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The Anticonvulsant Gabapentin (Neurontin) Does Not Act through γ-Aminobutyric Acid-B Receptors

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ABSTRACT

The actions of the anticonvulsant gabapentin [1-(aminomethyl)-cyclohexaneacetic acid, Neurontin] have been somewhat enigmatic until recently, when it was claimed to be a γ-aminobutyric acid-B ($GABA_B$) receptor agonist acting exclusively at a heterodimeric complex containing the $GABA_{B(1a)}$ splice variant (*Mol Pharmacol* 2001;**59:**144–152). In this study, we have investigated the effects of gabapentin on recombinant $GABA_{B(1a)}$ and $GABA_{B(1b)}$ receptors coexpressed with $GABA_{B(2)}$ in five different functional recombinant assays, its ability to inhibit [^{3}H]GABA binding in a $GABA_B$ receptor-selective binding assay using rat synaptic membranes, and its ability to inhibit transient lower esophageal sphincter relaxations in Labrador retriever dogs. Up to a concentration of 1 mM, gabapentin displayed no agonistic effects on either the $GABA_{B(1a,2)}$ or the $GABA_{B(1b,2)}$ heterodimer, when these were expressed in *Xenopus laevis* oocytes or mammalian cells and assayed by means of electrophysiology, calcium mobilization, inositol phosphate, and fluorometry assays. Gabapentin did not displace [^{3}H]GABA from $GABA_B$ receptor sites in rat synaptic membranes. Finally, in contrast to the classic $GABA_B$ receptor agonist baclofen, gabapentin was unable to inhibit transient lower esophageal sphincter relaxations in dogs. Because of high levels of $GABA_{B(1a)}$ in the canine nodose ganglion, this finding indirectly supports the inactivity of gabapentin on the $GABA_{B(1a,2)}$ heterodimer demonstrated in various in vitro assays. In light of these results, we find it highly questionable that gabapentin is a $GABA_B$ receptor agonist. Hence, the anticonvulsive effects of the compound have to arise from $GABA_B$ receptor-independent mechanisms. This also implies that the first $GABA_B$ receptor splice variant-selective ligand remains to be discovered.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system, where it exerts its effect through the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. The $GABA_B$ receptors belong to the family C of the G-protein-coupled receptor superfamily (Möhler and Fritschy, 1999; Marshall et al., 2000). Two receptors, $GABA_{B(1)}$ and $GABA_{B(2)}$, have recently been cloned, and several splice variants of both receptors have been identified (Kaupmann et al., 1997, 1998; Jones et al., 1998; White et al., 1998; Pfaff et al., 1999; Billinton et al., 2001).

$GABA_{B(1)}$ and $GABA_{B(2)}$ form heterodimers (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Gene knockout studies have shown that $GABA_{B(1)}$ is a necessary part for virtually all central $GABA_B$ receptors (Prosser et al., 2001; Schuler et al., 2001). The majority of the $GABA_B$ heterodimer complexes are either of a $GABA_{B(1a,2)}$ or a $GABA_{B(1b,2)}$ composition, and the two $GABA_{B(1)}$ splice variants differ in their expression pattern and their pre- and postsynaptic localization (Kaupmann et al., 1997; Benke et al., 1999; Poorkhalkali et al., 2000; Prosser et al., 2001; Schuler et al., 2001).

Agonist binding to the $GABA_{B(1,2)}$ heterodimer has been demonstrated to take place in the amino-terminal domain of the $GABA_{B(1)}$ subunit (Galvez et al., 1999, 2000; Malitschek et al., 1999). The major part of this region shares a weak amino acid sequence similarity with a family of bacterial periplasmic binding proteins, as is the case for other family C

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ABBREVIATIONS: GABA, γ−aminobutyric acid; VFT, Venus flytrap; TLESR, transient lower esophageal sphincter relaxation; AEBSF, 3,4-(2-aminoethyl)benzenesulfonylfluoride; $[Ca^{2+}]_i$, intracellular calcium concentration; FLIPR, fluorescence imaging plate reader; HBSS, Hanks' buffered saline solution; CHO, Chinese hamster ovary; HEK, human embryonic kidney; IP, inositol phosphate; TC, Tris-calcium; HPLC, high-performance liquid chromatography; CGP54626, [*S*-(R*,R*)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl)phosphinic acid; Kir, inwardly rectifying potassium channel.

receptors such as the metabotropic glutamate receptors and the calcium-sensing receptor (O'Hara et al., 1993; Kaupmann et al., 1997; Bräuner-Osborne et al., 1999b). This "Venus flytrap" (VFT) region is believed to consist of two globular lobes, and the endogenous agonist binds to residues in the cleft between these lobes (Galvez et al., 1999, 2000; Kunishima et al., 2000). The VFT region is conserved in its entirety in all $GABA_{B(1)}$ splice variants. Thus, the only molecular difference between splice variants $GABA_{B(1a)}$ and $GABA_{B(1b)}$ is the presence of two "Sushi domains" upstream of the VFT region in $GABA_{B(1a)}$ that are not found in $GABA_{B(1b)}$ (Kaupmann et al., 1997). The human $GABA_{B(1c)}$ is characterized by the absence of the second Sushi domain compared with $GABA_{B(1a)}$ (Billinton et al., 2001).

Gabapentin [1-(aminomethyl)cyclohexaneacetic acid, Neurontin] is a frequently administered anticonvulsant that has been shown to prevent partial seizures and generalized tonic-clonic seizures in epileptics (Taylor et al., 1998). Furthermore, the compound has displayed promising results in animal models of various forms of pain, amyotrophic sclerosis, bipolar disorder, and anxiety (Taylor et al., 1998). Although it was developed as an analog of GABA, gabapentin was originally claimed not to interact directly with either $GABA_A$ or $GABA_B$ receptors or with the high-affinity Na^+-dependent GABA transporters (Taylor, 1995; Taylor et al., 1998). Thus, the site of action of gabapentin has been somewhat of an enigma, although the compound displays high-affinity binding to the $\alpha_2\delta$ subunit of a calcium channel (Gee et al., 1996; Marais et al., 2001).

