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Title Page

Amperometric Measurement of Glutamate Release Modulation by Gabapentin and Pregabalin in Rat Neocortical Slices: Role of Voltage-Sensitive Ca^{2+} $\alpha_2\delta$-1 Subunit

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Running Title: $\alpha_2\delta$- ligand Modulation of Glutamate Release

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Abbreviations: aCSF, artificial cerebrospinal fluid; GBP, gabapentin; PGB, pregabalin; R-IBG, R-(-)-3-isobutylgaba; VSCC, voltage-sensitive calcium channel; MEAs, microelectrode arrays; K^+, potassium

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ABSTRACT

Gabapentin (GBP; Neurontin®) and pregabalin (PGB; Lyrica®, *S*-(+)-3-isobutylgaba) are used clinically to treat several disorders associated with excessive or inappropriate excitability, including epilepsy; pain from diabetic neuropathy, postherpetic neuralgia and fibromyalgia; and generalized anxiety disorder. The molecular basis for these drugs' therapeutic effects are believed to involve the interaction with the auxiliary $\alpha_2\delta$ subunit of voltage-sensitive Ca^{2+} channels (VSCC) translating into a modulation of pathological neurotransmitter release. Glutamate as the primary excitatory neurotransmitter in the mammalian central nervous system contributes, under conditions of excessive glutamate release, to neurological and psychiatric disorders. This study used enzyme-based microelectrode arrays to directly measure extracellular glutamate release in rat neocortical slices and determine the modulation of this release by GBP and PGB. Both drugs attenuated K^+-evoked glutamate release without affecting basal glutamate levels. PGB (0.1-100 μM) exhibited a concentration-dependent inhibition of K^+-evoked glutamate release with an IC_{50} of 5.3 μM. *R*-(-)-3-Isobutylgaba, the enantiomer of PGB, did not significantly reduce K^+-evoked glutamate release. The decrease of K^+-evoked glutamate release by PGB was blocked by the L-amino acid L-isoleucine, a potential endogenous ligand of the $\alpha_2\delta$ subunit. In neocortical slices from transgenic mice having a point mutation (i.e., R217A) of the $\alpha_2\delta$ -1 (subtype) subunit of VSCC, PGB did not affect K^+-evoked glutamate release yet inhibited this release in wild-type mice. The results show that GBP and PGB attenuated stimulus-evoked glutamate release in rodent neocortical slices and that the $\alpha_2\delta$ -1 subunit of VSCC appears to mediate this effect.

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Introduction

Several neurological and psychiatric disorders characterized by excessive or dysfunctional neurotransmitter release are routinely treated with gabapentin [GBP; Neurontin®, 1-(aminomethyl)cyclohexaneacetic acid] and pregabalin [PGB; Lyrica®, S-(+)-3-isobutylgaba, S-(+)-4-amino-3-(2-methylpropyl)butanoic acid] (Dooley et al., 2007). Although multiple mechanisms of action have historically been proposed to account for the preclinical and clinical profiles of these drugs, there is increasing evidence for a significant role of the auxiliary $\alpha_2\delta$ subunit of voltage-sensitive Ca^{2+} channels (VSCC) (Taylor et al., 1998; Taylor et al., 2007).

The binding of these ligands to the $\alpha_2\delta$ subunit is believed to be the source of their efficacy in treating epilepsy; pain from diabetic neuropathy, post-herpetic neuralgia, and fibromyalgia; and generalized anxiety disorder. With the recent availability of transgenic mice with point mutations of the $\alpha_2\delta$ subunit (i.e., $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 subtypes) (Bian et al., 2006; Bian et al., 2008), preclinical experiments can be designed to test for altered neurochemical and behavioral effects of GBP and PGB (Field et al., 2006).

In the present study, we used enzyme-based microelectrode arrays (MEA) that have micrometer-size platinum recording sites with sampling rates of > 1 Hz. These MEAs were developed in response to the limitations of other techniques or devices used to measure neurotransmitters, e.g. microdialysis/perfusate sampling coupled to high-performance liquid chromatography (Barnes et al., 1988; Shinohara et al., 1998; Burmeister et al., 2000). A drawback of microdialysis or perfusate sampling techniques is that they sample from a large area (Borland et al., 2005) and at relatively slow (minutes) sampling rates. Given the rapid nature of neurotransmission of chemical messengers such as glutamate, faster sampling rates and smaller sampling areas should be beneficial. Enzyme-coated MEAs have been extensively characterized in the brain of anesthetized and behaving animals to measure glutamate (Burmeister et al.,

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2002; Binns et al., 2005; Day et al., 2006; Nickell et al., 2006; Rutherford et al., 2007; Parikh et al., 2010) but not in brain slices.

We used these MEAs to directly measure K^+-evoked (extracellular) glutamate release in rat neocortical slices and to determine the modulation of this release by GBP and PGB. As the primary excitatory neurotransmitter in the mammalian CNS, glutamate has often been associated with a variety of pathological conditions (Meldrum, 2000), several of which are responsive to $\alpha_2\delta$-ligands like PGB. A reduction of excessive glutamate release by $\alpha_2\delta$ -ligands conceivably translates into clinically relevant therapeutic effects, especially considering the experimental evidence supporting a relationship between $\alpha_2\delta$ subunit binding and the modulation of processes subserving neurotransmitter release (Dooley et al., 2007).

