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RESEARCH PAPER

Subtype selectivity of α+β– site ligands of $GABA_A$ receptors: identification of the first highly specific positive modulators at α6β2/3γ2 receptors

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$GABA_A$ receptors; CGS 9895; pyrazoloquinolinones; pyrazolopyridinones; α+β– binding site; positive modulators; null modulators; benzodiazepine binding site

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BACKGROUND AND PURPOSE
$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the mammalian brain and the target of many clinically important drugs interacting with different binding sites. Recently, we demonstrated that CGS 9895 (2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one) elicits a strong and subtype-dependent enhancement of GABA-induced currents via a novel drug-binding site at extracellular αx+βy– (x = 1–6, y = 1–3) interfaces. Here, we investigated 16 structural analogues of CGS 9895 for their ability to modulate GABA-induced currents of various $GABA_A$ receptor subtypes.

EXPERIMENTAL APPROACH
Recombinant $GABA_A$ receptor subtypes were expressed in *Xenopus laevis* oocytes and investigated by the two-electrode voltage clamp method.

KEY RESULTS
Most of the compounds investigated were able to modulate GABA-induced currents of αβ and αβγ receptors to a comparable extent, suggesting that the effect of these drugs is not dependent on the benzodiazepine site of $GABA_A$ receptors. Steric hindrance experiments demonstrated that these compounds exert their action predominantly via the αx+βy– (x = 1–6, y = 1–3) interfaces. Whereas some compounds are unselectively modulating a broad range of receptor subtypes, other compounds feature remarkable functional selectivity for the α6β3γ2 receptor, or behave as null modulators at some receptor subtypes investigated.

CONCLUSION AND IMPLICATIONS
Pyrazoloquinolinones and pyrazolopyridinones represent the first prototypes of drugs exerting benzodiazepine-like modulatory effects via the α+β– interface of $GABA_A$ receptors. The discovery of modulators with functional subtype selectivity at this class of binding sites provides a highly useful tool for the investigation of α6β2/3γ2 receptor function, and may lead to novel therapeutic principles.

LINKED ARTICLE
This article is accompanied by Varagic *et al.*, pp. 371–383 of this issue. To view this article visit http://dx.doi.org/10.1111/bph.12151

Abbreviations
MTSEA-biotin (MB), N-Biotinylaminoethyl methanethiosulfonate; TEV, two-electrode voltage clamp

Introduction

$GABA_A$ receptors are the major inhibitory transmitter receptors in the brain. They are ligand-gated chloride channels composed of five subunits that can belong to different subunit classes. Six α, three β, three γ, one δ, one ε, one θ, one π, and three ρ subunits and their different regional, cellular and subcellular distribution (Wisden *et al.*, 1992; Pirker *et al.*, 2000) generate an enormous diversity of $GABA_A$ receptor subtypes with distinct pharmacological properties (Olsen and Sieghart, 2008). Drugs such as benzodiazepines, barbiturates, neuroactive steroids, anaesthetics and convulsants are mediating their action via allosteric binding sites at $GABA_A$ receptors (Sieghart, 1995) and it is now clear that $GABA_A$ receptors are modulating anxiety, the excitability of the brain, muscle tonus, vigilance, circadian rhythms, learning and memory (Sieghart, 1995).

While many receptor subtypes exist and mediate specific functions, GABAergic drugs often display very little selectivity among them. But, in contrast to classical benzodiazepines that stimulate all $GABA_A$ receptors composed of α1βγ2, α2βγ2, α3βγ2 and α5βγ2 receptors, several novel benzodiazepine-binding site ligands are able to selectively address some of these $GABA_A$ receptor subtypes (Sieghart and Ernst, 2005).

The high-affinity benzodiazepine-binding site is located in the extracellular domain of $GABA_A$ receptors, at the α+γ– interface (Sigel, 2002; Richter *et al.*, 2012). The action of benzodiazepines is thus strongly dependent on the types of α and γ subunits forming this interface. In addition, studies on genetically modified mice indicated that benzodiazepine-induced behavioural responses are mediated by $GABA_A$ receptor subtypes containing specific alpha subunits (Rudolph *et al.*, 1999). Drugs specifically interacting with $GABA_A$ receptor subtypes will thus exhibit quite selective behavioural and pharmacological effects (Sieghart and Ernst, 2005).

Recently, we demonstrated that the high-affinity benzodiazepine-binding site ligand CGS 9895 behaves as a null modulator via this site, and in addition, exerts a low-potency positive modulatory action at $GABA_A$ receptors via a newly discovered drug-binding site at the extracellular α+β– interface (Ramerstorfer *et al.*, 2011). This novel binding site is thus strongly influenced by the types of α and β subunits present in the receptor. Drugs selectively interacting with specific αx+βy– interfaces of $GABA_A$ receptors might therefore lead to novel therapeutic principles by addressing groups of receptors not accessible to drugs currently in use. (Sieghart *et al.*, 2012).

In the accompanying manuscript (Varagic *et al.*, 2013), we investigated 32 structural analogues of CGS 9895 for their interaction with the α1+β3– interface of $GABA_A$ receptors. Twenty-four of these compounds were able to enhance GABA-induced currents via the α1+β3– interface. Five structural analogues were null modulators at this interface and three compounds apparently did not interact with $GABA_A$ receptors (Varagic *et al.*, 2013). Here, we selected 16 of these 32 structural analogues of CGS 9895 and studied their subtype selectivity at αxβ3 and αxβ3γ2 receptors. We demonstrated that the action of some of these compounds can be strongly influenced by the α and β subunit type in these receptors and identified the first highly selective positive modulators of the α6β3γ2 $GABA_A$ receptor subtype.