In contrast to the previous beliefs, Ng et al. (2001) recently claimed that gabapentin is, in fact, a $GABA_B$ receptor agonist. Furthermore, the authors postulated that gabapentin acts exclusively on the $GABA_{B(1a,2)}$ heterodimer complex and has no effect on the $GABA_{B(1b,2)}$ and $GABA_{B(1c,2)}$ heterodimers (Ng et al., 2001). Therefore, gabapentin is the first $GABA_B$ receptor splice variant-selective compound to be identified in a published study. The $GABA_{B(1a,2)}$ activity of gabapentin was supported by a follow-up study in which the compound was reported to inhibit the high K^+-evoked activation of voltage-dependent calcium channels in a murine mIL-tsA58 cell line that endogenously expresses the $GABA_{B(1a,2)}$ heterodimer (Bertrand et al., 2001).

The proposed splice variant selectivity of gabapentin is quite remarkable, considering that the "Sushi domains" differentiating the $GABA_{B(1)}$ splice variants are located outside of the VFT region responsible for the binding of the endogenous agonist. Hence, to elucidate these observations further, we have characterized gabapentin pharmacologically at $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ heterodimers in five different functional recombinant assays, in a [^{3}H]GABA binding assay to rat synaptic membranes, and in a model measuring transient lower esophageal sphincter relaxations (TLESRs) in dogs.

Experimental Procedures

Materials. Culture media, serum, antibiotics, and buffers for cell culture were obtained from Invitrogen (Paisley, Scotland, UK). 3.4-(2-Aminoethyl)benzenesulfonylfluoride (AEBSF) was obtained from Calbiochem (La Jolla, CA). The gabapentin used in the study was obtained from Sigma-Aldrich (St. Louis, MO) or from Goedecke/Parke-Davis (Freiburg, Germany) (hereafter termed "Goedecke"), or it was extracted from Neurontin 100 capsules (Pfizer, New York, NY). *myo*-[2-^{3}H]Inositol and 4-amino-*N*-[2,3-^{3}H]butyric acid ([^{3}H]GABA) were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK and Uppsala, Sweden). Isoguvacine was purchased from Sigma/RBI (Natick, MA), and all other chemicals were obtained from Sigma-Aldrich. The tsA cells were a generous gift from Dr. Penelope S.V. Jones (University of California, San Diego, CA).

Electrophysiology on Oocytes. Oocyte preparation and injection were done essentially as described previously (Mosbacher et al., 1998; Lingenhoehl et al., 1999). In brief, ovarian lobes containing oocytes were surgically removed from anesthetized (1.2 g/l 3-aminobenzoic acid ethyl ester) female *Xenopus laevis* frogs. Oocytes were separated and defolliculated by treatment with collagenase type II (Sigma-Aldrich) and with a subsequent incubation in 4 mM EGTA, pH 8.5. They were injected 3 h later with 10 to 50 ng of mRNA coding for either of the two rat splice variants $GABA_{B(1a)}$ or $GABA_{B(1b)}$ (Kaupmann et al., 1997), together with $GABA_{B(2)}$ and rat inwardly rectifying potassium channels (Kir3.1, Kir3.2, and Kir3.4), and incubated at 18°C for 3 to 8 days. Two-electrode voltage-clamp recordings were made with a Geneclamp 500 amplifier (Axon Instruments, Union City, CA) using electrodes filled with 3 M KCl. Oocytes were continuously perfused with normal frog Ringer (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, and 1.8 mM $CaCl_2$, pH 7.2) or high-potassium Ringer (90 mM KCl, 27.5 mM NaCl, 10 mM HEPES, and 1.8 mM $CaCl_2$, pH 7.2). The compounds were dissolved in water at 100 mM and perfused in high-potassium Ringer at the given concentrations. No corrections for changes in the osmolarity were performed. Currents were recorded using LabVIEW-based software (KooL; New Vision Engineering, Winterthur, Switzerland) and analyzed using Prism 3.0 software (GraphPad, San Diego, CA). Baseline current drifts were corrected using linear interpolations. For concentration-response curves, the induced inward current was measured 2 s before the application of the next higher concentration. Data from different oocytes were pooled. Values are mean ± S.E.M.

Measurement of $[Ca^{2+}]_i$ by Fluorescence Imaging Plate Reader. For measurement of changes in intracellular calcium concentrations, human embryonic kidney (HEK) 293 cells were transiently transfected with rat or human $GABA_{B(1a,2)}$ or $GABA_{B(1b,2)}$. All transfections included Gαqzic to couple the receptors to phospholipase C (Franek et al., 1999) and were made as described in detail previously (Pagano et al., 2001). Transfected HEK 293 cells were plated into poly(D-lysine)-coated 96-well plates (BD Biosciences, San Jose, CA). At 24 to 72 h after transfection, cells were loaded for 45 min with 2 μM fluo-4 acetoxymethyl ester (Molecular Probes, Eugene, OR) in Hanks' buffered saline solution (HBSS) (Invitrogen, Basel, Switzerland) containing 50 μM probenecid (Sigma-Aldrich, Buchs, Switzerland). Plates were washed twice in HBSS and transferred to a FLIPR (Molecular Devices, Crawley, UK). Fluorescence was measured at room temperature for 3 min after the addition of agonists. Relative fluorescence changes over baseline (ΔF/F) were determined. Concentration-response curves were recorded with three to eight wells per concentration and experiment, and the data were pooled and fitted using Igor Pro (Wavemetrics, Lake Oswego, OR) with a logistic equation using weighted nonlinear regression.

The abilities of GABA and gabapentin to evoke changes in the intracellular calcium concentrations were also measured in Chinese hamster ovary (CHO-K1) cells stably expressing $GABA_{B(1a)}$ and $GABA_{B(2)}$ and in CHO-K1 cells stably expressing a $GABA_{B(1a)}$-Gαq-i5 fusion protein and $GABA_{B(2)}$. Fusion proteins of G-protein–coupled receptors and G-proteins have been demonstrated in numerous studies to exhibit pharmacological profiles similar to those of the wild-type receptors (Seifert et al., 1999). The cells were assayed in a FLIPR (Molecular Devices, Menlo Park, CA), using procedures similar to those described above.