An additional aspect of this study was to assess the effects of PGB on K^+-evoked (extracellular) glutamate release in neocortical slices from wild-type and $\alpha_2\delta$ -1 mutant mice. The $\alpha_2\delta$ -1 transgenic mice have a point mutation (viz., R217A) that markedly reduces [^{3}H]-GBP and [^{3}H]-PGB binding in CNS regions (e.g., neocortex) known to preferentially express the $\alpha_2\delta$ -1 protein (Bian et al., 2006; Field et al., 2006).

Methods

Animals. Male rats [Sprague-Dawley, 2-8 weeks old; Harlan Laboratories, Indianapolis, IN] and male mice [wild type and mutant $\alpha_2\delta$ -1 R217A (Bian et al., 2006; Field et al., 2006), 2-5 months old; Charles River Laboratories, Wilmington, MA] were housed in an AAALAC-accredited facility according to the standards outlined in the *Guide for the Use and Care of Laboratory Animals*.

Animals were under a 12-hr light-dark cycle, had *ad libitum* access to food and water, and were maintained for a minimum of 5 days before euthanasia by decapitation. The brains were removed by blunt dissection and placed in ice-cold buffer until slice

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preparation. The glutamate recordings occurred during the light phase of the light-dark cycle. All experimental protocols were approved by The Animal Care and Use Committee of the University of Kentucky.

Glutamate Release Measurements. Neocortical slices from rats and mice were prepared using standard protocols (Hascup et al., 2007). Briefly, coronal slices (0.35-0.4 mm thick), including the frontal and parietal areas exhibiting relatively high [^{3}H]-GBP and [^{3}H]-PGB binding (Bian *et al.*, 2006), were maintained for at least 1 h at room temperature in artificial cerebrospinal fluid (aCSF; composition (mM): NaCl (124), KCl (5), $CaCl_2$ (2.5), $MgCl_2$ (1.5), $NaHCO_3$ (26), NaH_2PO_4 (1.4), D-glucose (10); saturated with 95% O_2/5% CO_2; pH = 7.2–7.4] before the start of an experiment. The slices were transferred to immersion-style chambers (i.e., one slice/chamber), and superfused at a rate of 1.5-2.0 ml/min with aCSF (31-33°C). Each chamber was fitted with a Ag/AgCl reference electrode.

Ceramic-based MEAs (4 platinum sites in a row, 50 x 150 µm each) were assembled, coated with Nafion®, and subsequently coated with three layers of a 1% glutamate oxidase (Associates of Cape Cod, East Falmouth, MA)/1% bovine serum albumin/0.125% glutaraldehyde enzyme solution. Coated MEAs were allowed to cure a minimum of two days before use. Enzyme-based MEAs measure glutamate through the enzymatic breakdown of glutamate to yield a reporter molecule of hydrogen peroxide that is subsequently oxidized on the platinum recording surface to yield an oxidation current. The MEAs were then calibrated (*in vitro*) with glutamate in a phosphate-buffered solution (pH = 7.4) at 31-34°C to (a) generate a standard response curve (sensitivity > 2 pA/µM); (b) determine the limit of detection (≥ 3 times the signal-to-noise ratio; < 2.0 µM); and (c) assess the selectivity for glutamate relative to an endogenous electroactive compound, ascorbic acid (> 30:1). A MEA or MEA/micropipette assembly

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was lowered into the neocortical slice, and extracellular glutamate levels were measured once basal glutamate levels stabilized for at least 10 min.

Test substances were delivered through the superfusion system for a minimum of 15 min. before slice stimulation unless stated otherwise. The slices were stimulated twice (S_1, S_2) with high K^+ by one of two methods to evoke glutamate release: 1) direct, local application of 70 mM K^+ solution (composition (mM): KCl (70), NaCl (79), $CaCl_2$ (2.5); pH = 7.0-7.4) to depolarize the local glutamatergic network via pressure ejection; or 2) superfusion of 70 mM K^+ (i.e., increase of KCl in aCSF with corresponding decrease of NaCl (59 mM) to maintain iso-osmolarity) to depolarize the whole slice. Slices were allowed to recover a minimum of 20 minutes between stimulations, and drug solutions were delivered for a minimum of 15 minutes before stimulation. For local application, glass micropipettes (inside tip diameter of 10-15 µm) were formed from stock (1 mm o.d., 0.58 mm i.d.; A-M Systems, Everett, WA), attached, and the tip centered over the MEA recording site at a tip-to-tip distance of 70-110 µm. The 70 mM K^+ solution was applied at 1-min intervals until at least two to five reproducible glutamate responses were recorded. Delivery of solution volumes (i.e., 12.5-400 nL over 0.1-3.0 sec) was controlled by a pressure-ejection system (2-12 p.s.i.; Picospritzer II, Parker Hannifin Corp., Cleveland, OH), and monitored using a stereomicroscope fitted with a reticule (Gerhardt and Palmer, 1987). Extracellular glutamate levels were measured at 1 Hz using constant potential amperometry (+0.7 V *vs* Ag/AgCl reference) controlled by a FAST16 electrochemical recording system (Quanteon, LLC, Nicholasville, KY) and analyzed offline by customized Excel®- based software.

Calculations and statistics. Glutamate release amplitudes were calculated from the difference between maximum K^+-evoked glutamate release values and basal values. Values given are $X \pm$ S.E. ($n \geq 6$). In one set of experiments with PGB, a

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concentration-effect curve with corresponding IC_{50} value was calculated by nonlinear regression (Prism 4.0, GraphPad Software Inc., San Diego, CA). If appropriate, the results were analyzed using the t-statistic for group means, or one- or two-way analysis of variance followed by post-hoc comparisons using Dunnett's or Bonferroni multiple comparison statistic (InStat 3.0, GraphPad Software Inc., San Diego, CA). The minimal level of significance was $p \leq 0.05$ (two-tail criterion).

