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ORIGINAL PAPER

Unexpected Properties of δ-Containing $GABA_A$ Receptors in Response to Ligands Interacting with the α+ β− Site

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Abstract $GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the central nervous system and are the targets of many clinically important drugs, which modulate GABA induced chloride flux by interacting with separate and distinct allosteric binding sites. Recently, we described an allosteric modulation occurring upon binding of pyrazoloquinolinones to a novel binding site at the extracellular α+ β− interface. Here, we investigated the effect of 4-(8-methoxy-3-oxo-3,5-dihydro-2H-pyrazolo[4,3-c]quinolin-2-yl)benzonitrile (the pyrazoloquinolinone LAU 177) at several αβ, αβγ and αβδ receptor subtypes. LAU 177 enhanced GABA-induced currents at all receptors investigated, and the extent of modulation depended on the type of α and β subunits present within the receptors. Whereas the presence of a γ2 subunit within αβγ2 receptors did not dramatically change LAU 177 induced modulation of GABA currents compared to αβ receptors, we observed an unexpected threefold increase in modulatory efficacy of this compound at α1β2,3δ receptors. Steric hindrance experiments as well as inhibition by the functional α+ β− site antagonist LAU 157 indicated that the effects of LAU 177 at all receptors investigated were mediated via the α+ β− interface. The stronger enhancement of GABA-induced currents by LAU 177 at α1β3δ receptors was not observed at α4,6β3δ receptors. Other experiments indicated that this enhancement of modulatory efficacy at α1β3δ receptors was not observed with another α+ β− modulator, and that the efficacy of modulation by α+ β− ligands is influenced by all subunits present in the receptor complex and by structural details of the respective ligand.

Keywords $GABA_A$ · CGS 9895 · Pyrazoloquinolinones · α+ β− Binding site · Positive modulators · Null modulators · δ Subunit · Extrasynaptic receptor

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Introduction

$GABA_A$ receptors are ligand-gated chloride channels composed of five subunits. The existence of 6α, 3β, 3γ, δ, ε, π, θ, and 3ρ subunits gives rise to a multiplicity of $GABA_A$ receptor subtypes with distinct subunit composition and pharmacological and electrophysiological properties. Most of these receptors are composed however of one γ, two α and two β subunits. The δ, ε, and π subunits have been thought to replace the γ subunit in αβγ receptor subtypes [1]. However, the subunit stoichiometry and arrangement of receptors containing the δ or ε subunit have recently been questioned by demonstrating that several

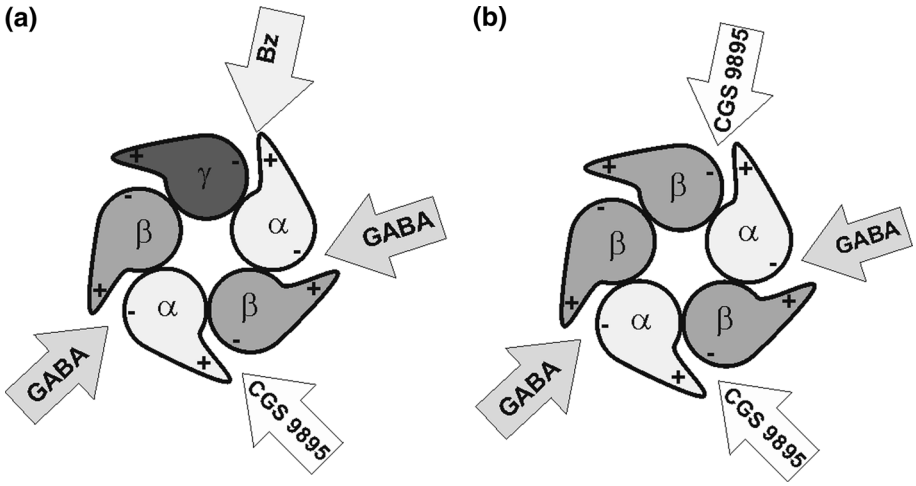

Fig. 1 Top view onto the extracellular domain of $GABA_A$ receptors composed of αβγ or αβ subunits. Each subunit has a plus (+) and a minus (−) side assigned. Binding sites for GABA are located at the interfaces formed by a "−" side of an alpha subunit and a "+" side of a beta subunit. **a** αβγ receptors composed of 2α, 2β and one γ subunit. The binding site for benzodiazepine site ligands (Bz) is located at the interface formed by the "+" side of an α and the "−" side of the γ subunit. The CGS 9895 binding site is located at the interface formed by the "+" side of an α and the "−" side of a β subunit. **b** αβ receptors formed of 2 α and 3 β subunits exhibit two CGS 9895 binding sites

recombinant concatenated δ- or ε-containing receptors with different subunit arrangements can be formed [2–6]. The ρ subunits can either form homo-oligomers or hetero-oligomers with other ρ subunits [1].

$GABA_A$ receptors are the site of action of a variety of clinically important drugs, such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics, convulsants, and others [7]. All these drugs seem to allosterically interact with binding sites that are partially or completely distinct from each other. Modelling studies have identified a total of at least 16 solvent accessible spaces within $GABA_A$ receptors that also could represent drug binding sites [8]. Thus, within the extracellular domain of $GABA_A$ receptors composed of 2α, 2β, and 1γ subunit, at least four binding sites seem to exist, each one located at a subunit interface. Each subunit per definition contains a plus (+) and a minus (−) side and the (+) side of one subunit forms an interface with the (−) side of the neighbouring subunit [9]. The two gamma-aminobutyric acid (GABA) binding sites are located at the two β+ α− interfaces [10], the benzodiazepine binding site is located at the α+ γ− interface [11] and a recently identified binding site for pyrazoloquinolinones such as CGS 9895 has been located at the α+ β− interface (Fig. 1a) [12]. It can be assumed that the remaining γ+ β− interface might also be the site of action of some drugs, but so far, direct evidence for this assumption is lacking. In addition to these extracellular binding sites at the subunit interfaces, another type of binding site has been identified within individual subunits of the extracellular domain of the GABA activated bacterial Erwinia chrysanthemi ligand-gated ion channel (ELIC) [13]. In the trans membrane (TM) domain, mutagenesis and photo labelling studies have suggested binding sites for volatile anesthetics, intravenous anesthetics, steroids, barbiturates, and ethanol [14–17]. These binding sites were partially assigned to subunit interfaces, or to the space inside the four helix bundle of each subunit of the $GABA_A$ receptor [18–22]. The actual existence of binding sites at these positions was demonstrated by crystallization studies using homologous proteins [23, 24]. Recently, a novel binding site for the endocannabinoid 2-arachidonyl glycerol was reported and localized between the TM3 and TM4 helices of the β2 subunit [25].

