Title page

Pregabalin modulation of neurotransmitter release is mediated by change in intrinsic activation/inactivation properties of Ca\textsubscript{v}2.1 calcium channels.

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Running Title page

Running title: Pregabalin modulation of neurotransmitter release.

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Abbreviations: PGB, pregabalin; EPSCs, excitatory postsynaptic currents; MNTB, Medial Nucleus of the Trapezoid Body; IpCa, presynaptic calcium currents; aCSF, artificial cerebrospinal fluid; QX-314, N-(2,6- iethylphenylcarbamoylmethyl)-triethyl-ammonium chloride; TTX, tetrodotoxin; TEA-Cl, Tetraethyl Ammonium Chloride; APs, action potentials; RM, Repeated Measures ANOVA.

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Abstract

In this work we studied the effects of the anticonvulsant and analgesic drug pregabalin (PGB) on excitatory postsynaptic currents (EPSCs) at principal neurons of the mouse Medial Nucleus of the Trapezoid Body (MNTB), as well as on presynaptic calcium currents ($I_{pCa}$) at the calyx of Held. We found that the acute application of PGB reduced the amplitude of EPSCs in a dose-dependent manner with a maximal blocking effect of about 30%. A clinical high-concentration dose of PGB (e.g., 500 μM) blocked $Ca_{V2.1}$ channel-mediated currents and decreased their facilitation during 100 Hz train, without changing their voltage-dependent activation. Furthermore, PGB also remove the inactivation of $Ca_{V2.1}$ channels at a clinically relevant low concentration of 100 μM. These results suggest novel modulatory mechanisms mediated by the acute administration of PGB on fast excitatory synaptic transmission and might contribute to better understanding PGB anticonvulsant/analgesic clinical effects.
Introduction

Pregabalin (PGB) [S-[+]-3-isobutylGABA] is a blood-brain barrier crossing drug with anticonvulsant and analgesic actions without any known effect on either γ-aminobutyric acid (GABA)A or GABAB receptors (Taylor et al., 1998; Maneuf et al., 2003). Although PGB is being used for the treatment of epilepsy and neuropathic pain, its mechanism of action is still not fully understood. PGB pharmacological actions (Belliotti et al., 2005; Taylor et al., 2007) are dependent on its high-affinity binding to α₂-δ auxiliary subunit of voltage-gated calcium channels (Gee et al., 1996; Taylor and Garrido, 2008). This mechanism is especially interesting considering that auxiliary subunits, like α₂-δ, are known to modulate membrane trafficking of calcium channels as well as their kinetic properties (Qin et al., 1998; Klugbauer et al., 2003). Also, an intracellular action of PGB, mediated by the L-amino acid transporter, has been suggested (Cunningham et al., 2004). Chronic PGB could play an active role on calcium channel trafficking (Hendrich et al., 2008). Using mutant subunits that do not bind gabapentin, Hendrich et al (2008) showed that the effect on channel trafficking were via α₂δ-1 and α₂δ-2. Furthermore, gabapentin has been involved in cortical synaptic maturation in mice (Eroglu et al., 2009), in such a way that gabapentin receptor, α₂-δ subunits, was required during early developmental stages of cortical excitatory synaptic transmission.

On the other hand, it has been shown that acute application of either PGB or gabapentin reduced calcium currents (Reviewed in Taylor et al., 2007), by interacting with α₂-δ auxiliary subunits (Stefani et al., 1998; Martin et al., 2002; Sutton et al., 2002). Concomitantly, PGB reduces evoked postsynaptic responses (Cunningham et al., 2004; Joshi and Taylor, 2006; Micheva et al., 2006). In contrast, studies with recombinant voltage-gated calcium channels have not shown any acute effect of PGB on channel function (Hendrich et al., 2008). Furthermore, electrophysiological recordings have
failed to describe acute gabapentin-mediated changes in calcium channel currents recorded from Purkinje cells or from human hippocampal neurons. Despite the contradictory effects of GBPs on calcium currents most of the studies where the effect of this compounds on transmitter release was analyzed showed a reduced release of various neurotransmitters from synapses in several neuronal tissues (Taylor et al. 2007). The aim of this work was to study acute PGB mechanism of action on excitatory neurotransmitter release at principal neurons of mouse Medial Nucleus of the Trapezoid Body (MNTB; Schneggenburger and Forsythe, 2006). This in vitro model consists of an axosomatic glutamatergic synapse (calyx of Held) onto MNTB neurons, which functions as a relay in the binaural auditory brainstem computing sound source localization (Schneggenburger and Forsythe, 2006). Due to the large size of presynaptic terminals and its accessibility to a patch clamp pipette, it is possible to directly measure both presynaptic calcium currents (IpCa) from the calyx of Held and excitatory postsynaptic currents (EPSCs) from the soma of the MNTB neurons (Schneggenburger and Forsythe, 2006). PGB-mediated modulation of both presynaptic CaV2.1 (P/Q-type) calcium currents and EPSCs was studied.