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, α2, α3, α4, α5, α6, β1, β2, β3 and γ2 (all from rat; Ramerstorfer *et al.*, 2010). After a linearization of cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the mMESSAGE mMACHINE® T7 transcription kit (Ambion, Austin, TX, USA). The capped transcripts were polyadenylated using yeast poly (A) polymerase (USB Corp., Cleveland, 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 (Sigel *et al.*, 1990) and were described elsewhere (Li *et al.*, 2003). Mature female *Xenopus laevis* (Nasco, Fort Atkinson, WI, USA) were anaesthetized in a bath of ice-cold 0.17% Tricain (Ethyl-m-aminobenzoat, Sigma-Aldrich, St. Louis, MO, USA) before decapitation and removal of the frog's ovary. Stage 5 to 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 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. Oocytes with follicle cell layer still around them were injected with an aqueous solution of mRNA. A total of 2.5 ng of mRNA per oocyte was injected. Subunit ratio was 1:1:5 for αxβ3γ2 (x = 1–3), 3:1:5 for αxβ3γ2 (x = 4–6) receptors and 1:1 for αxβ3 (x = 1, 2, 3 and 5) receptors consisting of wild-type or mutated subunits. After injection of mRNA, oocytes were incubated for at least 24 h for αxβ3 receptors and for at least 36 h for αxβ3γ2 receptors before the enveloping follicle cell layers were removed. Collagenase-treatment (type IA, Sigma) and mechanical defolliculation of the oocytes was performed as described previously (Li *et al.*, 2003).

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 (2–3 MΩ), which were filled with 2 M KCl. The oocytes were constantly washed by a flow of 6 $mL \cdot min^{-1}$ 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 microampere range for all subtypes of $GABA_A$ receptors. To test for modulation of GABA induced currents by compounds, a GABA concentration that was titrated to trigger 3% of the respective maximum GABA-elicited current of the individual oocyte (EC3) was applied to the cell together with various

concentrations of tested compounds. All recordings were performed at room temperature at a holding potential of –60 mV using a Warner OC-725C TEV (Warner Instrument, Hamden, CT, USA) or a Dagan CA-1B Oocyte Clamp or a Dagan TEV-200A TEV (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 analysed using GraphPad Prism (La Jolla, CA, USA). Data for GABA-dependent dose-response curve were fitted to the equation Y = Bottom + (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. Data are given as mean ± SEM from at least three oocytes of two and more oocyte batches. Statistical significance was calculated using unpaired and paired Student's *t*-test or a one-sample *t*-test comparing the mean with a hypothetical value of 100 (100% of GABA EC3 – control current) and 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-EC3 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 on 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, β3 and γ2S were cloned as described (Ebert *et al.*, 1996). cDNAs of the rat subunits α2, α3 and α5 were gifts from P. Malherbe and that of α6 was a gift of P. Seeburg. The mutated construct α1V211C was a gift from E Sigel (Institute of Biochemistry and Molecular Medicine, Bern, Switzerland). For the generation of mutated β3 and γ2 subunits, these subunits were subcloned into pCDM8 expression vectors (Invitrogen, San Diego, CA, USA) as described previously (Tretter *et al.*, 1997). 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 (Horton *et al.*, 1993). 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.

Investigated compounds

The following compounds were used and numbered according to the accompanying manuscript (Varagic *et al.*, 2013):

Compounds 1 (*CGS 8216*): 2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 2 (*CGS 9896*): 2-(4-chlorophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; and 30 (*CGS 20625*): 5,6,7,8,9,10-hexahydro-2-(4-methoxyphenyl)cyclohepta[*b*]pyrazolo[3,4-*d*]pyridin-3(2*H*)-one were kindly provided by Ciba-Geigy (Novartis, Basel, Switzerland); Compounds 5 (*PWZ-009A1*): 7-methoxy-2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 6 (*PZ-II-029*): 7-methoxy-2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 7 (*XHe-III-006c*): 7-bromo-2-(4-bromophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 8 (*XHe-II-087c*): 2-(4-bromophenyl)-6-(tert-butyl)-2H-pyrazolo[4,3-c]quinolin-3(5H)-one; 11 (*PZ-II-028*): 8-chloro-2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 17 (*PWZ-007A*): 8-methoxy-2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 20 (*XHe-III-24*): 8-*tert*-butyl-2-(4-fluorophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 22 (*XHe-II-17*): 8-*tert*-butyl-2-(4-ethynylphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 23 (*XHe-II-094*): 8-(tert-butyl)-2-(4-((trimethylsilyl)ethinyl)phenyl)-2H-pyrazolo[4,3-c]quinolin-3(5H)-one; 25 (*XHe-II-019*): 8-*tert*-butyl-2-(4-(5,5-dimethylhexa-1,3-diynyl)phenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 27 (*XHe-II-098b*): 2-(4-bromophenyl)-7,7,10,10-tetramethyl-7,8,9,10-tetrahydro-2*H*-benzo[*g*]pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; 32 (*XHe-III-67*): 2-(4-((trimethylsilyl)ethynyl)phenyl)-2H-pyrazolo[4,3-c]pyridin-3(5H)-one; 33 (*XHe-III-56*): 2-(4-bromophenyl)-2*H*-pyrazolo[4,3-*c*]pyridin-3(5*H*)-one were synthesized by the laboratory of Professor James Cook, Department of Chemistry and Biochemistry UW-Milwaukee, Milwaukee, WI, USA; *ROD 188*: (5R)-5-{(1R)-2-[(4-methylphenyl)sulfonyl]-1,2,3,4-tetrahydroisoquinolin-1-yl}dihydrofuran-2(3H)-one (gift from Prof. Robert Dodd, Institut de Chimie des Substances Naturelles, C.N.R.S. 91190, Gif-sur-Yvette, France); *Diazepam; Ro15-1788* (Sigma-Aldrich, St. Louis, MO, USA).