The α+ side not only contributes to the α+ β− interface but also to the α+ γ− interface. It thus was no surprise that the pyrazoloquinolinone CGS 9895 is able to interact with both interfaces. This compound acts as a high affinity null modulator at the benzodiazepine binding site (α+ γ− interface) and as a low potency positive allosteric modulator via the α+ β− interface [12]. These observations are consistent with previous findings indicating that many of the drugs interacting with $GABA_A$ receptors seem to do that via more than one binding site, as indicated by their different actions at different drug concentrations [7, 20].

In a subsequent study we identified 29 structural analogues of CGS 9895 that either behaved as positive allosteric modulators or null modulators via the α+ β− interface of $GABA_A$ receptors [26]. 16 of these compounds were then further investigated for their effects at $GABA_A$ receptor subtypes composed of α1,2,3,5β3 or α1–6β3γ2 subunits [27]. Results indicated that most of the compounds investigated exhibit comparable potency and

efficacy for αβ and αβγ receptors containing the same type of α or β subunit. Some small differences in the effects elicited in αβ and αβγ receptors were explained by a possible allosteric interaction of the compounds bound to the benzodiazepine site and the α+ β− site.

To possibly identify more receptor subtype-selective ligands in our compound library, in the present study we investigated some of the previously published compounds that so far have not been studied for their effects at various receptor subtypes [26, 27]. For that we started with LAU 177, one of the compounds exhibiting the highest efficacy at α1β3 or α1β3γ2 receptors [26]. Recently, evidence accumulated that extra synaptic receptors such as α1,4,6βδ and α5βγ receptors might have important functions in health and disease [28]. We therefore extended these studies by not only measuring the effects of this compound at receptors composed of α1,2,3,5β3 or α1–6β3γ2 subunits, but also at those composed of α1,4,6β3δ subunits. Surprisingly, LAU 177 exhibited pronounced differences in its efficacy between α1β3, α1β3γ2, and α1β3δ receptors. These and other results indicated that the presence of the γ2 or δ subunit in $GABA_A$ receptors somehow contribute to the efficacy of α+ β− site ligands.

Methods

Two Electrode Voltage Clamp (TEV)

In vitro transcription of mRNA was based on the cDNA expression vectors encoding for $GABA_A$ receptor subunits α1–6, β1–3, γ2 and δ (all from rat) [29]. After linearizing the cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the *mMESSAGE mMACHINE*® T7 transcription kit (Ambion, TX, USA). The capped transcripts were polyadenylated using yeast poly (A) polymerase (USB, OH, USA) and were diluted and stored in diethylpyrocarbonate-treated water at −70 °C.

The methods for isolating, culturing, injecting, and defolliculating of oocytes were identical with those described by E. Sigel [30]. Mature female *Xenopus laevis* (Nasco, WI, USA) were anaesthetized in a bath of ice-cold 0.17 % Tricain (Ethyl-m-aminobenzoat, Sigma, MO, USA) before decapitation and removal of the frog's ovary. Stage 5–6 oocytes with the follicle cell layer around them were singled out of the ovary using a platinum wire loop. Oocytes were stored and incubated at 18 °C in modified Barths' Medium [88 mM NaCl, 10 mM HEPES–NaOH (pH 7.4), 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.34 mM $Ca(NO_3)_2$] that was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Oocytes with follicle cell layer still around them were injected with an aqueous solution of mRNA. A total of 2.5–4 ng of mRNA per oocyte was injected. Subunit ratio was 1:1:5 for αxβ3γ2 receptors, 3:1:5 for αxβ3δ and 1:1 for αxβ3 receptors consisting of wild-type or mutated α subunits together with wild-type or mutated β3 subunits. After injection of mRNA, oocytes were incubated for at least 24 h for αβ and αβδ receptors and for at least 36 h for αβγ2 receptors before the enveloping follicle cell layers were removed. Collagenase-treatment (type IA, Sigma, MO, USA) and mechanical defolliculation of the oocytes was performed as described previously.

For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of Xenopus Ringer solution (XR, containing 90 mM NaCl, 5 mM HEPES–NaOH (pH 7.4), 1 mM $MgCl_2$, 1 mM KCl and 1 mM $CaCl_2$). For current measurements the oocytes were impaled with two microelectrodes (1–2 MΩ) which were filled with 2 M KCl. The oocytes were constantly washed by a flow of 6 ml/min XR that could be switched to XR containing GABA and/or drugs. Drugs were diluted into XR from DMSO-solutions resulting in a final concentration of 0.1 % DMSO perfusing the oocytes. Drugs were pre-applied for 30 s before the addition of GABA, which was then co-applied with the drugs until a peak response was observed. Between two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. Maximum currents measured in mRNA injected oocytes were in the published [26, 31] range for all wild type receptors. To test for modulation of GABA induced currents by compounds, a GABA concentration titrated to trigger 3–7 % of the respective maximum GABA-elicited current of the individual oocyte (=GABA EC_3) was applied to the cell together with various concentrations of compounds to be tested. All recordings were performed at room temperature at a holding potential of -60 mV using a Warner OC-725C two-electrode voltage clamp (Warner Instrument, Hamden, CT, USA) or a Dagan CA-1B Oocyte Clamp or a Dagan TEV-200A two-electrode voltage clamp (Dagan Corporation, Mineapolis, MN, USA). Data were digitized, recorded and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA, USA). Data were analyzed using GraphPad Prism. Data for GABA dependent dose–response curves were fitted to the equation $Y = \text{bottom} + (\text{top–bottom})/1 + 10^{(\text{LogEC50}-X)*nH}$, where EC_{50} is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50 %, and *n*H is the Hill coefficient. The bottom was restrained to 100 %, reflecting the GABA control current. Data are given as mean ± SEM (standard error of mean) from at least three oocytes of two or more oocyte batches. Statistical significance was determined by unpaired Student's *t* test and paired Student's *t* test for GABA concentration–response curves in the absence or

presence of modulator at α1β3δ receptors at a confidence interval of $P < 0.05$.

