauga, ON, Canada) filled with 1 ml of medium and 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% DMEM, 10% fetal bovine serum, and 8% sterile water (all from GIBCO). The medium was supplemented with 20 ng/ml NGF (Alomone Laboratories, Jerusalem, Israel) for the first 4 days and omitted thereafter. Antibiotic and antitoxicyc drugs (5 units/ml penicillin G, 5 units/ml streptomycin, and 12.5 ng/ml amphotericin B; GIBCO) were also included in the media during the first 4 days of culture. After 4 days in culture, DMOTC slices were treated with an antimitotic drug cocktail consisting of uridine, cytosine β-D-arabinofuranoside ( AraC), and 5-fluorodeoxyuridine (all at 10 μM) for 24 h to prevent the overgrowth of glial cells. During antimitotic treatment, the 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 Glutamaken-1 (all from GIBCO). The medium within these tubes was exchanged regularly with freshly prepared medium every 3–4 days.

After 15–21 days in vitro, DMOTC slices were treated for a period of 5–6 days with either GBP (1–100 μM) or PGB (1–100 μM) with or without 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH; 300 μM). 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; Alomone Labs, Jerusalem, Israel) for 4–5 days (Lu et al. 2007, 2009a). Other cultures were exposed acutely (for 90 s) to ATP (100 μM), (±)-1-amino cyclohexanol-trans-1,3-dicarboxylic acid (trans-ACPD; 25 μM; Tocris), or epinephrine (25 μM). Whole cell patch-clamp recordings were obtained from neurons in DMOTC immediately following 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 gelatiosa and up to a depth of 100 μm from the surface. Recordings were obtained using an NPI SEC-05L amplifier (npi Electronic, Tamm, Germany) in discontinuous current- or voltage-clamp mode. For recording, slices were superfused at room temperature (~22°C) with 95% O_2-5% CO_2-saturated artificial cerebrospinal fluid containing (in mM) 127 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 NaHCO_3, 1.3 MgSO_4·7H_2O, 2.5 CaCl_2, and 25 d-glucose, pH 7.4. Patch pipettes were pulled from thin-walled borosilicate glass (1.5/1.2 mm outer/inner diameter; WPI) to resistances of 5–10 MΩ when filled with an internal solution containing (in mM) 140 CsCl or 140 KCl, 5 HEPES, 2 Mg-ATP, and 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. 2006, 2007; Yasaka et al. 2010). In view of this, neurons were defined as high rheobase (>70 pA) or low rheobase (<30 pA). This afforded a method for distinguishing tonic firing inhibitory interneurons from higher rheobase, putative excitatory, delay firing neurons (Lu et al. 2007; Punakkal et al. 2013; Yasaka et al. 2010).

Spontaneous EPSCs were recorded for 3 min with the neuron clamped at −70 mV, and sIPSCs were recorded at 0 mV (Lu et al. 2009b, 2012). Data were acquired using pCLAMP 9.0 software (Axon Instruments). Synaptic events were analyzed using Mini Analysis software (Synaptosoft, Decatur, GA). Peaks of events were first automatically detected by the software according to a set of threshold criteria. All detected events were then visually reexamined and accepted only if they displayed a monophasic rise time to peak <25 ms, a smooth offset, and an amplitude more than three times the background noise. Organotypically cultured spinal cord cultures derived from the embryos of each dam were used over a 2-wk period. On the day of each experiment, studies were carried out on both 5–8 control slices and 5–8 slices 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 1 h before imaging with the membrane-permeant acetoxyethyl form of the fluorescent Ca^{2+} indicator dye fluo 4 (5 mM; Invitrogen Canada). The conditions for incubating the dye were standardized 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 (in mM) 131 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.3 MgSO_4·7H_2O, 25 d-glucose, and 2.5 CaCl_2 (20°C, flow rate 4 ml/min). Changes in Ca^{2+} fluorescence intensity were measured in dorsal horn neurons with a confocal microscope equipped with an argon (488 nm) laser and filters (XLUMPlanFI ×20, 0.95 NA objective, Olympus FV300; Olympus, Markham, ON, Canada). Changes were evoked by one of two methods: 1) application of high-K^{+} solution (20 or 35 mM) for 90 s or 2) electrical stimulation of the dorsal root entry zone (DRZ) (Biggs et al. 2012). Tungsten bipolar electrodes (WPI) were used with the following stimulating parameters: frequency, 50 Hz;
pulse duration, 100 μs; 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 60 V. A total of 8 stimulus trains (S1–S8) were delivered at 5-min intervals. Since the most stable response amplitudes were seen with the fifth and sixth stimuli (S5 and S6), responses to the fifth set of stimuli were used for all data comparisons (Biggs et al. 2012).