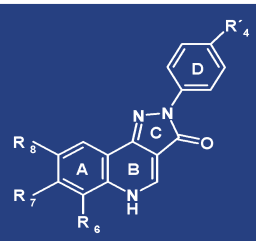
Results

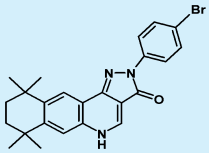
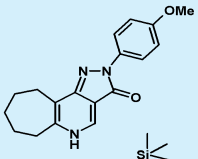
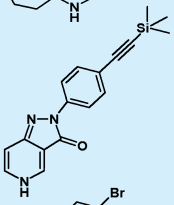
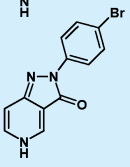
Effects of α1+β3– binding site ligands depend on the α subunit type

In the accompanying study (Varagic *et al.*, 2013), it was demonstrated that CGS 9895 (Ramerstorfer *et al.*, 2011) and 24 other pyrazoloquinolinones or pyrazolopyridinones could modulate GABA-induced currents at α1β3 receptors via the α1+β3– interface. Five additional structural analogues could inhibit the action of these modulators and thus acted as null modulators at the α1+β3– site. Here, 12 of these positive allosteric modulators as well as 4 null modulators at the α1+β3– interface (Table 1) were investigated for their effects at αxβ3 and αxβ3γ2 receptors containing different α (α1, α2, α3 and α5) subunits (Figures 1–4). Due to insufficient expression of α4β3 and α6β3 receptors, the effects of these compounds at α4- or α6- containing receptors could only be investigated at α4β3γ2 or α6β3γ2 receptors. All compounds studied here have been investigated previously for their apparent affinity for the high-affinity benzodiazepine-binding site of $GABA_A$ receptors (Brown *et al.*, 1984; Loo *et al.*, 1987; Williams *et al.*, 1989; He *et al.*, 1999; Yu *et al.*, 1999; Smith *et al.*, 2001; Ogris *et al.*, 2004), see Table 1. Whereas 10 of these compounds (1, 2, 5, 6, 7, 11, 17, 20, 22 and 30) exhibited a high affinity (nM) for the benzodiazepine-binding site of various $GABA_A$ receptor subtypes, compounds 8, 23, 25, 27, 32, 33, exhibited a low (300 nM-7 μM) affinity for these binding sites (Table 1).

Table 1

Structure and affinity of pyrazoloquinolinones and pyrazolopyridinones for the benzodiazepine-binding site at αxβ3γ2 GABA$_A$ receptors: top: Pyrazolo[4, 3-*c*]quinolin-3-one nucleus; left: different substituents and whole chemical structure of pyrazoloquinolinones or pyrazolopyridinones investigated; right: pyrazoloquinolinone or pyrazolopyridinone binding affinities for GABA$_A$ receptors (published data, see references in footnotes)



						αξ+/γ2– Ki (nM)					
	Compounds	**R_8**	**R_7**	**R_6**	**R_4'**	**α1β3γ2**	**α2β3γ2**	**α3β3γ2**	**α4β3γ2**	**α5β3γ2**	**α6β3γ2**
1	CGS 8216	H	H	H	H	0.17 ± 0.01[1]	0.49 ± 0.09[1]	0.3 ± 0.06[1]	35 ± 11[1]	1.3 ± 0.2[1]	N/D
2	CGS 9896	H	H	H	Cl	0.5 ± 0.1 (brain membranes)[2]					
5	PWZ-009A1	H	OMe	H	H	1.3[3]	1.3[3]	1.3[3]	N/D	0.8[3]	2[3]
6	PZ-II-029	H	OMe	H	OMe	0.3[3]	N/D	0.8[3]	N/D	0.5[3]	10[3]
7	XHe-III-006c	H	Br	H	Br	34[3]	44[3]	29[3]	210[3]	23[3]	319[3]
8	XHe-II-087c	H	H	tBu	Br	7000[3]	7000[3]	2000[3]	N/D	4000[3]	7000[3]
11	PZ-II-028	Cl	H	H	OMe	0.2[3]	N/D	0.2[3]	N/D	0.3[3]	1.9[3]
17	PWZ-007A	OMe	H	H	H	0.1[3]	0.1[3]	0.1[3]	N/D	0.2[3]	>10[3]
20	XHe-III-24	tBu	H	H	F	0.25[3]	N/D	8[3]	N/D	10[3]	328[3]
22	XHe-II-17	tBu	H	H	C≡CH	3.3[3]	10[3]	7[3]	258[3]	17[3]	294[3]
23	XHe-II-094	tBu	H	H	C≡CSiMe3	329[3]	1098[3]	1156[3]	>1000[3]	2462[3]	>3000[3]
25	XHe-II-019	tBu	H	H	C≡C–C≡CtBu	273[3]	428[3]	762[3]	N/D	1464[3]	>3000[3]
27	XHe-II-098b					7000[3]	8000[3]	3000[3]	N/D	7000[3]	4000[3]
30	CGS 20625					0.5 ± 0.1 (brain membranes)[2]					
32	XHe-III-67					>3000[3]	>3000[3]	>3000[3]	N/D	>3000[3]	>3000[3]
33	XHe-III-56					1010[3]	1640[3]	1930[3]	N/D	1300[3]	>3000[3]

[1]Displacement of [^{3}H]Ro15-1788 binding to human α1, α2, α3 and α5 containing receptors. In α4β3γ2 receptors, data represent displacement of specific [^{3}H]Ro15-4513 binding (means ± SEM, n = 3–6) (Smith *et al.*, 2001).
[2]Displacement of [^{3}H]flunitrazepam binding to mouse forebrain membranes (means ± SD, n = 6–9; Ogris *et al.*, 2004).
[3]Displacement of [^{3}H]Ro15-1788 (α*x*β3γ2; x = 1, 2, 3, 5 and 6) or [^{3}H]Ro15-4313 (α4β3γ2) binding to Ltk cells expressing human GABA$_A$ receptors. Data are the means of two determinations, which differed by less than 10% (He, 2000; He *et al.*, 1999; Yu *et al.*, 1999).
N/D, not defined.