MTSEA-Biotin—Steric Hindrance

2 mM MTSEA-biotin (*N*-Biotinylaminoethyl methanethiosulfonate) solution was freshly made in XR buffer containing the respective GABA-EC_3 concentration. Defolliculated oocytes were immediately immersed in the MTSEA-biotin solution for 3 min and washed with XR for 5 min. After the washing step, cells were used the same day for the electrophysiological recordings described above.

Materials

$GABA_A$ Receptor Subunits and Point Mutations

cDNAs of rat $GABA_A$ receptor subunits α1, α4, β1, β2, β3, and γ2S were cloned as described [32]. cDNAs of the rat subunits α2, α3, and α5 were gifts from P. Malherbe, that of α6 was a gift of P. Seeburg, and that of δ was a gift of C. Czajkowski. The mutated construct α1S204C was a gift from E. Sigel. For the generation of mutated β3 subunit, this subunit was subcloned into the pCDM8 expression vector (Invitrogen, San Diego, CA, USA) as described previously [33]. Mutated subunits were constructed by PCR amplification using the wild-type subunit as a template. For this, PCR primers were used to construct point mutations within the subunits by the 'gene splicing by overlap extension' technique [34]. The PCR primers for β3Q64C contained *XmaI* and *XhoI* restriction sites, which were used to clone the β3 fragments into pCI vector (Promega, Madison, WI, USA). The mutated subunits were confirmed by sequencing.

Compound Synthesis

Synthesis of *LAU* compounds was performed in analogy to previously outlined synthetic routes [35, 36].

Investigated Compounds

The following compounds were used: (LAU 177): 4-(8-methoxy-3-oxo-3,5-dihydro-2H-pyrazolo[4,3-c]quinolin-2-yl)benzonitrile. (LAU 157): 8-chloro-2-(4-nitrophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one. (PZ-II-028): 8-chloro-2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (was a gift from J. Cook). (DS2): 4-Chloro-*N*-[2-(2-thienyl)imidazo[1,-2-*a*]pyridin-3-yl]benzamide (Tocris, Bristol, UK). (Tracazolate): 4-(butylamino)-1-ethyl-6-methyl-1*H*-pyrazolo[3,4-b]pyridine-5-carboxylate (Sigma Aldrich, AT).

Results

In a search for $GABA_A$ receptor subtype-selective compounds we started to investigate the properties of the pyrazoloquinolinone LAU 177 (Fig. 2a) in more detail. This compound is a structural analogue of CGS 9895 [12], and as reported previously [26] and similar to CGS 9895, LAU 177 acts as a high affinity ligand at the benzodiazepine binding site of α1β3γ2 receptors (IC_{50} of [^{3}H]Ro15-1788 binding of 0.75 ± 0.81 nM), and as a strong positive allosteric modulator ($EC_{50} = 1$ μM, stimulation to 1063 ± 128 % of GABA EC_3 at 10 μM concentration) at α1β3 receptors. For the latter experiments α1β3 receptors were used, to exclude effects mediated via the high affinity benzodiazepine binding site at the α + γ- interface (Fig. 1, Supplementary Table 1). To confirm that LAU 177 mediates its effect at α1β3 receptors via the α1 + β3- interface, we here again employed the substituted cysteine accessibility method to introduce a steric hindrance into the α1+ β3− interface of α1β3 receptors. The point mutations α1S204C (loop C of the α1+ side) and β3Q64C (loop D of the β3− side) have been shown previously to not significantly change the potency or efficacy of GABA for enhancing GABA-induced currents at α1β3 or α1β3γ2 receptors [12]. Recombinant α1S204Cβ3Q64C receptors were expressed in Xenopus oocytes and the effects of various concentrations of LAU 177 were investigated and compared with those at α1β3 receptors. As shown in Fig. 2b, c, in agreement with previous results [26] LAU 177 enhanced GABA-induced currents at α1β3 receptors in a concentration dependent way up to 1152 ± 145 % of GABA EC_3. LAU 177 also enhanced GABA-induced currents in α1S204Cβ3Q64C receptors to a similar extent with a comparable potency. In the presence of MTSEA-biotin, however, current enhancement by LAU 177 was drastically reduced at the mutated receptor (Fig. 2b, c), indicating that LAU 177 exerted most of its action via the α1 + β3- binding site, as expected. In previous studies no change in potency and efficacy of a total of four different pyrazoloquinolinones has been observed when wild-type instead of mutated α1β3 receptors were incubated with MTSEA-biotin [12, 26].