Materials. Substances were either commercially available (Sigma-Aldrich) or donated (i.e., GBP, PGB, and R-(-)-3-isobutylgaba (R-IBG) (Pfizer)). Test compounds were dissolved directly in aCSF.

Results

The effects of GBP and PGB on resting glutamate levels were evaluated in initial experiments. Neither drug (0.1-100 µM) altered basal glutamate levels in neocortical slices (data not shown).

In the brains of anesthetized animals, delivery of high K^+ to stimulate glutamate release has been performed with local, pressure-ejected administration (Burmeister et al., 2002; Day et al., 2006; Quintero et al., 2007; Stephens et al., 2009). Meanwhile, studies in brain slices permit the use of two methods of delivering high K^+ solutions to activate neural networks: local delivery and superfusion. Local stimulation, using pressure delivery, produces a comparable stimulus to those used in previous studies with anesthetized animals. An additional benefit of brain slice recordings is the flexibility to also employ superfusion of high K^+ to evoke release. This sustained depolarization resembles prolonged or excessive excitability – a condition that characterizes some neurological disorders such as anxiety. We used both stimulation methods here to characterize the effects of $\alpha_2\delta$ subunit ligands and to compare glutamate measurements with MEAs to previous studies.

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The repeated pressure-ejection delivery of 70 mM K^+ solution yielded similar size glutamate signals with a mean amplitude of 3.9 ± 0.8 μM (Fig. 1A, B). In the presence of GBP (100 μM), the mean amplitude was significantly decreased by 46% to 2.1 ± 0.7 μM (Fig. 1A, B).

Because GBP was confirmed to modulate K^+-evoked glutamate release, the more recently developed $\alpha_2\delta$ ligand, PGB, was chosen for testing in additional experiments. PGB (100 μM) attenuated pressure-ejection delivery of 70mM K^+ solution (5.7 ± 1.5 μM glutamate, pre-PGB vs. 1.7 ± 1.2 μM glutamate, post-PGB; $t(4) = 3.59$, $p = 0.023$). We then transitioned to a paradigm of using repeated stimulation with superfused 70 mM K^+ (S_1, S_2). The S_2/S_1 ratio of control glutamate signals in rat neocortical slices was 0.97 (Figs. 2A, 3A); this ratio was markedly reduced by 78% to 0.21 by PGB (100 μM) (Figs. 2B, 3A). Other S_2/S_1 ratios for PGB include 0.80 (non-significant 14% inhibition) at 0.1 μM, 0.60 (non-significant 38% inhibition) at 1 μM, and 0.44 (54% inhibition) at 10 μM (Fig. 3A); an IC_{50} value of 5.3 μM was determined from the concentration-effect relationship (Fig. 3B). The enantiomer of PGB, R-IBG (100 μM), gave an S_2/S_1 ratio of 0.70 (non-significant 28% inhibition), contrasting sharply with the effect (78% inhibition) of an equimolar concentration of PGB (Fig. 3A).

PGB and the endogenous amino acid, L-isoleucine, are substrates for the system L-amino acid transporter and both have similar nanomolar affinity for the $\alpha_2\delta$ ligand binding site on the $\alpha_2\delta$ subunit. The S_2/S_1 ratio associated with the K^+-evoked glutamate signals in the presence of L-isoleucine (100 μM) was 0.84 (non-significant 13% inhibition), yet this compound reduced the effect (78% inhibition) of PGB (100 μM) as indicated by the S_2/S_1 ratio of 0.59 (non-significant 39% inhibition) (Fig. 3A).

An action of GBP and PGB at the $\alpha_2\delta$ -1 subtype, rather than the $\alpha_2\delta$ -2 subtype, has been proposed to account for the therapeutic effects of these drugs (Bian *et al.* 2006, 2008; Field *et al.* 2006). Using neocortical slices from the wild-type and $\alpha_2\delta$ -1

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transgenic mice (Bian *et al.* 2006; Field *et al.* 2006), PGB (100 μM) significantly decreased K^+-evoked glutamate signals (Fig. 4A and B) in wild-type by 36% [S_2/S_1 = 0.92 (control) *vs* 0.59 (PGB); n= 13 and 14, respectively] (Fig.5); in the $\alpha_2\delta$ -1 transgenic mice, the glutamate signals were unchanged by this drug [S_2/S_1 = 0.74 (control) *vs* 0.63 (PGB); n=9 each] (Fig. 4C and D).

Discussion

Aberrant glutamate neurotransmission is linked to a variety of neurological and psychological disorders. Thus, identifying mechanisms that could modulate abnormal glutamate release may provide an avenue for developing new therapeutics for modulating glutamate signaling. Here we used enzyme-based MEAs to directly measure extracellular glutamate and observed that both GBP and PGB attenuated the K^+-evoked glutamate release.

GBP has been used as an antiepileptic but its precise mechanism of action is unknown (Taylor et al., 2007). To help address this, we stimulated the neural network and measured synaptic spillover of glutamate employing a technique we have previously used in anesthetized animals of locally delivering high potassium solution to evoke depolarization and produce a release of glutamate (Burmeister et al., 2002; Day et al., 2006). We observed in these brain slices an attenuation of glutamate release by GBP after locally delivering high K^+; an effect on neurotransmitter release that is repeatedly observed after stimulus delivery (Dooley et al., 2000a; Dooley et al., 2000b; Dooley et al., 2002).

