ORIGINAL PAPER

The Influence of AA29504 on GABA$_A$ Receptor Ligand Binding Properties and Its Implications on Subtype Selectivity

Sylvia Sikstus[1] · Ali Y. Benkherouf[1] · Sanna L. Soini[1] · Mikko Uusi-Oukari[1]

Abstract

The unique pharmacological properties of δ-containing γ-aminobutyric acid type A receptors (δ-GABA$_A$Rs) make them an attractive target for selective and persistent modulation of neuronal excitability. However, the availability of selective modulators targeting δ-GABA$_A$Rs remains limited. AA29504 ([2-amino-4-(2,4,6-trimethylbenzylamino)-phenyl]-carbamic acid ethyl ester), an analog of K$^+$ channel opener retigabine, acts as an agonist and a positive allosteric modulator (Ago-PAM) of δ-GABA$_A$Rs. Based on electrophysiological studies using recombinant receptors, AA29504 was found to be a more potent and effective agonist in δ-GABA$_A$Rs than in γ2-GABA$_A$Rs. In comparison, AA29504 positively modulated the activity of recombinant δ-GABA$_A$Rs more effectively than γ2-GABA$_A$Rs, with no significant differences in potency. The impact of AA29504's efficacy- and potency-associated GABA$_A$R subtype selectivity on radioligand binding properties remain unexplored. Using [^{3}H]4'-ethynyl-4-n-propylbicycloorthobenzoate ([^{3}H]EBOB) binding assay, we found no difference in the modulatory potency of AA29504 on GABA- and THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol)-induced responses between native forebrain GABA$_A$Rs of wild type and δ knock-out mice. In recombinant receptors expressed in HEK293 cells, AA29504 showed higher efficacy on δ- than γ$_2$-GABA$_A$Rs in the GABA-independent displacement of [^{3}H]EBOB binding. Interestingly, AA29504 showed a concentration-dependent stimulation of [^{3}H]muscimol binding to γ$_2$-GABA$_A$Rs, which was absent in δ-GABA$_A$Rs. This was explained by AA29504 shifting the low-affinity γ$_2$-GABA$_A$R towards a higher affinity desensitized state, thereby rising new sites capable of binding GABA$_A$R agonists with low nanomolar affinity. Hence, the potential of AA29504 to act as a desensitization-modifying allosteric modulator of γ2-GABA$_A$Rs deserves further investigation for its promising influence on shaping efficacy, duration and plasticity of GABA$_A$R synaptic responses.

Keywords GABA · Muscimol · AA29504 · GABA$_A$ receptor · EBOB · Allosteric modulation

Introduction

γ-Aminobutyric acid type A receptors (GABA$_A$R), members of the Cys-loop ligand-gated ion channels superfamily, are the major sites for fast-acting synaptic inhibition in the mammalian brain [1, 2]. These heteropentameric protein complexes contain an inherent chloride channel that opens upon the binding of GABA, where chloride ion influx hyperpolarizes the membrane potential, thereby inhibiting the neuron [1, 3]. Numerous clinically significant drugs, including benzodiazepines, barbiturates, neurosteroids, and anesthetics, have been shown to positively modulate GABA$_A$R function [4]. GABA$_A$R protein subunits are encoded by 19 distinct genes: α1-α6, β1-β3, γ1-γ3, δ, ε, π, θ, and ρ1-ρ3 [1]. Each GABA$_A$R subunit is comprised of a large extracellular N terminus, four transmembrane domains (TM1–4), one extracellular TM2–3 loop, two intracellular loops (TM1–2 and TM3–4), and an extracellular C terminus [5]. The majority of receptor subtypes are composed of α, β and γ subunits with a stoichiometry of 2α:2β:1γ [6, 7]. These subtypes are mostly sensitive to benzodiazepines and reside in post-synaptic sites where they mediate fast synaptic phasic inhibition [1]. Receptor combinations in which γ2 is substituted with δ (2α:2β:1δ) are found in extra- and perisynaptic membranes. δ-containing GABA$_A$Rs (δ-GABA$_A$Rs) are

---

Sylvia Sikstus and Ali Y. Benkherouf contributed equally to this work.
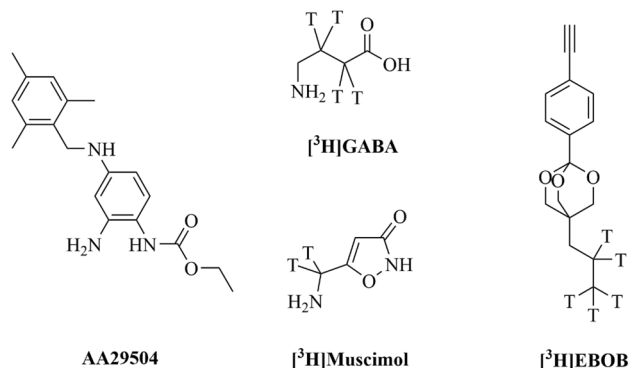
✉ Mikko Uusi-Oukari
mikko.uusi-oukari@utu.fi

1 Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20014 Turku, Finland

benzodiazepine-insensitive and display a high affinity for GABA, which enables them to be activated by low GABA concentrations to mediate a slow-desensitizing tonic inhibition [8–11]. Due to the unique functional and pharmacological properties of δ-GABA$_A$Rs, they represent an attractive drug target for selective and persistent modulation of neuronal excitability. The therapeutic potential of δ-GABA$_A$Rs has been studied in several disorders such as epilepsy [12, 13], schizophrenia [14], stroke [15], tremors [16], stress [17, 18] and alcohol withdrawal [19, 20]. However, in comparison to γ2-GABA$_A$Rs, the availability of selective allosteric modulators targeting δ-GABA$_A$Rs remains limited.

DS2 (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide), a selective positive allosteric modulator of α4/6βδ receptors, is a widely used pharmacological tool to probe δ-GABA$_A$R-mediated responses [21, 22]. It has been demonstrated to improve stroke recovery in vivo, but it showed limited brain bioavailability [23]. AA29504 ([2-amino-4-(2,4,6-trimethylbenzylamino)-phenyl]-carbamic acid ethyl ester) (Fig. 1), a structural analog of the voltage-gated potassium channel (KCNQ) opener retigabine, acts as an allosteric agonist and a positive allosteric modulator (Ago-PAM) of δ-GABA$_A$Rs at low micromolar concentrations [24–27]. In relation to DS2, AA29504 exhibits a superior brain permeability (60 min post 2 mg/kg, s.c.in mice resulted in 1 µM brain concentration) and has been shown to alleviate anxiety, stress and cognitive deficits in phencyclidine (PCP) rat model of schizophrenia [24, 25, 28]. These behavioral effects were associated with AA29504 modulation of extrasynaptic GABA$_A$Rs in the same studies, making it a promising tool to explore the role of extrasynaptic GABA$_A$R transmission in the CNS. The functional properties of AA29504 have been examined earlier using several electrophysiological techniques, including acutely prepared slices, cultured neurons and recombinant *Xenopus* oocyte/stable HEK293 Flp-In™ systems for various GABA$_A$R subtypes [24–27]. AA29504 at 1 µM concentration was found to

enhance both phasic and tonic currents induced by GABA$_A$R superagonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c] pyridin-3-ol) in rat cortical brain slices and mice dentate gyrus granule cells [24, 25]. In recombinant receptors, AA29504 was a more potent and effective agonist in δ-GABA$_A$Rs than in γ2-GABA$_A$Rs [27]. On the other hand, AA29504 positively modulated the activity of recombinant δ-GABA$_A$Rs more effectively than that of γ2-GABA$_A$Rs, but no significant differences were noted in terms of potency [24, 27]. However, ligand-receptor interactions governing the complex AA29504's efficacy- and potency-associated subtype selectivity remain unexplored. Radioligand binding assay is a valuable technique to elucidate this and to quantify the molecular parameters derived from single or multiple ligand-bound states [29, 30]. Earlier binding studies on native GABA$_A$Rs expressed in rat brain membranes reported that the analogue retigabine increased the binding affinity to GABA and vice versa [31]. Hence, we hypothesize AA29504's potential to selectively modulate the binding of specific receptor populations by shifting their binding affinity or by other mechanisms, which deserve experimental investigation. This would contribute to the pharmacological characterization of AA29504's selective modulatory activity and its interactions with GABA$_A$Rs agonists, as well as the interpretation of its GABA$_A$R-mediated effects in vivo.