In other experiments, the effects of LAU 177 were compared at various αβ and αβγ2 receptor subtypes (Fig. 3a, b). In agreement with previous results with other pyrazoloquinolinones [26] a similar concentration-dependent effect of LAU 177 at GABA EC_3 was obtained in α1β3 and α1β3γ2 receptors, (maximum stimulation at α1β3γ2 receptors to about 1179 ± 143 % of GABA EC_3 at 10 μM concentrations). The absence of strong LAU 177 effects at α1β3γ2 receptors at 10–100 nM concentrations, that are sufficient to saturate the benzodiazepine binding site of these receptors, suggested that this compound acts as

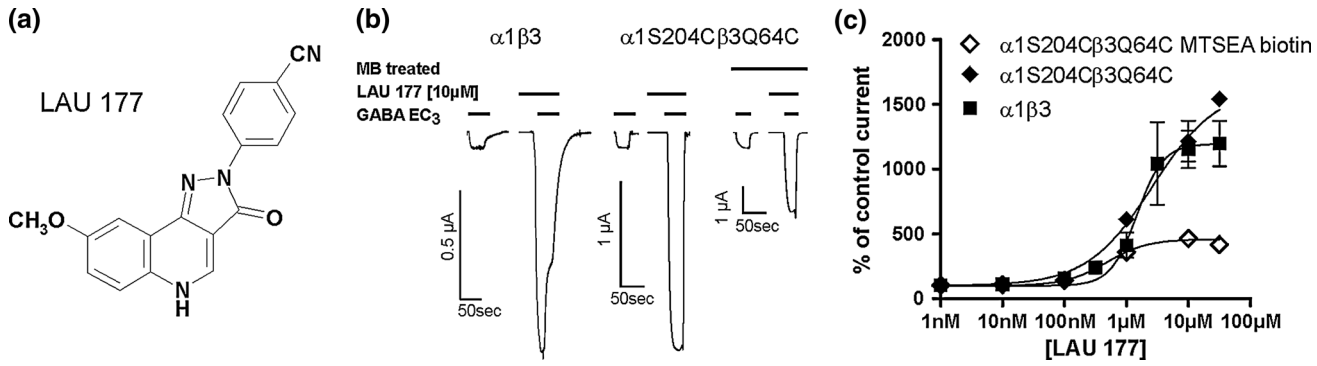

Fig. 2 Steric hindrance at the α+ β− interface drastically reduces the effects of LAU 177 at α1β3 receptors. **a** Structure of LAU 177. **b** Representative current traces of GABA EC_3 in the absence or presence of 10 μM LAU 177 at α1β3 or α1S204Cβ3Q64C receptors without or with steric hindrance (MB treated). **c** Concentration—response effects of LAU 177 at α1β3 (n = 3) or α1S204Cβ3Q64C receptors (n = 4) in the absence or presence of MTSEA-biotin. MTSEA-biotin significantly reduced the effect of 10 μM LAU 177 at α1S204Cβ3Q64C receptors ($p < 0.005$; unpaired Student's t test; n = 4). Data are mean values ± SEM

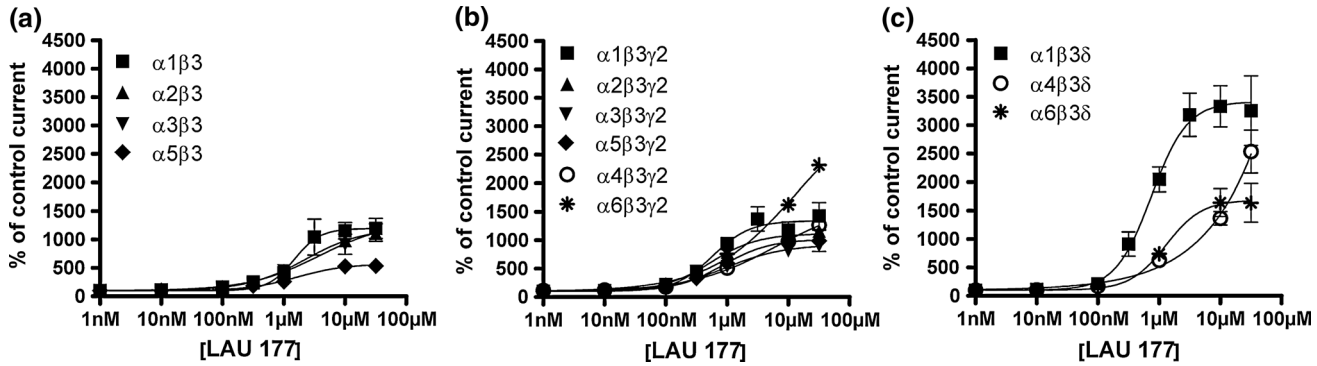

Fig. 3 Effects of LAU 177 at different $GABA_A$ receptor subtypes. **a** Concentration–response curves of LAU 177 at α1β3, α2β3, α3β3 and α5β3 receptors (n = 3). **b** Concentration–response curves of LAU 177 at α1-6β3γ2 receptors (n = 3–7). **c** Concentration–response curves of LAU 177 at α1β3δ, α4β3δ and α6β3δ receptors (n = 4–7). Data are mean values ± SEM

a high affinity null modulator at the benzodiazepine binding site of $GABA_A$ receptors, similar to CGS 9895 and other pyrazoloquinolinones [26, 27]. The similarity in the dose response curves between α1β3 and α1β3γ2 receptors (Fig. 3a, b, Supplementary Tables 1 and 2) suggested that LAU 177 mediates most of its effect at α1β3γ2 receptors via the α1+ β3− interface.

To investigate a possible receptor subtype-selectivity, the effects of LAU 177 were also investigated at α2β3, α3β3, and α5β3 receptors (Fig. 3a, Supplementary Table 1). LAU 177 was a comparably strong modulator of GABA EC_3 at α2β3 and α3β3 receptors, reaching a stimulation of up to 981 ± 20 and 913 ± 178 % of GABA EC_3 at 10 μM concentrations. The effects of LAU 177 at α5β3 receptors, however, were weaker and resulted in an enhancement of the GABA-induced current to about 528 ± 103 % GABA 10 μM. Whereas in α2β3γ2 and α3β3γ2 receptors the effects of LAU 177 were comparable to those of α2β3 and α3β3 receptors, the effects of this compound at α5β3γ2 was stronger than at α5β3 receptors (Fig. 3b, Supplementary Tables 1 and 2). As with several other pyrazoloquinolinones [27], LAU 177 exhibited a stronger stimulation at α6β3γ2 receptors than at all other receptors investigated. The effects of LAU 177 at α4β3γ2 receptors, however, were comparable to those observed at all the other αxβ3γ2 receptors (Fig. 3b, Supplementary Table 2).