We observed that PGB reduced the amplitude of EPSCs at the calyx of Held synapses with a dose-dependence blocking effect that reached a maximum plateau of ~30%. The amplitude of presynaptic CaV2.1 (P/Q-type) calcium currents decreased although to lesser extend, in the presence of PGB, while no differences were observed in their voltage activation properties. A larger rescue from inactivation of CaV2.1 presynaptic channels was induced by both 100 μM and 500 μM PGB (i.e., plasma concentrations expected during single and repetitive clinically administrations of PGB, respectively). Our results indicate that PGB significantly alters synaptic glutamatergic neurotransmission by modulation of presynaptic calcium channels.
Methods

Preparation of brainstem slices

Experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Fifty 11–15 days old mice were used in this study. Brain was removed rapidly after decapitation, and placed into an ice-cold low-sodium artificial cerebrospinal fluid (aCSF). The brainstem was glued on a Peltier chamber of an Integraslice 7550PSDS vibrating microslicer (Campden Instruments Ltd, UK). Transverse slices containing the MNTB were cut sequentially and transferred to an incubation chamber containing normal aCSF with low calcium/high magnesium (0.5mM CaCl\(_2\) and 3mM MgCl\(_2\)) at 37°C for 1 h. Slices were then allowed to rest at room temperature. Slices of 200 μm and 300 μm thickness were used for presynaptic Ca\(^{2+}\) current and EPSC recording experiments, respectively. Normal aCSF contained (mM): NaCl 125, KCl 2.5, NaHCO\(_3\) 26, NaH\(_2\)PO\(_4\) 1.25, glucose 10, ascorbic acid 0.5, myo-inositol 3, sodium pyruvate 2, MgCl\(_2\) 1 and CaCl\(_2\) 2. Low sodium aCSF was obtained from normal aCSF replacing NaCl by 250mM sucrose and changing both MgCl\(_2\) and CaCl\(_2\) concentrations to 2.9mM and 0.1mM, respectively. The pH was 7.4 when gassed with 95% O\(_2\)–5% CO\(_2\).

Whole-cell patch-clamp recordings

Slices were transferred to an experimental chamber continuously aerated with 95% O\(_2\)-5% CO\(_2\) saturated normal aCSF at room temperature (22-25°C). MNTB neurons were visualized using Nomarski optics of an Eclipse E600-FN (Nikon, Japan) microscope, and a 60X, 1 NA water immersion objective lens (Nikon). Whole-cell voltage clamp recordings were made with patch pipettes pulled from thin-walled borosilicate glass (Harvard Apparatus, GC150F-15, UK). Electrodes had resistances of 3.6-4.2 MΩ for presynaptic recordings and 3.0-3.5 MΩ for postsynaptic recordings, when filled with
internal solution. Patch solutions for voltage clamp recordings contained (mM): CsCl 110, Hepes 40, TEA-Cl 10, Na₂phosphocreatine 12, EGTA 1, MgATP 2, LiGTP 0.5 and MgCl₂ 1. The pH was adjusted to 7.3 with CsOH. To block Na⁺ currents and avoid postsynaptic action potentials, 10 mM N-(2,6- iethylphenylcarbamoylmethyl)-triethyl-ammonium chloride (QX-314) was added to the when recording EPSCs. Lucifer yellow (1-3 mg/ml) was also included into pipette solution to visually confirm that presynaptic terminals were recorded.

Whole-cell patch clamp recordings were made using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier, a Digidata 1200 (Axon Instruments), and pClamp 9.0 software (Axon Instruments). Electrophysiology data were sampled at 50 kHz and filtered at 4–6 kHz (Low pass Bessel). Series resistance values ranged 6-15 MΩ, for both pre and postsynaptic recordings and were compensated by up to 60%. Whole-cell membrane capacitances (15–25 pF) were obtained from the amplifier “whole-cell capacitance” knob values after total compensation of cell and pipette transients generated by a 10 ms voltage step. Leak currents were subtracted on-line with a P/4 protocol. Calcium currents were recorded in the presence of extracellular TTX (1 μM) and TEA-Cl (10 mM).

EPSCs were evoked by stimulating the globular bushy cell axons in the trapezoid body at the slices’s midline using a bipolar platinum electrode and an isolated stimulator (0.1 ms duration and 4–7 V amplitude). Strychnine (1 μM) was added to the external solution to block glycinergic synaptic responses. Presynaptic action potentials (APs) were measured in whole-cell configuration under current clamp mode. Current clamp intracellular solution contained (mM): K-gluconate 110, KCl 30, Hepes 10, Na₂phosphocreatine 10, EGTA 0.2, MgATP 2, LiGTP 0.5 and MgCl₂ 1. Resting membrane potentials ranged from -68 mV to -78 mV. Electrode series resistance and
electrode capacitance were compensated electronically. APs were elicited using 0.1-2 nA/0.25 ms step current pulses. Pregabalin (Gador S.A., Argentina) and L-Isileucine were bath applied.

**Data presentation and fitting analysis**

Activation curves were obtained from tail currents recorded after depolarizing pulses were repolarized to holding potentials, and fitted to a Boltzmann equation:

\[ I(V) = I_{\text{max}}/(1+\exp(V_{1/2}-V)/k). \]

The inactivation rate of the presynaptic calcium currents was studied using a paired square pulses protocol (Patil et al., 1998) and calculated as \( I_{2\text{PP}}/I_{1\text{PP}} \), corresponding to calcium current inactivation during pre-pulse (i.e., PP from -75 to -10 mV, applied prior to an interpulse) or \( I_{2\text{TP}}/I_{1\text{TP}} \) during test-pulse (i.e., TP, applied after a depolarizing interpulse from -100 to -10 mV, in 10 mV increments). The suffix 1 or 2 for both pre-pulse and test-pulses correspond to the initial or the last 4 ms of the elicited calcium current, respectively (see protocol shown in Fig. 5A).