In this study, we implemented radioligand binding assays to examine the allosteric modulatory behavior of AA29504 and its influence on agonist binding properties in native and recombinant GABA$_A$Rs. The selectivity of AA29504 to δ-GABA$_A$Rs was confirmed using wild-type (WT) and δ subunit knockout (δKO) C57BL/6 J mice forebrains, as well as recombinant receptors expressed in human embryonic kidney 293 (HEK293) cell line. Radioligands employed (Fig. 1) were [³H]4'-ethynyl-4-n-propylbicycloorthobenzoate ([³H]EBOB), a non-competitive blocker of GABA-gated chloride channel [32, 33], the neurotransmitter [³H]GABA, and [³H]muscimol, a universal GABA$_A$R agonist with exceptionally high affinity to δ-GABA$_A$Rs [34–36].

Materials and Methods

Animals

Wild-type (C57BL/6J, WT; RRID: IMSR JAX:000,664), and GABA$_A$R δ subunit knockout (C57BL/6J, δKO; RRID: MGI:3,639,693) mice (age: 3–12 months, both sexes; weight: 19–32 g) were a kind gift from Dr. Martin Wallner (UCLA: University of California, Los Angeles, Los Angeles, CA). The δKO mice were originally produced and validated at Harmonics Lab by injecting ES cells into C57BL/6J blastocysts and backcrossing them with C57BL/6J mice for at least ten generations (Jackson Laboratories, stock

Fig. 1 Chemical structures of AA29504 and the radioligands used in this study. T indicates the tritium (³H) radiolabel position

No. 000664) [12]. The mice were maintained on a hybrid C57BL/6–129 Sv background and genotyped by Southern blot analysis as previously reported [12]. Briefly, BamHI-digested mouse tail DNA samples were hybridized with 830-bp PCR product (probe D). Southern blot of BamHI digested DNA indicated that probe D hybridized to a 6.6-kb BamHI fragment from the wild-type δ gene and a 7.7-kb BamHI fragment from the targeted allele. All animals were housed in standard conditions (12:12 h light: dark cycle at 21 ± 1 °C and humidity 65%) with access to Rodent Lab Chow #5001 and filtered tap water ad libitum. Mice were euthanized by decapitation, their fore/midbrains were dissected (loosely referred as forebrain), frozen on dry ice, and stored at -70 °C. All experimental procedures in this study complied with protocols approved by the Animal Experiment Board in Finland and UCLA Chancellor's Animal Research Committee (Animal Welfare Approval Number: A3196-01).

Reagents

[³H]Muscimol (22 Ci/mmol) and [³H]EBOB (48 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). [³H]GABA (30 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Unlabeled GABA and picrotoxin were from Sigma Chemicals Co. (St. Louis, MO, USA). AA29504 and THIP were from Tocris Biosciences (Bristol, UK).

Preparation of Brain Membranes

WT and δKO forebrain membranes were prepared using the method of Squires and Saederup [37] as modified by Uusi-Oukari et al. [38]. Homogenized and washed membranes were suspended in 10 mM Tris–HCl, pH 7.4 and stored at -70 °C. Prior to binding experiments, the frozen membrane suspensions were thawed, centrifuged at 20,000 g for 10 min at $+4$ °C, and resuspended in assay buffer.

Recombinant GABA_A Receptor Expression in HEK293 cells

Human embryonic kidney (HEK) 293 cells were maintained at $+37$ °C/10% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD, USA), supplemented with 3.7 g/L $NaHCO_3$, 10% Fetal Bovine Serum (FBS) (Gibco, Gaithersburg, MD, USA), 50,000 U/L penicillin and 50 mg/L streptomycin (Sigma-Aldrich, St Louis, MO, USA). The cells were divided and plated on 10 cm culture dishes for binding assays 24 h before transfection. HEK293 cells were transfected with rat cDNAs (α1, α6, β2, β3, γ2S, δ) in pRK5 plasmids [39] under the control of cytomegalovirus (CMV) promoter using calcium phosphate

transfection method essentially as described by Lüddens and Korpi [40]. The plasmids were used in 1:1 and 1:1:1 ratios for transfections containing 2 [(α6)+(β3)] or 3 [(α1 or α6)+(β2 or β3)+(γ2S or δ)] different subunits, respectively (5 μg of each plasmid DNA for a 10 cm plate). The cells were incubated at 37 °C/10% CO_2 for 24 h post-transfection. Old culture medium was replaced with fresh medium and the incubation resumed for another 24 h. The cells were harvested 48 h post-transfection. Culture medium was removed and the cells were detached from the plates by pipetting in ice-cold buffer containing 50 mM Tris-citrate or 10 mM Tris–HCl, 0.15 M NaCl, 2 mM EDTA, pH 7.4 and centrifuged at 20,000×g for 10 min at $+4$ °C. The resulting pellets were finally suspended in assay buffer and used directly in binding assays.

Measurement of [³H]muscimol and [³H]GABA Binding

The binding of [³H]muscimol (2 nM) and [³H]GABA (5 nM) were measured in assay buffer (10 mM Tris–HCl, pH 7.4) at room temperature (22 ºC) in a total volume of 300 μl. Individually pooled triplicate membrane samples (3 total binding and 3 non-specific binding) were incubated with shaking for 15 min. The effect of AA29504 on binding was determined in the presence of various concentrations of AA29504. Non-specific binding was determined in the presence of 100 μM GABA. The incubation was terminated by filtration of the samples with a Brandel Cell Harvester (model M-24, Gaithersburg, MD, USA) onto Whatman GF/B filters (Whatman International Ltd., Maidstone, UK).

The samples were rinsed twice with 4–5 ml of ice-cold assay buffer. Filtration and rinsing steps took a total time of 15 s. The filters were air-dried and immersed in 3 ml of Optiphase HiSafe 3 scintillation fluid (Wallac, Turku, Finland) and vortexed. The radioactivity was determined in a Wallac model 1410 liquid scintillation spectrometer (Wallac, Turku, Finland). The average specific counts per minute (CPM) and % specific [³H]muscimol (2 nM) binding to the membrane homogenates were as follows: WT (1649 CPM, 88%), δKO (1566 CPM, 84%), α1β2γ2 (366 CPM, 58%), α6β2γ2 (256 CPM, 54%) and α6β2δ (191 CPM, 47%). For [³H]GABA (5 nM) the binding values were: WT (516 CPM, 68%) and δKO (292 CPM, 55%).

The effect of AA29504 on the association and dissociation of [³H]muscimol binding was measured essentially as described by Benkherouf et al. [41]. Saturation analysis of [³H]muscimol was performed essentially as described by Uusi-Oukari and Korpi [42]. Triplicate samples of the membranes were incubated in assay buffer with a concentration series of [³H]muscimol (1–50 nM) at room temperature (22 ºC) for 60 min in the absence and presence of 10 μM AA29504. Non-specific binding was determined in the

presence of 100 μM GABA. The incubation was terminated by filtration and the radioactivity of the air-dried filters was measured using a scintillation spectrometer as described above.

Measurement of [³H]EBOB Binding

The displacement of 1 nM [³H]EBOB binding was measured in [³H]EBOB assay buffer (50 mM Tris–HCl, pH 7.4, 120 mM NaCl) at room temperature (22 °C) in a total volume of 400 μl in the absence and presence of various concentrations of GABA or THIP with and without 10 μM AA29504. Triplicate samples were incubated with shaking for 2 h. Non-specific binding was determined in the presence of 100 μM picrotoxin. The incubations were terminated as described above for [³H]muscimol binding. The average CPM and % specific [³H]EBOB (1 nM) binding to the membrane homogenates were as follows:WT (889 CPM, 77%), δKO (1020 CPM, 79%), α6β3γ2 (1055 CPM, 89%), α6β3δ (402 CPM, 66%), α6β3: (1200 CPM, 90%).

Protein Measurement

In all radioligand binding experiments, the average protein concentrations were 0.8 mg/ml for WT and 0.9 mg/ml for δKO forebrain membranes. These were determined with Bio-Rad Coomassie blue dye-based protein assay kit (Hercules, CA, USA) as per the manufacturer's protocol.

Data Analysis

GraphPad Prism software (GraphPad, San Diego, CA, USA) was used for nonlinear least squares curve-fitting and statistical testing of association, dissociation, saturation binding and radioligand displacement data. The association and dissociation curves were used for the estimation of association (K_{on}) and dissociation (K_{off}) rate constants. Saturation binding curves were used for the estimation of total number of high-affinity binding sites (B_{max}), and equilibrium dissociation constants (K_D). Radioligand displacement values were fitted to a sigmoidal dose–response (variable Hill Slope) curve for the estimation of the half-maximal inhibitory concentration (IC_{50}):

$$Y = Bottom + (Top - Bottom)/(1 + (IC50/X)^{HillSlope})$$

where Y is the percentage of control binding, Bottom = 0 when non-specific binding is subtracted from all binding values, Top is the maximum radioligand binding in the absence of test compound, and X is the test compound concentration. Statistical comparisons were made with One-way ANOVA, Two-way ANOVA or Brown-Forsythe and Welch ANOVA followed by the relevant (Tukey's or Dunnett's) post hoc

tests for multiple comparisons. All data were expressed as means ± SEM and p-values of less than 0.05 were considered significant. The study samples were not randomized and analysis was performed in a parallel unblinded mode.