So far, we did not investigate the effects of pyrazoloquinolinones at $GABA_A$ receptors containing a δ instead of a γ2 subunit. Such receptors are located exclusively extra- and peri-synaptically, exhibit a high sensitivity to GABA, show little desensitization and are believed to be one of the primary mediators of tonic inhibition [37]. So far, only α1βδ [38], α4βδ [39] or α6βδ receptors [40] have been more or less unequivocally identified in the mammalian brain. We therefore investigated only these receptor subtypes in the present study. To confirm the actual incorporation of the delta subunit into αβδ receptors, GABA-induced currents at all Xenopus oocytes injected with mRNAs of α, β and δ subunits were tested by the addition of 10 μM of the imidazopyridine DS2 [41]. At this concentration DS2 dramatically enhanced GABA-induced currents at αβδ, but not at αβ

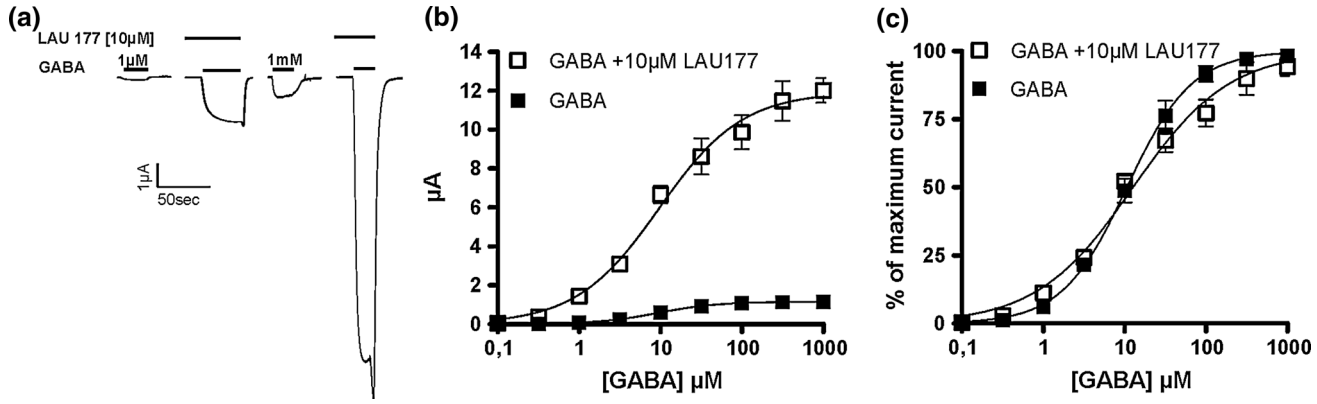

Fig. 4 LAU 177 strongly enhances GABA evoked currents at α1β3δ receptors without changing GABA-potency. **a** Representative traces of a low GABA concentration (1 μM) and a high GABA concentration (1 mM) in absence or presence of 10 μM LAU 177. **b** Concentration dependent currents of GABA (*filled square*, n = 4) and GABA plus 10 μM LAU 177 (*open square*, n = 4). **c)** Effects of GABA and GABA plus 10 μM LAU 177 are normalized to maximum evoked current. A concentration of 10 μM LAU 177 showed no significant change of GABA EC_{50} ($p > 0.05$; paired student's *t* test; n = 4). Data are mean values ± SEM

or αβγ2 receptors [41] (experiments not shown). Since the pyrazoloquinolinones mediate their effects via the α+ β− interface, it was tacitly assumed that the effects of these compounds would be comparable whether a δ or a γ2 subunit is present in the receptors. Surprisingly, however, LAU 177 exhibited a much stronger effect at α1β3δ (stimulation of GABA EC_3 to 3285 ± 257 % at 10 μM concentration) than at α1β3γ2 receptors (stimulation of GABA EC_3 to 1179 ± 143 % at 10 μM concentration) (Fig. 3b, c, Supplementary Tables 2 and 3). Similarly, the effects of LAU 177 at α4β3δ receptors (stimulation of GABA EC_3 to 1365 ± 117 % at 10 μM concentration) were stronger than at α4β3γ2 receptors (stimulation of GABA EC_3 to 981 ± 52 % at 10 μM concentration), whereas the effects of this compound at α6β3γ2 (stimulation of GABA EC_3 to 1622 ± 87 % at 10 μM concentration) and α6β3δ (stimulation of GABA EC_3 to 1639 ± 244 % at 10 μM concentration) receptors were comparable (Supplementary Tables 2 and 3).

The strong potentiation of the GABA current by LAU 177 at α1β3δ receptors was similar to the effects of other compounds observed at these receptors. Specifically, it has been demonstrated that neurosteroids, tracazolate, and DS2 are able to strongly enhance GABA-induced currents at δ-containing receptors compared to the much weaker effects at receptors not containing the δ-subunit. [31, 41] To further investigate this strong effect of LAU 177, a GABA concentration-effect curve was generated at α1β3δ receptors in the absence or presence of LAU 177 (Fig. 4a, b). In agreement with previous results [31], GABA elicited currents at α1β3δ receptors are very small. Whereas GABA was able to induce currents of 8 μA in α1β3 or of 16 μA at α1β3γ2 receptors, the maximal GABA-induced effect at α1β3δ receptors was only about 1 μA. In the presence of LAU 177, however, the GABA-induced current was

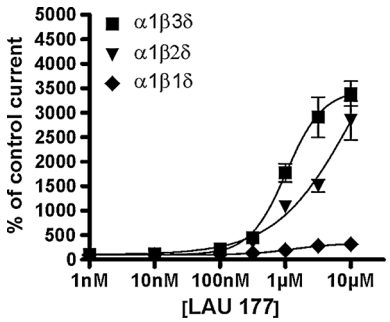

Fig. 5 β-subunit dependent effects of LAU 177 at α1βδ receptors. The effects of LAU 177 at α1β3δ (n = 13) and α1β2δ (n = 9) receptors are comparable (stimulation to 3285 ± 257 % at α1β3δ and to 2843 ± 396 % at α1β2δ at 10 μM concentrations) although LAU 177 exhibits a reduced potency at α1β2δ receptors. However, the effects of LAU 177 at α1β1δ receptors (n = 9) are drastically reduced. Data are mean values ± SEM

dramatically potentiated up to 13 μA at 1 mM GABA. This effect was similar to that of tracazolate or THDOC described previously [31], indicating that LAU 177 also is able to dramatically enhance the efficacy of GABA for opening the $GABA_A$ receptor-associated chloride channel. Interestingly, however, in contrast to the effects observed with tracazolate or THDOC [31], the potency of GABA for enhancing chloride currents was not significantly changed by LAU 177 (Fig. 4c).