Calcium Measurements by Fluorometer. HEK 293 cells (1.5×10^6) were electroporated (250 V, 300 μF; Gene Pulser; Bio-Rad, Hercules, CA) with 5 μg of rat $GABA_{B(1a)}$ or $GABA_{B(1b)}$ cDNA with

$GABA_{B(2)}$ and Gαqzic (Franek et al., 1999) cDNAs in a total volume of 150 μl of buffer (50 mM K_2HPO_4, 20 mM CH_3COOK, and 20 mM KOH, pH 7.4). Transfected cells were resuspended in culture medium and plated on poly(D-lysine)-coated glass coverslips. Twenty-four hours after transfection, the cells were incubated at room temperature for 1 h in a HEPES buffer, pH 7.6 (Invitrogen), containing the calcium indicator fura-2 acetoxymethyl ester (10 μg/ml), 0.5% Pluronic F-127 (Molecular Probes), and 1% (v/v) dimethyl sulfoxide. Glass coverslips carrying dye-loaded cells were mounted into a perfusing cuvette (2 ml/min) in a fluorescence spectrophotometer (F-4500; Hitachi, Yokohama, Japan). Changes in $[Ca^{2+}]_i$ were recorded as the fluorescence ratio at 380 nm versus that at 360 nm. The viability of transfected cells was tested by application of 10 μM ATP.

Inositol Phosphate Assay. The tsA cells [a transformed HEK 293 cell line (Chahine et al., 1994)] were maintained at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% calf serum. tsA cells (3×10^5) were plated in a 6-cm tissue culture plate and transfected the following day with 0.25 μg of $GABA_{B(1a)}$-pCDNA3.1 or $GABA_{B(1b)}$-pCDNA3.1, 1.2 μg of $GABA_{B(2)}$-pCDNA3.1, and 0.25 μg of Gαq-z5-pCDNA3.1, using Polyfect as DNA carrier according to the protocol of the manufacturer (QIAGEN GmbH, Hilden, Germany). The day after transfection, the cells were transferred to 24 wells in a 96-well cluster plate in growth medium containing 2 μCi/ml *myo*-[2-^{3}H]inositol. After 16 to 20 h, the cells were washed in HBSS and incubated for 20 min in HBSS supplemented with 0.9 mM $CaCl_2$ and 1.05 mM $MgCl_2$. The cells were then incubated for another 20 min in phosphate-buffered saline supplemented with 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$, and 10 mM LiCl. Finally, the cells were incubated for 40 min in phosphate-buffered saline supplemented with 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$, 10 mM LiCl, and various concentrations of GABA and gabapentin (from Sigma-Aldrich or Goedecke). The reactions were stopped by exchanging the buffer with 200 μl of ice-cold 20 μM formic acid, and separation of total [^{3}H]inositol phosphates was carried out by ion-exchange chromatography as described previously (Nanevicz et al., 1996; Bräuner-Osborne et al., 1999a).

[^{3}H]GABA Filtration Binding Assay. Rat synaptic membranes were prepared using the method described by Zukin et al. (1974), with some modifications. Whole brains from Sprague-Dawley male rats (about 300 g) were homogenized in 10 volumes of ice-cold buffer containing 0.32 M sucrose, 10 mM Tris, 0.1 mM AEBSF, and 20 μg/ml bacitracin, pH 7.4. The homogenate was centrifuged at 1,000*g* for 10 min, and the supernatant was then centrifuged at 20,000*g* for 20 min. The pellet was resuspended (by vortex) in 6 volumes of ice-cold distilled water containing 0.1 mM AEBSF and 20 μg/ml bacitracin (pH set to 7.4), and centrifuged at 8,000*g* for 20 min. The supernatant and the upper layer of the pellet were centrifuged at 33,000*g* for 20 min. The pellet was resuspended in 6 volumes of 50 mM Tris, pH 7.4, containing 1 mM AEBSF and 20 μg/ml bacitracin and centrifuged at 33,000*g* for 20 min. The last centrifugation step was repeated one more time, and finally the pellet was snap-frozen in methanol/dry ice and stored overnight at −70°C. The frozen pellet was thawed and washed six times in 6 volumes of 50 mM Tris, pH 7.4, by centrifugation at 8,000*g* for 10 min at 18°C. The resulting pellet was resuspended in TC buffer (50 mM Tris/2.5 mM $CaCl_2$, pH 7.4), snap-frozen in methanol/dry ice, and stored at −70°C. Membranes to be used in the radioligand binding assay were further treated (washed) as follows. The membranes were thawed in lukewarm water followed by resuspension in TC buffer and homogenization using a Polytron PT 3000 from Kinematika AG (Basel, Switzerland) five times for 5 s each. The membranes were washed three times in TC buffer by centrifugation at 8,000*g* for 10 min, resuspended in TC buffer, and homogenized 10 times in a Teflon/glass homogenizer. The membranes were suspended in aliquots, snap-frozen in methanol/dry ice, and stored at −70°C. Protein concentration was determined according to the method of Bradford (1976) using the Bio-Rad protein assay kit with bovine γ-globulin as a standard.

The [^{3}H]GABA competition assay, modified from the method of Olpe et al. (1990), was performed in 96-well plates in 200 μl of TC-isoguvacine buffer (TC buffer supplemented with 40 μM isoguvacine to saturate the $GABA_A$ receptor sites) containing 20 nM [^{3}H]GABA (3.48 TBq/mmol), 80 μl of test compound (at indicated concentrations, diluted in water), and 80 μg of synaptic membrane (diluted in TC-isoguvacine buffer) and incubated for 10 min at room temperature before addition). The mixture was then incubated on a microplate shaker (Denley Instruments, Billinghurst-West Sussex, UK) for 20 min at room temperature, followed by rapid filtration through a glass fiber filter (Printed Filtermat B filters; PerkinElmer Wallac, Gaithersburg, MD) that had been presoaked in 0.3% polyethyleneimine, followed by a wash with TC buffer using a Tomtec cell harvester (Tomtec, Orange, CT). The filters were dried in a microwave at maximal effect for 1.5 min followed by incubation at 55°C for 45 min. MeltiLex B/HS scintillation sheets from PerkinElmer Wallac (Turku, Finland) were melted onto the filter, and radioactivity was determined in a Microbeta scintillation counter (PerkinElmer Wallac).