Videos 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 version 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 2-wk period. Five to eight 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, GBP, capsaicin, BCH, or BDNF), and data were collected from at least five cells in each slice.

**Immunohistochemistry.** 3,3′-Diaminobenzidine (DAB) immunohistochemistry was adapted from previously published methods used in organotypic cultures (Avossa et al. 2003). Spinal cord DMOTC were maintained in vitro for 2 wk and then fixed with 4% paraformaldehyde in PBS overnight at 4°C. The following day, cultures were rinsed 3 times in 1% PBS for 10 min 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 incubation in 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 h. 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 100 mg/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, ~18 h, at room temperature in a humid chamber. Following incubation, the cultures were washed 3 times with 1% PBS at room temperature with gentle 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 100 mg/ml bovine serum albumin solution as the primary antibodies for 1.5 h. The cultures were then washed 3 times in 1% PBS for 10 min each time at room temperature with gentle rocking and then incubated with the Vectastain Elite ABC kit (Vector Laboratories) diluted at 1:100 in PBS for 2 h. Cultures were then washed again 3 times for 10 min each time in 1% PBS and then incubated for ~45 s in the developing DAB kit (Vector). The reaction was stopped by washing 3 times for 10 min each time in 1% PBS. The cultures were dehydrated by subsequent 1-min treatments in 50, 70, and 90% ethanol, 50/50% ethanol and xylene, and 100% xylene and then mounted with Permount mounting medium. Images were captured using a Zeiss AxioCam MRm camera with a Zeiss Observer Z1 inverted fluorescence microscope (both from Carl Zeiss, Oberkochen, Germany).

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

**Statistical analysis.** Statistical analysis was carried out using Student’s two-tailed paired t-test or one-way ANOVA with Tukey-Kramer multiple comparisons test as appropriate. Cumulative probability plots for sEPSCs and sIPSCs were analyzed using Kolmogorov-Smirnov (KS) statistics.

**RESULTS**

**Gabapentinoids exert preferential long-term effects on HVA I_{Ca} in medium-sized and small IB_{4−} 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} by 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 −10 mV) was determined from current-voltage plots.

Exposure to 10 μM PGB for 5–6 days reduced maximal HVA I_{Ba} density in small IB_{4−} 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 IB_{4+} neurons. Peak current density in control small IB_{4+} 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 I_{Ba} density to 40% of control amplitude. Thus maximal HVA I_{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 ± 8.7 pA/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 I_{Ba} in medium neurons was prevented in the presence of BCH (300 μM), a specific blocker of this transporter (Hendrich et al. 2008). GBP (100 μM) reduced maximal HVA I_{Ba} density in medium neurons from 27.0 ± 7.4 (n = 13) to 6.8 ± 3.9 pA/pF (n = 13, P < 0.02; Fig. 2E). Maximal I_{Ba} density at −10 mV in BCH-treated medium neurons was 41.0 ± 2.4 pA/pF (n = 12), and this was unchanged in BCH plus 100 μM GBP (39.1 ± 3.8 pA/pF, n = 14, P > 0.65; Fig. 2F).

Figure 2G illustrates a typical family of HVA I_{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 100 μM 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. Figure 2I illustrates normalized and superimposed currents recorded at −10 mV from Fig. 2, G 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 10 μM PGB were highly significant for medium neurons and significant for
A–D: HVA $I_{\text{Ba}}$ density-voltage plots for small isolectin B4-negative (IB4−; A), small IB4-positive (IB4+; B), medium (C), and large DRG neurons (D) in the presence or absence of $10 \mu M$ PGB (applied for 3–4 days). Note profound suppression of current in small IB4− and medium-sized neurons but not in small IB4+ or large neurons. 

