Research paper

Gabapentin increases expression of δ subunit-containing GABA_A receptors

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ARTICLE INFO

ABSTRACT

Background: Gabapentin is a structural analog of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Its anticonvulsant, analgesic and anxiolytic properties suggest that it increases GABAergic inhibition; however, the molecular basis for these effects is unknown as gabapentin does not directly modify GABA type A (GABA_A) receptor function, nor does it modify synaptic inhibition. Here, we postulated that gabapentin increases expression of δ subunit-containing GABA_A (δGABA_A) receptors that generate a tonic inhibitory conductance in multiple brain regions including the cerebellum and hippocampus.

Methods: Cell-surface biotinylation, Western blotting, electrophysiologic recordings, behavioral assays, high-performance liquid chromatography and gas chromatography-mass spectrometry studies were performed using mouse models.

Findings: Gabapentin enhanced expression of δGABA_A receptors and increased a tonic inhibitory conductance in neurons. This increased expression likely contributes to GABAergic effects as gabapentin caused ataxia and anxiolysis in wild-type mice but not δ subunit null-mutant mice. In contrast, the antinociceptive properties of gabapentin were observed in both genotypes. Levels of GABA_A receptor agonists and neurosteroids in the brain were not altered by gabapentin.

Interpretation: These results provide compelling evidence to account for the GABAergic properties of gabapentin. Since reduced expression of δGABA_A receptor occurs in several disorders, gabapentin may have much broader therapeutic applications than is currently recognized.

Fund: Supported by a Foundation Grant (FDN-154312) from the Canadian Institutes of Health Research (to B.A.O.); a NSERC Discovery Grant (RGPIN-2016-05538), a Canada Research Chair in Sensory Plasticity and Reconsolidation, and funding from the University of Toronto Centre for the Study of Pain (to R.P.B.).

1. Introduction

Gabapentin (1-(aminomethyl)cyclohexaneacetic acid) was approved by the US Food and Drug Administration, initially for the treatment of seizures in 1993 and subsequently for the treatment of postherpetic neuralgia in 2002 [1]. Over the past two decades, the indications for gabapentin have expanded widely and it is now one of the most commonly prescribed medications in the United States [1]. Gabapentin is primarily used "off-label" for the treatment of acute and neuropathic pain [2]. Also, due to its anxiolytic and sedative effects, gabapentin is widely prescribed for sleep disturbances, bipolar disorder and restless leg syndrome [3]. Despite its widespread use, gabapentin causes serious adverse effects including drowsiness, ataxia, mental confusion and respiratory depression [3,4]. Given its increasing use and multiple adverse effects, it is important to understand gabapentin's molecular mechanisms of action [1,4,5].

The behavioral properties of gabapentin have been attributed to inhibition of the auxiliary α_2δ subunit of voltage-dependent calcium channels, which reduces calcium channel function and the release of

excitatory neurotransmitters from presynaptic terminals [6,7]. Interestingly, many of the behavioral properties of gabapentin suggest that it increases the function of inhibitory γ-aminobutyric acid (GABA) type A (GABA_A) receptors. Similar to many drugs that are positive allosteric modulators of GABA_A receptors including benzodiazepines, ethanol and many general anesthetic drugs, gabapentin causes sedation, somnolence, hypnosis, memory deficits, ataxia and respiratory depression [3,4]. Gabapentin is a structural analog of the inhibitory neurotransmitter GABA, yet it has no direct effects on GABA_A receptor function, nor does it increase inhibitory synaptic transmission [1,8]. Thus, the molecular basis of gabapentin's GABAergic properties has remained enigmatic.

GABA_A receptors are pentameric transmitter-gated ion channels formed from 19 different subunits [9,10]. The combinations of subunits produce receptor subtypes with different pharmacological and physiological properties. We hypothesized that gabapentin selectively modifies a subtype of GABA_A receptors through mechanisms that have not been identified.

GABA_A receptors are classified into two major groups: synaptic and extrasynaptic where synaptic receptors generate transient inhibitory postsynaptic current and extrasynaptic receptors generate a tonic inhibitory current that regulates neuronal excitability and network plasticity [9,10]. Extrasynaptic GABA_A receptors usually contain either a δ subunit or α5 subunits in combination with β2 or β3 subunits [9–11].

Results from our previous study of hippocampal neurons suggested that gabapentin targets extrasynaptic GABA_A receptors [8]. In that earlier study, treating cultured hippocampal neurons with gabapentin for 36 to 48 h increased a tonic inhibitory current but did not change the amplitude, frequency or time course of miniature inhibitory postsynaptic currents. These results were unexpected as the tonic inhibitory current in hippocampal neurons is primarily generated by α5 subunit-containing GABA_A receptors and gabapentin did not up-regulate the function or expression of these receptors. In that report, it was assumed that the increase in GABA_A receptor activity was caused by increasing levels of endogenous agonists, as suggested by others [7,12,13].

In the current study, we consider an alternative novel hypothesis. We postulate that gabapentin increases the cell-surface expression of extrasynaptic δ subunit-containing GABA_A (δGABA_A) receptors in neurons. We focus on δGABA_A receptors because these receptors are the most widely expressed subtype of extrasynaptic GABA_A receptors in the mammalian brain [9,10]. These receptors are normally expressed at low levels in cultured hippocampal neurons. However, the localization of GABA_A receptors on the cell surface can be dynamically regulated by complex trafficking mechanisms that can be pharmacologically modified [14]. The net movement of GABA_A receptors to and from the plasma membrane determines the number of receptors that accumulate on the cell surface, and these processes could be altered by gabapentin [15].

Our results first demonstrate that gabapentin increases expression of δGABA_A receptors, an effect that would account for at least some of the drug's GABAergic properties. Given that reduced expression of δGABA_A receptors contributes to a variety of cognitive and psychiatric disorders [16], these results have broad implications.

2. Methods

2.1. Experimental animals

Experiments were approved by the Animal Care Committee of the University of Toronto. All studies were performed in accordance with guidelines from the Canadian Council on Animal Care. GABA_A receptor δ subunit null-mutant (Gabrd−/−) mice and wild-type (WT) mice (C57BL/6J × 129Sv/SvJ) aged 2.5 to 4.5 months old were used. These mice were bred from a stock that was generously provided by Dr. Gregg E. Homanics (University of Pittsburgh, Pittsburgh, Pennsylvania, USA). The generation, genotyping, and characterization of Gabrd−/− mice have been previously described [17]. In addition, adult Swiss Webster mice (3–4 months old, Charles River, Montreal, Quebec, Canada) were used for measuring the levels of agonists and neurosteroids that modify GABA_A receptors. Only male mice were selected for the studies because the estrous cycle alters the expression of extrasynaptic δGABA_A receptors [18,19]. Mice were housed in a pathogen-free facility at the University of Toronto (25 ± 1 °C) on a 14-h light/10-h dark cycle (lights on 6:00 AM to 8:00 PM). Experimenters were blinded to genotypes and treatment conditions.

2.2. Preparation of brain slices

After live decapitation, the brain was removed from 10- to 14-week-old WT mice and placed in ice-cold, oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) that contained (in mM): 124 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.6 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 D-glucose with solution osmolarity adjusted to 300–310 mOsm. Coronal brain slices (350 μm) were prepared with a VT1200S vibratome (Leica, Deerfield, Illinois, USA). Cerebellar and hippocampal slices were then dissected out in ice-cold oxygenated ACSF. For *ex vivo* experiments, the slices were collected 2 h post-treatment and transferred to small chambers that were filled with ice-cold oxygenated ACSF. Slices were equilibrated for 1 h before undergoing cell-surface biotinylation and Western blotting procedures. For *in vitro* experiments, individual cerebellar or hippocampal slices were equilibrated at room temperature (23–25 °C) for 1 h. Slices were then treated with gabapentin (300 μM) that was dissolved in oxygenated ACSF or vehicle control at 35–37 °C. This concentration of gabapentin increases the tonic current after a prolonged treatment of cultured hippocampal neurons [8].