PGB is structurally related to GBP but with greater reported efficacy in clinical studies (Taylor et al., 2007). Using these MEAs, we used a whole slice superfusion of high K^+ to stimulate neurotransmitter release similar to Dooley et al. (Dooley et al., 2000b). We have observed that this type of depolarization evokes glutamate release

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that is calcium-dependent (J.E. Quintero and G.A. Gerhardt, unpublished observations). The dose dependent inhibition of K^+-evoked glutamate release by PGB resulted in an IC_{50} = 5.3μM compared to an IC_{50} = 11.8 μM of K^+-evoked [^{3}H]Norepinephrine release (Dooley et al., 2002).

The magnitude of the attenuation of evoked glutamate release was larger than the reports in some studies that have examined GBP or PGB on stimulus evoked neurotransmission (Dooley et al., 2000a; Dooley et al., 2000b; Dooley et al., 2002; Brown and Randall, 2005). One apparent factor that may influence the effectiveness of these compounds is the type of stimulus; such that GBP and PGB may exert an effect on the prolonged, depolarization- induced neurotransmitter release that more closely resembles hyperexcitability as found in pathological states rather than the normal physiological neurotransmission (Dooley et al., 2000a; Maneuf et al., 2001; Dooley et al., 2007). Additionally, some of the GBP or PGB effects on stimulus-evoked neurotransmitter release may have been diluted in studies on slices or synaptic endings where the whole chamber perfusate is sampled (Dooley et al., 2000a; Fink et al., 2000; Fink et al., 2002) versus the limited focal area sampled by the 50 x 150 μm size of these MEAs in slices. For example, with the high resolution technique of whole-cell patch clamp in cortical slices, GBP and PGB show an effect as high as ~80% on parameters related to glutamate neurotransmission (Cunningham et al., 2004). Nonetheless, the effect of GBP and PGB on neurotransmitter release remains controversial given reports that GBP and PGB have no effect on K^+ evoked glutamate release from human synaptosomes (Brawek et al., 2009) or that the GBP and PGB effect may be linked to the trafficking of calcium channels to the cell surface (Hendrich et al., 2008; Mich and Horne, 2008; Bauer et al., 2009; Thorpe and Offord, 2010). The full effect of these ligands may result both from altering calcium channel trafficking and also from the more rapid modulation of synaptic function (Taylor, 2009).

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Meanwhile, the pharmacological effects of PGB are stereoselective and this was borne out by the non-significant changes to the S_2/S_1 with the enantimore, R-IBG. The α amino acids, L-isoleucine and L-leucine, have been proposed as potential endogenous ligands for the $\alpha_2\delta$ subunit (Thurlow et al., 1993). While L-isoleucine did not produce a significant change to K^+-evoked glutamate release, L-isoleucine did inhibit the PGB attenuation of K^+-evoked glutamate release similar to what had been previously described with L-isoleucine and GBP in cortical brain slices (Cunningham et al., 2004). A more complex role in neurotransmission and GBP and PGB effectiveness may be the case for these α amino acids where these endogenous ligands may act as "positive modulators required for full functionality of the $\alpha_2\delta$ subunit" (Hendrich et al., 2008).

Previously, Wang and Offord (1999) showed that the arginine at position 217 in the α region of the $\alpha_2\delta$ subunit is critical for GBP binding. Subsequently, in a R217A knockin mouse that was developed, [^{3}H]GBP and [^{3}H]PGB binding to neocortical membranes was greatly reduced in R217A mice compared to wild-type mice (Bian et al., 2006; Field et al., 2006). Accordingly, in slices from R217A mice we concluded that a functional $\alpha_2\delta$ subunit is necessary for PGB to attenuate K^+ -evoked glutamate release. While the mean S_2/S_1 ratio (0.74) in slices from the R217A mice was lower than the S_2/S_1 ratio of slices from wild-type mice (0.92), the means were not significantly different. However, we cannot rule out the possibility of a change in excitability properties of the neurons and synapses in these animals given that the $\alpha_2\delta$ subunit is critical for normal synapse formation or function (Eroglu et al., 2009).

In summary, we showed that GBP and PGB can modulate stimulus-evoked glutamate release in rat neocortical brain slices and that the $\alpha_2\delta$ subunit of the VSCC is involved in the inhibitory effects of these ligands. This ability to modulate excitatory neurotransmitter release may, in part, explain the efficacy of these molecules in the clinic. The application of slice recording methodology coupled to the MEA recording

technology establishes a new means to better assess drugs mechanisms of action by the direct measurement of neurotransmitter release.

Authorship contributions:

Participated in research design: Quintero, Dooley, Pomerleau, Huettl, Gerhardt.

Conducted experiments: Quintero.

Contributed new reagents or analytic tools: Gerhardt.

Performed data analysis: Quintero, Dooley, Pomerleau, Gerhardt.

Wrote or contributed to the writing of the manuscript: Quintero, Dooley, Pomerleau, Huettl, Gerhardt

Other: Directed research efforts; Gerhardt.

References

Barnes S, Leighton GE and Davies JA (1988) A novel superfusion chamber for the measurement of endogenous glutamate release from cerebellar slices. *Journal of Neuroscience Methods* **23**:57-61.

Bauer CS, Nieto-Rostro M, Rahman W, Tran-Van-Minh A, Ferron L, Douglas L, Kadurin I, Sri Ranjan Y, Fernandez-Alacid L, Millar NS, Dickenson AH, Lujan R and Dolphin AC (2009) The Increased Trafficking of the Calcium Channel Subunit {alpha}2{delta}-1 to Presynaptic Terminals in Neuropathic Pain Is Inhibited by the {alpha}2{delta} Ligand Pregabalin. *J. Neurosci.* **29**:4076-4088.