E: HVA $I_{\text{Ba}}$ density-voltage plots for control medium neurons and neurons exposed to 100 $\mu M$ GBP for 3–4 days. F: HVA $I_{\text{Ba}}$ density-voltage plots for control medium neurons and medium neurons exposed to 300 $\mu M$ 2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) or 300 $\mu M + 100 \mu M$ GBP for 5–6 days. Note lack of effect of GBP on BCH-treated neurons. G: family of HVA $I_{\text{Ba}}$ evoked in a control small IB4− neuron in response to a series of voltage commands. Voltage recordings are shown in bottom trace. H: family of HVA $I_{\text{Ba}}$ evoked in another small IB4− neuron recorded after 6 days in the presence of 100 $\mu M$ GBP. Note reduction in current amplitude and attenuation of inactivation. J: current recordings obtained at $-10$ mV, 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 $I_{\text{Ba}}$. Error bars in A–F define SE.

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 EPSCs evoked by stimulation of the DRZ. 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 μM PGB on sEPSCs. These responses were completely eliminated by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; data not shown). Figure 4A illustrates typical recordings of sEPSCs at −70 mV. The main effect of 5–6 days of exposure to 10 μM PGB was to reduce sEPSC amplitude in high-threshold (putative excitatory) neurons by ~25% (P < 0.0001 for KS test, Fig. 4B; P < 0.0001 for t-test on average amplitude, Fig. 4B; inset). All other effects of PGB were much smaller. Thus the interevent interval (IEI) of sEPSCs in high-threshold neurons was only slightly affected and was actually decreased by ~12% (P < 0.0001 for KS test and P < 0.05 for t-test on average IEI; Fig. 4C and inset). 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 t-test on average amplitude; 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).

Effects of Mn²⁺ on excitatory synaptic transmission in dorsal horn. If PGB inhibition of excitatory neurotransmission in substantia gelatinosa can be ascribed to a decrease in presynaptic Ca²⁺ influx as a result of impaired Ca²⁺ channel function, similar or even larger effects should be seen with 20 μM Mn²⁺ because this is more effective than 10 μM PGB in reducing I_{Na} in DRG cell bodies (Fig. 3B). However, data pooled from two high-threshold and three low-threshold neurons show that 20 μM Mn²⁺ had no significant effect on sEPSC amplitude or IEI (Fig. 4, F and G). This suggests that the ability of gabapentinoids to suppress excitatory synaptic transmission in substantia gelatinosa cannot be attributed to decreased Ca²⁺ channel expression in primary afferent terminals.

Long-term effects of PGB and GBP 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 0 mV are completely eliminated by a mixture of bicuculline and strychnine (Lu et al. 2012). They thus represent sIPSCs with no contamination from sEPSCs. PGB was generally more effective in altering inhibitory synaptic transmission in low-threshold (putative inhibitory) neurons than in high-threshold (putative excitatory) neurons. PGB (10 μM) reduced the amplitude of sIPSCs in low-threshold neurons by ~39% (P < 0.0001 for KS test, Fig. 5B). This change was highly significant when mean event amplitudes were compared (P < 0.0001 for t-test; Fig. 5B, inset). PGB also substantially increased IEI to 280% of control (decreased sIPSC frequency) of control in low-threshold neurons (P < 0.0001 for KS test and t-test on mean data values; Fig. 5C and inset).

sIPSCs were quite infrequent in high-threshold neurons. PGB (10 μM) altered the amplitude distribution of sIPSCs with a tendency toward more small events (P < 0.0001 for 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). PGB (10 μM) also tended to increase the IEI of sIPSC in high-threshold neurons (P < 0.02 for KS test; Fig. 5E, 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 antiallodynic 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 sIPSCs (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 Ca²⁺ imaging.