2.3. Cell-surface biotinylation and western blotting

Slices were placed on ice and incubated twice for 30 min with 0.75 mg/ml NHS-SS-biotin (Thermo Scientific, Rockford, Illinois, USA) that was dissolved in DPBS (Gibco, Burlington, Ontario, Canada). Excess biotin was quenched and removed by washing slices 6 times with ice-cold modified TBS solution containing (in mM): 25 Tris-Cl, 137 NaCl, 1 KCl, 2.3 $CaCl_2$, pH 7.4. Slices were then placed in lysis buffer (pH 7.4) containing complete protease inhibitor cocktail (Roche, Laval, Quebec, Canada) for homogenization. Insoluble material was removed by centrifugation. Bicinchoninic acid assay (Bio-Rad, Hercules, California, USA) was performed to determine the protein concentration. Supernatant lysates were incubated with Hi-Capacity NeutrAvidin beads (Thermo Scientific, Rockford, Illinois, USA) for 16–18 h at 4 °C. The beads were washed with PBS containing 0.05% SDS. Bound material was eluted with elution buffer containing (in mM): 50 Tris-Cl, 2% SDS, 2 DTT; protein concentration was determined using DC™ Protein Assay (Bio-Rad, Hercules, California, USA) and subjected to SDS-PAGE analysis. Western blot analyses with antibodies for δ (Millipore, Billerica, Massachusetts, USA), α1 (Abcam, Cambridge, Massachusetts or Millipore, Billerica, Massachusetts, USA), and α5 (PhosphoSolutions, Aurora, Colorado, USA) $GABA_A$ receptor subunits were performed. Anti-β-actin antibody (Millipore, Billerica, Massachusetts, USA) and anti-Na^+/K^+ ATPase antibody (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) were also used. Blots were imaged using the Chemidoc XRS+ system (Bio-Rad, Hercules, California, USA) and quantified using Image Lab software (Bio-Rad, Hercules, California, USA). All receptor bands were normalized to the loading control, Na^+/K^+ ATPase. Blots containing surface protein were probed for β-actin to determine the purity of the isolated biotinylated surface protein. Samples for surface proteins were analyzed using two replicate blots to control for transfer and loading errors. The two normalized values were averaged to obtain a single value for each sample. Data for each sample were presented as a percentage of the mean of the control sample.

2.4. Whole-cell voltage clamp recordings from cerebellar slices

After live decapitation, the cerebellum was quickly removed from 10- to 14-week-old mice and submerged in oxygenated (95% O_2, 5% CO_2) ice-cold cutting solution, which contained (in mM): 235 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgSO_4$, 28 D-glucose (pH 7.4; 305–315 mOsm). The tissue was maintained in ice-cold solution while sagittal slices of cerebellar vermis (300 μm) were cut using a VT1200S vibratome. The slices were transferred to oxygenated ACSF and allowed to recover for at least 1 h at room temperature before being transferred to a submersion recording chamber, where they were perfused with ACSF at 3–4 ml/min.

Experiments were performed on an Olympus BX51 upright microscope equipped with differential interference contrast/infrared optics. Conventional whole-cell voltage clamp recordings were carried out at room temperature from granule neurons located in the granule cell layer, which were visually identified. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and (2R)-amino-5-phosphonovaleric acid (APV, 20 μM) were added to the extracellular solution to block ionotropic glutamate receptors and tetrodotoxin (0.3 μM) was used to block voltage-dependent sodium channels. Patch pipettes (4–6 MΩ) were fabricated from borosilicate glass capillary tubing and filled with an intracellular solution containing (in mM): 140 CsCl, 10 HEPES, 11 EGTA, 4 MgATP, 2 $MgCl_2$, 1 $CaCl_2$ and 10 TEA (pH 7.3 with CsOH, 285–295 mOsm). Whole-cell currents were recorded using the MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, California, USA) that was controlled with pClamp 9.0 software via a Digidata 1322 interface (Molecular Devices). Membrane capacitance was measured using the membrane test protocol in pClamp 9.0. Currents were sampled at 10 kHz and filtered at 2 kHz with an eight-pole low-pass Bessel filter. All cells were recorded at a holding potential of −60 mV. To measure tonic current, the $GABA_A$ receptor competitive antagonist bicuculline (10 μM) was applied. Tonic current was quantified by measuring the change in the holding current calculated from a Gaussian fitting of 30-s segments.

2.5. Rotarod assay

The rotarod assay is commonly used to assess motor coordination in rodents. The rotarod apparatus (Economex, Columbus Instruments, Columbus, Ohio, USA) consists of an elevated rotating rod. Mice placed on the rod must actively move in a coordinated manner to prevent themselves from falling. Mice were placed in the experimental room 1 h prior to training or testing in order to acclimatize to the environment. Prior to the testing day, the mice were trained on the rotarod for 4 times a day with 15-min intervals between trials, until they could stay on the rod consistently for a minimum of 300 s. The rotation speed of the rod was set to 12 rotations per minute. Training took approximately 2 weeks. For testing, each mouse was placed on the rotarod and the time spent on the rotating rod was recorded. The maximally allowed time on the rod was 300 s, and each mouse was given 4 trials with 15-min intervals. The time spent on the rotarod (latency to fall) from the 4 trials was averaged.

2.6. Elevated plus maze assay

The elevated plus maze assay is used to test anxiety-like behavior in rodents [20]. The maze contains four arms (14 in. × 2 in.) that were made of transparent plexiglass. Two arms were open and two were enclosed (6-in.-high walls). The arms were joined by a middle square (2 in. × 2 in.). The entire maze was positioned 50 cm above the floor. Mice generally consider the dark or enclosed space as "safe" and tend to stay in these areas when anxious. Exploration of the open areas was deemed to reflect low levels of anxiety. Each mouse was placed in the center square facing an open arm, in the opposite position to where the experimenter was located and was allowed to explore the maze for 5 min. The time spent and number of entries in open and closed arms were manually scored in real-time using custom written scripts in Microsoft Excel.

2.7. Tail flick assay

The tail flick assay is used to measure acute nociception in rodents. Acute thermal nociception was assessed using a radiant heat tail flick apparatus (Columbus Instruments, Columbus, Ohio, USA). Each mouse was gently restrained and the midpoint of the tail was placed over the apparatus. The light intensity was fixed to produce an average tail-flick latency of 3 to 4 s in control mice. The latency to flick the tail away from the activated light source (latency to tail flick) was automatically measured to the nearest 1/100th of a second using an integrated light beam detector. A maximal cutoff time to respond was set at 10 s to prevent tissue damage.

2.8. Formalin nociception assay

The formalin assay was used to assess nociception-related behaviors in freely moving mice [21]. Briefly, mice were acclimatized to the testing chamber for 20 min before receiving an injection of formalin. The chamber consisted of a round, acrylic cylinder (15 cm diameter and 30 cm tall). The cylinder was suspended above an angled mirror to facilitate observation from all vantage points. Formalin (5%, 20 μl) was injected subcutaneously into the plantar surface of the right hind paw, and the time spent licking the injected hind paw was recorded. The first two minutes immediately after the formalin injection were not scored. Subsequently, the mice were observed for 2-min intervals every 4 min for the next 44 min. Two mice were tested concurrently and staggered 2 min apart. Nociceptive hind paw licking was classified as occurring in 1 of

2 phases: Phase 1 from 0 to 6 min and Phase 2 from 6 to 46 min after injection of formalin.