Measurements of TLESRs in Dogs. TLESRs were measured in adult Labrador retriever dogs using Dentsleeve manometry as described previously (Lehmann et al., 1999). Gabapentin (20 mg/kg) was administered directly into the stomach through the manometric assembly. Thirty minutes after administration, TLESRs were stimulated by liquid nutrient infusion and air insufflation and quantitated during a 45-min period. The effect of the compound was compared with the average of the five preceding control experiments for each individual dog. Gabapentin used for these experiments was obtained from Neurontin capsules (Pfizer), and a suspension was made in 0.9% NaCl immediately before the experiment.

Chemical Analyses of Gabapentin. Melting points of the gabapentin samples obtained from Sigma-Aldrich and Goedecke were determined in capillary tubes. ^{1}H NMR spectra of both samples were recorded on a 300-MHz Varian Gemini-2000 BB spectrometer (Varian, Palo Alto, CA) in CD_3OD using the solvent residual peak as internal standard. Elemental analyses of the same gabapentin samples were performed at the Department of Physical Chemistry, University of Vienna, Austria, and were within ±0.4% of the theoretical values for zwitterionic gabapentin.

HPLC analyses of the gabapentin sample from Sigma-Aldrich were performed on a Knauer Vertex Spherisorb ODS2 column (5 μm, 4.0 × 120 mm) using a TSP HPLC system (Bie & Berntsen A/S, Copenhagen, Denmark) consisting of a P2000 pump, an AC 3000 autoinjector, and an SM 5000 PDA detector. The column was eluted at 1.0 ml/min with aqueous trifluoroacetic acid, pH 2.0.

Results

We first attempted to reproduce the reported agonistic effects of gabapentin under the same experimental conditions as Ng et al. (2001). GABA (2.5 μM) elicited a solid response at both rat $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ heterodimers expressed in *X. laevis* oocytes together with K^+ channel Kir3 subunits (Fig. 1A, inset). In contrast, application of 1 mM gabapentin (extracted from Neurontin capsules or the pure compound provided by Goedecke) did not give rise to any response (Fig. 1). Both samples of gabapentin were tested as agonists as well as antagonists at $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$, and no significant change of control was obtained in any of the experiments (Fig. 1). Ng et al. (2001) found gabapentin to exhibit an efficacy at the $GABA_{B(1a,2)}$ heterodimer similar to that of baclofen and determined the EC_{50} value of gabapentin at this heterodimer to be 15 μM.

We also compared responses of human and rat $GABA_B$

receptors in experiments measuring calcium mobilization using a FLIPR. Only at the highest concentration tested (10 mM) did gabapentin (from Goedecke) give rise to weak agonistic responses in HEK 293 cells expressing the rat and human $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ heterodimers (Fig. 2, A and B). These small responses could be antagonized with CGP54626 (data not shown). GABA activated both heterodimers with EC_{50} values in the low micromolar range (Fig. 2, A and B). To investigate whether gabapentin had modulatory or antagonistic effects at $GABA_B$ receptors, it was tested in the presence of 3 μM GABA as well. In these experiments, gabapentin displayed neither antagonistic nor positive modulatory properties on $GABA_{B(1a,2)}$ or $GABA_{B(1b,2)}$ (Fig. 2, C and D).

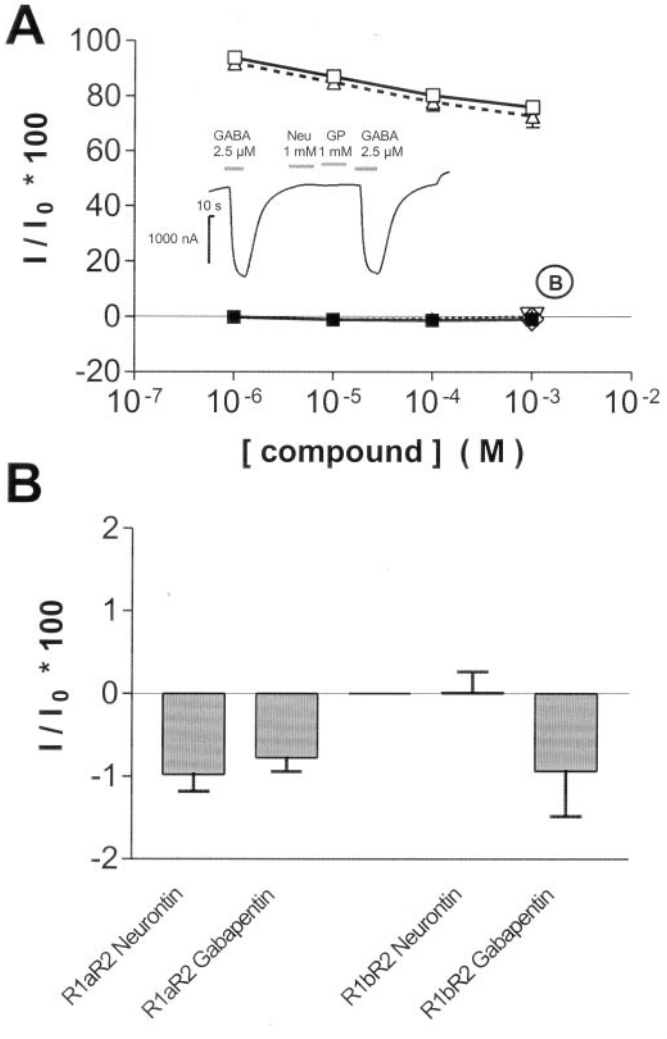

Fig. 1. Gabapentin and "Neurontin" (gabapentin extracted from Neurontin capsules) had no effects on *X. laevis* oocytes expressing rat $GABA_{B(1a,2)}$ or $GABA_{B(1b,2)}$ heterodimers and Kir3 channels. A, Neurontin showed no clear dose-dependent antagonistic effects in oocytes expressing $GABA_{B(1a,2)}$ (□, solid line) or $GABA_{B(1b,2)}$ (▷, dotted line). The linear reduction in the response probablyis caused by a run-down of the response. Neither Neurontin ($GABA_{B(1a,2)}$, ■, solid line; $GABA_{B(1b,2)}$, ▲, dotted line) nor gabapentin ($GABA_{B(1a,2)}$, ◇; $GABA_{B(1b,2)}$, ◁) showed an agonistic activity in oocytes. The effects at 10^{-3} M (indicated by circled B) are shown at a different scale in B. Data were normalized to the GABA EC_{80} response (I_0). For the $GABA_{B(1a,2)}$ and the $GABA_{B(1b,2)}$ heterodimers, the GABA concentrations used were 5.38 μM and 2.52 μM, respectively. Inset, a current record at −70 mV from an oocyte expressing $GABA_{B(1b,2)}$ and Kir 3.1/2/4 channels. Bars indicate the application of GABA (2.5 μM), Neurontin (Neu, 1 mM), and gabapentin (GP, 1 mM). B, no agonistic effects of Neurontin and gabapentin were observed at 1 mM (n = 3) in oocytes.