Challenge with high-K⁺ solution (20 or 35 mM) produces robust increases in the concentration of intracellular Ca²⁺ in dorsal horn neurons in DMOTC (Biggs et al. 2012; Lu et al. 2009a). Typical recordings are illustrated in Fig. 6A, top trace. K⁺-evoked Ca²⁺ responses were reduced in ampli-

Table 1. Effect of PGB on ratio of peak to end-of-pulse HVA I_{Na} in DRG neurons

<table>
<thead>
<tr>
<th>Control ratio</th>
<th>Small I_{Na}−</th>
<th>Small I_{Na}+</th>
<th>Medium</th>
<th>Large</th>
</tr>
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<tbody>
<tr>
<td>Ratio in PGB (10 μM)</td>
<td>2.94 ± 0.68 (5)</td>
<td>1.94 ± 0.32 (6)</td>
<td>1.57 ± 0.17 (6)</td>
<td>2.35 ± 0.30 (n = 6)</td>
</tr>
<tr>
<td>P value</td>
<td>P &lt; 0.03</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.0001</td>
<td>P &gt; 0.45</td>
</tr>
</tbody>
</table>

Values are ratios (means ± SE; no. of neurons in parentheses) of peak to end-of-pulse high-voltage-activated Ca²⁺ channel currents measured using Ba²⁺ as a charge carrier (HVA I_{Na}) in control and pregabalin (PGB)-treated dorsal root ganglion (DRG) neurons. Decreased ratio = decreased inactivation; refer to Fig. 2F.
Fig. 4. A: sample recording of spontaneous excitatory postsynaptic current (sEPSC) from a high-threshold neuron in the substantia gelatinosa region of a spinal DMOTC (V_h = −70 mV). B and C: cumulative probability plots of sEPSC amplitude (B) and interevent interval (IEI; C) from high-threshold neurons in the substantia gelatinosa region of a spinal DMOTC showing comparison of control neurons with those exposed to 10 µM PGB for 5–6 days. Data represent 3,692 events from 6 neurons in control slices (black) and 9,825 events from 10 neurons in PGB-treated slices (gray). *P < 0.0001 for amplitude and IEI, Kolmogorov-Smirnoff (KS) test. Insets display mean amplitudes and IEIs from the same data. D and E: cumulative probability plots of sEPSC amplitude (D) and IEI (E) from low-threshold neurons showing comparison of control neurons with those exposed to 10 µM PGB for 5–6 days. Data represent 11,139 events from 14 neurons in control slices (black) and 9,680 events from 8 neurons in PGB-treated slices (gray). P < 0.0004 for amplitude and P < 0.0001 for IEI, KS test. Insets display mean amplitudes and IEIs from the same data. F and G: lack of effect of 20 µM Mn^{2+} on amplitude (F) and IEI (G) of sEPSC data pooled from 3 low-threshold and 2 high-threshold neurons (black, control; gray, Mn^{2+}).

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 100 µM GBP had no significant effect on the Ca^{2+} responses evoked with 35 mM K^{+} (Table 2).

By contrast, when slices were exposed to GBP for 5–6 days, Ca^{2+} responses evoked by 35 mM K^{+} were reduced in a concentration-dependent fashion (Fig. 6, B and C). Whereas 1 and 10 µM GBP were without effect, pronounced reduction of the amplitude and AUC was seen with 100 µM GBP (P < 0.01).

Similar effects were seen with PGB, which depressed Ca^{2+} responses evoked by stimulation of the DRZ (Fig. 6D). PGB was more effective than GBP, because clear depression of
stimulation-evoked Ca\(^{2+}\) responses was seen with concentrations as low as 1 \(\mu M\) (\(P < 0.0001\) for 1, 10, or 100 \(\mu M\) PGB for both amplitude and AUC; Fig. 6, E and F).

**Cellular origin of \(K^+\)-evoked Ca\(^{2+}\) signals.** Because glutamate is known to increase intracellular Ca\(^{2+}\) levels in cultured spinal astrocytes (Ahmed et al. 1990), there is a possibility that \(K^+\)-evoked Ca\(^{2+}\) signals in spinal DMOTC originate from astrocytes rather than neurons. This seems unlikely, because the cells generating Ca\(^{2+}\) signals in response to 35 mM K\(^+\) (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 nonstimulated 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\(^{2+}\) responses (Fig. 7F). The cell represented in Fig. 7E responds to both norepinephrine (NE; 25 \(\mu M\)) and ATP (100 \(\mu M\)), whereas that represented in Fig. 7F does not. Since it has been suggested that NE and ATP evoke Ca\(^{2+}\) 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.