Bian F, Hannah D and Campbell B (2008) Calcium channel alpha2-delta type 2 subunit is the major binding protein for pregabalin in cerebellum and septum: an ex vivo autoradiographic study in wild-type and genetically modified mice, in *2008 Society for Neuroscience* p Program No. 845.813, Washington, D.C.

Bian F, Li Z, Offord J, Davis MD, McCormick J, Taylor CP and Walker LC (2006) Calcium channel alpha2-delta type 1 subunit is the major binding protein for pregabalin in neocortex, hippocampus, amygdala, and spinal cord: an ex vivo autoradiographic study in alpha2-delta type 1 genetically modified mice. *Brain Res* **1075**:68-80.

Binns BC, Huang Y, Goettl VM, Hackshaw KV and Stephens RL, Jr. (2005) Glutamate uptake is attenuated in spinal deep dorsal and ventral horn in the rat spinal nerve ligation model. *Brain Res* **1041**:38-47.

Borland LM, Shi G, Yang H and Michael AC (2005) Voltammetric study of extracellular dopamine near microdialysis probes acutely implanted in the striatum of the anesthetized rat. *J Neurosci Methods.* **146**:149-158.

Brawek B, Löffler M, Weyerbrock A and Feuerstein T (2009) Effects of gabapentin and pregabalin on K+-evoked 3H-GABA and 3H-glutamate release from human neocortical synaptosomes. *Naunyn-Schmiedeberg's Archives of Pharmacology* **379**:361-369.

Brown JT and Randall A (2005) Gabapentin fails to alter P/Q-type Ca2+ channel-mediated synaptic transmission in the hippocampus in vitro. *Synapse* **55**:262-269.

Burmeister J, Pomerleau F, Palmer M, Day B, Huettl P and Gerhardt G (2002) Improved ceramic-based multisite microelectrode for rapid measurements of L-glutamate in the CNS. *J.Neurosci.Methods* **119**:163-171.

Burmeister JJ, Moxon K and Gerhardt GA (2000) Ceramic-based multisite microelectrodes for electrochemical recordings. *Anal.Chem.* **72**:187-192.

Cunningham MO, Woodhall GL, Thompson SE, Dooley DJ and Jones RS (2004) Dual effects of gabapentin and pregabalin on glutamate release at rat entorhinal synapses in vitro. *Eur.J Neurosci* **20**:1566-1576.

Day BK, Pomerleau F, Burmeister JJ, Huettl P and Gerhardt GA (2006) Microelectrode array studies of basal and potassium-evoked release of L-glutamate in the anesthetized rat brain. *J Neurochem* **96**:1626-1635.

Dooley DJ, Donovan CM, Meder WP and Whetzel SZ (2002) Preferential action of gabapentin and pregabalin at P/Q-type voltage-sensitive calcium channels: inhibition of K+-evoked [3H]-norepinephrine release from rat neocortical slices. *Synapse* **45**:171-190.

Dooley DJ, Donovan CM and Pugsley TA (2000a) Stimulus-dependent modulation of [(3)H]norepinephrine release from rat neocortical slices by gabapentin and pregabalin. *J Pharmacol.Exp.Ther.* **295**:1086-1093.

Dooley DJ, Mieske CA and Borosky SA (2000b) Inhibition of K(+)-evoked glutamate release from rat neocortical and hippocampal slices by gabapentin. *Neurosci Lett.* **280**:107-110.

Dooley DJ, Taylor CP, Donevan S and Feltner D (2007) Ca2+ channel alpha2delta ligands: novel modulators of neurotransmission. *Trends Pharmacol Sci* **28**:75-82.

Eroglu Ç, Allen NJ, Susman MW, O'Rourke NA, Park CY, Özkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF and Barres BA (2009) Gabapentin Receptor [alpha]2[delta]-1 Is a Neuronal Thrombospondin Receptor Responsible for Excitatory CNS Synaptogenesis. *Cell* **139**:380-392.

Field MJ, Cox PJ, Stott E, Melrose H, Offord J, Su TZ, Bramwell S, Corradini L, England S, Winks J, Kinloch RA, Hendrich J, Dolphin AC, Webb T and Williams D (2006) Identification of the alpha2-delta-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. *Proc Natl Acad Sci U S A* **103**:17537-17542.

Fink K, Dooley DJ, Meder WP, Suman-Chauhan N, Duffy S, Clusmann H and Gothert M (2002) Inhibition of neuronal Ca(2+) influx by gabapentin and pregabalin in the human neocortex. *Neuropharmacology* **42**:229-236.

Fink K, Meder W, Dooley DJ and Gothert M (2000) Inhibition of neuronal Ca(2+) influx by gabapentin and subsequent reduction of neurotransmitter release from rat neocortical slices. *Br.J Pharmacol.* **130**:900-906.

Hascup KN, Rutherford EC, Quintero JE, Day BK, Nickell JR, Pomerleau F, Huettl P, Burmeister J, Gerhardt GA, Michael AC and Borland LM (2007) Second-by-Second Measures of L-Glutamate and Other Neurotransmitters Using Enzyme-Based Microelectrode Arrays, in *Electrochemical methods for neuroscience* (Michael AC and Borland LM eds) pp 407-450, CRC Press, Boca Raton, FL.