The agonistic properties of GABA and gabapentin on $GABA_B$ receptor-dependent release of intracellular calcium were also characterized in CHO-K1 cells stably expressing the human $GABA_{B(1a,2)}$ heterodimer. GABA stimulated intracellular calcium release in these cells with an EC_{50} of 380 ± 32 nM (n = 3), whereas gabapentin (up to 1 mM as the final concentration) had no effect on the calcium release (data not shown). A similar result was obtained in CHO cells stably expressing a $GABA_{B(1a)}$-Gqi5 fusion protein and $GABA_{B(2)}$. Despite the high potency of GABA (EC_{50} = 180 ± 34 nM; n = 3), gabapentin showed no effect at concentrations up to 1 mM (data not shown).

To investigate further the lack of agonistic effects of gabapentin on $GABA_B$ receptors, we characterized the compound in an assay for Ca^{2+} mobilization in a continuous perfusion chamber using fura-2-loaded cells. In this fluorometry assay, 10 mM gabapentin (from Goedecke) elicited weak agonistic responses in HEK 293 cells expressing the $GABA_{B(1a,2)}$ or $GABA_{B(1b,2)}$ heterodimer together with the chimeric G-protein Gq-zic (Fig. 3A). The agonist response in $GABA_{B(1a,2)}$-transfected cells could be antagonized with CGP54626 (Fig. 3A). GABA activated both heterodimers (Fig. 3B).

Because the FLIPR and fluorometer assays are sensitive to rapid but not to slow changes in $[Ca^{2+}]_i$, we also characterized the effects of gabapentin on $GABA_B$ receptors in a traditional second-messenger assay (Fig. 4). In the inositol phosphate (IP) assay, the $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits are coexpressed with the chimeric G-protein Gαq-z5, which enables us to measure $GABA_B$ receptor function as IP accumulation (Bräuner-Osborne and Krogsgaard-Larsen, 1999). In this assay GABA displayed EC_{50} values of 3.2 ± 0.5 μM at $GABA_{B(1a,2)}$ and 3.5 ± 0.7 μM at $GABA_{B(1b,2)}$ (n = 3 in both cases). In contrast to the effects of GABA, no response was observed in cells transfected with $GABA_{B(1a,2)}$ or $GABA_{B(1b,2)}$ upon application of gabapentin (from Sigma-Aldrich or Goedecke) in concentrations up to 100 mM (Fig. 4).

Gabapentin has previously been reported not to influence radioligand binding to native $GABA_B$ receptors (Taylor, 1995; Taylor et al., 1998). To verify this, we characterized the effects of the compound on [^{3}H]GABA binding to $GABA_B$ receptor sites in rat brain membranes in an assay in which the $GABA_A$ receptor sites were blocked by coincubation with 40 μM isoguvacine (Fig. 5). Gabapentin (in concentrations up to 1 mM) did not have any effect on the [^{3}H]GABA binding, whereas the IC_{50} for GABA was 90 ± 16 nM (n = 3).

For the functional assessment of gabapentin on native $GABA_B$ receptors, we investigated the well documented inhibition of TLESR by activation of $GABA_B$ receptors at the vagal afferent pathway. At 20 mg/kg, gabapentin did not significantly affect TLESRs in dogs. The average inhibition was 14 ± 23% of controls (n = 4). Likewise, in two experiments in which gabapentin was given i.v. at 50 mg/kg, there was no effect on TLESRs (11% inhibition and 17% stimulation, respectively).

To exclude the possibility that our negative findings arose from testing the wrong compound, we analyzed two of the samples used in the functional assays described above. Melting point determinations and elemental analyses of the gabapentin samples from Sigma-Aldrich and Goedecke were performed and found to be in agreement with the data expected

for zwitterionic gabapentin (Satzinger, 1994). Furthermore, ^{1}H NMR spectra of both batches were identical to those previously reported (Griffiths et al., 1991), thus confirming the authenticity of the gabapentin samples used. Under the conditions used in the HPLC analyses, GABA eluted almost at the front (retention time = 1.3 min). Based on peak areas, at 200 nm, of peaks eluting with retention time < 1.5 min, the gabapentin sample from Sigma-Aldrich contained less than 4% (w/w) (detection limit) of GABA.

Discussion

The recent cloning of $GABA_{B(1)}$ and $GABA_{B(2)}$ has brought about a substantially better understanding of the pharmacology and medicinal chemistry aspects of the $GABA_B$ receptors (Kaupmann et al., 1997, 1998; Jones et al., 1998; White et al., 1998). The majority of $GABA_B$ receptors exist as either $GABA_{B(1a,2)}$ or $GABA_{B(1b,2)}$ heterodimers (Benke et al., 1999; Prosser et al., 2001; Schuler et al., 2001), and the wish to understand the physiological roles of these two heterodimers has prompted the search for $GABA_{B(1)}$ splice variant-selective ligands. Considering that the VFT domain responsible for binding of the endogenous agonist is conserved within the $GABA_{B(1)}$ splice variants, this quest has seemed to be quite difficult. Recently, however, the anticonvulsant drug gabapentin was claimed to be a selective agonist at the $GABA_{B(1a,2)}$ heterodimer with an EC_{50} value in the low micromolar range (Bertrand et al., 2001; Ng et al., 2001). We were puzzled by the proposed involvement of regions outside of the VFT region in the binding of gabapentin, considering its GABA-template, and decided to verify the data.