2.9. Measurement of GABA_A receptor agonist levels with high-performance liquid chromatography

Adult Swiss Webster mice (3–4 months old, Charles River, Montreal, Quebec, Canada) were treated with gabapentin (30 mg/kg, I.P.) or normal saline (0.9% w/v of NaCl, I.P.) every 12 h for 4 consecutive days. Two hours after the final treatment, the whole brain and spinal cord extracts were collected for measurements of GABA, taurine and alanine concentrations using high-performance liquid chromatography (HPLC), as previously described [22,23]. The mice were anesthetized with isoflurane using a bell jar apparatus and decapitated. The whole brain was removed and flash frozen in isopentane that was cooled with a slurry of dry ice and ethanol. The spinal column was cut at the end of the thoracic and mid-sacral segments, then was extracted by hydraulic extrusion using ice-cold saline and snap frozen in an Eppendorf tube using isopentane that was cooled with a slurry of dry ice and ethanol. The frozen tissues were homogenized in 80% (v/v) methanol at 0 °C. An aliquot of the homogenate was diluted a further 5× in ice-cold methanol and left on ice for 10 min prior to centrifugation at 12,000 g for 4 min at 4 °C. The supernatant was diluted ten-fold with ice-cold deionized water and transferred to a HPLC vial.

Derivatizing reagent (5 μl) containing 2-mercaptoethanol and o-phthaldialdehyde was mixed with the 5 μl of sample or standard, held in the instrument sample loop for 1.5 min, and injected into an Alliance 2690XE HPLC system (Waters, Mississauga, Ontario, Canada). The system employed a Symmetry C18 column (4.5 × 100 mm, 5 μm) that was coupled to a guard column and a Waters 474 programmable fluorescence detector (Waters, Mississauga, Ontario, Canada). All the data were acquired and processed using Empower Pro software (Waters, Mississauga, Ontario, Canada).

A mobile phase gradient consisting of varying amounts of sodium phosphate buffer and methanol was used to separate the amino acids. The samples were maintained at 4 °C and the column compartments were maintained at 30 °C. The excitation and emission wavelengths were 260 nm and 455 nm, respectively.

2.10. Measurement of neurosteroid levels with gas chromatography-mass spectrometry

Alphaxalone was added to the residual 80% methanol homogenate as an internal standard. The mixture was vortexed and left on ice for 10 min prior to centrifugation at 12,000 g for 5 min at 4 °C. Solid-phase extractions on the supernatants were performed using an Oasis HLB 30 mg extraction plate (Waters, Mississauga, Ontario, Canada). The wells were washed with 5% (v/v) methanol and the neurosteroids were eluted using a 9:1 (v/v) methylene dichloride:methanol solution. The eluents were dried and the analytes were derivatized for 1 h at 45 °C with 10 μl of n-heptafluorobutyrylimidazole in 50 μl of ethyl acetate. The samples were dried and reconstituted in 50 μl of toluene, washed with 50 μl of deionized water, and centrifuged at 12,000 g for 2 min. The toluene layer was analyzed with gas chromatography using an Agilent 6890 gas chromatograph (Mississauga, ON, Canada) with a HP-MS5 5% phenylmethylsiloxane column (30 cm × 250 μm, 0.25 μm film thickness) coupled to an Agilent 5973 mass spectrometer (Mississauga, Ontario, Canada) in the negative ion-chemical ionization mode. The inlet temperature was 250 °C. The oven temperature was initialized at 100 °C, increased to 295 °C at a rate of 8 °C/min, and maintained at 295 °C for 5 min.

The mass spectrometer was operated in the single ion monitoring mode and standard curves were run in parallel with each assay. The capture and analysis of data were performed using Agilent MSD Chemstation software (Mississauga, Ontario, Canada). Tissue concentrations of neurosteroids that were below the limit of detection in a sample were assigned a value of 1 pg steroid/g tissue.

2.11. Drugs and chemicals

Gabapentin, CNQX, APV and bicuculline were purchased from Abcam (Cambridge, United Kingdom). Tetrodotoxin was purchased from Alomone Labs (Jerusalem, Israel), and formalin from Sigma Aldrich (St. Louis, Missouri, USA).

2.12. Statistical analysis

Data are presented as mean ± SEM. An unpaired Student t-test was used to compare two groups. The two-way analysis of variance (ANOVA, treatment x genotype) followed by the Tukey's multiple comparisons post hoc test was used to compare more than two groups. All analyses were performed using GraphPad Prism 6.01 (Graphpad Software Inc., La Jolla, California, USA). A two-tailed hypothesis test was used, and statistical significance was set at $P < 0.05$. No outliers were excluded from the statistical analyses.

2.13. Data availability

Data supporting the findings of this manuscript are available from the corresponding author upon request.

3. Results

### 3.1. Gabapentin increases δGABA_A receptor expression in the cerebellum

To determine whether gabapentin modifies the expression of δGABA_A receptors, mice were treated with gabapentin (100 mg/kg, I.P.) or vehicle (0.9% w/v of NaCl, I.P.) and 2 h later, *ex vivo* brain slices were harvested and processed for biotinylation and Western blotting (Fig. 1a). Studies were first performed with tissue from the cerebellum as this brain region has the highest expression levels of δGABA_A receptors and cerebellar dysfunction causes ataxia [16].

Gabapentin increased cell-surface expression of δ subunits to 136.3% ± 5.4% of control (n = 6 per condition, P = 0.0005; Fig. 1b) whereas total expression levels were unchanged (109.1% ± 4.4% of control, n = 6 per condition, P = 0.16). We also studied the expression levels of α1 subunits as these subunits primarily contribute to synaptic GABA_A receptors. Gabapentin did not modify cell-surface expression or total expression levels of α1 subunits, suggesting that it selectively modifies expression of δGABA_A receptors (Fig. 1c).

Gabapentin could increase cell-surface expression of δGABA_A receptors by exerting either a direct effect on the brain or by stimulating systemic changes, such as an increase in levels of circulating neurosteroids or other paracrine factors that enter the brain [18,24]. To determine whether gabapentin up-regulates the expression of δGABA_A receptors, through mechanisms that are independent of systemic actions, slices of cerebellum harvested from drug-naive mice were directly incubated with gabapentin (300 μM) for 6 h (Fig. 1d) [25]. Gabapentin increased both cell-surface expression (167.7% ± 3.6% of control, n = 3 each, P = 0.0003) and total expression levels (179.2% ± 5.9% of control, n = 3 each, P = 0.0002) of δ subunits (Fig. 1e). Thus, treatment of the brain slices recapitulated the effect of administering gabapentin systemically. The expression of α1 subunits was unchanged in cerebellar slices treated under similar experimental conditions (Supplementary Fig. 1).