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

**Gabapentinoid effects on substantia gelatinosa neurons require entry via the system L amino acid transporter.** Blockade of the long-term effects of gabapentinoids on HVA \(I_{\text{Ba}}\) in DRG neurons by BCH (Fig. 2, E 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 α2δ-subunits (Eroglu et al. 2009).

Addition of 300 μM BCH to spinal cord DMOTC together with 100 μM GBP prevented its action. The two left columns of Fig. 8, A and B, show the extent of suppression of K⁺-evoked Ca²⁺ responses by 100 μM GBP (P < 0.01 for amplitude and P < 0.001 for AUC compared with control). By contrast, 100 μM GBP failed to decrease K⁺-evoked Ca²⁺ responses in the presence of 300 μM BCH. In fact, responses evoked by BCH plus GBP were larger than those evoked by BCH alone (P < 0.05 for amplitude and P < 0.001 for AUC, right columns of Fig. 8, A and B).

Experiments using 10 μM PGB and Ca²⁺ responses evoked by DRZ stimulation yielded similar but not identical data. PGB strongly depressed response amplitude and AUC (P < 0.001 for both compared with control; Fig. 8, C 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 (P < 0.01 and P < 0.001, respectively; Fig. 8, C and D).

Gabapentinoid effects on BDNF-treated slices. Since gabapentinoids are primarily antiallodynic agents rather than analgesics, it is important to determine their actions in “neuropathic” slices. Since BDNF is strongly implicated in the central

Table 2. Lack of effect of acutely applied GBP on K⁺-evoked Ca²⁺ responses in dorsal horn neurons

<table>
<thead>
<tr>
<th></th>
<th>100 μM GBP</th>
<th>Control</th>
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<tbody>
<tr>
<td>Amplitude, au</td>
<td>863.7 ± 166.8</td>
<td>649.8 ± 145.7</td>
</tr>
<tr>
<td>AUC, au</td>
<td>104,734 ± 13,742</td>
<td>98,676 ± 19,158</td>
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Values are means ± SE of amplitude and area under the curve (AUC) in arbitrary units (au) in control and gabapentin (GBP)-treated dorsal horn neurons (n = 19 neurons, 10 μM GBP; n = 13 neurons, 100 μM GBP). P values are from comparisons with respective controls.
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, 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 200 ng/ml BDNF for 5–6 days (Lu et al. 2007, 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 200 ng/ml BDNF for 5–6 days (Lu et al. 2007, 2009a).

By contrast with the lack of effect of a low concentration of GBP (10 μM; Fig. 6, B and C) in control cultures, this concentration was quite effective in attenuating K⁺-evoked Ca²⁺ responses in DMOTC slices cultured with BDNF. Thus responses recorded in 10 μM GBP plus BDNF were of significantly lower amplitude than those recorded in BDNF alone (P < 0.001, Fig. 9A), as was the AUC (P < 0.05, 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, because small, IB₄⁻ neurons are more sensitive to the action 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
Actions of the Ca$^{2+}$ channel blocker Mn$^{2+}$ argued against this possibility. Third, we asked whether gabapentinoids have preferential effects on excitatory neurons and processes in dorsal horn. In general, this seemed to be the case. We went 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 I$_{Ba}$ in DRG neurons.** According to the classical literature, Aδ- and C-fibers, with medium-sized and small 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 for transfer of innocuous information. Over the last 10 years, however, it has become apparent that the situation is far more complex; some Aβ-fibers may carry nociceptive information, whereas not all C-fibers 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 nonpeptidergic, whereas IB₄ neurons contain neuropeptides (Braz et al. 2014). Despite this, the assumption that IB₄ - neurons are always nociceptors has been challenged (Braz et al. 2014; Fang et al. 2005). Thus, when DRG neurons are studied 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 permits the straightforward conclusion that not all afferents are equally sensitive to gabapentinoids. This explains why they lack a simple anesthetic effect. The antiallodynic as opposed to
analgesic properties of gabapentinoids may also reflect their increased effectiveness in "neuropathic" BDNF-treated slices (Fig. 9). As discussed below and as illustrated in Fig. 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 amino acid transporter, absence of this transporter in large and small IB₃₊ neurons may provide an explanation for their selective action on medium and small IB₃₋ neurons. Another possibility relates to differences in the properties of αδ-subunits in the different neuron types. This is supported by the recent description of two splice variants of the αδ-1 subunits in DRG neurons (Lana et al. 2013). One, designated αδ-1 ΔA+B+C, is found in many brain regions, whereas the other, αδ-1 ΔA+BAC, is upregulated in small DRG neurons following nerve injury. Interestingly, the αδ-1 ΔA+BΔC variant expresses low affinity for gabapentinoids. Perhaps the relative abundance of αδ-1 ΔA+BΔC in different subpopulations of DRG neurons relates to their differential sensitivity to gabapentinoids.