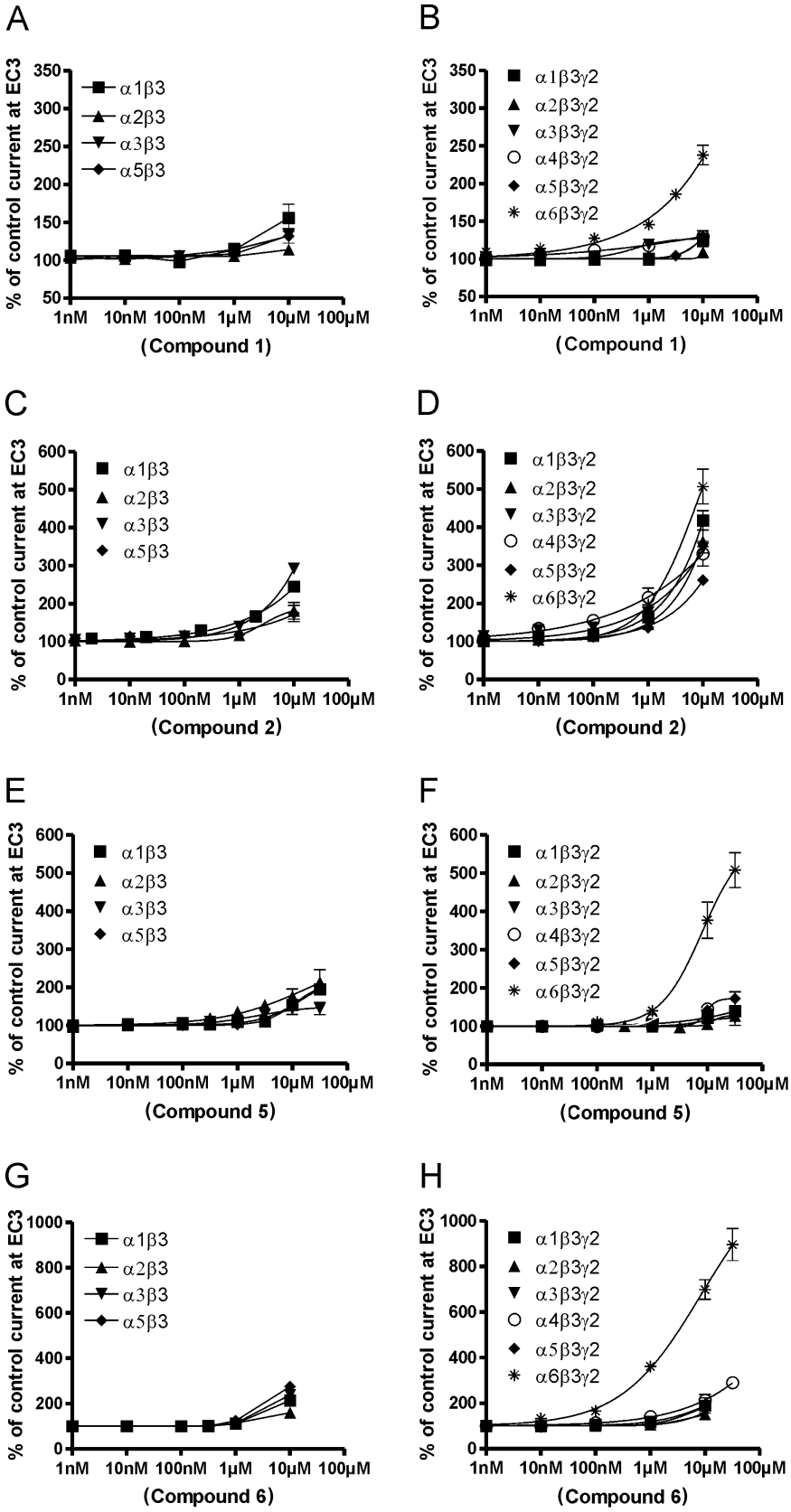


Figure 1

Alpha selectivity of compounds 1, 2, 5 and 6 at αxβ3 and αxβ3γ2 $GABA_A$ receptors: (A), (C), (E), (G) Concentration-dependent modulation of GABA EC3 current at α1β3 (■), α2β3 (▲), α3β3 (▼) and α5β3 (◆) receptors. (B), (D), (F), (H) Concentration-dependent modulation of GABA EC3 current at α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (▼), α4β3γ2 (○), α5β3γ2 (◆) and α6β3γ2 (*) receptors. Data represent means ± SEM (n = 4–6).