3.2. Gabapentin increases a tonic inhibitory current in cerebellar granule neurons

We next asked whether increased cell-surface expression of δ subunits was sufficient to increase the amplitude of the tonic current in cerebellar neurons. Whole-cell currents were recorded from cerebellar

Ex vivo cerebellar slices

In vitro cerebellar slices
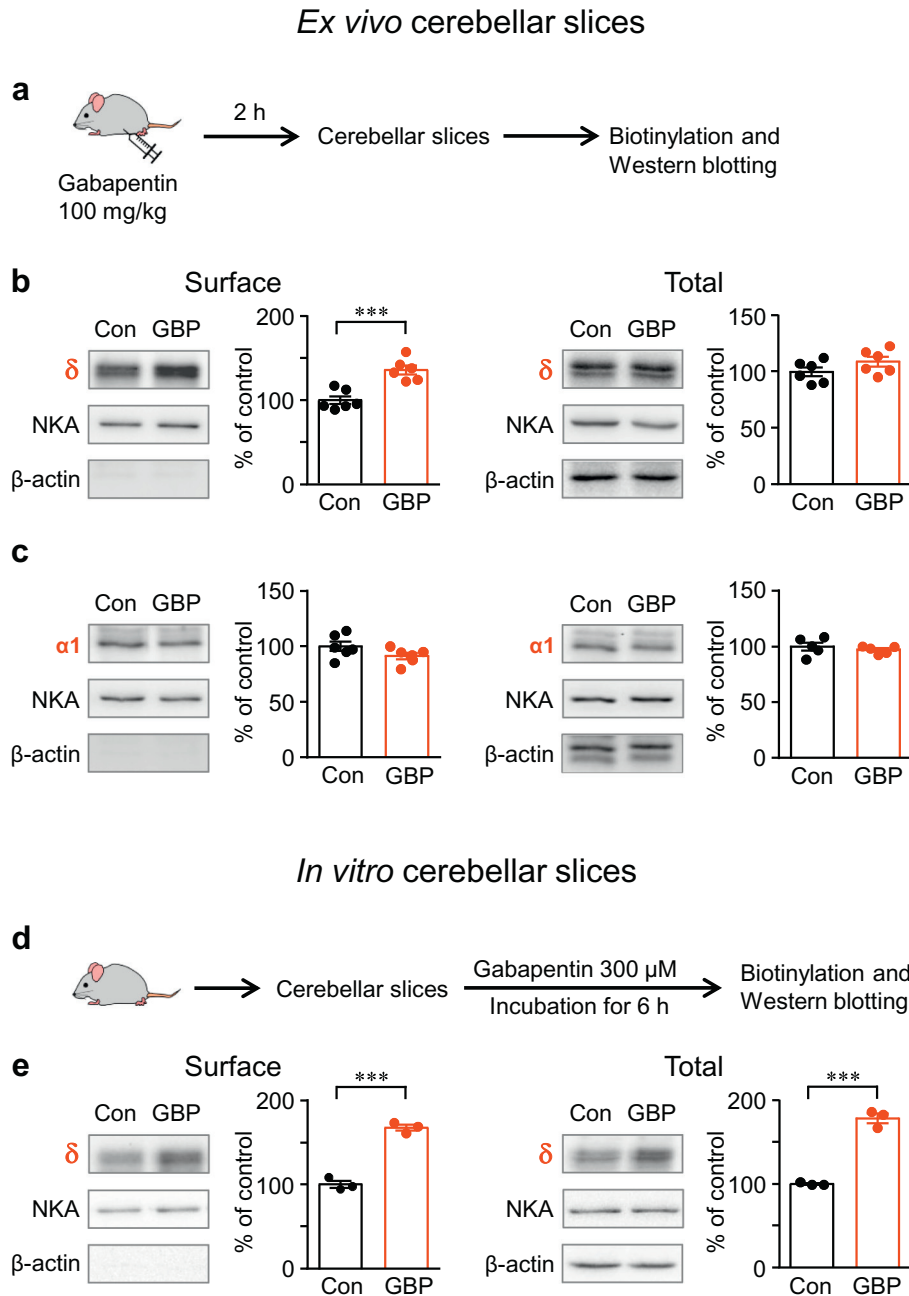
Fig. 1. The expression of δ subunits in the cerebellum is increased by gabapentin. (a) A schematic diagram shows the experimental timeline. (b, c) Representative Western blots and summarized data of cell-surface and total expression of δ subunits (b) and α1 subunits (c) in the cerebellum. b: n = 6 for both surface and total, c: n = 6, 5 for surface and total, respectively. (d) A schematic diagram shows the experimental timeline. (e) Representative Western blots and summarized data of the cell-surface and total expression of δ subunits after the cerebellar slices were treated with gabapentin. n = 3 for each condition. ***P < 0.001, unpaired Student t-test. Data are Mean ± SEM. Con = control, GBP = gabapentin, NKA = Na/K-ATPase.

granule neurons with patch clamp technique, as previously described [26]. Cerebellar slices were harvested from mice that lacked the δGABA$_A$ receptors (*Gabrd* −/− mice) and wild-type (WT) mice. The mice were treated with gabapentin (100 mg/kg, I.P.) then sacrificed 2 h later (Fig. 2a). To measure the amplitude of the tonic current, the competitive GABA$_A$ receptor antagonist bicuculline (10 μM) was applied to the *ex vivo* brain slices. Current amplitude was quantified by measuring the change in the holding current before and after the application of bicuculline [27,28].

Gabapentin increased the tonic current in WT granule neurons to 153.9% of control (Gabapentin: 8.0 ± 0.8 pA/pF, n = 11 vs. Control: 5.2 ± 0.6 pA/pF, n = 12, P = 0.02; Fig. 2b & c) but had no effect on the tonic current in *Gabrd* −/− neurons (Gabapentin: 2.3 ± 0.6 pA/pF,

n = 8 vs. Control: 2.2 ± 0.5 pA/pF, n = 7, P = 1.0; Fig. 2b & c). We observed that the baseline tonic current was lower in *Gabrd* −/− neurons than in WT neurons (*Gabrd* −/−: 2.2 ± 0.5 pA/pF, n = 8 vs. WT: 5.2 ± 0.6 pA/pF, n = 12, P = 0.02), as previously reported by others [29]. These results provide evidence that gabapentin increases a tonic inhibitory current in cerebellar granule neurons that is primarily generated by δGABA$_A$ receptors [16].

3.3. Gabapentin increases δGABA$_A$ receptor cell-surface expression in the hippocampus

Clinical and preclinical studies have shown that gabapentin causes memory loss, sedation and anxiolysis [3,4]. These diverse effects suggest
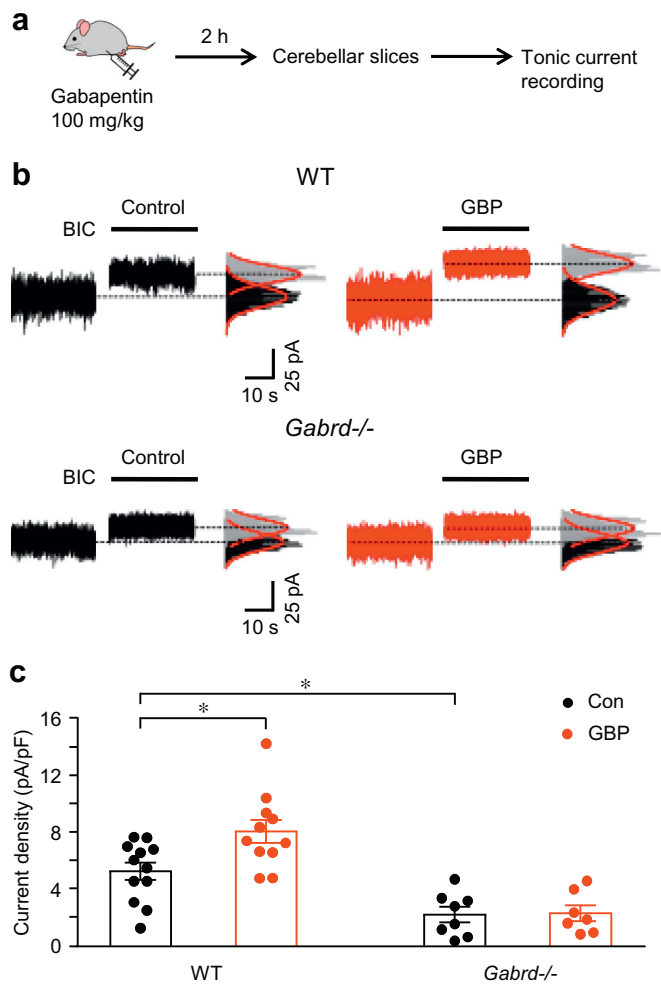
Fig. 2. Tonic inhibitory current in cerebellar granule neurons is enhanced by gabapentin. (a) A schematic diagram shows the experimental timeline. (b, c) Representative traces (b) and summarized data (c) for the tonic current recorded in cerebellar granule cells from WT and $Gabrd-/-$ slices. WT: n = 12, 11 for control and gabapentin, respectively. $Gabrd-/-$: n = 8, 7 for control and gabapentin, respectively. Two-way ANOVA, effect of gabapentin treatment: $F_{(1,34)} = 4.3$, P = 0.046; effect of genotype: $F_{(1,34)} = 39.5$, P < 0.0001; effect of interaction: $F_{(1,34)} = 3.8$, P = 0.06; *P < 0.05, Tukey's multiple comparisons test. Data are Mean ± SEM. BIC = bicuculline, Con = control, GBP = gabapentin.

that gabapentin modifies δGABA$_A$ receptors in regions of the central nervous system other than cerebellum. Thus, we next studied whether gabapentin-induced increased expression of δGABA$_A$ receptors generalizes to another brain region.