Activation time constants (\( \tau_{\text{on}} \)) were obtained by fitting the time course of activation of presynaptic calcium currents evoked by depolarizing voltage steps, while the time constant of deactivation was measured fitting the decaying phase of tail currents. Both time courses were fitted by a single exponential function.

PGB dose-response curves were obtained by averaging the percentage of EPSC amplitude reduction at MNTB neurons from different slices. In few cases, increasing concentrations of PGB were tested in the same neuron. In our hands, the steady-state blocking effect of PGB was observed after 15 minutes of slice perfusion for all tested concentrations. Dose response values were fitted to a Hill equation: \( I(C)=I_{\text{max}}/(1+C/IC_{50})^{nH} \) using SigmaPlot 10.
Statistical differences were evaluated using SigmaPlot 10 and Statistica 7. Average data are expressed and plotted as mean±sem. Statistical significance was determined using either Repeated Measures (RM) ANOVA or Student’s t test. The significance of ANOVA comparison was considered significant when \( p<0.05 \) and the Student-Newman-Keuls post hoc test \( r<0.05 \).

**Drugs and reagents**

All substances used for the preparation of aCSF and recording solutions were purchased from either Sigma-Aldrich or Merck (USA).
Results

Acute effects of pregabalin (PGB) on evoked EPSCs

A broad range of concentrations of pregabalin (PGB) have been used in previous reports (Beydoun et al., 2005; McClelland et al., 2004; Micheva et al., 2006). After a single in vivo administration of PGB, its relevant clinical concentration in plasma was described to reach up to 120 µM (Johannessen et al., 2003), while higher concentrations such as 500 µM were expected after multiple doses (plasma half-life ~6 hours, Beydoun et al., 2005). In order to determine the maximal PGB concentrations with a clear synaptic effect in vitro, we first studied the dose-response curve of PGB on EPSCs recorded from the principal neurons of MNTB (Fig. 1A). We recorded EPSCs (which amplitudes were independent of stimulus intensity above threshold) during acute slice bathing with aCSF solutions containing 10, 100, 500 µM and 1 mM PGB. Although 100 µM did not produce a significant effect on synaptic responses in vitro (Fig. 1A, Repeated Measures ANOVA, $p>0.05$), EPSCs were significantly reduced by both 500 µM and 1 mM PGB (Fig. 1A; Repeated Measures ANOVA $F_{[2,16]}= 10.60$, $p<0.003$, Student-Newman-Keuls post hoc test, $t<0.05$). Indeed, mean EPSCs amplitudes were reduced by 30±3% in the presence of 500 µM PGB (Fig. 1A, B, 10.1±0.6 nA, $n = 12$ and 7.1±0.2 nA; $n = 7$, for controls and after 15 minutes of 500 µM PGB, respectively, Student’s t test, $p = 0.024$).

No significant changes on EPSCs amplitudes were observed during 20 minutes of perfusion with aCSF solution (i.e., under vehicle perfusion condition). The reduction of EPSC amplitudes was partially rescued (~10%) by L-Isoleucine (1.5 mM: Fig. 1C empty circles and Fig. 1D, light grey line and bar). L-Isoleucine is a pharmacological tool used extensively to reverse PGB effects due to its known high affinity for PGB-binding site at $\alpha_2$-δ subunits (Stewart et al., 1993; Su et al., 2005).
Amplitudes of miniature EPSCs (mEPSCs) in the presence of TTX (1μM) can be considered good estimates of quantal size (Fig. 2A, B). We found that the frequency of spontaneous events was drastically reduced in the presence of PGB (Fig. 2B; 0.5±0.1 Hz + PGB 500 μM compared to 1.7±0.4 Hz –PGB condition, Student’s t test, p = 0.004) while no differences were found in their mean amplitudes (Fig. 2C; 39±2 pA in –PGB, n = 11 and 38±2 pA in +PGB, n = 10). These results suggest a presynaptic acute action of PGB, reducing spontaneous neurotransmitter release at the calyx of Held-MNTB synapses.

**Acute modulation of presynaptic Ca\(^{2+}\) currents (I\(_{pCa}\)) by Pregabalin**

Based on PGB dose-response presented above, we decided to investigate the effect of PGB (500 μM) on presynaptic Ca\(^{2+}\) currents (I\(_{pCa}\)). We evoked I\(_{pCa}\) with 50 ms depolarizing voltage ramps using normal aCSF solution (Fig. 3A, voltage range:-75 mV to +60 mV). This protocol allowed us to study the effects of PGB on both amplitude and voltage-dependent activation of calcium currents while minimizing its run-down. At the age period used in this work (P11-15), only P/Q type calcium channels mediate neurotransmitter release at the presynaptic calyx of Held (Iwasaki and Takahashi, 1998, Inchauspe et al., 2004 Fedchyshyn and Wang, 2005). Peak I\(_{pCa}\) amplitudes observed after 15 minutes bath application of PGB were reduced by 30% (Fig. 3A, filled grey circles), and were partially rescued up to 10% by L-Isoleucine (1.5 mM, Fig. 3A, empty grey circles). Additionally, L-Isoleucine itself did not affect calcium currents (Supplementary Fig. 1A, B). Therefore, acute PGB was able to reduce calcium currents by a mechanism involving interactions with PGB and L-Isoleucine receptor, the α2-δ auxiliary subunits of the calcium channel.
Furthermore, we examined the effects of PGB on the current–voltage (I–V) relationship of \( I_{\text{pCa}} \). We observed that calcium current density values (peak values at -15 mV) were significantly reduced by PGB (500 \( \mu \)M; Fig. 3B left, representative current traces on the right). Mean calcium current densities were -28.3±3.9 pA/pF for –PGB and -16.5±5.0 pA/pF for +PGB conditions (Repeated Measures ANOVA, \( F_{[2, 214]} = 19.594, p<0.001 \), Student-Newman-Keuls post hoc test, \( t<0.01 \)). Steady-state activation curves, obtained by plotting tail current peak amplitudes vs. step pulse voltage (Fig. 3C), were not significantly different when comparing –PGB and +PGB conditions. Half-activation (\( V_{1/2} \)) voltages were -28.9±0.4 mV (n = 11) in –PGB and -28.3±0.5 mV (n = 11) in +PGB conditions and slope factors were also similar: \( k = 5.7±0.4 \) mV (-PGB) and 5.4±0.4 mV (+PGB) (Student’s t-test; \( p>0.05 \), n = 11).