Results

AA29504 Modulation of GABA- and THIP-Induced [³H]EBOB Displacement

[³H]EBOB binding assay was initially carried out to evaluate AA29504's allosteric modulatory activity on native GABA$_A$Rs expressed in WT and δKO forebrain membranes. A concentration series of GABA- and THIP-induced [³H]EBOB displacement was performed in the absence or presence of AA29504. As highlighted in Fig. 2, the inclusion of 10 μM AA29504 produced a leftward shift of [³H]EBOB displacement curves in both mouse lines. AA29504 decreased the IC_{50} of GABA-induced [³H]EBOB displacement from 15.9 ± 5.0 μM to 1.0 ± 0.2 μM in WT mice (p < 0.05) and from 8.4 ± 1.8 μM to 1.2 ± 0.5 μM in δKO mice (p < 0.05). Furthermore, AA29504 decreased the IC_{50} of THIP-induced [³H]EBOB displacement from 0.20 ± 0.11 mM to 15.8 ± 6.5 μM in WT mice (p < 0.01) and from 0.22 ± 0.05 mM to 12.1 ± 1.5 μM in δKO mice (p < 0.05, One-way ANOVA followed by Tukey's post hoc test). There were no significant differences between WT and δKO mouse binding values in the effects of GABA or THIP on forebrain GABA$_A$Rs in the absence or presence of AA29504 (p > 0.05, Two-way ANOVA followed by Tukey's post hoc test).

The Direct Actions of AA29504 on [³H]EBOB Binding to Recombinant GABA$_A$ Receptors

We assessed the potential of AA29504 to directly displace [³H]EBOB binding to recombinant GABA$_A$Rs expressed in HEK293 cells as an indicator for allosteric agonist activity. The receptor subunit combinations α6β3γ2, α6β3δ, and α6β3 were selected to further examine the influence of γ and δ subunits on [³H]EBOB binding displacement by AA29504. The results indicate that AA29504 was able to displace [³H]EBOB binding to all three receptor subtypes in a concentration-dependent manner (Fig. 3). This GABA-independent radioligand displacement was evident already at a nanomolar range for α6β3 (≥ 100 nM), α6β3δ (≥ 100 nM) and α6β3γ2 (≥ 300 nM) receptors where the calculated IC_{50} values for AA29504 were 0.4 ± 0.02 μM, 1.1 ± 0.2 μM and 11.1 ± 1.4 μM, respectively. Statistical analysis revealed a significant difference with regard to AA29504 potency on the tested recombinant receptors as it followed the rank
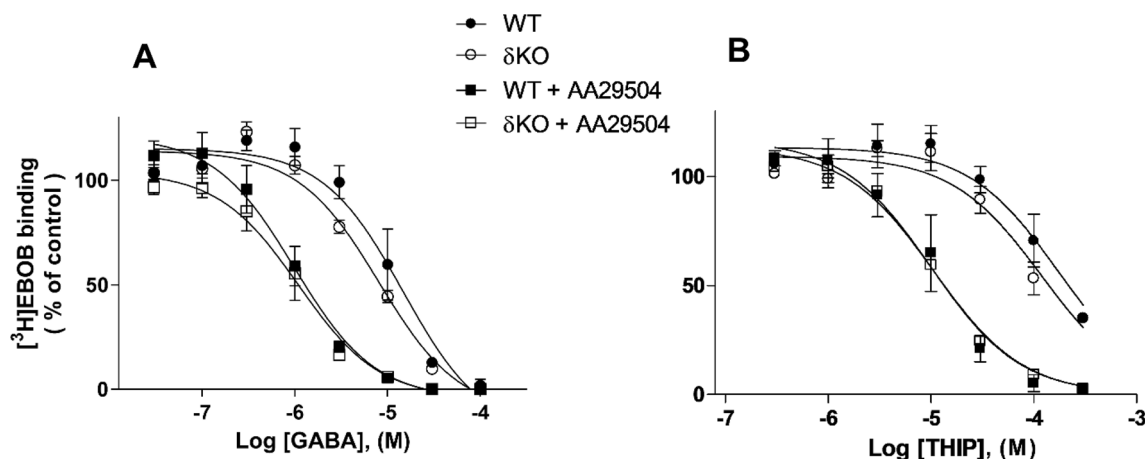
Fig. 2 AA29504 positive modulation of GABA- and THIP- induced 1 nM [³H]EBOB displacement in WT and δKO mice forebrain membranes. Displacement curves of [³H]EBOB binding as % of control with 7–8 concentrations of GABA (**A**) and THIP (**B**) in the absence or presence of 10 μM AA29504. The control is basal [³H]EBOB binding in the absence of GABA, THIP and AA29504. The radioligand displacement by AA29504 was significant in WT ($p < 0.05$) and δKO mice ($p < 0.05$), with no potency difference between the mouse lines ($p > 0.05$). All values represent the mean ± SEM, n = 3 independent experiments using triplicate membrane samples pooled individually from each mouse line's forebrain
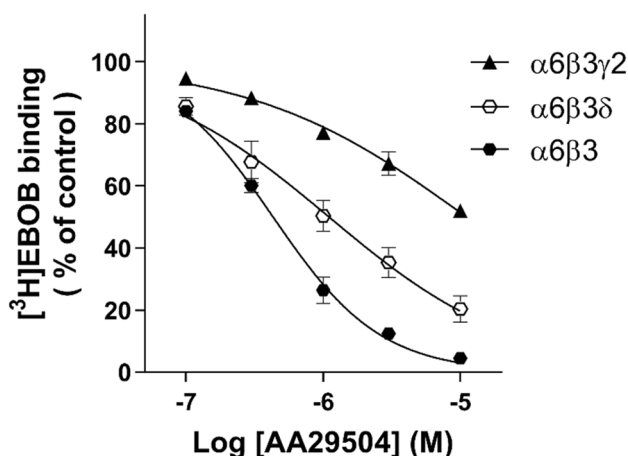
Fig. 3 GABA-independent displacement curves of 1 nM [³H] EBOB binding to recombinant α6β3, α6β3γ2 and α6β3δ GABA_ARs expressed in HEK293 cells with 5 concentrations of AA29504. The control is basal [³H]EBOB binding in the absence of AA29504. AA29504 displacement potency followed the rank order: α6β3 > α6β3δ > α6β3γ2 ($p < 0.05$). All values represent means ± SEM, n = 3–6 independent transfections and experiments performed in triplicate

order: α6β3 > α6β3δ > α6β3γ2 ($p < 0.05$, Brown-Forsythe and Welch ANOVA followed by Dunnett's post hoc test).

AA29504 Stimulation of [³H]muscimol and [³H]GABA Binding to Native GABA_A Receptors

We further examined the modulatory effects of AA29504 on the high-affinity agonist binding to native GABA_ARs expressed in WT and δKO mice. The binding of [³H]

muscimol and [³H]GABA was measured at room temperature (22 °C) with increasing concentrations of AA29504, where individually pooled forebrain membrane samples were incubated for 15 min. Figure 4 shows that AA29504 produced a concentration-dependent stimulation in [³H]muscimol and [³H]GABA binding to both WT and δKO mice forebrains. Two-way ANOVA followed by Tukey's post hoc analysis indicated that AA29504 was more potent in stimulating [³H]muscimol ($p < 0.01$) and [³H]GABA ($p < 0.001$) binding in δKO than in WT mice.

AA29504 Stimulation of [³H]muscimol Binding to Recombinant GABA_A Receptors

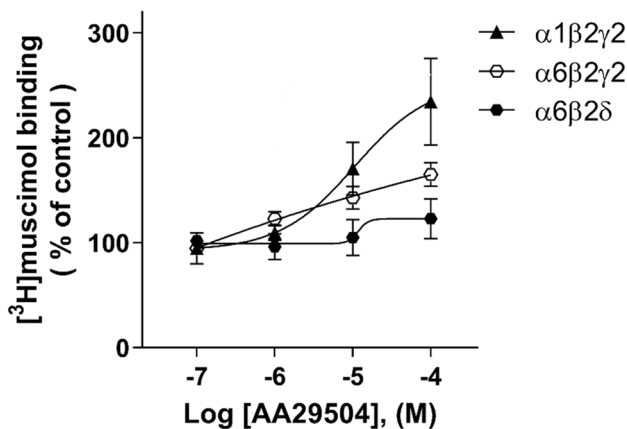Under the same conditions performed for native GABA_ARs expressed in WT and δKO mice forebrains, we compared the role of γ and δ subunits on AA29504 modulation of [³H]muscimol binding in recombinant α1β2γ2, α6β2γ2, and α6β2δ receptors expressed in HEK293 cells. As illustrated in Fig. 5, AA29504 stimulated [³H]muscimol binding to α1β2γ2 and α6β2γ2 receptor subtypes in a concentration-dependent manner ($p < 0.05$). In contrast, it had no significant effect on the binding to α6β2δ receptor subtype (Two-way ANOVA followed by Tukey's post hoc test).