δGABA$_A$ receptors are highly expressed in the dentate gyrus [9,16]. Indeed, autoradiographic studies have demonstrated high levels of gabapentin binding in this subregion of the hippocampus [9,30]. To determine whether *in vivo* treatment with gabapentin increased expression levels of δGABA$_A$ receptors in the hippocampus, mice were injected with gabapentin (100 mg/kg, I.P.) and 2 h later, *ex vivo* hippocampal slices were harvested and processed for biotinylation analyses (Fig. 3a). Cell-surface expression of δ subunit was increased (122.4% ± 3.8% of control, n = 6 each, P = 0.0013; Fig. 3b) whereas the total expression levels were unchanged (101.5% ± 0.6% of control, n = 6 each, P = 0.7). Gabapentin did not alter expression levels of α1 subunits (Fig. 3c).

Since extrasynaptic α5GABA$_A$ receptors are highly expressed in the hippocampus [11], we next studied whether gabapentin increased the expression levels of this receptor subtype. Gabapentin did not modify the expression levels of α5 subunits (Fig. 3d),

consistent with our previous findings from studies of cultured hippocampal neurons [8].

Finally, we harvested hippocampal slices from drug-naïve WT mice and treated them directly with gabapentin (300 μM) for 6 h (Supplementary Fig. 2a). Interestingly, cell-surface expression (132.6% ± 3.3% of control, n = 4 each, P = 0.0002) but not the total expression level of δ subunits (106.9% ± 6.7% of control, n = 4 each, P = 0.4) was increased (Supplementary Fig. 2b). Despite similar experimental conditions, the latter finding was inconsistent with those in cerebellar slices where total expression levels were increased. In other studies of hippocampal slices, direct application of gabapentin did not change the expression levels of α1 and α5 subunits (Supplementary Fig. 2c&d). Thus, the selective increase in expression of δGABA$_A$ receptors is not restricted to the cerebellum.

### 3.4. Gabapentin causes ataxia and reduces anxiety in WT mice but not Gabrd−/− mice

We next asked whether increased expression of δGABA$_A$ receptors contributes to some of the behavioral properties of gabapentin. Behaviors including ataxia, anxiety and nociception, which are mediated by different regions of the central nervous system, were studied in WT and $Gabrd-/-$ mice (Fig. 4a). Ataxia, a lack of voluntary coordination of muscle movement that can result from cerebellum dysfunction [31], is a common adverse effect of gabapentin. Ataxia is frequently studied using the rotarod assay [31]. Mice were treated with gabapentin or vehicle and 2 h later, placed on a rotating rod. The latency to fall off the rotarod indicated voluntary motor coordination. Under baseline conditions, performance on the rotarod was similar in WT and $Gabrd-/-$ mice (Fig. 4b). Gabapentin (30–100 mg/kg) impaired the performance of WT mice, as evidenced by a decrease in latency time (Fig. 4b). In contrast, the same doses had no significant effects on motor performance in $Gabrd-/-$ mice. While there was a slight reduction at the highest dose of gabapentin in $Gabrd-/-$ mice, the change was not significant.

Gabapentin is often prescribed for its anxiolytic and sedative properties. Indeed, in a clinical study we showed that gabapentin reduces anxiety scores in highly anxious patients prior to surgery [32]. To probe the mechanisms of gabapentin-induced anxiolysis, WT and $Gabrd-/-$ mice were studied with the elevated plus maze assay. The time spent in the open arms of the maze provided a surrogate measure of anxiety caused by open spaces. Mice were treated with gabapentin (30 mg/kg, I.P.), and 2 h later were placed on the elevated plus maze. Baseline performance was similar between genotypes (P = 0.8, Fig. 4c). Gabapentin-treated WT mice exhibited an 11-fold increase in the time spent in the open arms (Gabapentin: 11.3 ± 3.2%, n = 10 vs. Control: 1.0 ± 0.5%, n = 9, P = 0.007; Fig. 4c). In contrast, gabapentin did not increase the open arm time of $Gabrd-/-$ mice (Gabapentin: 3.2 ± 1.1%, n = 11 vs. Control: 3.7 ± 2.0%, n = 11, P = 1.0). Similarly, the gabapentin-treated WT mice, but not the gabapentin-treated $Gabrd-/-$ mice, showed a significant increase in the number of entries into the open arms (Supplementary Fig. 3a). In both genotypes, gabapentin treatment did not alter the time spent in the closed arms, or the number of entries into the closed arms (Supplementary Fig. 3b). Thus, while baseline anxiety was not influenced by the expression of δGABA$_A$ receptors, δGABA$_A$ receptors were necessary for the anxiolytic effects of gabapentin.

3.5. Gabapentin modulation of nociception in WT mice and Gabrd−/− mice

We and others have shown that δGABA$_A$ receptors regulate nociception [33,34]. We next asked whether gabapentin modulates nociception in WT and $Gabrd-/-$ mice. Tail flick and formalin assays were used to study acute and persistent nociception, respectively. The tail flick assay assesses nociceptive threshold by measuring the latency