We analyzed the voltage dependence of \( I_{\text{pCa}} \) time constants of activation (tau-on) and deactivation (tau-off) obtained from the initial part of square depolarizing pulses or their repolarizing phase of tail currents, respectively (see Figure 4). Both activation and deactivation kinetics were faster in the presence of PGB 500 \( \mu \)M. Thus, suggesting a dual PGB effect by reducing \( I_{\text{pCa}} \) amplitudes while accelerating calcium channel kinetics of opening and closing.

**Presynaptic Ca\(^{2+}\) currents (\( I_{\text{pCa}} \)) inactivation properties are targeted by PGB**

Initially, acute PGB was observed to remove current inactivation during depolarizing square pulses (see Fig. 3B right, +PGB). We continued characterizing acute PGB effects on inactivation properties of \( \text{Ca}_V2.1 \) calcium channel mediated \( I_{\text{pCa}} \) by comparing the rate of inactivation using a three square pulses protocol (Fig. 5A; see Methods, Patil et al, 1998). A first pre-pulse (i.e., PP) generated calcium currents that inactivated along the pulse. Thus, calcium currents ratio measured at the beginning and at the end of the
pre-pulse (I1 & I2 PP, respectively) gave us information about the calcium channels inactivation during pre-pulse (see Fig. 5B). Similarly to pre-pulse, we compare calcium currents at the beginning and at the end of the test-pulse (I1 & I2 TP, respectively). Second pulse or inter-pulse (IP) was used to modulate desinactivation/inactivation of calcium channels prior test-pulse (TP). We measured calcium currents at the end of inter-pulse (Fig. 5C).

In control condition there was a 10% pre-pulse inactivation rate (I2PP/I1PP), which was drastically reduced in the presence of PGB (Fig. 5B, 0.90±0.03 for –PGB, n = 16 and 0.99±0.01 for +PGB, n = 6, Student’s t-test; p = 0.042). Such effect was further characterized by analyzing the test-pulse inactivation rate (I2TP/I1TP). While a clear inactivation was detected in the absence of PGB, in the presence of 500 μM of the drug a larger difference in current inactivation was observed (Fig. 5D, squares plot vs. close triangles plot). Significant differences in the voltage dependent I2TP/I1TP ratio were observed (slopes: -PGB = 9±3x10⁻⁴ mV⁻¹ and +PGB = 2±1x10⁻⁴ mV⁻¹, Student t-test, p = 0.025). Furthermore, 100 μM of PGB (a plasma concentration within clinical range) was also capable of rescuing IloCa from inactivation (slope: -6±3x10⁻⁴ mV⁻¹, Student’s t-test, p = 0.001, Fig. 5D, open triangles). The inactivation previously observed in control conditions was abolished when barium replaced calcium as the charge carrier (Supplemental Fig. 2A).

The similarity of mean steady state current-voltage (I-V) relationships observed during the inter-pulse for control (Fig. 5C, -PGB, squares) and +PGB (Fig. 5C, 100 and 500 μM; triangles, repeated Measures ANOVA, p>0.05) supports the hypothesis of a dual blocking/recovering of IloCa by acute PGB. Indeed, the loss of calcium channels by the blocking effect of PGB can be promptly compensated by the rescue of other calcium channels from the inactivation due to PGB.
We continued using a long conditioning pre-pulse protocol to further characterize PGB effects on calcium channels steady-state inactivation. We used depolarizing pre-pulses from -75 mV to -15 mV (2.5 mV steps) of 2.5 s duration to allow calcium channel inactivation (Figure 6A), followed by a 50 ms test pulse to quantify the ratio of channels still available to be open. Figure 6A shows representative $I_{\text{pCa}}$ from calyx of Held terminals in –PGB (Figure 6A, upper black traces) and 500 μM PGB conditions (Figure 6A, bottom grey traces). $I_{\text{pCa}}$ generated by the test voltage step were normalized to the maximum peak amplitude, plotted against the pre-pulse voltage and fitted by the Boltzmann’s distribution function (Fig. 6B). Half-inactivation voltages ($V_{1/2}$) were similar for both conditions (-34.1±0.9 mV, n = 9 for –PGB and -35.9±1.4 mV, n = 6 for +PGB, Student’s t-test, $p$>0.05) but slope factors $k$ were significantly reduced in the presence of PGB: -4.8±0.4 mV and -4.1±0.2 mV for -PGB and +PGB, respectively (Student’s t-test, $p$ = 0.05). Moreover, in the presence of PGB, peak currents elicited by pre-pulse depolarizations above -25 mV reached a “plateau” of bigger $I_{\text{pCa}}$ amplitudes (Fig. 6B). This result indicates that PGB did not affect half-inactivation voltage but allowed more calcium channels to remain open, in agreement with our previous results.