The Effect of AA29504 on [³H]muscimol Binding Kinetics

We questioned whether AA29504 stimulation of [³H]muscimol binding is due to alterations in receptor-ligand binding kinetics as we assessed the influence of AA29504 on [³H]muscimol association and dissociation rates in

A

B
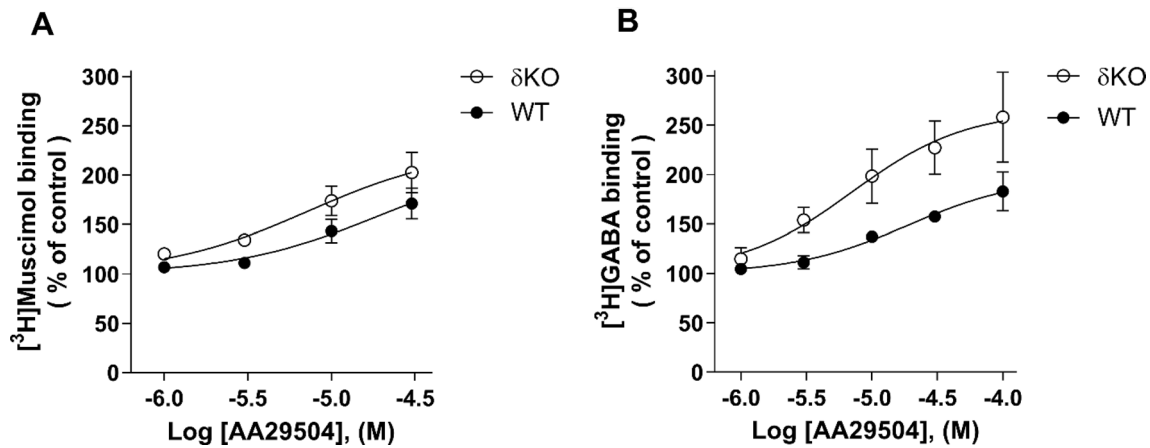
Fig. 4 The effects of AA29504 on 2 nM [³H]muscimol (**A**) and 5 nM [³H]GABA (**B**) binding to WT and δKO mice forebrain membranes. AA29504 was significantly more potent in stimulating [³H]muscimol ($p < 0.01$) and [³H]GABA ($p < 0.001$) binding in δKO than in WT mice. The values represent [³H]muscimol binding as % of control with 4 concentrations of AA29504, where the control is basal [³H] muscimol (**A**) or [³H]GABA (**B**) binding in the absence of AA29504 (mean ± SEM, n = 3 independent experiments using triplicate membrane samples pooled individually from each mouse line's forebrain)

Fig. 5 The effect of AA29504 on 2 nM [³H]muscimol binding to recombinant α1β2γ2, α6β2γ2 and α6β2δ GABA$_A$Rs expressed in HEK293 cells. AA29504 potentiation of [³H]muscimol binding was significant in α1β2γ2 and α6β2γ2 ($p < 0.05$), while absent in α6β2δ recombinant GABA$_A$Rs ($p > 0.05$)**.** The values represent [³H]muscimol binding as % of control with 4 concentrations of AA29504, where the control is basal [³H]muscimol binding in the absence of AA29504 (mean ± SEM, n = 3–4 independent transfections and experiments performed in triplicate)

WT and δKO forebrain membranes. The results indicate that [³H]muscimol association at 22 °C was faster in δKO compared to WT in the absence of AA29504, where the calculated association rate constants K_{on} were $5.9 \pm 0.6 \times 10^8\,M^{-1} \times min^{-1}$ and $2.2 \pm 0.3 \times 10^8\,M^{-1} \times min^{-1}$, respectively (mean ± SEM, n = 3) ($p < 0.05$, One-way ANOVA followed by Tukey's post hoc test). Co-incubation with AA29504 did not significantly affect [³H]muscimol K_{on} in either δKO ($4.8 \pm 0.9 \times 10^8\,M^{-1} \times min^{-1}$) or WT mice ($3.9 \pm 1.1 \times 10^8\,M^{-1} \times min^{-1}$) (mean ± SEM, n = 3),

but it notably increased the amount of specific radioligand binding in both mouse lines ($p < 0.001$). The increased binding was maximally $240 \pm 23\%$ of control binding without AA29504 in δKO mice, significantly higher than that in WT mice ($166 \pm 5\%$ of control) ($p < 0.001$, Two-way ANOVA followed by Tukey's post hoc test) (Fig. 6A).

Similar to the association, the dissociation rate constant K_{off} of [³H]muscimol binding at 22 °C was higher in δKO ($0.66 \pm 0.03\,min^{-1}$) than in WT ($0.38 \pm 0.02\,min^{-1}$) (mean ± SEM, n = 3), reflecting a faster radioligand dissociation in the former mouse line ($p < 0.05$, One-way ANOVA followed by Tukey's post hoc test). However, no evident effects were observed with AA29504 on [³H]muscimol.

K_{off} in δKO ($0.64 \pm 0.05\,min^{-1}$) and WT forebrains ($0.43 \pm 0.02\,min^{-1}$) (mean ± SEM, n = 3) ($p > 0.05$, One-way ANOVA followed by Tukey's post hoc test) (Fig. 6C).

Saturation Analysis of [³H]muscimol Binding

As a probable mechanism for AA29504-induced stimulation of [³H]muscimol binding to GABA$_A$ receptors, we hypothesized AA29504's potential to modulate agonist binding by shifting the binding affinity of specific receptor populations. Hence, we assessed the effects of AA29504 on the total number of high-affinity [³H]muscimol binding sites and binding affinity by analyzing the saturation kinetics of the high-affinity [³H]muscimol binding to WT and δKO mouse forebrain membranes at 22 °C (Fig. 7). Using increasing concentrations of [³H]muscimol (1–50 nM), in the absence (control) or presence of AA29504, all tested groups except control WT membranes were best fit to the one-site binding model. Binding to control WT membranes was best fit to the two-site binding model [$p = 0.0013$, F
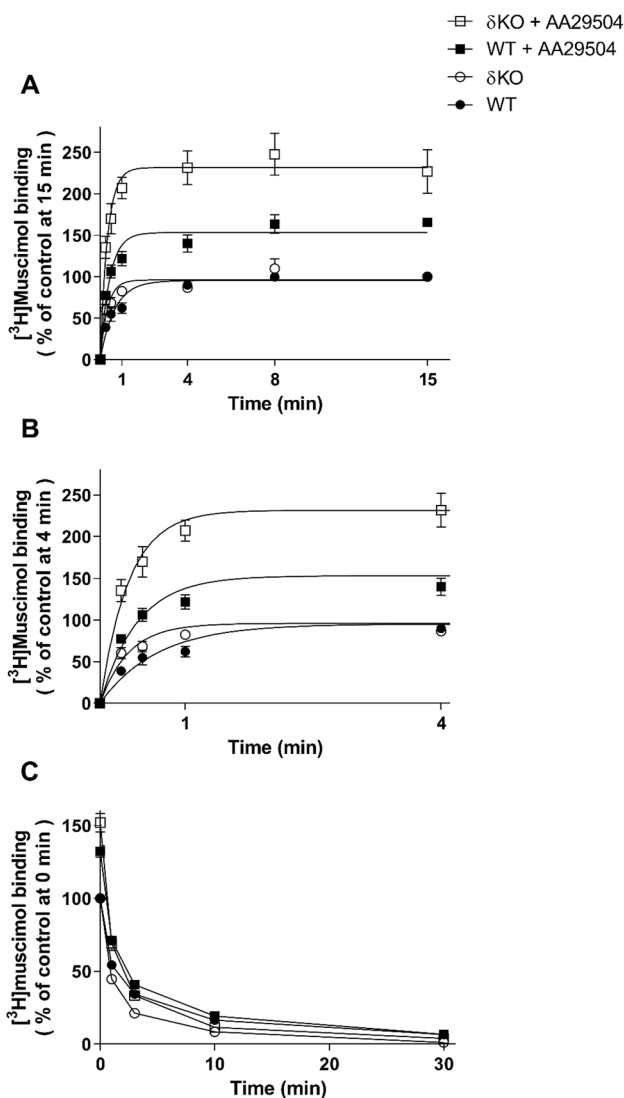
Fig. 6 The association (**A**) and dissociation (**C**) of 2 nM [³H]muscimol with WT and δKO mice forebrain membranes in the absence or presence of 10 μM AA29504. Insert of Fig. 6A association analysis where 0–4 min time range is highlighted (**B**). The co-incubation with AA29504 did not produce significant changes in the association (K_{on}) and dissociation (K_{off}) rate constants in either mouse line ($p > 0.05$). The values are expressed as % of control binding at 15 min for association and 0 min for dissociation, where the control is the maximal [³H]muscimol basal binding in the absence of AA29504 (mean ± SEM, n = 3 independent experiments using triplicate membrane samples pooled individually from each mouse line's forebrain)