Ex vivo hippocampal slices
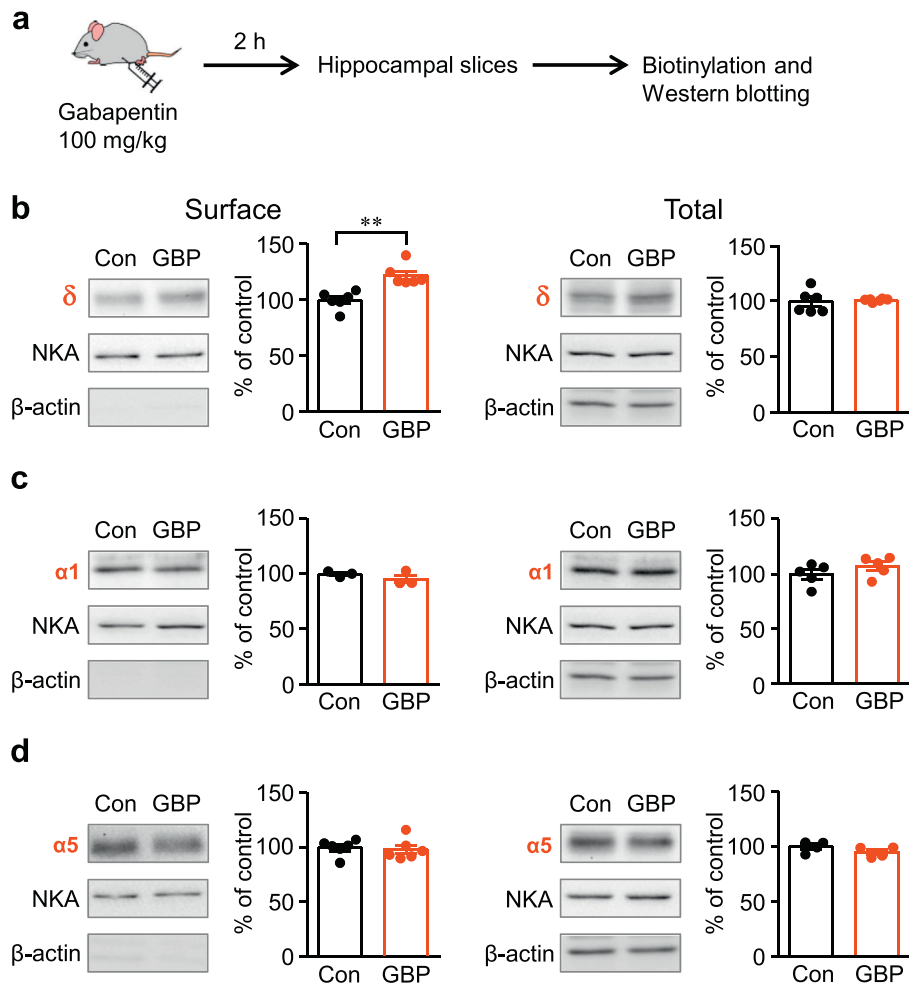
Fig. 3. The cell-surface expression of δ subunits in hippocampus is increased by gabapentin. (a) A schematic diagram shows the experimental timeline. (b, c, d) Representative Western blots and summarized data of the cell-surface and total expression of the δ (b), α1 (c), and α5 (d) subunits in the hippocampus. b: n = 6 for both surface and total; c: n = 3, 5 for surface and total, respectively; d: n = 6, 4 for surface and total, respectively. **P < 0.01, unpaired Student *t*-test. Data are Mean ± SEM. Con = control, GBP = gabapentin, NKA = Na/K-ATPase.

time to tail flick in response to a thermal stimulus. The formalin assay involves both acute and tonic phases. The acute phase (Phase 1) occurs within the first 6 min after formalin injection and responses are thought to reflect the direct activation of nociceptors by the noxious chemical. The tonic phase (Phase 2) is measured 6 min to 46 min after the formalin injection. The licking behavior exhibited by the mice is thought to result from central sensitization and inflammation at the level of the spinal dorsal horn [33].

Under baseline conditions, the latency to tail flick was similar in the two genotypes (Fig. 4d). Gabapentin (60 mg/kg, I.P.), administered at a dose that was anxiolytic in WT mice, caused no change in the tail flick response in either group (Fig. 4d). For Phase 1 of the formalin test, under baseline conditions, no differences were observed between genotypes for the licking time. Gabapentin (60 mg/kg, I.P.) did not alter the licking time in either genotype (Fig. 4e). For Phase 2, the baseline response of *Gabrd*−/− mice was greater than WT mice (WT control: 99.0 ± 9.4 s, n = 10 vs. *Gabrd*−/− control: 135.1 ± 5.4 s, n = 8, P = 0.006; Fig. 4e). Gabapentin reduced the Phase 2 response of both WT and *Gabrd*−/− mice and abolished the differences between the two genotypes (Fig. 4e). These results suggest that gabapentin attenuation of chronic or inflammatory nociception is independent of the expression of δGABA$_A$ receptors.

3.6. Longer-term administration of gabapentin increases expression of δGABA$_A$ receptors in the cerebellum

In the studies described above, the mice were treated with a single dose of gabapentin. However, patients are typically treated with gabapentin for days to months [4]. Such long-term exposure to other GABAergic drugs including benzodiazepines and barbiturates can cause tachyphylaxis or drug resistance due to internalization of GABA$_A$ receptors from the cell surface [35]. Thus, we next studied whether a longer exposure to gabapentin would trigger a sustained increase in the cell-surface expression of δGABA$_A$ receptors.