As shown for α1β3γ2 receptors and CGS 9895 [12] or PZ-II-028 [27], the effects of LAU 177 at α1β3δ receptors also strongly depended on the type of beta subunit present in the receptors (Fig. 5). LAU 177 exhibited similar efficacy but reduced potency at α1β2δ receptors ($EC_{50} > 10.7$ μM) when compared with α1β3δ receptors ($EC_{50} = 1.0$ μM). The efficacy of this compound at α1β1δ receptors, however, was drastically reduced (Fig. 5, Supplementary Table 4) compared to α1β2,3δ receptors.

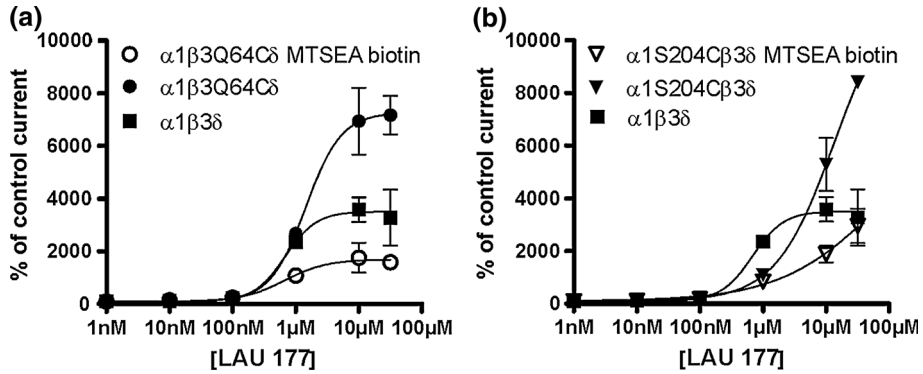

Fig. 6 Steric hindrance at α1β3δ receptors via the β− or α+ side. **a** Introduction of point mutation Q64C at the "−" side of the β subunit strongly enhances the effects of LAU 177 (to 6941 ± 1268 % at 10 μM; n = 3) compared to α1β3δ wild-type receptors (3372 ± 455 % at 10 μM; n = 5). Incubation with MTSEA biotin dramatically reduces this effect to 1754 ± 568 % (at 10 μM; n = 3) ($p < 0.05$; unpaired Student's t test). **b** Introduction of the point mutation S204C at the "+" side of the α subunit strongly enhances the effects of LAU 177 (to 5278 ± 1008 % at 10 μM; n = 3) as compared to α1β3δ receptors (3327 ± 455 % at 10 μM; n = 5). Incubation with MTSEA biotin dramatically reduces this effect to 1884 ± 311 % (at 10 μM; n = 4) ($p < 0.05$; unpaired Student's t test). Data are mean values ± SEM

Thus, the effects of LAU 177 on $GABA_A$ receptors depend on all subunits in the receptor complex—not only the subtype of α and β subunits, but also the presence of a γ or a δ subunit effect potency and efficacy to some extent. To investigate further whether the strong modulatory effects of LAU 177 at α1β3δ receptors were also mediated via the pyrazoloquinolinone binding site at the α1+ β3− interface, steric hindrance experiments were again performed. Here, it was interesting to investigate whether the effect of LAU 177 could be inhibited via both the α1+ and the β3− side. As shown in Fig. 6a, LAU 177 exhibited a comparable effect at 100 nM and 1 μM concentrations at α1β3δ or α1β3Q64Cδ receptors. This point mutation, however, dramatically enhanced the effect of LAU 177 in α1β3Q64Cδ receptors at 10 and 30 μM concentration as compared to α1β3δ receptors. On incubation with MTSEA-biotin this effect of LAU 177 was drastically reduced at all LAU 177 concentrations investigated. Similarly, the point mutation α1S204C dramatically enhanced the effect of LAU 177 at 10 and 30 μM concentration, but at 1 μM concentration the effect of LAU 177 was smaller in the mutated receptor as compared to the wild-type receptor (Fig. 6b). On incubation of α1S204Cβ3δ receptors with MTSEA-biotin, the effects of LAU 177 were also dramatically reduced at 1, 10, and 100 μM concentrations. Together, these data indicate that the effects of LAU 177 can be reduced by steric hindrance introduced via the α1+ as well as via the β3− side of the interface, indicating that both sides contributed to the binding of LAU 177. These data indicated that most of the effects of LAU 177 at α1β3δ receptors are mediated via an interaction of this compound with the α1+ β3− site of $GABA_A$ receptors.

An alternative way to demonstrate that the effects of LAU 177 were mediated via the α1+ β3− interface of α1β3δ receptors is to use a compound acting as a null modulator (antagonist) of this binding site. LAU 157 is one of the five null modulators for α1β3 receptors previously identified [26]. So far however, this compound was not investigated for its effect at other receptors subtypes. We thus investigated whether LAU 157 (Fig. 7a) behaves as a null modulator at αβδ receptors. Results shown in Fig. 7b indicate that LAU 157 did not significantly modulate GABA EC_3 at α1β3, α1β3δ, α1β2δ receptors. To investigate whether this lack of effect was due to LAU 157 behaving as a null modulator or whether LAU 157 did not bind at all at the α+ β− interface of these receptors, experiments were performed investigating the inhibition of the effects of LAU 177 at α1β3δ, α1β2δ, or α1β3 receptors by LAU 157. As shown in Fig.7c, results indicated that LAU 157 was able to completely inhibit the effects of LAU 177 in all receptors investigated, suggesting that it is a null modulator at the α+ β− interface and was also able to inhibit the effects of LAU 177 at α1β3δ receptors.