In conclusion, both low (100 μM) and high (500 μM) PGB concentrations accelerate CaV2.1 calcium channel recovery from steady state inactivation, allowing them to be ready to open during successive depolarizations.

**Acute PGB effects on $I_{\text{pCa}}$ elicited by AP waveforms**

In order to assess the effect of PGB on calcium influx during presynaptic nerve action potentials (APs), we studied the acute effect of PGB on the calcium currents evoked by AP waveforms previously recorded under current clamp mode (Fig. 7A). We found that the mean calcium density evoked by a single AP was reduced 14±5% in the presence of
PGB (31.5±3.4 pA/pF for –PGB and 26.4±2.7 pA/pF for +PGB, paired Student’s t-test, p = 0.006, n = 14). Also, I_{pCa} have been shown to facilitate during repetitive stimulation (Cuttle et al., 1998; Borst and Sakmann, 1998; Inchauspe et al. 2004). We continue studying the effects of acute PGB bath application on I_{pCa} facilitation during either paired pulses or 100 Hz trains of APs (Fig. 7B, C). I_{pCa} paired pulse facilitation was slightly reduced by 500 μM PGB (Mean I_{pCa} facilitation ratios: 1.05±0.01 and 1.03±0.01 for –PGB and +PGB, respectively; Student’s t-test, p = 0.03; Fig. 7B), while a clear reduction on the facilitation rate was observed during 100 Hz trains stimulation. Figure 7D shows the time course of calcium current amplitudes vs. number of AP, normalized to the amplitude of the first I_{pCa} evoked by the train. In control conditions, a clear I_{pCa} facilitation was observed (112±2%, n = 15), which was reduced by PGB (106±4%, n = 9; Repeated Measures ANOVA F_{[2, 284]} = 36.99, p<0.001, Student-Newman-Keuls post hoc test, t<0.001; Fig. 7D). However, similar I_{pCa} facilitation was obtained at 100 Hz for both Ca^{2+} with PGB and Ba^{2+} mediated currents (Supplementary Figure 2B). Furthermore, the barium current facilitation was similar in the absence (maximum at 104±3%, n=3) or in the presence of 500 μM PGB (106±6%, n = 3). These results suggest that PGB-mediated reduction of intrinsic short-term facilitation of Ca_{V}2.1 channels was calcium-dependent.
Discussion

Our results suggest that PGB modulates glutamatergic neurotransmission at the calyx of Held-MNTB synapse in three ways: 1) reducing presynaptic Ca\(^{2+}\) influx through CaV2.1 channels, 2) decreasing the number of inactivated presynaptic CaV2.1 channels, 3) decreasing short-term facilitation of presynaptic CaV2.1 channels and 4) accelerating the tau-on of calcium channels.

Few reports have been published showing acute PGB blocking effects on calcium currents at either cultured neurons (Martin et al., 2002; Sutton et al., 2002) or heterologous systems (Hendrich et al., 2008), as well as PGB mediated reduction of synaptic transmission at both cultured hippocampal neurons (Micheva et al., 2006) and neuromuscular junction (Joshi and Taylor, 2006). Recently, Eroglu et al., (2009) demonstrated that \(\alpha_2\delta\) subunit is involved in excitatory synapse formation and suggested a therapeutic role for gabapentin (a PGB analogue) mediated by the blocking of new synapse formation. However, no clear mechanism for PGB has been proposed to explain its cortical antiepileptic effects.

The results presented here provide a novel mechanism of action underlying acute PGB effects on synaptic transmission. Firstly, PGB was shown to block presynaptic CaV2.1 channel mediated currents, and therefore, EPSC amplitudes. In agreement with a previous report (Sutton et al., 2002), blocking calcium channels did not shift the voltage value corresponding to peak of \(I_{\text{pCa}}\) or the steady state activation curves. Secondly, a significant rescue from inactivation was induced by PGB not only at 500 \(\mu\)M (within clinical plasma concentration range expected after multiple PGB doses) but also at 100 \(\mu\)M (within clinical range after a single dose). We consider that PGB acts as a neuromodulator instead of a classical channel blocker of calcium for three reasons: a) the presence of large tail currents observed during stimulation using square pulses (see
Fig. 3B, C, right panels) as well as the results obtained from the double pulse inactivation protocol (Fig. 5A); b) the large amount of $I_{\text{pCa}}$ (Hori and Takahashi, 2009), remaining even in the presence of PGB; and 3) because PGB maintained the steady state half-inactivation voltage of P/Q calcium channels unchanged (Fig. 6B) but modified their kinetics of activation, inactivation and deactivation (Figs. 5D, 6B).