**Presynaptic Ca²⁺ channels and gabapentinoid action.** Mn²⁺ (20 μM) was more effective than PGB (10 μM) in blocking HVA IₓBa in DRG neurons (Fig. 3B). By contrast, this concentration of PGB was more effective than Mn²⁺ 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 Ca²⁺-channels and reduced voltage-gated Ca²⁺ influx into presynaptic nerve terminals. If this were the case, 20 μM Mn²⁺ would be more effective than 10 μM PGB. Although in expression systems and in DRG cell bodies, interaction of gabapentinoids with αδ impedes trafficking of pore-forming Cavα₂,1 α-subunits of voltage-gated Ca²⁺ 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 α-subunits of voltage-gated Ca²⁺ 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 Ca²⁺-channels and release sites (Cao and Tsien 2010), the strength of synaptic transmission is normally saturated with regard to Ca²⁺-channel expression. Thus large decreases in Ca²⁺ influx in nerve terminals, as would be seen in the presence of 20 μM Mn²⁺, 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 αδ-subunits interrupts the interaction between Ca²⁺-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 Ca²⁺-independent miniature EPSCs in hippocampal pyramidal neurons (Cheng et al. 2006).

**Effects of gabapentinoids in substantia gelatinosa.** The αδ-1 subunit is expressed as a transmembrane protein with the α-subunit of the mature Ca²⁺ channel (Cassidy et al. 2014; Dolphin 2012). This and other data have 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 retrace (Bailey and Ribeiro-da-Silva 2006).

Because we had used intracellular Cs⁺ to improve the signal-to-noise ratio of sEPSCs, 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 green fluorescent protein-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 sEPSCs in excitatory neurons (Fig. 4B) with only a small and unexpected decrease in IEI (~12%; Fig. 4C). Because the Ca²⁺ uptake 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 IEI of inhibitory events is up to tens of seconds (Fig. 5, C and E). This is far greater than that of excitatory events (always <1.5 s; Fig. 4, C and E), 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 their effect on sIPSCs and play a major role in their 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 \(\delta\)-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 sEPSCs (Fig. 4) and sIPSCs (Fig. 5), they do not preferentially reflect the release of glutamate vs. GABA. Their selectivity, which leads to an overall decrease in dorsal horn excitability, may reflect their aforementioned preferential effect on sEPSC on excitatory (Fig. 4, B and C) vs. inhibitory neurons (Fig. 4, D and E).

This selectivity of action of gabapentinoids toward excitatory terminals on excitatory substantia gelatinosa neurons may relate to their actions in the periphery (Fig. 10). Since gabapentinoid-sensitive, IB\(_4\)– neurons project primarily to excitatory vertical neurons in outer lamina II (Braz et al. 2014), this may explain the stronger effect of \(\alpha_2\beta_3\) 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, because A\(\delta\)-fibers also appear to selectively project to excitatory dorsal horn neurons (Lu and Perl 2005). By contrast, putative nonnociceptive, IB\(_4^+\) C-fiber 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 the relatively weak effects of gabapentinoids at excitatory synapses onto inhibitory neurons (Fig. 4D). Spontaneous synaptic activity in inhibitory neurons may also arise from low-threshold A\(\beta\)-fiber 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.

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 min 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 intraperitoneal administration of GBP attenuates mechanical allodynia 3 h after administration. Interestingly, the amount of depression of HVA/\(J_{\text{Ca}}\), which we see with chronic application of 10 \(\mu\)M PGB in medium and IB\(_4\)– DRG neurons is comparable with the 40% depression seen with acute application of 100 \(\mu\)M GBP or 10 \(\mu\)M 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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