Hendrich J, Van Minh AT, Heblich F, Nieto-Rostro M, Watschinger K, Striessnig J, Wratten J, Davies A and Dolphin AC (2008) Pharmacological disruption of calcium channel trafficking by the α2δ ligand gabapentin. *Proceedings of the National Academy of Sciences* **105**:3628-3633.

Maneuf YP, Hughes J and McKnight AT (2001) Gabapentin inhibits the substance P-facilitated K(+)-evoked release of [(3)H]glutamate from rat caudial trigeminal nucleus slices. *Pain* **93**:191-196.

Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr* **130**:1007S-1015S.

Mich PM and Horne WA (2008) Alternative Splicing of the Ca2+ Channel β4 Subunit Confers Specificity for Gabapentin Inhibition of Cav2.1 Trafficking. *Molecular Pharmacology* **74**:904-912.

Nickell J, Salvatore MF, Pomerleau F, Apparsundaram S and Gerhardt GA (2006) Reduced plasma membrane surface expression of GLAST mediates decreased glutamate regulation in the aged striatum. *Neurobiol Aging*.

Parikh V, Ji J, Decker MW and Sarter M (2010) Prefrontal beta2 subunit-containing and alpha7 nicotinic acetylcholine receptors differentially control glutamatergic and cholinergic signaling. *J Neurosci* **30**:3518-3530.

Quintero JE, Day BK, Zhang Z, Grondin R, Stephens ML, Huettl P, Pomerleau F, Gash DM and Gerhardt GA (2007) Amperometric measures of age-related changes in glutamate regulation in the cortex of rhesus monkeys. *Exp Neurol* **208**:238-246.

Rutherford EC, Pomerleau F, Huettl P, Stromberg I and Gerhardt GA (2007) Chronic second-by-second measures of L-glutamate in the central nervous system of freely moving rats. *J Neurochem* **102**:712-722.

Shinohara K, Honma S, Katsuno Y, Abe H and Honma K (1998) Circadian release of amino acids in the suprachiasmatic nucleus in vitro. *Neuroreport* **9**:137-140.

Stephens ML, Quintero JE, Pomerleau F, Huettl P and Gerhardt GA (2009) Age-related changes in glutamate release in the CA3 and dentate gyrus of the rat hippocampus. *Neurobiol Aging*.

Taylor CP (2009) Mechanisms of analgesia by gabapentin and pregabalin - Calcium channel [alpha]2-[delta] [Cav[alpha]2-[delta]] ligands. *Pain* **142**:13-16.

Taylor CP, Angelotti T and Fauman E (2007) Pharmacology and mechanism of action of pregabalin: The calcium channel [alpha]2-[delta] (alpha2-delta) subunit as a target for antiepileptic drug discovery. *Epilepsy Research* **73**:137-150.

Taylor CP, Gee NS, Su TZ, Kocsis JD, Welty DF, Brown JP, Dooley DJ, Boden P and Singh L (1998) A summary of mechanistic hypotheses of gabapentin pharmacology. *Epilepsy Res.* **29**:233-249.

Thorpe AJ and Offord J (2010) The alpha2-delta protein: an auxiliary subunit of voltage-dependent calcium channels as a recognized drug target. *Curr Opin Investig Drugs* **11**:761-770.

Thurlow RJ, Brown JP, Gee NS, Hill DR and Woodruff GN (1993) [3H]Gabapentin may label a system-L-like neutral amino acid carrier in brain. *European Journal of Pharmacology: Molecular Pharmacology* **247**:341-345.

Wang M, Offord J, Oxender DL and Su TZ (1999) Structural requirement of the calcium-channel subunit alpha2delta for gabapentin binding. *Biochem J* **342 (Pt 2)**:313-320.

Footnotes:

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Figure Legends

Fig. 1. Effect of GBP(100 μM) on K^+-evoked glutamate release from rat neocortical slices. In (A), glutamate release evoked by repeated pressure ejection delivery of 70 mM K^+ solution (arrowheads on abscissa) in the absence (first three traces) and presence of GBP (last three traces). In (B), the amplitude of K^+-evoked glutamate release [as derived from (A)] was decreased by GBP. Values given are X ± S.E. (n = 6). The paired t-statistic gave t(5) = 2.930 (p =0.0326). A significant difference from the control value is indicated by an asterisk (*$p \leq 0.05$).

Fig. 2. Effect of PGB (100 μM) on K^+-evoked glutamate release in rat neocortical slices. In (A), detection of glutamate release by MEAs after repeat superfusion with 70 mM K^+ (arrowheads; S_1, S_2) for 50 sec. In (B), PGB (closed bar), present 15 min before S_2, attenuated glutamate release.

Fig. 3. Effects of PGB, (0.1-100 μM), R-(-)-3-isobutylgaba (100 μM), and L-isoleucine (100 μM) to inhibit K^+-evoked glutamate release in rat neocortical slices. In (A), concentration-effect relationship of PGB and inactivity of R-(-)-3-isobutylgaba, L-isoleucine, and PGB (100 μM) and L -isoleucine (100 μM) combination after repeat superfusion with 70 mM K^+ (S_1, S_2) for 50 sec. Substances were present 15 min before S_2. Values given are X ± S.E. (n = 7). Analysis of variance of S_2/S_1 values for control and PGB concentrations gave $F(4,30) = 5.17$ (p = 0.003). A significant difference from the control value is indicated by an asterisk (*$p \leq 0.05$, ***$p \leq 0.001$). The S_2/S_1 values obtained for the other substances, including the PGB and L -isoleucine combination, were not significantly different from the control value. The transformed data (B) from (A)

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depict inhibition (%) by PGB relative to the mean control S_2/S_1 value of 0.97 normalized to 1.0; the corresponding IC_{50} value was 5.3 μM.