(DFn, DFd) = 11.11 (2, 14)], displaying two binding affinities at distinguishable receptor densities (Table 1). The sum of high affinity Bmax values ($Bmax_{(1)} + Bmax_{(2)}$) in WT membranes, however, was equivalent to control δKO Bmax (no AA29504). The presence of 10 μM AA29504 in WT membranes rendered [³H]muscimol binding more favorable to one-site model as it displayed a single apparent affinity that was intermediate between the affinities obtained

in control WT membranes. On the other hand, AA29504 significantly decreased the equilibrium dissociation constant (K_D) reflecting an enhancement of [³H]muscimol binding affinity in δKOs ($p < 0.001$). Moreover, AA29504 increased [³H]muscimol Bmax in δKO ($p < 0.05$) as well as WT mouse lines ($p < 0.01$) (Two-way ANOVA followed by Tukey's post hoc test). The calculated [³H]muscimol Bmax and K_D values are summarized in Table 1 (Fig. 7).

Discussion

This paper probes into the complex action of the retigabine synthetic analogue, AA29504, on $GABA_A R$ binding properties and function. Using radioligand binding assays, we demonstrate the positive allosteric modulation of AA29504 on GABA- and THIP- induced responses in native $GABA_A Rs$ expressed in C57BL/6 J mouse forebrains. These modulatory activities are evident in WT and δKO mice with no differences in terms of potency. The results are not surprising as the ion-channel-site and allosteric modulation of the GABA and ion-channel coupling are relatively little changed in δKO mice [43]. The non-differential AA29504 potency between native $GABA_A Rs$ expressed in WT and δKO mice corresponds with the earlier observed modulation in αβ and αβγ recombinant expression systems [24, 27], leading to the conclusion that AA29504 is not particularly selective to αβδ $GABA_A Rs$. However, as demonstrated with [³H]EBOB binding to recombinant $GABA_A Rs$ in this study, and similar to the earlier findings with electrophysiological measurements [27], AA29504 agonist efficacy is higher in α6β3δ than in α6β3γ2 receptors as it was more efficient in displacing [³H]EBOB directly (in the absence of GABA) in the former subtype (Fig. 3). Receptor desensitization was found to be a key factor determining $GABA_A Rs$ response efficacy

Table 1 Values of equilibrium dissociation constants (K_D) and total amount of high-affinity binding sites (B_{max}), calculated from the saturation analysis of [³H]muscimol binding to WT and δKO mouse forebrain membranes at room temperature (22 °C)

Mouse line	AA29504 (10 μM)	K_D (nM)	B_{max} (pmol/mg protein)
WT	–	4.1 ± 3.6 (1), 40 ± 15.5 (2)	0.7 ± 0.7 (1), 5 ± 0.3 (2)
	+	23 ± 4	7.6 ± 0.9**
δKO	–	53 ± 6	6.1 ± 0.6
	+	30 ± 3***	8.1 ± 0.6*

K_D and B_{max} values represent means ± SEM, n = 3 independent experiments using triplicate membrane samples pooled individually from each mouse line's forebrain

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from the corresponding control values without AA29504 (Two-way ANOVA followed by Tukey's post hoc test)
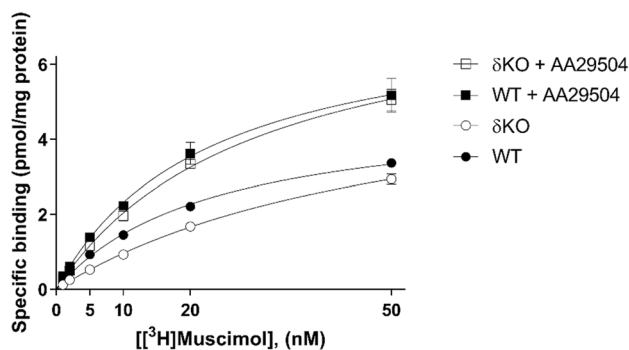
Fig. 7 Saturation analysis of [³H]muscimol (1–50 nM) binding to WT and δKO mice forebrain membranes in the absence or presence of 10 μM AA29504. The values are expressed as pmol/mg protein (mean ± SEM, n = 3 independent experiments using triplicate membrane samples pooled individually from each mouse line's forebrain). See Table 1. for detailed statistical comparisons and significance

[44–46]. The fact that δ-GABA$_A$Rs display a slow desensitization rate and high open-channel stability [8, 47, 48] may contribute to the higher efficacy of AA29504 in relation to γ2-GABA$_A$Rs. AA29504 agonist behavior, nevertheless, was not dependent on the presence of γ2 and δ subunits and even displaced [³H]EBOB with higher efficiency in α6β3 compared to α6β3δ and α6β3γ2 receptors. This further suggests the role of GABA$_A$R's transmembrane β + /α − interface in exerting AA29504 pharmacological activity [27, 49], as similarly found for neurosteroids [50, 51] and general anesthetics such as etomidate and propofol [52, 53]. Functional assessment at numerous mutant GABA$_A$Rs and on in silico analysis of its low-energy conformations indicated that AA29504 and etomidate exert their effects through the same site or overlapping binding sites between α-TM1 and β-TM3 transmembrane domains [27]. Propofol and potentiating neurosteroids also bind between the same domains but at distinct binding pockets [54]. This inter-transmembrane binding site is in the vicinity of the physical desensitization gate at the intracellular end of the GABA$_A$R channel [55–57] suggesting that the site could act as a target for modulating desensitization by AA29504, a mechanism of action already established for desensitization-modifying allosteric modulators (DAM) such as etomidate [58, 59], propofol [60, 61] and neurosteroids [62, 63].

Modulating GABA$_A$R desensitization involves alterations in ligand binding properties as receptor affinity depends on its channel physical state according to the following order: resting state < open state < desensitized state [64, 65]. Thus, we examined AA29504 modulation of agonist binding to GABA$_A$Rs where AA29504 increased the high-affinity [³H]muscimol and [³H]GABA binding to native GABA$_A$Rs expressed in WT and δKO mouse forebrains. Mammalian WT fore/midbrain contain up to 10% of δ-GABA$_A$Rs [66, 67] and the deletion of δ subunit in δKO mice leads to an

increase in αβγ2 receptor expression since δ subunit does not compete with γ2 in receptor assembly with α and β subunits [68]. Despite the well-established high-affinity muscimol and GABA binding to δ-GABA$_A$Rs [8, 10, 36, 41], the enhancements of GABA$_A$R agonists binding were higher in δKO than in WT mice. The effect of AA29504 on [³H] muscimol binding was in fact absent in α6β2δ, while evident in α1β2γ2 and α6β2γ2 recombinant GABA$_A$Rs (Fig. 5), suggesting the involvement of γ2-GABA$_A$Rs in this enhancement. In binding kinetics assays, [³H]muscimol association and dissociation rates are exceptionally low in αβδ receptors, reflecting the slow binding and unbinding kinetics of muscimol in WT compared to δKO forebrain and cerebellar membranes (Fig. 6; [41]). This behavior was not altered upon co-incubation with AA29504 as we did not observe any significant changes in the association (K_{on}) and dissociation (K_{off}) rate constants in either mouse line. Hence, the link between AA29504-induced stimulation of [³H]muscimol binding and alterations in receptor-ligand binding kinetics was not established.