Mice were treated with gabapentin (100 mg/kg/day, I.P.) or vehicle for 6 consecutive days. Two hours after the final injection, *ex vivo* cerebellar slices were harvested and processed for biotinylation and Western blotting (Supplementary Fig. 4a). Gabapentin increased cell-surface expression (144.4% ± 17.4% of control, n = 4 each, P = 0.046; Supplementary Fig. 4b). The total expression was also increased but the change did not reach statistical significance (149.3% ± 23.9% of control, n = 4 each, P = 0.1; Supplementary Fig. 4b). Thus, similar to acute gabapentin treatment, longer-term treatment increased expression of δGABA$_A$ receptors.
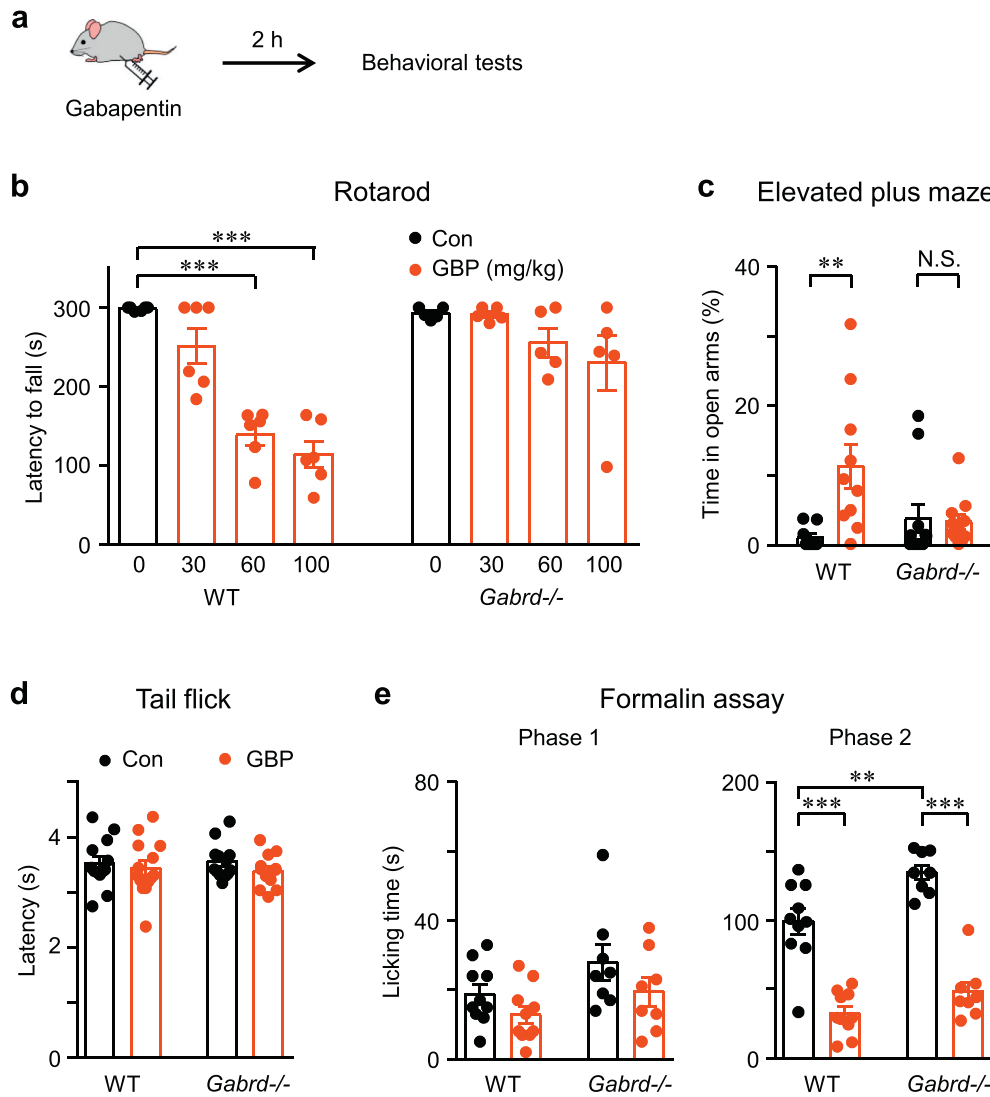
Fig. 4. δGABA$_A$ receptors contribute to the ataxic and anxiolytic, but not the analgesic, properties of gabapentin. (a) A schematic diagram shows the experimental timeline. (b) Gabapentin dose-dependently reduces the latency to fall in WT but not in *Gabrd*−/− mice. n = 6 for each group in WT mice and n = 5 for each group in *Gabrd*−/− mice. Two-way ANOVA, effect of gabapentin treatment: $F_{(3,36)} = 22.7$, $P < 0.0001$; effect of genotype: $F_{(1,36)} = 29.0$, $P < 0.0001$; effect of interaction: $F_{(3,36)} = 5.9$, $P = 0.002$. ***$P < 0.001$ compared with control, Tukey's multiple comparisons test. (c) Gabapentin increases the time that WT but not *Gabrd*−/− mice spent in the open arms. n = 9, 10 for control and gabapentin, respectively in WT mice and n = 11 for each group in *Gabrd*−/− mice. Two-way ANOVA, effect of gabapentin treatment: $F_{(1,37)} = 5.9$, $P = 0.02$; effect of genotype: $F_{(1,37)} = 1.7$, $P = 0.2$; effect of interaction: $F_{(1,37)} = 7.2$, $P = 0.01$. **$P < 0.01$, N.S.: no significance, Tukey's multiple comparisons test. (d) gabapentin does not change the latency to tail flick in either genotype. n = 13, 14 for control and gabapentin, respectively in WT mice and n = 13, 12 for control and gabapentin, respectively in *Gabrd*−/− mice. Two-way ANOVA, effect of gabapentin treatment: $F_{(1,48)} = 1.3$, $P = 0.3$; effect of genotype: $F_{(1,48)} = 0.005$, $P = 0.9$; effect of interaction: $F_{(1,48)} = 0.16$, $P = 0.7$. (e) Gabapentin does not alter licking time in either genotype for Phase 1 but reduced licking time for both genotypes for Phase 2. n = 10 for each treatment group in WT mice and n = 8 for each treatment group in *Gabrd*−/− mice. Phase 1: Two-way ANOVA, effect of gabapentin treatment: $F_{(1,32)} = 4.1$, $P < 0.05$; effect of genotype: $F_{(1,32)} = 4.8$, $P = 0.04$; effect of interaction: $F_{(1,32)} = 0.12$, $P = 0.73$. Phase 2: Two-way ANOVA, effect of gabapentin treatment: $F_{(1,32)} = 114.4$, $P < 0.0001$; effect of genotype: $F_{(1,32)} = 12.9$, $P = 0.001$; effect of interaction: $F_{(1,32)} = 2.0$, $p = 0.16$. **$P < 0.01$, ***$P < 0.001$, Tukey's multiple comparisons test. Data are Mean ± SEM. Con = control, GBP = gabapentin.
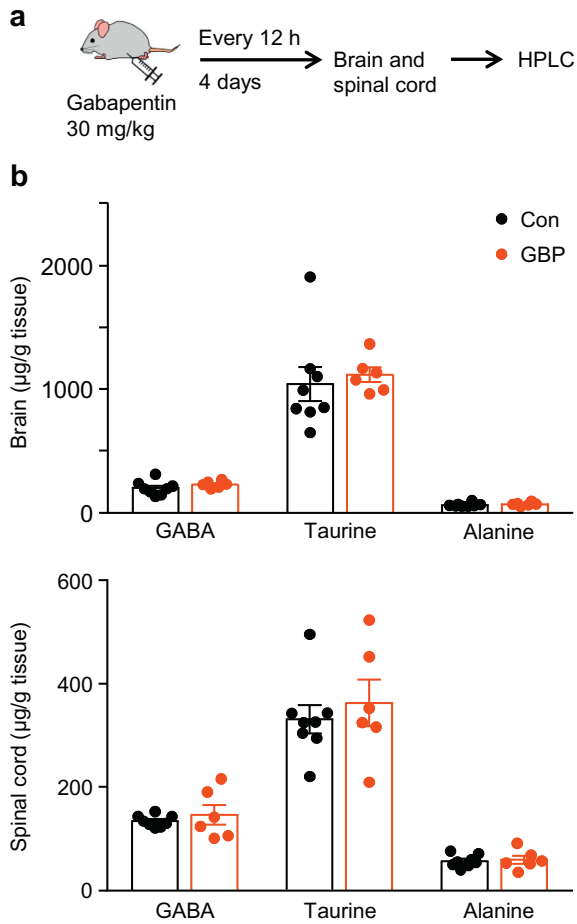
3.7. Gabapentin does not increase the levels of GABA$_A$ receptor agonists in the brain or spinal cord

Several endogenous agonists including the primary neurotransmitter GABA, as well as taurine and alanine, activate GABA$_A$ receptors. δGABA$_A$ receptors have a high affinity for GABA compared to other subtypes of native GABA$_A$ receptors [36]. Furthermore, taurine is a potent agonist of extrasynaptic GABA$_A$ receptors [37]. Alanine, which is also present at relatively high concentrations in the brain [38–40], evokes bicuculline-sensitive Cl⁻ currents in hippocampal neurons (unpublished results). Thus, gabapentin could stimulate small increases in levels of GABA, taurine and alanine in the brain or spinal cord and thereby increase the function of high-affinity δGABA$_A$ receptors. Such small changes in agonist levels could contribute to the gabapentin's behavioral properties, as suggested by previous studies [7,12,13].

We next measured the levels of GABA, taurine and alanine in the brain or spinal cord of gabapentin- and vehicle-treated mice. A 4-day treatment paradigm was used for these studies to reduce the risk of failing to detect any transient or modest changes in neurotransmitter levels. Mice were treated with gabapentin (30 mg/kg, I.P.) or vehicle every 12 h for 4 consecutive days (Fig. 5a). Two hours after the final treatment, the whole brain, and the lumbar and upper sacral regions of the spinal cord were extracted. Levels of GABA, taurine and alanine were measured using high-performance liquid chromatography. The results showed that gabapentin failed to modify the levels of GABA, taurine and alanine (Fig. 5b), suggesting that gabapentin does not increase agonist concentrations after chronic treatment.

a

Gabapentin 30 mg/kg → Every 12 h, 4 days → Brain and spinal cord → HPLC

b

Brain (μg/g tissue): GABA, Taurine, Alanine

Spinal cord (μg/g tissue): GABA, Taurine, Alanine

● Con
● GBP

Fig. 5. Levels of GABA$_A$ receptor agonists are not increased by gabapentin. (a) A schematic diagram shows the experimental timeline. (b) Gabapentin does not change levels of GABA$_A$ receptor agonists in the brain and spinal cord. n = 8 and 6 for control and gabapentin, respectively; P > 0.1 for each agonist, unpaired Student t-test. Data are mean ± SEM. Con = control, GBP = gabapentin.

An increase in the levels of neurosteroids could also account for gabapentin-induced increase in the function of δGABA$_A$ receptors, as these receptors are highly sensitive to certain neurosteroids [41,42]. We next measured the levels of the neurosteroids 5-dehydroepiandrosterone (DHEA), 3α,5α-THPROG, 3α,5β-THPROG, pregnenolone, 3β,5α-THPROG, and THDOC in the brain after 4 days of same treatment with gabapentin. Gabapentin did not alter the levels of these neurosteroids in the brain (Supplementary Fig. 5).