To investigate whether this strong modulation of α1β3δ receptors by LAU 177 as compared to α1β3γ2 receptors was a general property of pyrazoloquinolinones, the effects of PZ-II-028 [26, 27], were also analyzed at γ2 and δ containing receptors in analogous experiments. In contrast to LAU 177, the modulation of GABA-induced currents by PZ-II-028 at α1β3δ receptors (stimulation to 1186 ± 89 % at 10 μM concentration) was only slightly enhanced compared to that at α1β3γ2 receptors (stimulation to 939 ± 64 at 10 μM concentration [27]. The modulation by this compound of GABA-induced currents at α4β3γ2 receptors (226 ± 14 %) [27] was also enhanced at α4β3δ receptors (425 ± 26 %). But the strong stimulation of GABA-induced current at α6β3γ2 receptors by PZ-II-028 (stimulation to 1871 ± 28 % at 10 μM concentration, [27]) no

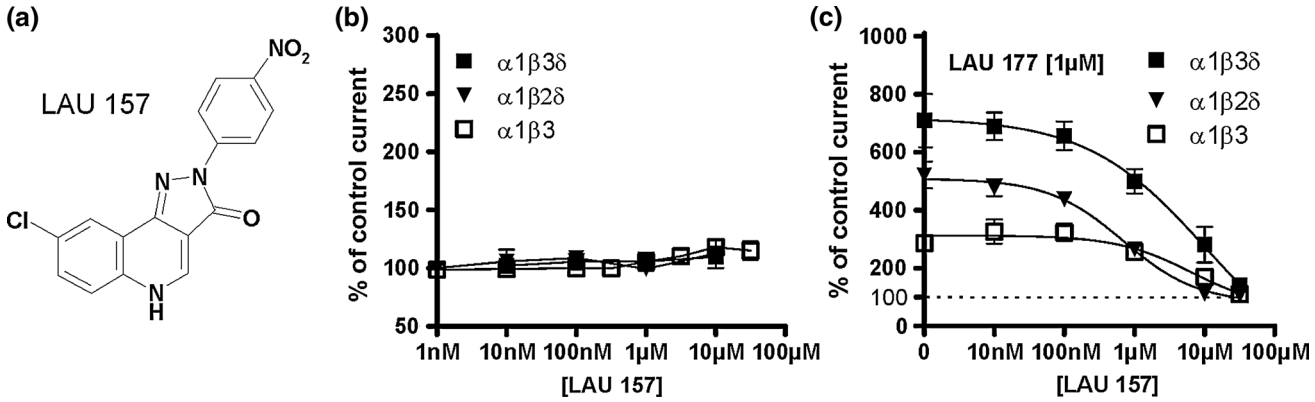

Fig. 7 LAU 157 inhibits effects of LAU 177. **a** Structure of LAU 157. **b** Concentration-dependent effect of LAU 157 at α1β3δ (n = 3), α1β2δ (n = 3) and α1β3 (n = 3). **c** Increasing concentrations of LAU 157 dose dependently inhibit the effect of 1 μM LAU 177 at α1β3δ (n = 4), α1β2δ (n = 3) and α1β3 (n = 4), reaching complete inhibition. Data are mean values ± SEM

longer was observed in α6β3δ receptors (stimulation to 1152 ± 106 % at 10 μM concentration). The effects of this compound class are thus not only dependent on the molecular structure of the compound but also on the receptor composition (α, β subtypes, γ2, or δ subunit). Further experiments will have to be performed to investigate the effects of PZ-II-028 and those of other pyrazoloquinolinones in αβ, αβγ2 and αβδ receptors in more detail.

Tracazolate [31] and the imidazopyridine DS2 [41] also display strong enhancement of GABA currents in δ-containing receptors, and the efficacy is determined by all subunits present in the receptor complex. This analogy prompted us to investigate whether tracazolate or DS2 might exert their action via the extracellular α+ β− binding site. Our experiments however indicated that increasing concentrations (up to 30 μM) of the α+ β− site antagonist LAU 157 did not inhibit the effects of 1 μM DS2 (stimulation to 400 % of GABA EC_3) or of 3 μM tracazolate (stimulation to 900 % of GABA EC_3) at α1β3δ receptors (experiments not shown). This seems to indicate that neither tracazolate nor DS2 exert their actions via the pyrazoloquinolinone binding site at the α+ β− interface.

Discussion

In this study, we investigated the effects of the pyrazoloquinolinone LAU 177 in more detail. The nM affinity of this compound for the benzodiazepine binding site at α1β3γ2 receptors, its lack of modulation of GABA-induced currents at this receptor in the 10–100 nM range, as well as the comparable concentration-effect curve at α1β3 and α1β3γ2 receptors indicate that this compound, similar to other pyrazoloquinolinones [12, 26, 27], is a high affinity null modulator at the benzodiazepine binding site and a low potency high efficacy modulator via the extracellular α1+ β3− interface. This conclusion was supported by steric hindrance experiments at α1β3 receptors as well as by the use of LAU 157, an α1+ β3− site antagonist, that was able to completely inhibit the action of LAU 177 at α1β3 receptors.

Modulation by LAU 177 of $GABA_A$ receptor subtypes composed of α1,2,3,5β3 or α1–6β3γ2 subunits, was slightly different in potency and efficacy, indicating that the modulation of this compound is dependent on the type of the α subunit present in the receptor. This again is consistent with LAU 177 eliciting modulation by binding to the α+ β− interface. The extent of modulation of GABA currents was comparable in α1–3β3 or α1–3β3γ2 receptors containing the same α subunit type. Only at α5β3γ2 receptors we observed a stronger modulation of LAU 177 than at α5β3 receptors, As with several other pyrazoloquinolinones [27], LAU 177 exhibited a distinctly stronger effect at receptors composed of α6β3γ2 subunits than at receptors containing other α subunit types together with β3 and γ2.