A broad range of concentrations (from 0.25 μM to 1 mM) has been used in previous reports (Bayer et al., 2004; McClelland et al., 2004; Micheva et al., 2006; Hendrich et al., 2008). Furthermore, it was previously described that gabapentin (a PGB analog) may be 4 to 8-fold more concentrated in the brain than in blood plasma (Taylor et al., 1998; Blake et al., 2007). In our hands, dose-response curves for PGB in vitro reached a maximum blocking “plateau” of synaptic responses at 500 μM. Likewise, PGB rescued Cav2.1 presynaptic channels from its inactivated state. A bath concentration of 500 μM was further used since this concentration showed a maximum synaptic effect without any drug associated toxicity. Nevertheless, PGB accumulation can be expected after multiple administrations (i.e., 100 and 500 μM, Johannessen et al., 2003; Beydoun et al., 2005). The discrepancies between the extracellular PGB concentrations used in this work (i.e., using MNTB mice slices) and the ones using in other preparations (hippocampus, trigeminal nucleus, heterologous systems, etc.) might be related to particular interactions of α2-δ auxiliary subunits with its cellular environment. In this sense, different studies with recombinant calcium channels have failed to show any acute effects of gabapentin on channel function (Taylor, 2009) as expected by the fact that recombinant channels lack many interacting proteins normally found at synapses (e.g., syntaxin, synaptotagmin, etc.).

PGB (500 μM) reduced AMPA-mediated EPSCs amplitudes by a 30% during low frequency stimulation without affecting their time course as previously observed at both
culture DRGs (Sutton et al., 2002) and neuromuscular junction (Joshi and Taylor, 2006). On the other hand, no differences were observed on cumulative histograms of rise and decay times of mEPSCs between either condition (data not shown) suggesting a lack of postsynaptic effect of PGB. Thus, given the calcium co-operativity on glutamatergic neurotransmission at the calyx of Held-MNTB synapse (Fedchyshyn and Wang, 2005), the PGB-mediated reduction of EPSCs amplitudes was consistent with the little \( I_{\text{pCa}} \) decrease (~10-14%; see Figure 7A and B).

PGB action on calcium currents (i.e. inhibitory effect on calcium current amplitudes vs. its ability to reduced channel inactivation) appeared to be related to the population of calcium channels being activated. Total number of activated calcium channels using square pulses protocol was larger compared to AP waveforms. Using the former protocol PGB had predominant effect on the recue from inactivation of \( \text{CaV2.1} \) presynaptic channels (see Fig. 5D) while using the later PGB effects on the inhibition of the calcium current is more relevant (see Fig. 7A). Calcium currents elicited by an action potential are “tail currents”-like, less affected by PGB than those recorded with long depolarizing pulses. One possible explanation underlying this effect would be related to the observed reduction in calcium currents activation time constants after PGB treatment. Moreover, a minor contribution could also result from the reduction in steady state inactivation observed with PGB. In fact, a 30% reduction in EPSCs amplitude and a 10% reduction in action potential triggered calcium currents are consistent with a co-operativity of 3 (Schneggenburger and Forsythe, 2006). In addition, both the increase in the activation speed of the \( I_{\text{pCa}} \) and the less inactivation observed for \( \text{CaV2.1} \) channels might justify the decrease of presynaptic short-term facilitation.

We consider that PGB is an important tool to understand the physiological role of the \( \alpha 2-\delta \) auxiliary subunit of voltage-gated calcium channels. Several calcium-dependent
processes like calcium current inactivation (see Fig. 5A) and facilitation (see Fig. 7D) was abolished using barium as the charge carrier (see Supplementary Fig. 2). These results shed light on the effects of α2-δ subunits on the biophysical properties of calcium channels and their physiological function.

**Possible antiepileptic actions of pregabalin**

Several papers have reported a close relationship between alterations on ion channels and different neurological pathologies (channelopathies). Focusing on calcium channels, pathologies like migraine, ataxia or epilepsy have been associated with mutations on the alpha subunit of Cav2.1, P/Q type calcium channels (Terwindt et al., 1997; Burgess and Noebels, 1999; Pietrobon, 2005). Therefore, migraine and epilepsy might be closely related not only in their etiology (i.e. genetic; Fletcher et al., 1996; Terwindt et al., 1997; Noebels, 2001) but also in their treatment (Welch, 2005; Masdrakis et al., 2008).

Thus, a direct modulation of acute PGB on calcium channel α2δ subunits might have an impact in reducing Ca2+-dependent potassium currents and might also have antiepileptic effects. In the hippocampus dentate gyrus, alterations of the existing fine tune between big- (BK) and small-conductance (SK) Ca2+-dependent potassium channels might induce hippocampal synchronization, which leads to temporal lobe epilepsy (Brenner et al., 2005). Indeed, an enhancement of BK over SK channels would preclude dentate granule cells from acting as a “low-pass filter” (i.e., ultimately preventing frontal lobe seizures; see Brenner et al., 2005). Since SK channels are more sensitive to intracellular Ca2+ than BK, results presented here fit into a possible PGB-mediated antiepileptic action through the reduction of BK activation by blocking calcium channels during action potentials while enhancing SK channels activation by preventing calcium channels inactivation during long periods of time (Fig 6B). At the same time, the PGB-
dependent elimination of Cav2.1 channels from inactivation would secondarily favor the recruitment of SK channels, reducing neuronal firing rates. All together, PGB modulation of activation/inactivation properties of Cav2.1 calcium channels is in accordance with previously observed PGB clinical antiepileptic effects (Taylor et al., 2007).

Although our results describe novel acute PGB mechanisms, one can also speculate that PGB chronic actions on cortical areas would control excitation by partially blocking Cav2.1-mediated excitatory efferent from pyramid cells, while preventing Cav2.1 channels from being inactivated during interneuron high frequency repetitive action potential discharges. Thus, PGB might prevent epilepsy-mediated unbalances on cortical excitation/inhibition ratio. Finally, further characterization of PGB actions on both hippocampal and cortical circuits will be central to understand its pharmacological action to treat pathologies such as epilepsy and migraine.