In agreement with AA29504 stimulation of the agonist binding, [³H]muscimol saturation analysis revealed AA29504-induced GABA$_A$R shift to the high-affinity states. In WT mice, [³H]muscimol displayed two high-affinity receptor populations in the absence of AA29504: low-nanomolar ($K_D = 4.1 \pm 3.6$ nM) for δ-GABA$_A$Rs as earlier reported [41, 69], and intermediate-nanomolar ($K_D = 40 \pm 15.5$ nM) for non-δ-GABA$_A$Rs. The intermediate-nanomolar affinity in WT, comparable to δKO mice ($K_D = 53 \pm 6$), was found to represent < 10% of total non-δ-GABA$_A$Rs when occupied by 5 nM [³H]muscimol [41]. This non-δ-GABA$_A$R population was suggested to rise from desensitized γ2-GABA$_A$Rs [70], pre-frozen brain membranes at − 70 °C [71] and trace residual [GABA] from adequately washed brain membranes. Previous autoradiography and membrane homogenate binding assays showed that the deletion of δ subunit in δKO mice leads to a substantial loss of high-affinity [³H]muscimol binding, especially in the forebrain region [12, 41, 43, 72], whereas in recombinant GABA$_A$Rs, the replacement of γ2 subunit by δ in α6β2γ2 receptors abolished AA29504 enhancement in [³H]muscimol binding (Fig. 5). Therefore, the increase in [³H]muscimol Bmax in WT forebrain may be attributed to AA29504-induced alteration of non-δ-GABA$_A$Rs towards a higher affinity state. This was confirmed upon co-incubation of AA29504 with δKO forebrain membranes which displayed an increase in the receptor sites available for high-affinity [³H]muscimol binding. These additional sites that were undetectable in the absence of AA29504 appeared as a result of an enhancement in [³H]muscimol binding affinity. It was reported earlier that δKO dentate gyrus granule cell macrocurrents exhibit considerably higher channel desensitization compared to WT [73]. Hence, a plausible

explanation for this subtype-dependent [^{3}H]muscimol binding is that αβδ receptors already exhibit high affinity to [^{3}H]muscimol and undergo minor desensitization [41, 62, 74, 75] that is unaltered by AA29504. On the other hand, a part of low-affinity αβγ2 receptors with micromolar K_D desensitize upon AA29504 exposure and shift to a high-affinity state [70, 76] resulting in increased [^{3}H]muscimol binding that can be measured in the nanomolar range (Fig. 4; [70]). These high-affinity [^{3}H]muscimol-bound receptors display a desensitized non-functional state that is impermeable to chloride influx [69, 77]. However, this state is not permanent as ligand-bound desensitized receptors may re-sensitize and shift to a functional open state [78, 79]. This re-sensitization was found to increase the probability and mean time of GABA$_A$R's open state, which contributes to the prolongation of the inhibitory postsynaptic currents (IPSCs) [3, 80, 81]. Recent evidence has shown that desensitization promotes GABA$_A$R phosphorylation by protein kinase C (PKC) leading to the rise of a new receptor population that induces long-term potentiation at the inhibitory synapses [82]. The phosphorylation by PKC was also reported to decrease GABA$_A$R sensitivity to ethanol and benzodiazepines [83]. Hence, the influence of AA29504 on phosphorylation as a consequence of receptor desensitization needs to be examined for its potential role in regulating the allosteric modulatory effects on GABA$_A$Rs.

Conclusion

This study sheds light on AA29504's modulatory activity, its direct actions and interactions with agonists in GABA$_A$R complex. Using [^{3}H]EBOB radioligand as a unique probe for assessing drug enhancement of GABA$_A$R function, we demonstrated for the first time the non-differential AA29504 modulatory potency on native GABA$_A$Rs expressed in WT and δKO C57BL/6J mice. We further displayed AA29504's GABA-independent activity on recombinant GABA$_A$Rs expressed in HEK293 cells, indicating higher selective agonist efficacy on δ-GABA$_A$Rs in relation to γ$_2$-GABA$_A$Rs. Interestingly, AA29504 showed a concentration-dependent stimulation of GABA$_A$ agonist binding to γ2 GABA$_A$Rs but not to δ-GABA$_A$Rs. This newly revealed selective modulation by AA29504 is attributed to its ability to shift the low-affinity γ$_2$-GABA$_A$Rs towards a higher affinity desensitized state, thereby rising new sites capable of binding GABA$_A$R agonists with low nanomolar affinity. Hence, the potential of AA29504 to act as a desensitization-modifying allosteric modulator (DAM) of γ2-GABA$_A$Rs deserves further investigation for its promising influence on shaping efficacy, duration and plasticity of GABA$_A$R synaptic responses [46, 62, 81, 82, 84, 85].

References

1. Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric acidA receptors: classification on the basis of subunit composition, pharmacology, and function. Update Pharmacol Rev 60:243–260. https://doi.org/10.1124/pr.108.00505

2. Sigel E, Steinmann ME (2012) Structure, function, and modulation of GABAA receptors. J Biol Chem 287:40224–40231. https://doi.org/10.1074/jbc.R112.386664

3. Macdonald RL, Olsen RW (1994) GABA(A) receptor channels. Annu Rev Neurosci 17:569–602. https://doi.org/10.1146/annurev.ne.17.030194.003033

4. Sieghart W (1995) Structure and pharmacology of γ-aminobutyric acid(A) receptor subtypes. Pharmacol Rev 47:181–234.

5. Feng HJ, Macdonald RL (2010) Barbiturates require the N terminus and first transmembrane domain of the δ subunit for enhancement of α1β3δGABAa receptor currents. J Biol Chem 285:23614–23621. https://doi.org/10.1074/jbc.M110.122564

6. McKernan RM, Whiting PJ (1996) Which GABAA-receptor subtypes really occur in the brain? Trends Neurosci 19:139–143. https://doi.org/10.1016/S0166-2236(96)80023-3

7. Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant GABA(A) receptor subtype. J Neurosci 17:2728–2737. https://doi.org/10.1523/jneurosci.17-08-02728.1997

8. Nusser Z, Sieghart W, Somogyi P (1998) Segregation of different GABA(A) receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 18:1693–1703. https://doi.org/10.1523/jneurosci.18-05-01693.1998

9. Brickley SG, Cull-Candy SG, Farrant M (1999) Single-channel properties of synaptic and extrasynaptic GABA(A) receptors suggest differential targeting of receptor subtypes. J Neurosci 19:2960–2973. https://doi.org/10.1523/jneurosci.19-08-02960.1999

10. Nusser Z, Mody I (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. J Neurophysiol 87:2624–2628. https://doi.org/10.1152/jn.2002.87.5.2624

11. Semyanov A, Walker MC, Kullmann DM, Silver RA (2004) Tonically active GABAA receptors: modulating gain and maintaining

the tone. Trends Neurosci 27:262–269. https://doi.org/10.1016/j.tins.2004.03.005

12. Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW, Homanics GE (1999) Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. Proc Natl Acad Sci USA 96:12905–12910. https://doi.org/10.1073/pnas.96.22.12905

13. Lewis RW, Mabry J, Polisar JG, Eagen KP, Ganem B, Hess GP (2010) Dihydropyrimidinone positive modulation of δ-subunit-containing γ-aminobutyric acid type a receptors, including an epilepsy-linked mutant variant. Biochemistry 49:4841–4851. https://doi.org/10.1021/bi100119t

14. Marx CE, Stevens RD, Shampine LJ, Uzunova V, Trost WT, Butterfield MI, Massing MW, Hamer RM, Morrow AL, Lieberman JA (2006) Neuroactive steroids are altered in schizophrenia and bipolar disorder: relevance to pathophysiology and therapeutics. Neuropsychopharmacology 31:1249–1263. https://doi.org/10.1038/sj.npp.1300952

15. Clarkson AN, Huang BS, MacIsaac SE, Mody I, Carmichael ST (2010) Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. Nature 468:305–309. https://doi.org/10.1038/nature09511

16. Handforth A, Kadam PA, Kosoyan HP, Eslami P (2018) Suppression of harmaline tremor by activation of an extrasynaptic gabaa receptor: implications for essential tremor. Tremor Hyperkinet Mov 8:546. https://doi.org/10.7916/D8JW9X9K

17. Maguire JL, Stell BM, Rafizadeh M, Mody I (2005) Ovarian cycle-linked changes in GABAA receptors mediating tonic inhibition alter seizure susceptibility and anxiety. Nat Neurosci 8:797–804. https://doi.org/10.1038/nn1469

18. Wiltgen BJ, Sanders MJ, Ferguson C, Homanics GE, Fanselow MS (2005) Trace fear conditioning is enhanced in mice lacking the δ subunit of the GABAA receptor. Learn Mem 12:327–333. https://doi.org/10.1101/lm.89705

19. Bowen MT, Peters ST, Absalom N, Chebib M, Neumann ID, McGregor IS (2015) Oxytocin prevents ethanol actions at δ subunit-containing GABAA receptors and attenuates ethanol-induced motor impairment in rats. Proc Natl Acad Sci USA 112:3104–3109. https://doi.org/10.1073/pnas.1416900112

20. Chen J, He Y, Wu Y, Zhou H, Su LD, Li WN, Olsen RW, Liang J, Zhou YD, Shen Y (2018) Single ethanol withdrawal regulates extrasynaptic δ-GABAA receptors via PKCδ activation. Front Mol Neurosci 11:141. https://doi.org/10.3389/fnmol.2018.00141

21. Wafford KA, van Niel MB, Ma QP, Horridge E, Herd MB, Peden DR, Belelli D, Lambert JJ (2009) Novel compounds selectively enhance δ subunit containing GABAA receptors and increase tonic currents in thalamus. Neuropharmacology 56:182–189. https://doi.org/10.1016/j.neuropharm.2008.08.004