4. Discussion

The results show that gabapentin increases expression of δGABA$_A$ receptors in the cerebellum and hippocampus. This enhanced expression of δGABA$_A$ receptors is associated with an increase in the tonic current in cerebellar neurons. Behavioral studies show that δGABA$_A$ receptors are necessary for the ataxia and anxiolysis but not anti-nociceptive properties of gabapentin. Collectively, these results provide the first evidence of a clinically-approved drug that rapidly increases cell-surface expression levels of δGABA$_A$ receptors within a period of hours. The findings challenge the widely accepted notion that modulation of α$_2$δ subunits of voltage-dependent calcium channels alone accounts for many of the behavioral effects of gabapentin.

Increased expression of δGABA$_A$ receptors on the cell surface is due to either enhanced trafficking of δGABA$_A$ receptors and/or stabilization of receptors at the plasma membrane. The increase in total levels of δGABA$_A$ receptors suggests *de novo* synthesis of new receptors [43].

Thus, more than one mechanism could account for the increase in receptor expression. Interestingly, whether gabapentin increases cell-surface as well as total expression of δGABA$_A$ receptors depends on the brain region and experimental paradigm. As an example, treatment *in vivo* with gabapentin increases cell-surface expression but not total expression levels in the cerebellum, whereas direct application of gabapentin to cerebellar slices *in vitro* increases both cell-surface and total expression. The factors that account for such differences in cell-surface and total expression of δGABA$_A$ receptors are uncertain. Little is known about regulation of the expression and trafficking of δGABA$_A$ receptors. The signaling pathways, transcription factors, kinases and phosphatases that regulate the gene encoding the δ subunits (*Gabrd*) could differ between brain regions. Interestingly, the trafficking and expression of synaptic GABA$_A$ receptors are differently modified by protein kinase A, protein kinase C, p38 mitogen-activated protein kinases and phosphatases [15,43]. Additional studies are needed to determine how gabapentin modifies cell-surface and total expression of δGABA$_A$ receptors.

Several lines of evidence support the notion that increased activity of δGABA$_A$ receptors contributes to gabapentin-induced ataxia and anxiolysis. Modulators that increase the function of δGABA$_A$ receptors, including increased endogenous hormones in the estrus cycle, exogenous neurosteroids and ethanol all cause ataxia [44] and anxiolysis [18,24]. Similarly, the δGABA$_A$ receptor-selective super-agonist 4,5,6,7-tetrahydroisoxazolo(5,4-c) pyridin-3-ol (gaboxadol or THIP) causes ataxia and anxiolysis. Gaboxadol is considered to be a "super-agonist" as it generates a current that is larger than current evoked by a saturating concentration of GABA [36]. In both humans and laboratory animals, gaboxadol, similar to gabapentin, produces anxiolysis and impairs motor coordination [45–49].

The results show that δGABA$_A$ receptor do not contribute to gabapentin's antinociceptive properties. Gabapentin does not modify responses in the tail flick assay or Phase 1 of the formalin assay. In a previous study, we showed that gaboxadol attenuates responses in Phase 1 of the formalin test in WT but not *Gabrd*−/− mice [33]. These differences in behavioral properties may be due to differences in the efficacy of gaboxadol versus gabapentin. Gaboxadol, which acts as an allosteric modulator and super-agonist, may cause a greater increase in δGABA$_A$ receptor function than gabapentin, which is not a positive allosteric modulator but rather increases cell-surface expression. In addition, under baseline conditions, expression levels of δGABA$_A$ receptors are low in dorsal root ganglion neurons and the spinal cord [16]. Thus, the gabapentin-induced increase in cell-surface expression may not be sufficient to modify antinociception. We also showed that gabapentin reduces responses in Phase 2 of the formalin test in both WT and *Gabrd*−/− mice. This effect does not depend on δGABA$_A$ receptors but may involve other receptor targets, including α$_2$δ subunit of voltage-dependent calcium channels, α2δ-NMDA receptor complexes, hyperpolarization-activated cation channels or sodium channels [6,50–52].

Gabapentin caused ataxia in WT but not *Gabrd*−/− mice raising the concern that ataxia potentially confounded the effects of gabapentin on anxiety and nociception. However, the low dose of gabapentin (30 mg/kg, I.P.) that was selected for elevated plus maze studies did not impair performance on the rotarod. Also, gabapentin increased the number of entries into the open arms. Ataxia would likely have produced the opposite effect and reduced the number of entries. Finally, while gabapentin produced ataxia only in WT mice, it had similar antinociceptive effects in WT and *Gabrd*−/− mice. These results are consistent with previous findings showing that deficits in motor coordination do not impair primary sensory function [53]. Overall, it is unlikely that gabapentin-induced ataxia confounded the responses in the elevated plus maze and formalin assays.

Gabapentin does not increase levels of GABA, alanine, taurine or neurosteroids in the brain or spinal cord. Similar results of GABA levels have been reported by others [54–56]. However, in other studies

[7,12,13], gabapentin increases GABA levels in both rodents and humans, either by increasing GABA synthesis via the activation of GABA-synthesizing enzyme glutamic acid decarboxylase or by decreasing GABA metabolism via the inhibition of GABA transaminase. Even small increases in agonist levels could enhance activity of high-affinity δGABA$_A$ receptors. One limitation of our study is that agonist and neurosteroid levels were measured in the whole brain and spinal cord. This approach may not be sufficiently sensitive to detect small changes in discrete, yet functionally important pools of agonists or neurosteroids. Indeed, gabapentin increases GABA turnover in some, but not all, brain regions [57].

Our results raise several questions that are worthy of future study. It would be of interest to investigate whether δ subunit binding partners including the $\alpha 4$ and $\alpha 6$ subunits are also upregulated by gabapentin. In cerebellum, the predominant form of δGABA$_A$ receptors is $\alpha 6\beta 2/3\delta$, with low expression levels of $\alpha 4\beta 2/3\delta$. In the hippocampus and thalamus, $\alpha 4\beta 2/3\delta$ GABA$_A$ receptors are predominantly expressed [9,10,16]. $\beta 2$ or $\beta 3$ subunits can also partner with subunits other than the δ subunit, to form either synaptic ($\alpha 1\beta 2/3\gamma$) or extrasynaptic ($\alpha 5\beta 3\gamma$) GABA$_A$ receptors [9–11]. Interestingly, the effects of gabapentin are selective for the δ subunit as expression of both $\alpha 1$ subunit and $\alpha 5$ subunit was unchanged, through mechanisms that are unknown.

Finally, at a broader translational level, one of the most serious and potentially life-threatening adverse effects of gabapentin is respiratory depression [58–60]. The association of the use of gabapentin and a 60% increase in narcotic-related death is of great concern [59]. In a collaborative preclinical study, we showed that increased δGABA$_A$ receptor function reduces ventilation and upper airway muscle tone [61]. Thus, it will be of interest to determine whether respiratory depression due to coadministration of gabapentin and a narcotic is mediated by increased expression of δGABA$_A$ receptors.

Overall, the results identify increased expression of δGABA$_A$ receptors as a novel mechanism to account for gabapentin's GABAergic properties. Rapid upregulation of δGABA$_A$ receptors may be helpful in treating disorders associated with reduced expression of δGABA$_A$ receptors including alcoholism, fragile X syndrome and neuropsychiatric disorders including depression and schizophrenia [16].

Conflict of interest

The authors declare no conflict of interest.

Author contributions

J.Y., R.P.B., G.B.B., and B.A.O. designed research; J.Y., R.P.B., A.P., A.A.G., A.A.Z., and G.R. performed research; J.Y., D.S.W., R.P.B., A.P., and G.R. analyzed data; D.S.W. and B.A.O wrote the manuscript.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.008.

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