Fig. 4. Effects of PGB (100 μM) on K^+-evoked glutamate release in neocortical slices from wild-type mice and transgenic mice having a point mutation (i.e., R217A) of the voltage-sensitive Ca^{2+} channel $\alpha_2\delta$-1 subunit. Glutamate release was assessed with MEAs after repeated superfusion with 70 mM K^+ (S_1, S_2) for 50 sec. Slices from wild-type mice were (A) treated as control and (B) exposed to PGB (15 min before S_2). Meanwhile, slices from transgenic mice (R217A) were (C) treated as control and (D) exposed to PGB (15 min before S_2). Open bars: 70 mK K^+; closed bars: PGB.

Fig. 5. The point mutation, R217A, prevents PGB from attenuating K^+-evoked glutamate release in slices. The S_2/S_1 ratio derived from repeated K^+ superfusion of slices was decreased by PGB. A main effect of PGB treatment was identified with a two-way ANOVA [$F(1,41) = 5.57$ ($p = 0.023$)], and a post-hoc Bonferroni test revealed a significant effect ($p < 0.05$) of PGB only in slices from wild-type animals. A significant difference from the control value is indicated by the asterisk (*: $p \leq 0.05$). Values given are X ± S.E.

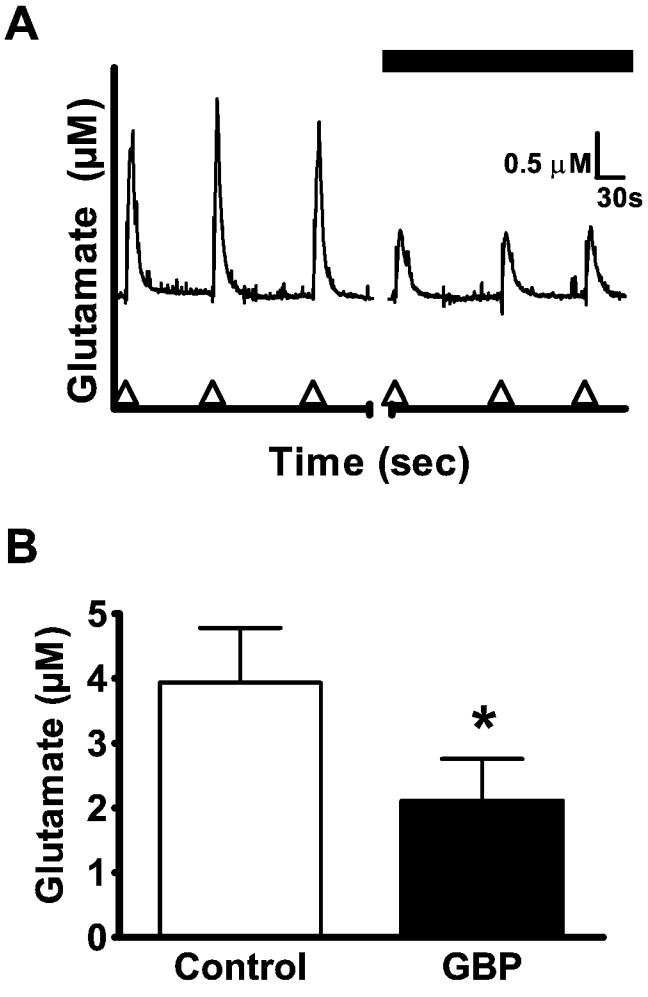

A
Glutamate (μM)
0.5 μM
30s
Time (sec)
B
Glutamate (μM)
5
4
3
2
1
0
*
Control
GBP

Fig. 1

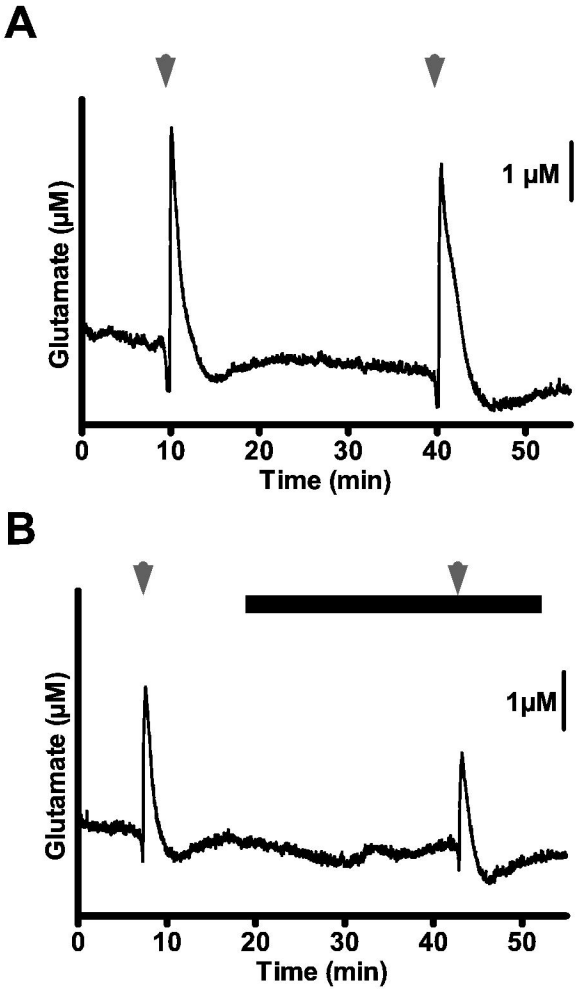
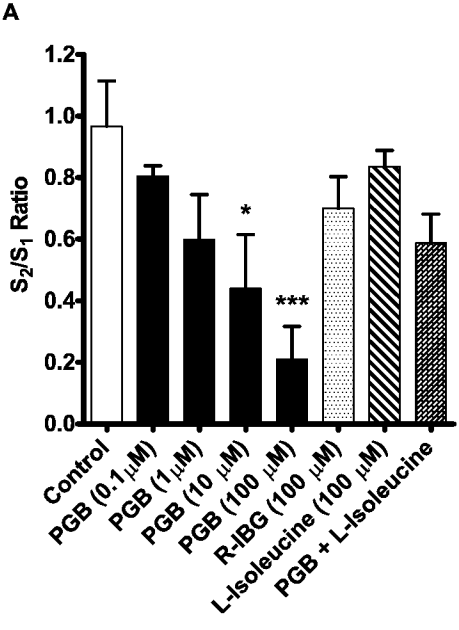