22. Jensen ML, Wafford KA, Brown AR, Belelli D, Lambert JJ, Mirza NR (2013) A study of subunit selectivity, mechanism and site of action of the delta selective compound 2 (DS2) at human recombinant and rodent native GABA A receptors. Br J Pharmacol 168:1118–1132. https://doi.org/10.1111/bph.12001

23. Neumann S, Boothman-Burrell L, Gowing EK, Jacobsen TA, Ahring PK, Young SL, Sandager-Nielsen K, Clarkson AN (2019) The delta-subunit selective GABAA receptor modulator, DS2, improves stroke recovery via an anti-inflammatory mechanism. Front Neurosci 13:1133. https://doi.org/10.3389/fnins.2019.01133

24. Hoestgaard-Jensen K, Dalby NO, Wolinsky TD, Murphey C, Jones KA, Rottländer M, Frederiksen K, Watson WP, Jensen K, Ebert B (2010) Pharmacological characterization of a novel positive modulator at α4β3δ-containing extrasynaptic GABAA receptors. Neuropharmacology 58:702–711. https://doi.org/10.1016/j.neuropharm.2009.12.023

25. Vardya I, Hoestgaard-Jensen K, Nieto-Gonzalez JL, Dósa Z, Boddum K, Holm MM, Wolinsky TD, Jones KA, Dalby NO, Ebert B, Jensen K (2012) Positive modulation of δ-subunit containing GABA A receptors in mouse neurons. Neuropharmacology 63:469–479. https://doi.org/10.1016/j.neuropharm.2012.04.023

26. Falk-Petersen CB, Søgaard R, Madsen KL, Klein AB, Frølund B, Wellendorph P (2017) Development of a robust mammalian cell-based assay for studying recombinant α4β1/3δ GABAA receptor subtypes. Basic Clin Pharmacol Toxicol 121:119–129. https://doi.org/10.1111/bcpt.12778

27. Olander ER, Madjroh N, Bunch L, Söderhielm PC, Jensen AA (2018) Delineation of the functional properties and the mechanism of action of AA29504, an allosteric agonist and positive allosteric modulator of GABAA receptors. Biochem Pharmacol 150:305–319. https://doi.org/10.1016/j.bcp.2018.02.015

28. Damgaard T, Plath N, Neill JC, Hansen SL (2011) Extrasynaptic GABAA receptor activation reverses recognition memory deficits in an animal model of schizophrenia. Psychopharmacology 214:403–413. https://doi.org/10.1007/s00213-010-2039-9

29. Hulme EC, Trevethick MA (2010) Ligand binding assays at equilibrium: validation and interpretation. Br J Pharmacol 161:1219–1237. https://doi.org/10.1111/j.1476-5381.2009.00604.x

30. Dong C, Liu Z, Wang F (2015) Radioligand saturation binding for quantitative analysis of ligand-receptor interactions. Biophys Rep 1:148–155. https://doi.org/10.1007/s41048-016-0016-5

31. Van Rijn CM, Willems-van Bree E (2003) Synergy between retigabine and GABA in modulating the convulsant site of the GABAA receptor complex. Eur J Pharmacol 464:95–100. https://doi.org/10.1016/S0014-2999(03)01426-2

32. Cole LM, Casida JE (1992) GABA-gated chloride channel: binding site for 4′-ethynyl-4-n-[2,3–3H2]propylbicycloorthobenzoate ([3H]EBOB) in vertebrate brain and insect head. Pestic Biochem Physiol 44:1–8. https://doi.org/10.1016/0048-3575(92)90002-H

33. Huang J, Casida JE (1996) Characterization of [3H]ethynylbicycloorthobenzoate ([3H]EBOB) binding and the action of insecticides on the γ-aminobutyric acid-gated chloride channel in cultured cerebellar granule neurons. J Pharmacol Exp Ther 279:1191–1196.

34. Krogsgaard-Larsen P, Hjeds H, Curtis DR, Lodge D, Johnston GAR (1979) Dihydromuscimol, thiomuscimol and related heterocyclic compounds as GABA analogues. J Neurochem 32:1717–1724. https://doi.org/10.1111/j.1471-4159.1979.tb02284.x

35. DeFeudis FV, Ossola L, Schmitt G, Mandel P (1980) Substrate specificity of [3H] muscimol binding to a particulate fraction of a neuron-enriched culture of embryonic rat brain. J Neurochem 34:845–849. https://doi.org/10.1111/j.1471-4159.1980.tb09656.x

36. Quirk K, Whiting PJ, Ian Ragan C, McKernan RM (1995) Characterisation of δ-subunit containing GABAA receptors from rat brain. Eur J Pharmacol Mol Pharmacol 290:175–181. https://doi.org/10.1016/0922-4106(95)00061-5

37. Squires RF, Saederup E (2000) Additivities of compounds that increase the numbers of high affinity [3H]muscimol binding sites by different amounts define more than 9 GABAA receptor complexes in rat forebrain: Implications for sichizophrenia and clozapine research. Neurochem Res 25:1587–1601. https://doi.org/10.1023/A:1026666419725

38. Uusi-Oukari M, Vähätalo L, Liljeblad A (2014) Modifications of diflunisal and meclofenamate carboxyl groups affect their allosteric effects on GABAA receptor ligand binding. Neurochem Res 39:1183–1191. https://doi.org/10.1007/s11064-014-1351-x

39. Uusi-Oukari M, Kleinz R, Mäkelä R, Lüddens H, Korpi ER (2000) Quantification of GABA(A) receptor subunit mRNAs by non-radioisotopic competitive RT-PCR utilizing plate-based EIA methodology. J Neurosci Methods 95:65–73. https://doi.org/10.1016/S0165-0270(99)00158-2

40. Lüddens H, Korpi ER (1997) Methods for transient expression of hetero-oligomeric ligand-gated ion channels. Methods Mol Biol Clifton NJ 83:55–63. https://doi.org/10.1385/0-89603-495-x:55

41. Benkherouf AY, Taina KR, Meera P, Aalto AJ, Li XG, Soini SL, Wallner M, Uusi-Oukari M (2019) Extrasynaptic δ-GABA A receptors are high-affinity muscimol receptors. J Neurochem 149:41–53. https://doi.org/10.1111/jnc.14646

42. Uusi-Oukari M, Korpi ER (1989) Cerebellar GABAA receptor binding and function in vitro in two rat lines developed for high and low alcohol sensitivity. Neurochem Res 14:733–739. https://doi.org/10.1007/BF00964950

43. Korpi ER, Mihalek RM, Sinkkonen ST, Hauer B, Hevers W, Homanics GE, Sieghart W, Lüddens H (2002) Altered receptor subtypes in the forebrain of GABAA receptor δ subunit-deficient mice: recruitment of γ2 subunits. Neuroscience 109:733–743. https://doi.org/10.1016/S0306-4522(01)00527-9

44. Frosch MP, Lipton SA, Dichter MA (1992) Desensitization of GABA-activated currents and channels in cultured cortical neurons. J Neurosci 12:3042–3053. https://doi.org/10.1523/jneurosci.12-08-03042.1992

45. Oh DJ, Dichter MA (1992) Desensitization of GABA-induced currents in cultured rat hippocampal neurons. Neuroscience 49:571–576. https://doi.org/10.1016/0306-4522(92)90227-S

46. Bianchi MT, Macdonald RL (2002) Slow phases of GABAA receptor desensitization: structural determinants and possible relevance for synaptic function. J Physiol 544:3–18. https://doi.org/10.1113/jphysiol.2002.020255

47. Mortensen M, Ebert B, Wafford K, Smart TG (2010) Distinct activities of GABA agonists at synaptic- and extrasynaptic-type GABAA receptors. J Physiol 588:1251–1268. https://doi.org/10.1113/jphysiol.2009.182444

48. Ahring PK, Bang LH, Jensen ML, Strøbæk D, Hartiadi LY, Chebib M, Absalom N (2016) A pharmacological assessment of agonists and modulators at α4β2γ2 and α4β2δ GABAA receptors: the challenge in comparing apples with oranges. Pharmacol Res 111:563–576. https://doi.org/10.1016/j.phrs.2016.05.014

49. McGrath M, Tolia M, Raines DE (2020) The effects of a competitive antagonist on GABA-evoked currents in the presence of sedative-hypnotic agents. Pharmacol Rep 72:260–266. https://doi.org/10.1007/s43440-019-00031-y

50. Hosie AM, Wilkins ME, Da Silva HMA, Smart TG (2006) Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. Nature 444:486–489. https://doi.org/10.1038/nature05324

51. Miller PS, Scott S, Masiulis S, De Colibus L, Pardon E, Steyaert J, Aricescu AR (2017) Structural basis for GABA A receptor potentiation by neurosteroids. Nat Struct Mol Biol 24:986–992. https://doi.org/10.1038/nsmb.3484