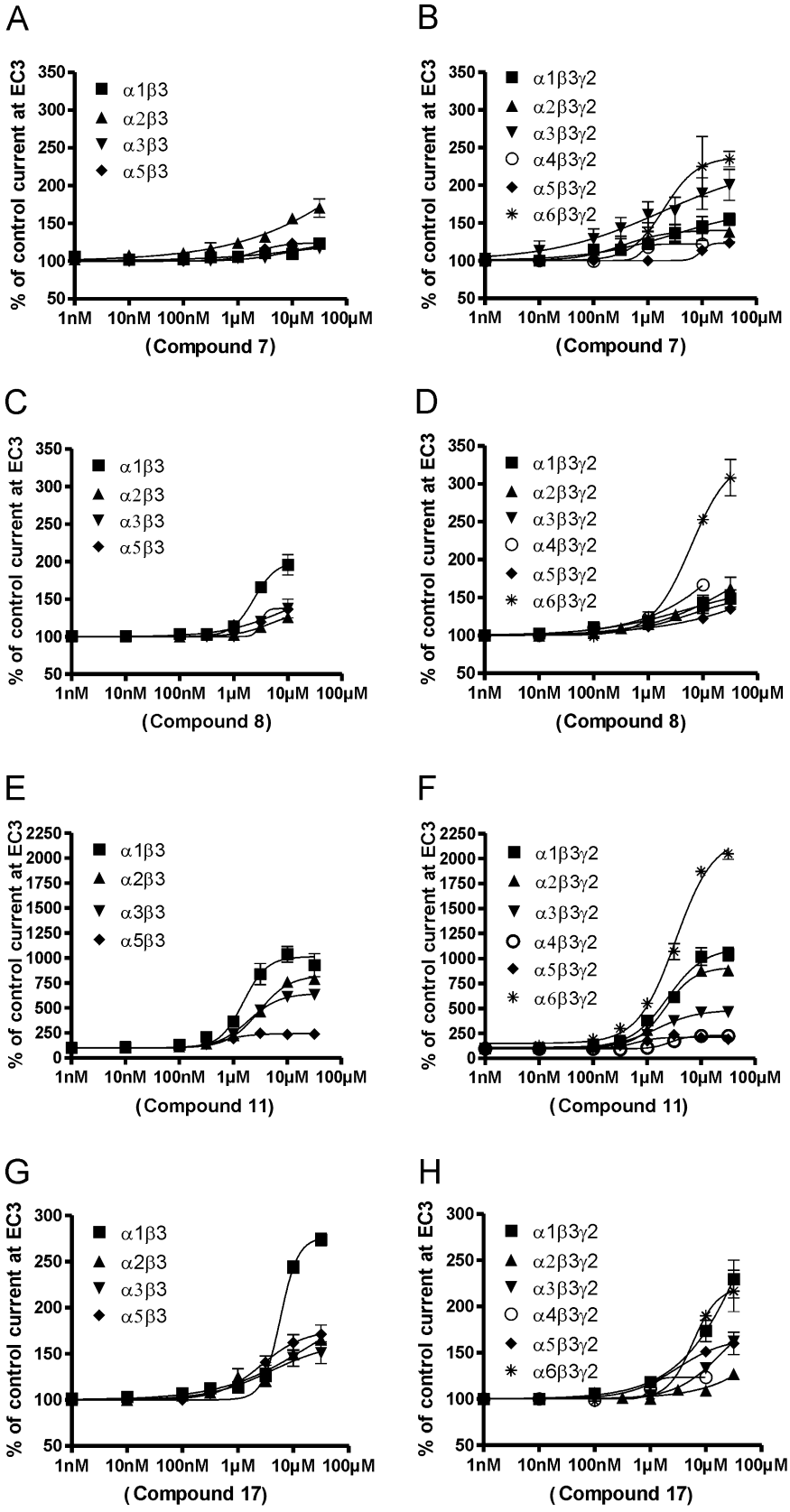


Figure 2

Alpha selectivity of compounds 7, 8, 11 and 17 at αxβ3 and αxβ3γ2 GABA$_A$ receptors: (A), (C), (E), (G) Concentration-dependent modulation of GABA EC3 current at α1β3 (■), α2β3 (▲), α3β3 (▼) and α5β3 (◆) receptors. (B), (D), (F), (H) Concentration-dependent modulation of GABA EC3 current at α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (▼), α4β3γ2 (○), α5β3γ2 (◆) and α6β3γ2 (*) receptors. Data represent means ± SEM (n = 4–6).

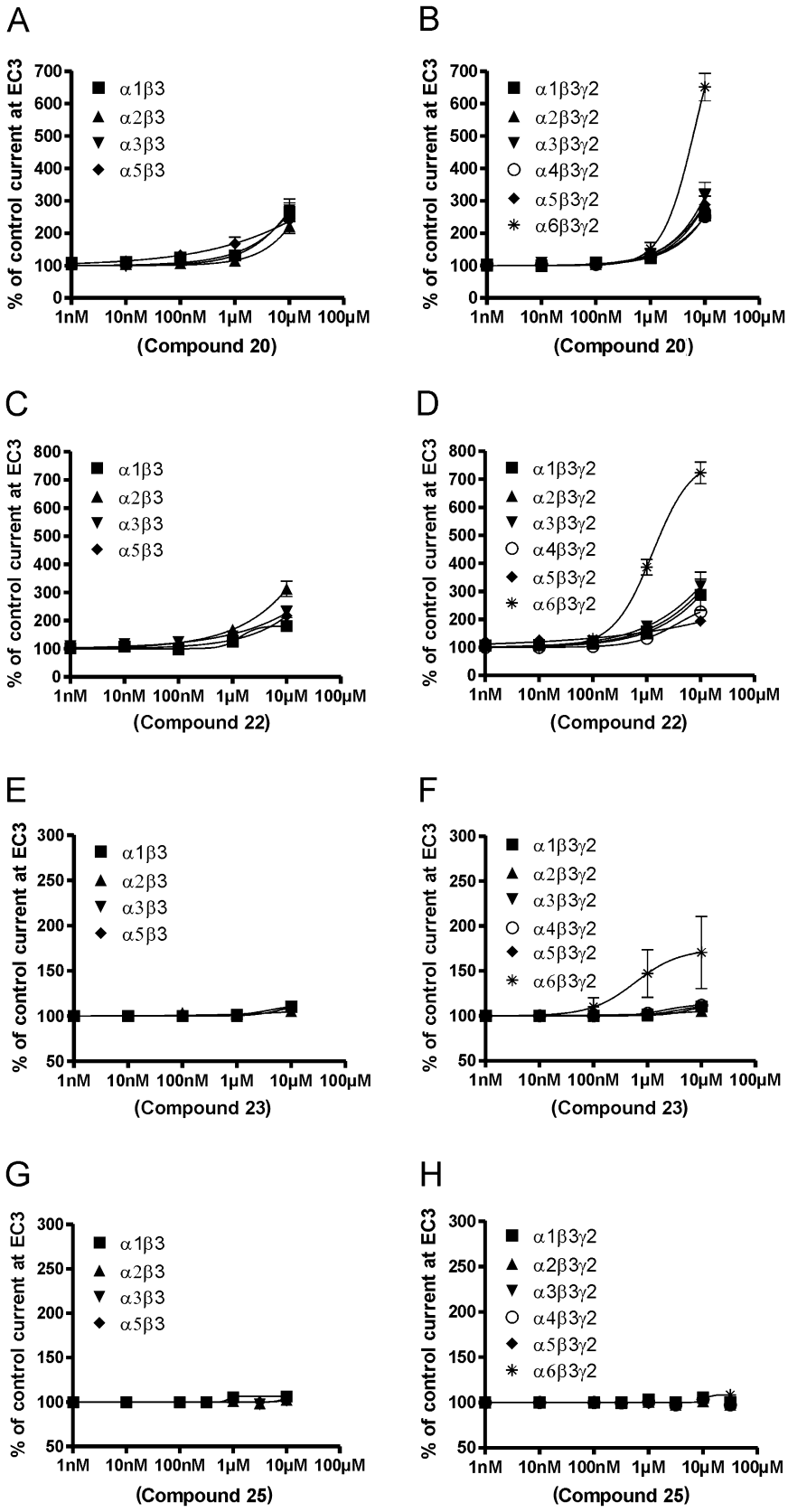


Figure 3

Alpha selectivity of compounds 20, 22, 23 and 25 at αxβ3 and αxβ3γ2 $GABA_A$ receptors: (A), (C), (E), (G) Concentration-dependent modulation of GABA EC3 current at α1β3 (■), α2β3 (▲), α3β3 (▼) and α5β3 (◆) receptors. (B), (D), (F), (H) Concentration-dependent modulation of GABA EC3 current at α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (▼), α4β3γ2 (○), α5β3γ2 (◆) and α6β3γ2 (*) receptors. Data represent means ± SEM ($n = 4–6$).