In our hands, gabapentin did not display any agonistic effects at either rat or human variants of the $GABA_{B(1a,2)}$ or the $GABA_{B(1b,2)}$ heterodimer, when characterized by means of electrophysiology in oocytes (up to 1 mM), in FLIPR experiments (up to 1 mM), or in an IP assay (up to 100 mM). The only gabapentin responses we observed were in the FLIPR and fluorometer assays of transiently transfected HEK 293 cells at concentrations of 10 mM. These weak agonistic responses took place at a concentration 1,000- to 10,000-fold higher than the concentrations used by Ng and coworkers to obtain an effect (Fig. 2A) (Bertrand et al., 2001; Ng et al., 2001) and is much too high to explain the therapeutic benefits of the compound. Furthermore, these effects of gabapentin were observed for both the $GABA_{B(1a,2)}$ and the $GABA_{B(1b,2)}$ heterodimers; thus, it was clearly not a receptor splice variant-specific effect. A 1:10,000 contamination of gabapentin with GABA would be sufficient to elicit these responses. We cannot exclude the possibility, given the purity profiles of the gabapentin samples obtained from the chemical analyses, that the agonist responses are

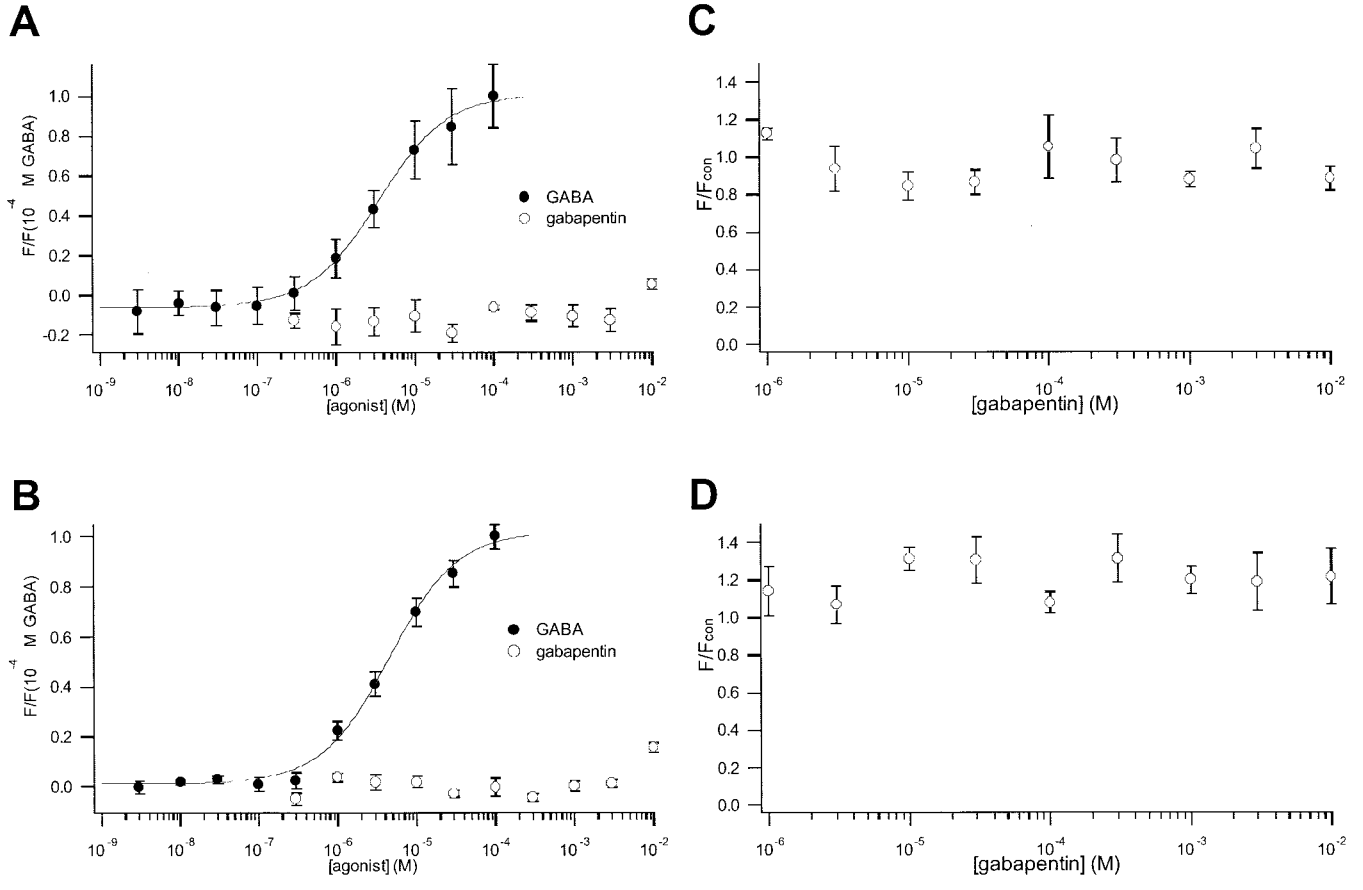

Fig. 2. Comparison of the effects of GABA and gabapentin on human $GABA_{B(1a,2a)}$ and $GABA_{B(1b,2a)}$ heterodimers in the FLIPR. Concentration-response curves of GABA (●) and gabapentin (○) tested for agonism at $GABA_{B(1a,2a)}$ (A) and $GABA_{B(1b,2a)}$ (B). Data are normalized to the average response of 10^{-4} M GABA; error bars are S.E.M. from quadruplicate determinations. Lines are weighted fits of Hill equations to the data points. EC_{50} values are 3.5 (2.6, 4.8) μM and 4.6 (2.7, 7.9) μM for $GABA_{B(1a,2a)}$ and $GABA_{B(1b,2a)}$, respectively (mean; lower and upper 95% confidence interval). Antagonism of gabapentin (○) against 3 μM GABA at human $GABA_{B(1a,2a)}$ (C) and $GABA_{B(1b,2a)}$ (D). Data are normalized to the response induced by 3 μM GABA alone, and error bars are S.E.M. from quadruplicate determinations.

caused by traces of GABA or other impurities in the gabapentin.