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References


Footnotes

Figure legends

Figure 1. Pregabalin (PGB) reduces excitatory postsynaptic currents.

A. Dose-response relationship fitted to a Hill equation (IC$_{50}$=161.13 μM, Hill slope: -2.4663). EPSC amplitudes after the inhibitory effect of PGB at both 500 μM and 1 mM concentrations, were statistically different respect to control conditions (Repeated Measures ANOVA $F_{[2,16]}= 10.60$, $p<0.003$, Student-Newman-Keuls post hoc test, $t<0.05$). **Top inset.** Representative traces of EPSCs in control conditions and after 15 min incubation with two different PGB concentrations. **B.** Time plot of EPSC normalized amplitudes from MNTB neurons (-75 mV holding potential) before and during bath perfusion with 500 μM PGB (top black solid bar). Mean EPSCs amplitude was reduced by a 30±3% (Student’s t test, $p = 0.024$). Note how EPSCs maximum inhibition reached a steady state value after 10 min of PGB bath application. **C.** EPSC amplitudes in the absence (black dots) or in the presence of PGB (500 μM, grey dots) and after L-Isoleucine application (1.5 mM, open grey dots). **D.** PGB effect was partially rescued (up to 10%) by L-Isoleoeucine. Values are represented as mean±sem.

Figure 2. Frequencies, but not amplitudes of spontaneous EPSCs are reduced by pregabalin (PGB).

**A.** Representative traces of miniatures EPSCs (mEPSPs, in the presence of 1μM TTX) in the absence (top) or presence of 500 μM PGB (bottom). **B, C.** Mean mEPSCs frequencies and amplitudes, respectively. mEPSPs were recorded during 3 minutes. Mean amplitudes were 39±2 pA in -PGB (n=11) and 38±2 pA in +PGB (n =10), while frequencies were 1.7±0.4 Hz and 0.5±0.1 Hz, respectively (*, Student’s t-test, $p = 0.004$). Values are represented as mean±sem.
Figure 3. Acute reduction of presynaptic calcium currents ($I_{\text{pCa}}$) by pregabalin (PGB).

A. Peak current amplitudes observed for 50 ms depolarizing voltage ramps either after bath perfusion with PGB 500 $\mu$M (filled grey circles, n = 7) or with the combination of PGB + Isoleucine 1.5 mM (empty grey circles, n = 3). PGB reduced calcium currents ($I_{\text{pCa}}$) by a 30% while in the presence of PGB+Isoleucine, $I_{\text{pCa}}$ recovered 10% above control amplitudes. Stimulus ramps protocol with their representative $I_{\text{pCa}}$ are shown on the right inset. Numbers from 1 to 3 indicate the specific time-points of representative traces illustrated. B. Current density-voltage relationships for $I_{\text{pCa}}$ before (-PGB) and after 15 min bath perfusion with PGB (+PGB). The $I_{\text{pCa}}$ started activating at -35 mV with an apparent reversal potential at +40 mV. Peak inward current density was reached at -15 mV with mean values of -28.3±3.9 pA/pF for -PGB and -16.5±5.0 pA/pF for +PGB (*, Repeated Measures ANOVA, $F_{[2,214]} = 19.594$, $p<0.001$, Student-Newman-Keuls post hoc test, $t<0.01$). Stimulus waveform for I-V protocol (holding potential -75 mV, voltage square pulses ranging from -60 to +50 mV, 5 mV steps, 20 ms duration) is shown together with representative recordings of $I_{\text{pCa}}$ -PGB (top, black) and +PGB (bottom, grey). Current amplitudes are the mean during the last 5 ms of the recordings for each potential. C. $I_{\text{pCa}}$ activation curves, obtained from tail currents (see representative tails currents shown on right panels). Activation curves were fitted using a Boltzmann’s equation. $I_{\text{pCa}}$ activated at the same voltages at both conditions. Half-activation voltages ($V_{1/2}$) were 28.9±0.4 mV for -PGB (n=11) and 28.3±0.5 mV for +PGB (n=11, Student’s t-test, $p>0.05$). Slopes (k) were 5.7±0.4 mV and 5.4±0.4 mV (Student’s t-test, $p>0.05$) for -PGB and +PGB, respectively. Values are represented as mean±sem.
Figure 4: Effect of PGB (500 μM) on calcium currents activation and deactivation time courses.

A. Representative traces (dotted lines) of $I_{\text{pCa}}$ at -30 mV without (black) and with (grey) pregabalin obtained from the I-V protocol. Current traces were fitted (filled line) with a single exponential function. B. $I_{\text{pCa}}$ activation time constants (tau-on) plotted against voltage command step. There are significant differences between –PGB (n = 10) and +PGB (n = 5; 500 μM) all over the voltage range from -30 mV to +10 mV (* Student t-test, p<0.05). C. Representative traces (dotted lines) of tail currents after repolarizing to -75 mV from a depolarizing pulse at -10 mV without (black) and with (grey) pregabalin, obtained from the I-V protocol. Currents were fitted (filled line) with a single exponential function. D. Deactivation time constant at -10 mV (tau-off) obtained from tail current decaying phase is plotted. Significant differences between –PGB (n =12) and +PGB (n = 4) conditions was found (* Student t-test, p<0.05, n = 12. Values are represented as mean±sem.

Figure 5. PGB eliminates the recovery from inactivation of presynaptic calcium currents, without affecting their voltage-dependent activation.