A
Glutamate (μM)
1 μM
0
10
20
30
40
50
Time (min)
B
Glutamate (μM)
1μM
0
10
20
30
40
50
Time (min)

Fig. 2


A
1.2
1.0
0.8
0.6
0.4
0.2
0.0
S2/S1 Ratio
*
***
Control
PGB (0.1 μM)
PGB (1 μM)
PGB (10 μM)
PGB (100 μM)
R-IBG (100 μM)
L-Isoleucine (100 μM)
PGB + L-Isoleucine

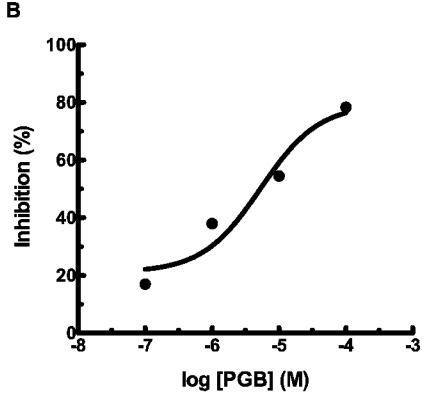

B
100
80
60
40
20
0
Inhibition (%)
-8
-7
-6
-5
-4
-3
log [PGB] (M)

Fig. 3

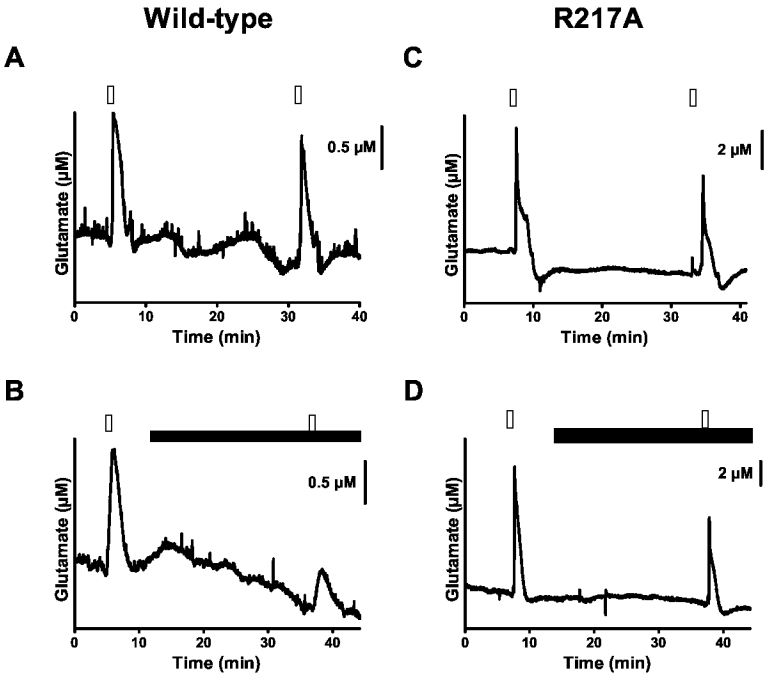

Wild-type
R217A
A
B
C
D
Glutamate (μM)
0.5 μM
2 μM
Time (min)
0
10
20
30
40

Fig. 4

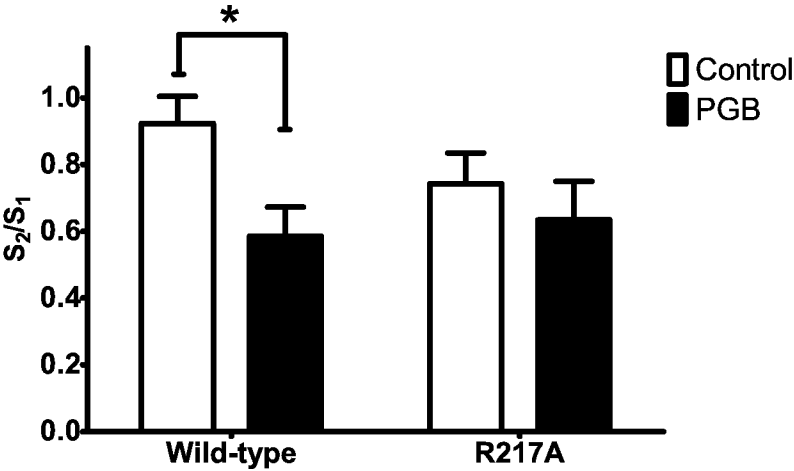


Fig. 5