The results from the functional in vitro assays are supported by the lack of inhibition displayed by gabapentin in the TLESR model. It is well documented that $GABA_B$ receptor agonists are powerful inhibitors of TLESRs in dogs (Lehmann et al., 1999), ferrets (Blackshaw et al., 1999), and humans (Lidums et al., 2000). Although the identity of the splice variant(s) mediating this effect has (have) not been determined, it is likely that $GABA_{B(1a)}$ plays an important role. This assumption rests on the observation that high levels of $GABA_{B(1a)}$ are found in the canine nodose ganglion (J. Ekstrand, N. Poorkhalkali, and A. Lehmann, unpublished observations) supplying vagal afferents that represent the major site of action with regard to inhibitory effects on TLESRs of $GABA_B$ receptor agonists (Partosoedarso et al., 2001). Hence, the observation that gabapentin does not inhibit TLESRs in dogs indirectly supports the notion that gabapentin does not activate the native $GABA_{B(1a,2)}$ heterodimer.

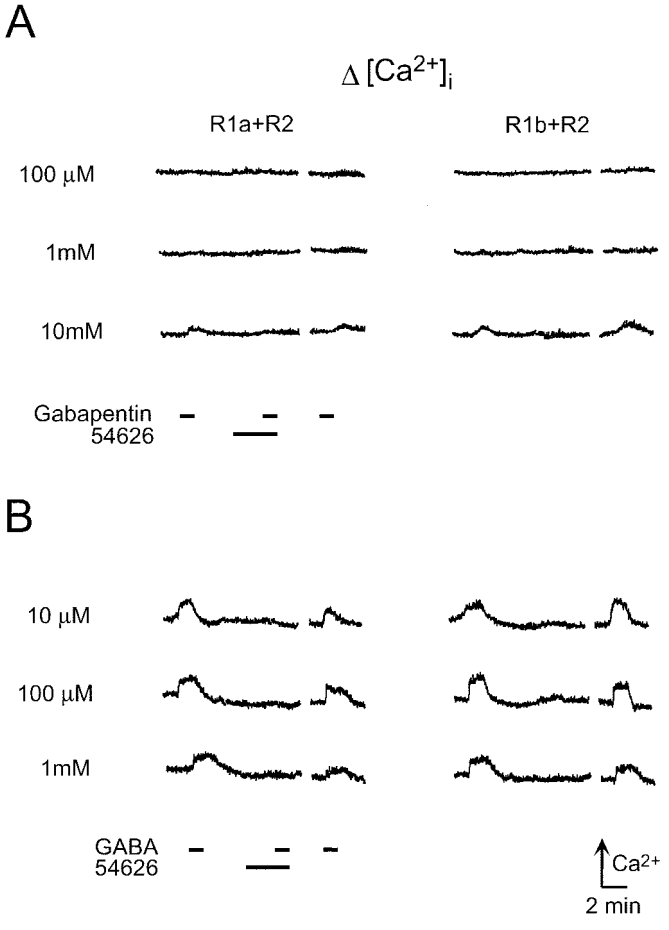

Fig. 3. Calcium measurements by fluorometer on HEK 293 cells transfected with $GABA_{B(1a)}$ or $GABA_{B(1b)}$ and $GABA_BR2$ plus chimeric G-protein (Gαqzic). Functional coupling to phospholipase C shows Ca^{2+} transients as measured by changes in fura-2 fluorescence intensity ratios [$R_{340/380}$, $(\Delta[Ca^{2+}])_i$]. Bars below traces indicate drug application. B, one-minute application of agonists gabapentin (A; 100 μM, 1 mM, 10 mM) or GABA B; 10 μM, 100 μM, 1 mM), 2-min application of antagonist CGP54626 (10 μM), and 1-min application of both agonists and antagonist. Both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ present functional coupling only with 10 mM gabapentin, but subtype $GABA_{B(1b,2)}$ shows higher $(\Delta[Ca^{+2}])_i$ response. The transfections with both subtypes show functional response with all concentrations of GABA used.

Ng et al. (2001) used recombinant human and murine $GABA_B$ receptors in their study of gabapentin. The experiments in the present study were performed on recombinant rat and human receptors (summarized in Table 1). We do not have the murine mIL-tsA58 cell line, in which gabapentin is reported to have inhibited the high K^+-evoked calcium mobilization (Bertrand et al., 2001), nor have we tested the effects of gabapentin at recombinant murine $GABA_B$ receptors. A comparison of the amino acid sequence of the Sushi domains in the rat, human, and murine $GABA_{B(1a)}$ receptors reveals that only five residues are not identical throughout the three species. Val^7, Leu^{14}, Ala^{24}, Glu^{127}, and Val^{143} in rat $GABA_{B(1a)}$ corresponds to Ala^8, Pro^{15}, Ala^{25}, Asp^{128}, and

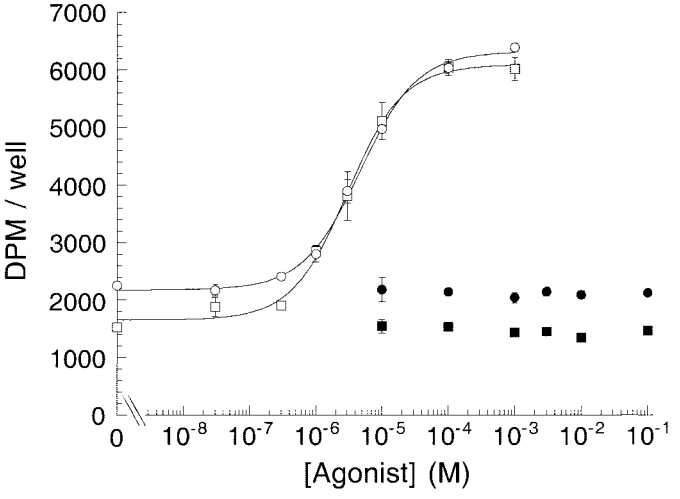

Fig. 4. The effects of GABA and gabapentin (from Goedecke) on IP accumulation in tsA cells transfected with $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ heterodimers and Gαq-z5. Three individual experiments were performed in triplicate as described under *Experimental Procedures*. □, GABA at $GABA_{B(1a,2)}$; ○, GABA at $GABA_{B(1b,2)}$; ■, gabapentin at $GABA_{B(1a,2)}$; and ●, gabapentin at $GABA_{B(1b,2)}$. Data are given as disintegration per minute (DPM) per well, and lines are weighted fits of Hill equations to the data points. Gabapentin from Sigma-Aldrich was also inactive at $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ heterodimers in concentrations up to 100 mM (data not shown).