A. Inactivation protocol (top) consisting of paired square pulses separated by depolarizing voltage steps (inter-pulse voltage $V_{\text{IP}}$ from -75 mV to -10 mV, 10 mV increments) and representative calcium currents (bottom) are shown for –PGB (black) and + PGB 500 μM (grey) conditions. Note how steady-state inactivation during both pre- and test-pulses was reduced by PGB. B. Inactivation rate during the pre-pulse ($I_{\text{2PP}}/I_{\text{1PP}}$) was 10% in normal conditions and was largly reduced by PGB bath application. C. Mean steady-state current vs. interpulse voltage relationship for control (-PGB, squares) and +PGB 100 μM (open triangles) or 500 μM (close triangles). Note
the similarity of curves under the 3 different conditions ($p>0.05$, Repeated Measures ANOVA). D. Inactivation rate during the test-pulse ($I_{2TP}/I_{1TP}$) versus $V_{ip}$. The slope of the lineal fitting for -PGB was $9 \times 10^{-4} \pm 3 \times 10^{-4}$ mV$^{-1}$, $-6 \times 10^{-4} \pm 3 \times 10^{-4}$ mV$^{-1}$ for +PGB 100 μM (Student’s t-test, $p = 0.001$) and $2 \times 10^{-4} \pm 1 \times 10^{-4}$ mV$^{-1}$ for +PGB 500 μM (Student’s t-test, $p = 0.025$).

Figure 6. Presynaptic calcium currents ($I_{pCa}$) steady-state inactivation is modulated by PGB.

A. Stimulation protocol and sample traces of $I_{pCa}$ with (grey) and without PGB (black), evoked by a 20 ms voltage step to the potential corresponding to the peak of the I-V curve, after conditioning prepulses of 1.5 s to voltages ranging from -75 mV to -10 mV (10 mV steps). B. Steady state inactivation from presynaptic terminals with or without PGB. Test $I_{pCa}$ are normalized to the maximum peak amplitude evoked after the -60 mV conditioning pulse, plotted against the conditioning voltage and fitted by a Boltzmann’s distribution function. Half activation voltage are $V_{1/2} = -34.1 \pm 0.9$ mV for -PGB ($n = 9$) and $-35.9 \pm 1.4$ mV for +PGB ($n = 6$, Student’s t-test, $p>0.05$). The slope factor is significantly lower in the presence of PGB: $-4.1 \pm 0.2$ mV and $-4.8 \pm 0.4$ mV for -PGB and +PGB, respectively (Student’s t-test, $p = 0.05$). Values are represented as mean±sem.

Figure 7. PGB decreases $I_{pCa}$ facilitation during paired-pulse and high frequency trains of action potentials.

A. Representative traces of $I_{pCa}$ (left, bottom traces) evoked by a single AP waveform (left, upper trace) recorded at the calyx of Held presynaptic terminals. Mean AP evoked $I_{pCa}$ density (right) are $31.5 \pm 3.4$ pA/pF for –PGB and $26.4 \pm 2.7$ pA/pF for +PGB (500
μM). $I_{pCa}$ density decreases 14±5% in the presence of PGB (Paired Student’s t-test, $p = 0.006, n = 14$). B. Representative paired $I_{pCa}$ traces evoked by paired action potentials (at 100Hz) recorded in current clamp configuration (top), in both absence (middle left, black traces) and presence of PGB (500 μM, middle right grey traces). Plot of $I_{pCa}$ ratios (bottom) in –PGB (squares, mean: 1.05±0.01) and. +PGB (triangles, mean:1.03±0.01, Student’s t-test, $p = 0.03$). C. Representative $I_{pCa}$ traces generated by 100 Hz trains of AP before (top) or after (bottom) PGB bath application. D. Normalized current amplitudes during 100 Hz. train of APs. $I_{pCa}$ facilitation observed in the absence of PGB (maximum at 112±2%, n=15) was attenuated in the presence of 500 μM PGB (106±4% after the 3rd shock, n = 9; Repeated Measures ANOVA, $F_{[2, 284]}= 36.99, p<0.001$, Student-Newman-Keuls post hoc test, $t<0.001$).
Figure 1

A

EPSCs Inhibition (%)

PGB concentration (µM)

B

Normalized EPSCs Amplitudes

Time (min)

C

EPSCs Amplitude (pA)

Time (min)

D

EPSCs Amplitude (pA)

-PGB

+PGB (500 µM)

+PGB (500 µM) + Isoleucine (1.5mM)

-PGB

+PGB 500 µM

+PGB+ISOL
Figure 2

A

-PGB

+ PGB (500 µM)

30 pA

100 ms

B

2.5

2.0

1.5

1.0

0.5

0.0

mEPSC Frequency (Hz)

-PGB

+PGB

C

60

50

40

30

20

10

0

mEPSC Amplitudes (pA)

-PGB

+PGB

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Figure 5

A

Pre-Pulse  Inter-Pulse  Test-Pulse
-10 mV
-75 mV
-100 mV

-PGB

+PGB (500 μM)

10 ms
500 pA

B

C

Inter-Pulse Voltage

D

Inter-Pulse Voltage (mV)

+PGB
-PGB

Inter-Pulse Voltage

1.05
1.00
0.95
0.90
0.85
0.80
0.75

-100 -80 -60 -40 -20 0

100

-100

-100 -80 -60 -40 -20 0

1.1
1.0
0.9
0.8
0.7

+PGB (100 μM)
+PGB (500 μM)

-100 -80 -60 -40 -20 0

0

0.6

0.5

0.4

0.3

0.2

0.1