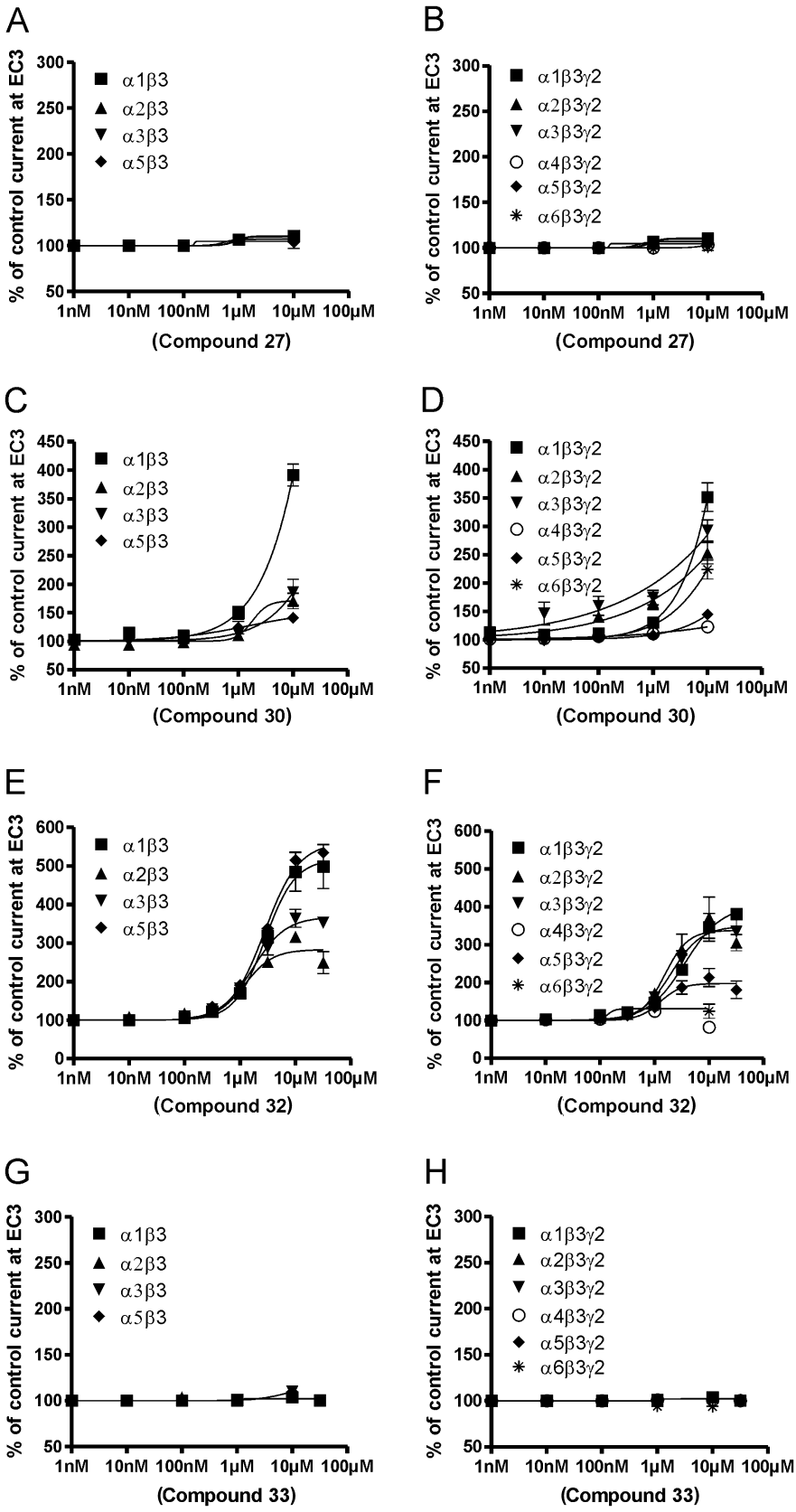


Figure 4

Alpha selectivity of compounds 27, 30, 32 and 33 at αxβ3 and αxβ3γ2 GABA$_A$ receptors: (A), (C), (E), (G). Concentration-dependent modulation of GABA EC3 current at α1β3 (■), α2β3 (▲), α3β3 (▼) and α5β3 (◆) receptors. (B), (D), (F), (H) Concentration-dependent modulation of GABA EC3 current at α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (▼), α4β3γ2 (○), α5β3γ2 (◆) and α6β3γ2 (*) receptors. Data represent means ± SEM (n = 4–6).