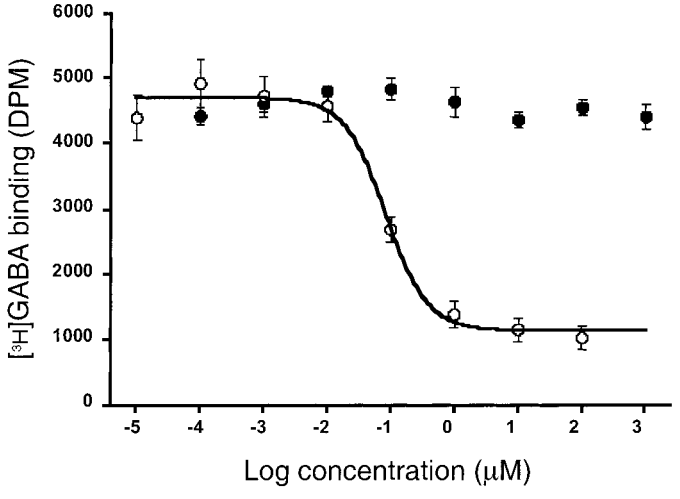

Fig. 5. Effects of GABA and gabapentin on displacement of [^{3}H]GABA binding at $GABA_B$ receptor sites in rat brain membranes. The effects of GABA (○) and gabapentin (●) on [^{3}H]GABA binding at $GABA_B$ receptor sites in rat brain membranes were measured as detailed under *Experimental Procedures*. The results shown are the mean ± S.E.M. of three independent experiments.

TABLE 1

Pharmacological characteristics of gabapentin at recombinant GABAB receptors

The EC_{50} values obtained for gabapentin derived from three different sources at $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ heterodimers in five different functional recombinant assays. The two FLIPR assays were performed in two different laboratories. The species of the $GABA_B$ receptors used in the respective assays are given. The value given for each experiment represents both the $GABA_{B(1a,2)}$ and the $GABA_{B(1b,2)}$ heterodimers. GABA was able to activate both heterodimers in all five assays.

Assay	Species	Gabapentin		
		Sigma	Goedeke	Neurontin
			μM	
Oocytes	Rat	N.T.	>1,000	>1,000
FLIPR (I)	Rat and human	N.T.	>10,000	>10,000
FLIPR (II)	Human	>1,000	N.T.	>1,000
Fluorometry	Rat	N.T.	>10,000	N.T.
IP	Rat	>100,000	>100,000	N.T.

N.T., not tested.

Ile^{144} in human $GABA_{B(1a)}$ and to Val^7, Leu^{14}, Val^{24}, Asp^{127}, and Ile^{143} in murine $GABA_{B(1a)}$. The first three of these five residues are located in the signal peptides of the receptors and cannot explain any species differences. Because the last two residues are identical in murine and human $GABA_{B(1a)}$, it is reasonable to assume that gabapentin would be inactive at the murine $GABA_{B(1a,2)}$ heterodimer as well.

In principle, the idea that gabapentin is a $GABA_B$ receptor agonist is not far-fetched, because $GABA_B$ agonists like baclofen and 4-amino-3-(5-chloro-2-thienyl)-butanoic acid possess bulky side chains on the β-carbon of their GABA-template. However, because gabapentin does not influence [^{3}H]GABA binding to $GABA_B$ receptors in rat brain tissue (Fig. 5), any putative binding site of the compound clearly is located outside of the endogenous agonist binding pocket in the VFT region of the receptor. If gabapentin were indeed a $GABA_{B(1a,2)}$-selective agonist, residues in Sushi domains in the amino-terminal domain of $GABA_{B(1a)}$ would have to constitute a major contribution to the binding of the ligand (Kaupmann et al., 1997). If the binding site of gabapentin were that different from that of the endogenous agonist, the GABA-like structure of gabapentin would have to be purely coincidental.

In conclusion, we have been unable to find agonistic effects of gabapentin on recombinant and native $GABA_B$ receptors, as shown by Ng and coworkers (Bertrand et al., 2001; Ng et al., 2001). Gabapentin originating from three different sources has been tested on recombinant rat and human $GABA_B$ receptors in three laboratories using five different functional recombinant assays, and in none of these assays have we been able to detect any significant agonistic responses of up to 1 mM gabapentin (Table 1). In an in vivo model, gabapentin was unable to inhibit TLESRs in dogs, which indirectly supports the notion that the compound is inactive at the $GABA_{B(1a,2)}$ heterodimer. Gabapentin was not active at the $GABA_{B(1b,2)}$ heterodimer either; thus, we find it highly questionable that gabapentin should exert its effects through direct activation of $GABA_B$ receptors. In the study by Bertrand et al. (2001), gabapentin was also reported to be an agonist at native $GABA_B$ receptors in neurons of hippocampal slices, where the depression of Ca^{2+} responses brought on by application of 2 mM gabapentin or 40 μM baclofen could be blocked with the $GABA_B$ receptor antagonist CGP55845 (Bertrand et al., 2001). In light of the very high concentration of gabapentin used in this experiment, contamination of GABA cannot be ruled out as the explanation for this apparent $GABA_B$ effect. Alternatively, indirect effects leading to increased extracellular GABA concentration such as enhanced GABA release, or altered GABA metabolism or uptake inhibition could be the explanation (Taylor et al., 1998). This could also explain why CGP55845 partially antagonizes the anticonvulsive effect of gabapentin as shown by Cao et al. (2001). In any case, the mechanism of action for gabapentin remains elusive, and future studies will have to address the basis for the enigmatic anticonvulsant effects of the compound. Furthermore, the first $GABA_B$ receptor splice variant-selective ligand remains to be identified.

During the peer review of this article, another group (Lanneau et al., 2001) reached the same conclusion that we have. Interestingly, they used different assays than we did: guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate and [^{3}H] CGP 54626A binding assays to recombinant cell lines and electrophysiology on CA1 and CA3 hippocampal neurons.

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