Interestingly, however, LAU 177 exhibited a much stronger modulation of GABA-induced currents at α1β3δ receptors than at α1β3 or α1β3γ2 receptors. Steric hindrance experiments indicated that the effects of this compound at α1β3δ receptors were also mediated via the α+ β− interface and this conclusion was supported by the use of the α+ β− site antagonist LAU 157, that was able to completely inhibit the effects of LAU 177 at α1β3δ receptors. This conclusion was further supported by the finding that the extra stimulation of LAU 177 at δ-containing receptors also depended on the type of α and β subunits. The α subunit dependence, however, was much stronger than in αβ or αβγ2 receptors. Thus, whereas the effects of LAU 177 were strongly enhanced at α1β3δ and α4β3δ as compared to α1β3γ2 and α4β3γ2 receptors, there was no significant change of efficacy at α6β3δ receptors as

compared to α6β3γ2 receptors. In addition, LAU 177 strongly modulated GABA-induced currents at α1β3δ and α1β2δ receptors, with a lower potency at α1β2δ receptors. In contrast, LAU 177 exhibited a quite low modulation at α1β1δ receptors.

Taken together, all evidence indicates that LAU 177 exerts its modulatory effects in α1β3, α1β3γ2 and α1β3δ receptors by binding to the extracellular pocket at the α1+ β3− interface. Nevertheless, the extent of modulation was much stronger at α1β3δ receptors than at the other two receptors. The differences in the effects of this compound can thus only have been induced by the presence of the delta subunit and its contribution to state transitions or state stabilization.

The much stronger modulation by LAU 177 of α1β3δ compared to α1β3γ2 receptors, however, was not observed with PZ-II-028, another pyrazoloquinolinone. Screening experiments indicated that this compound exhibited no or at most a weak, statistically marginal, increase in the modulation of α1β3δ over α1β3γ2 receptors. In contrast, PZ-II-028 exhibited a large decrease in the modulation of α6β3δ as compared to α6β3γ2 receptors, whereas LAU 177 exhibited no difference in the enhancement of GABA-induced currents at α6β3δ and α6β3γ2 receptors. From a different point of view it could also be stated that the γ2 subunit in this case could enhance the effects of PZ-II-028 as compared to δ-containing receptor subtypes. As demonstrated previously, the superstimulation of α6β3γ2 by PZ-II-028 was additionally dependent on the β3 subunit and was absent in β2 containing receptors [27]. Since it is assumed that αβ receptors are composed of 2 α and 3 β subunits [33, 42, 43], a replacement of a β3 by a γ2 or a δ subunit in α1,6β2,3, α1,6β2,3γ2, or α1,6β2,3δ receptors changes the efficacy of compounds differentially, depending on the structure of the pyrazoloquinolinone as well as on the types of α and β subunits present in the receptors. The actual effects of the pyrazoloquinolinones thus clearly depend on all subunits present in the receptors, although the effects of these compounds are mediated via the α+ β− interface. The mechanisms of these changes in efficacy currently are not known.

In agreement with previous results [31] we demonstrated that GABA only weakly activated α1β3δ receptors, indicating that GABA is a partial agonist at these receptors. The addition of 10 μM LAU 177 dramatically (about 12 times) enhanced the conductance of α1β3δ receptors induced by maximal GABA concentrations. A similar effect has been demonstrated previously using tracazolate or THDOC [31], or DS2 [41]. In contrast to these compounds that also were able to enhance the potency of GABA consistently, LAU 177 only enhanced the efficacy of GABA but not its potency, suggesting a difference in the actions of LAU 177, tracazolate, THDOC, or DS2. This conclusion was supported by our finding that the α+ β− site antagonist LAU 157 was unable to block the effects of DS2 or tracazolate (experiments not shown). From these observations we conclude that LAU 177 has a unique profile of action in αβ, αβγ and αβδ receptors that results from its binding to the pyrazoloquinolinone binding site at the extracellular α+ β− interface. The efficacy of LAU 177 is determined by ligand specific features, by the binding site forming subunits, and by the nature of the third subunit in the complex. In addition, the site of action and mechanism by which the third subunit and LAU 177 act together is distinct from that of DS2 and tracazolate. Whereas the site of action of DS2 and tracazolate is not known, LAU 177 is acting via the extracellular α+ β− interface.

Investigations with recombinant concatenated δ-containing receptors have indicated that several types of δ receptors can be formed, in which the δ subunit can assume different positions and replace either the γ2 or one of the β subunits of α1βγ2 receptors [3, 4]. Each one of these receptors displayed a specific set of properties, and there are also receptors that obviously cannot be activated by GABA in the absence of the steroid THDOC, suggesting that these receptors are essentially silent in the absence of the steroid [3, 4]. If these different receptors also can be formed from non-concatenated subunits, it can be assumed that a mixture of δ-receptors with different subunit arrangements might be formed on injection of *Xenopus* oocytes with $GABA_A$ receptor subunit mRNAs and that the low GABA activation of this receptor mixture could have been caused by a low percentage of receptors that can be directly activated by GABA. Depending on the type of α subunit, the composition of the receptor mixture could have changed possibly explaining the different allosteric modulation of δ-containing receptor subtypes by LAU 177. Further experiments would have to be performed with concatenated receptors to investigate this possibility.

In summary, LAU 177 is a modulator of $GABA_A$ receptors which can uncover extrasynapic α1β2,3δ receptors that due to their low GABA-induced currents are functionally more or less silent in the absence of modulatory agents such as neurosteroids [4, 31]. In contrast to previously described modulators exhibiting similar effects, such as tracazolate or DS2, LAU 177 interacts with a known binding site, the extracellular α+ β− site, making it more suitable for a rational design of drugs exhibiting such an action profile. The existence of the functional α+ β− site antagonist LAU 157 not only is useful for in vivo studies as it can terminate the effect of LAU 177 or similar compounds, but also can help to identify compounds from different structural classes interacting with this binding site. Thus, the path forward is to use LAU 177 and related pyrazoloquinolinones as a pharmacophore template to

develop more compounds acting as superstimulators on $\alpha1\beta2,3\delta$ receptors and to further improve their pharmacological profiles.

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