To obtain a rapid overview on the effects of these compounds at different $GABA_A$ receptor subtypes, concentration-activity curves were performed at αxβ3 and αxβ3γ2 receptors. Due to the relatively low potency as well as solubility problems of most of these compounds, it was not always possible to reach saturation of the effects observed (Figures 1–4). All positive α1+β3– modulators investigated were able to stimulate GABA-induced currents at αxβ3 as well as αxβ3γ2 receptors at concentrations ≥1 μM. In many cases, the extent of stimulation was comparable at αβ and αβγ receptors containing the same α subunit, indicating that stimulation by these compounds was not dependent on the presence of a benzodiazepine-binding site at the α+γ– interface. Interestingly, several compounds (1, 5, 6, 8, 11, 20, 22, 23) exhibited a much stronger stimulation at α6β3γ2 than at other receptor subtypes (Figures 1–3). Specifically, modulation by compound 5, or compound 6, (Figure 1F, H) of αxβ3γ2 receptors was functionally highly selective for α6β3γ2 receptors. Thus, compounds 5 and 6, to the best of our knowledge, are the first strongly α6β3γ2 receptor-selective ligands available. Compound 11, also displaying a relative preference for α6β3γ2 over other αxβ3γ2 receptors, was unique in possessing the highest efficacy at most receptors (Figure 2E, F, note the different scale). Compound 23, one of the α1+β3– site null modulators investigated, also exhibited no modulatory effects at all other receptors except α6β3γ2, where it was a weak positive modulator (Figure 3F). This functional selectivity for α6β3γ2 receptors was much weaker in compounds 2 and 7, and was lacking in compounds 17, 30 and 32. Interestingly, compound 32 was the only compound stimulating all subtypes except the α6β3γ2 receptors. Three of the four investigated α1+β3– null modulators (compounds 25, 27 and 33; Figures 3G, H; 4A, B and G, H, respectively) also did not modulate GABA-induced currents at all other receptors investigated. Details on the percentage of stimulation of GABA EC3 currents by all these compounds at all receptor subtypes are given in Supporting Information Table S1.

Effects of compounds on α6β3γ2 receptors are mediated via the α6+/β3– interface

The functional selectivity of compounds 1, 5, 6, 8, 11, 20, 22 and 23 for α6β3γ2 over other receptor subtypes was investigated further for compounds 1, 6 and 11. The strongest efficacy at α6β3γ2 receptors was observed by 10 μM of compound 11 (to 1871 ± 28% of GABA EC3). The most potent selective compound, however, was compound 6. This compound started to significantly stimulate GABA-induced currents at 10 nM concentration (133 ± 4%), and at 1 μM concentration, compound 6 stimulated α6β3γ2 receptors to 372 ± 13% of GABA EC3, whereas other receptors were barely modulated (Supporting Information Table S1).

To investigate whether the effects of compounds 11, 6 or 1 were mediated via the α6+β3– or the α6+γ2– interface, again steric hindrance studies were performed (Ramerstorfer *et al.*, 2011; Varagic *et al.*, 2013). Since both of these interfaces contain the α6 subunit, the steric hindrance could only be applied at the γ2– or the β3– side of the interface to distinguish between the two binding sites. Covalent labelling by MTSEA-biotin of the amino acid residue γ2M130C, located at the γ2– side of the benzodiazepine-binding pocket of α6β3γ2M130C receptors, had no effect on compound 11 action (Figure 5A), but drastically inhibited the slight modulatory effect of the benzodiazepine site ligand Ro15-1788 at α6β3γ2M130C receptors (Figure 5B). These data indicate that the modulatory effect of compound 11 at α6β3γ2 receptors (Figure 5A) is not mediated via the benzodiazepine-binding site. This conclusion was confirmed by steric hindrance experiments at the α+/β– interface. Introducing the β3Q64C mutation at the β– side of the α+/β– interface already reduced the effect of compound 11 at α6β3Q64Cγ2 receptors (Figure 5A). The subsequent covalent modification of the cysteine with the cysteine reactive reagent MTSEA-biotin significantly diminished the potentiation of α6β3Q64Cγ2 receptors by compound 11 even further (Figure 5A). On the other hand, the α6 modulatory effect of Ro15-1788 remained unaltered by both the β3Q64C mutation and its subsequent covalent modification by MTSEA-biotin (Figure 5B). These data indicate that the effects of compound 11 at α6β3γ2 receptors are mediated by its interaction with the α6+β3– interface, whereas the effects of Ro15-1788 are mediated via the benzodiazepine-binding site of these receptors.

Basically, compound 6 behaves as compound 11 in these experiments: covalent labelling by MTSEA-biotin of the amino acid residue γ2M130C, located at the γ2– side of the benzodiazepine-binding pocket of α6β3γ2M130C receptors could not inhibit the compound 6 effect (Figure 5C), while introducing the β3Q64C mutation at the β– side of the α+/β– interface already reduced the effect of compound 6 at α6β3Q64Cγ2 receptors (Figure 5C). The subsequent covalent modification of the cysteine with the cysteine reactive reagent MTSEA-biotin again significantly diminished the potentiation of α6β3Q64Cγ2 receptors by compound 6 (Figure 5C). The effect of compound 1 at α6β3γ2 receptors was also investigated in this manner, and was also largely reduced by steric hindrance via MTSEA-biotin modification of α6β3Q64Cγ2 receptors (Figure 5D).

To further investigate the modulatory effect of compound 11 and its dependence on the GABA concentration, we performed GABA concentration-response curves in the presence or absence of 10 μM compound 11 at α1β3γ2 and at α6β3γ2 receptors (Figure 6). GABA induced α1β3γ2 receptor currents with an EC_{50} of 36 μM and a Hill coefficient of 1.1. Interestingly, the shape of the GABA concentration-response curve was different at α6β3γ2 receptors, resulting in an EC_{50} of 28 μM and a Hill coefficient of 0.7. The difference in the shape of the concentration-response curves at the different receptor subtypes might have been caused by a heterogeneous population of receptors formed in α6β3γ2-injected *Xenopus* oocytes exhibiting distinct activation properties by GABA. Alternatively, GABA might have been able to distinguish between the two GABA-binding sites of α6β3γ2 receptors (Hadley and Amin, 2007). In any case, compound 11 caused a left shift in the GABA dose-response curves at α1β3γ2 as well as at α6β3γ2 receptors and this left shift, as expected, was more prominent at α6β3γ2 (EC_{50} = 4 μM; nH = 0.8) than at α1β3γ2 (EC_{50} = 14 μM μM; nH = 1.1) receptors (Figure 6A, B). Moreover, the percentage of stimulation by compound 11 was much stronger at GABA EC3 than at GABA EC20 at both receptors.