52. Siegwart R, Jurd R, Rudolph U (2002) Molecular determinants for the action of general anesthetics at recombinant α2β3γ2 γ-aminobutyric acidA receptors. J Neurochem 80:140–148. https://doi.org/10.1046/j.0022-3042.2001.00682.x

53. Maldifassi MC, Baur R, Sigel E (2016) Functional sites involved in modulation of the GABAA receptor channel by the intravenous anesthetics propofol, etomidate and pentobarbital. Neuropharmacology 105:207–214. https://doi.org/10.1016/j.neuropharm.2016.01.003

54. Jayakar SS, Zhou X, Chiara DC, Jarava-Barrera C, Savechenkov PY, Bruzik KS, Tortosa M, Miller KW, Cohen JB (2019) Identifying drugs that bind selectively to intersubunit general anesthetic sites in the α1β3γ2 GABAAR transmembrane domain. Mol Pharmacol 95:615–628. https://doi.org/10.1124/mol.118.114975

55. Gielen M, Thomas P, Smart TG (2015) The desensitization gate of inhibitory Cys-loop receptors. Nat Commun 6:6829. https://doi.org/10.1038/ncomms7829

56. Gielen M, Corringer PJ (2018) The dual-gate model for pentameric ligand-gated ion channels activation and desensitization. J Physiol 596:1873–1902. https://doi.org/10.1113/JP275100

57. Masiulis S, Desai R, Uchański T, Serna Martin I, Laverty D, Karia D, Malinauskas T, Zivanov J, Pardon E, Kotecha A, Steyaert J, Miller KW, Aricescu AR (2019) GABAA receptor signalling mechanisms revealed by structural pharmacology. Nature 565:454–459. https://doi.org/10.1038/s41586-018-0832-5

58. Liu K, Jounaidi Y, Forman SA, Feng HJ (2015) Etomidate uniquely modulates the desensitization of recombinant α1β3δ GABAA receptors. Neuroscience 300:307–313. https://doi.org/10.1016/j.neuroscience.2015.05.051

59. Liao Y, Liu X, Jounaidi Y, Forman SA, Feng HJ (2019) Etomidate effects on desensitization and deactivation of α4β3δ GABA A receptors inducibly expressed in HEK293 TetR cells. J Pharmacol Exp Ther 368:100–105. https://doi.org/10.1124/jpet.118.252403

60. Orser BA, Wang LY, Pennefather PS, MacDonald JF (1994) Propofol modulates activation and desensitization of GABA(A) receptors in cultured murine hippocampal neurons. J Neurosci 14:7747–7760. https://doi.org/10.1523/jneurosci.14-12-07747.1994

61. Baker PM, Pennefather PS, Orser BA, Skinner FK (2002) Disruption of coherent oscillations in inhibitory networks with anesthetics: Role of GABAA receptor desensitization. J Neurophysiol 88:2821–2833. https://doi.org/10.1152/jn.00052.2002

62. Wohlfarth KM, Bianchi MT, Macdonald RL (2002) Enhanced neurosteroid potentiation of ternary GABAA receptors containing the δ subunit. J Neurosci 22:1541–1549. https://doi.org/10.1523/jneurosci.22-05-01541.2002

63. Sugasawa Y, Cheng WWL, Bracamontes JR, Chen ZW, Wang L, Germann AL, Pierce SR, Senneff TC, Krishnan K, Reichert DE, Covey DF, Akk G, Evers AS (2020) Site-specific effects of neurosteroids on gabaa receptor activation and desensitization. eLife 9:1–32. https://doi.org/10.7554/ELIFE.55331

64. Colquhoun D (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. Br J Pharmacol 125:923–947. https://doi.org/10.1038/sj.bjp.0702164

65. Xu XJ, Roberts D, Zhu GN, Chang YC (2016) Competitive antagonists facilitate the recovery from desensitization of α1β2γ2 GABA A receptors expressed in Xenopus oocytes. Acta Pharmacol Sin 37:1020–1030. https://doi.org/10.1038/aps.2016.50

66. Whiting PJ (2003) The GABAA receptor gene family: new opportunities for drug development. Curr Opin Drug Discov Devel 6:648–657.

67. Hörtnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G (2013) Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. Neuroscience 236:345–372. https://doi.org/10.1016/j.neuroscience.2013.01.008

68. Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, Houser CR (2002) GABAA receptor changes in δ subunit-deficient mice: altered expression of α4 and γ2 subunits in the forebrain. J Comp Neurol 446:179–197. https://doi.org/10.1002/cne.10210

69. Chandra D, Halonen LM, Linden AM, Procaccini C, Hellsten K, Homanics GE, Korpi ER (2010) Prototypic GABAA receptor agonist muscimol acts preferentially through forebrain high-affinity binding sites. Neuropsychopharmacology 35:999–1007. https://doi.org/10.1038/npp.2009.203

70. Chang YC, Ghansah E, Chen Y, Ye J, Weiss DS (2002) Desensitization mechanism of GABA receptors revealed by single oocyte binding and receptor function. J Neurosci 22:7982–7990. https://doi.org/10.1523/jneurosci.22-18-07982.2002

71. Yang JS, Olsen RW (1987) gamma-Aminobutyric acid receptor binding in fresh mouse brain membranes at 22 degrees C: ligand-induced changes in affinity. Mol Pharmacol 32:266–277.

72. Olsen RW, Snowman AM (1982) Chloride-dependent enhancement by barbiturates of γ-aminobutyric acid receptor binding. J Neurosci 2:1812–1823. https://doi.org/10.1523/jneurosci.02-12-01812.1982

73. Carver CM, Reddy DS (2016) Neurosteroid structure-activity relationships for functional activation of extrasynaptic δGABAA receptors. J Pharmacol Exp Ther 357:188–204. https://doi.org/10.1124/jpet.115.229302

74. Saxena NC, Macdonald RL (1994) Assembly of GABA(A) receptor subunits: role of the δ subunit. J Neurosci 14:7077–7086. https://doi.org/10.1523/jneurosci.14-11-07077.1994

75. Haas KF, Macdonald RL (1999) GABA(A) receptor subunit γ2 and δ subtypes confer unique kinetic properties on recombinant GABA(A) receptor currents in mouse fibroblasts. J Physiol 514:27–45. https://doi.org/10.1111/j.1469-7793.1999.027af.x

76. Glen Newell J, Dunn SMJ (2002) Functional consequences of the loss of high affinity agonist binding to γ-aminobutyric acid type A receptors. Implications for receptor desensitization. J Biol Chem 277:21423–21430. https://doi.org/10.1074/jbc.M110312200

77. Agey MW, Dunn SMJ (1989) Kinetics of [3H]muscimol binding to the GABAA receptor in bovine brain membranes. Biochemistry 28:4200–4208. https://doi.org/10.1021/bi00436a012

78. Jones MV, Westbrook GL (1995) Desensitized states prolong GABAA channel responses to brief agonist pulses. Neuron 15:181–191. https://doi.org/10.1016/0896-6273(95)90075-6

79. Kang Y, Saito M, Toyoda H (2020) Molecular and regulatory mechanisms of desensitization and resensitization of GABAa receptors with a special reference to propofol/barbiturate. Int J Mol Sci 21:563–563. https://doi.org/10.3390/ijms21020563

80. Zhang ZX, Lü H, Dong XP, Liu J, Xu TL (2002) Kinetics of etomidate actions on GABAA receptors in the rat spinal dorsal horn neurons. Brain Res 953:93–100. https://doi.org/10.1016/S0006-8993(02)03274-2

81. Jones MV, Westbrook GL (1996) The impact of receptor desensitization on fast synaptic transmission. Trends Neurosci 19:96–101. https://doi.org/10.1016/S0166-2236(96)80037-3

82. Field M, Dorovykh V, Thomas P, Smart TG (2021) Physiological role for GABAA receptor desensitization in the induction of long-term potentiation at inhibitory synapses. Nat Commun 12:2112. https://doi.org/10.1038/s41467-021-22420-9

83. Qi ZH, Song M, Wallace MJ, Wang D, Newton PM, McMahon T, Chou WH, Zhang C, Shokat KM, Messing RO (2007) Protein kinase Cε regulates γ-aminobutyrate type A receptor sensitivity to ethanol and benzodiazepines through phosphorylation of γ2 subunits. J Biol Chem 282:33052–33063. https://doi.org/10.1074/jbc.M707233200

84. Zhu WJ, Vicini S (1997) Neurosteroid prolongs GABA(A) channel deactivation by altering kinetics of desensitized states. J Neurosci 17:4022–4031. https://doi.org/10.1523/jneurosci.17-11-04022.1997

85. Gielen M, Barilone N, Corringer PJ (2020) The desensitization pathway of GABAA receptors, one subunit at a time. Nat Commun 11:5369. https://doi.org/10.1038/s41467-020-19218-6