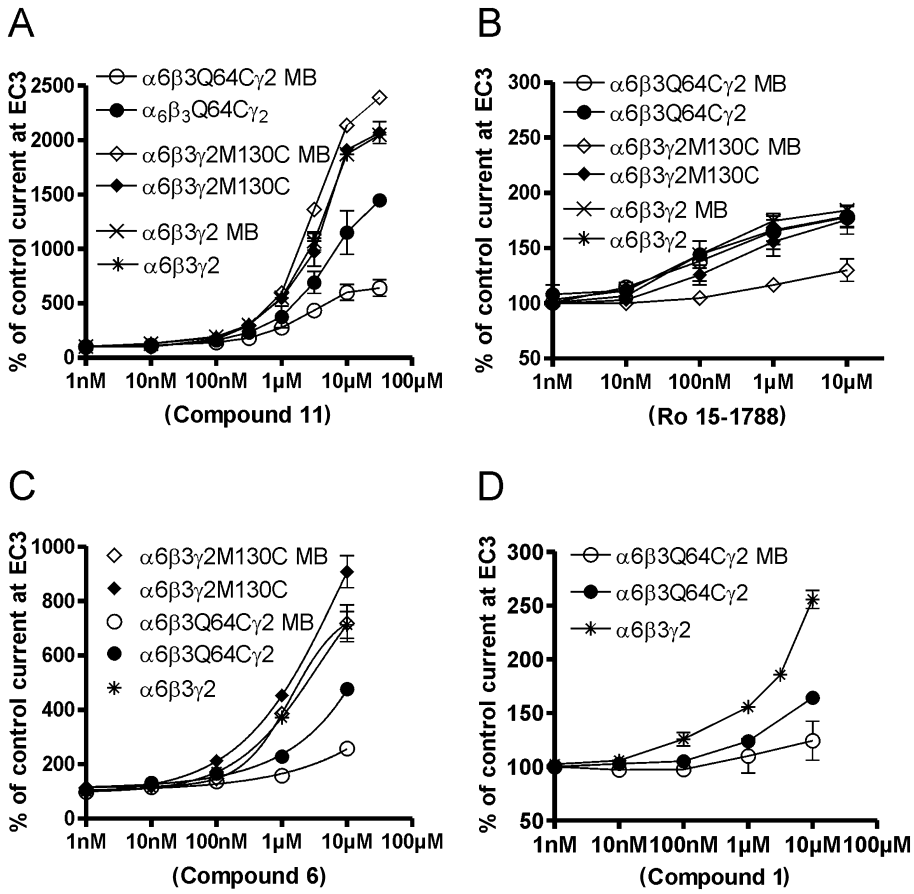


Figure 5

Evidence for the α6+/β3– binding site action of pyrazoloquinolinones: (A) Concentration-dependent modulation of GABA EC3 current by compound 11 at α6β3γ2 (*), α6β3Q64Cγ2 (●) and α6β3γ2M130C (◆) receptors before and after incubation with MTSEA-biotin: α6β3γ2 (x), α6β3Q64Cγ2 (○) and α6β3γ2M130CMB (◇). Significant reduction of the compound 11 effect on GABA EC3 was observed at α6β3Q64Cγ2 (10 nM–30 μM, $P < 0.05$) (●) as compared with wild-type α6β3γ2, which was more profound after incubation with MTSEA-biotin (○) (10 nM–30 μM, $P < 0.001$). Data represent means ± SEM ($n = 4$–11). (B) Concentration-dependent modulation of GABA control current by Ro15-1788 at α6β3γ2 (*), α6β3Q64Cγ2 (●) and α6β3γ2M130C (◆) receptors in the absence and presence of MTSEA-biotin (MB). A significant reduction of Ro15-1788 effects on GABA EC3 (100 nM–10 μM, $P < 0.05$) was observed only at α6β3γ2M130C labelled with MTSEA-biotin (◇). Data represent means ± SEM ($n = 5$–6); (C) Concentration-dependent modulation of GABA EC3 current by compound 6 at α6β3γ2 (*), α6β3Q64Cγ2 (●) and α6β3γ2M130C (◆) receptors and at α6β3Q64Cγ2 (○) and α6β3γ2M130C (◇) receptors after incubation with MTSEA-biotin (MB). Significant reduction of the effects of compound 6, as compared with wild-type α6β3γ2 was observed at α6β3Q64Cγ2 (100 nM–10 μM, $P < 0.05$) (●), which was more profound after the incubation with MTSEA-biotin (○) (100 nM–10 μM, $P < 0.001$). Data represent means ± SEM ($n = 4$–7). (D) Concentration-dependent modulation of GABA EC3 by compound 1 at α6β3γ2 or α6Q64Cβ3γ2 in the absence or presence of MTSEA-biotin. There is a significant difference between effects of compound 1 at α6β3γ2 (*) and α6Q64Cβ3γ2 (●) receptors (100 nM–10 μM, $P < 0.05$). The GABA EC3-enhancing effect of compound 1 was almost completely abolished at MTSEA-biotin-labelled α1Q64Cβ3γ2 MB receptors (100 nM–10 μM, $P < 0.001$) (○), whereas no changes were observed in the control experiment at wild α6β3γ2 receptors pre-incubated with MTSEA-biotin (MB; not shown). Data represent means ± SEM ($n = 4$–6).

Some of the compounds are null modulators at all receptor subtypes investigated

Compounds 25, 27 and 33 were not able to modulate GABA-induced currents at any one of the investigated receptors. These compounds, thus, are either non-binders or inactive binders (null modulators). In the accompanying manuscript (Varagic *et al.*, 2013), it was demonstrated that these compounds act as null modulators capable of inhibiting the effect of compound 11 at α1β3 receptors. To investigate whether these compounds are also null modulators at other receptor subtypes, compound 11 again was used as positive modulator, as it is stimulating GABA EC3 at all investigated receptor subtypes at 300 nM (α(1, 6)β3γ2) or at 1 μM (α(2, 3, 5)β3γ2) to a sufficient extent to clearly indicate inhibition or lack of inhibition when a candidate inhibitor is co-applied (Figure 7). α4β3γ2 receptors were not investigated as they are only weakly modulated by only two of the ligands investigated. It was demonstrated that 60 μM of compound 25 significantly inhibited the effects of compound 11 at all receptors investigated. The strongest inhibition was observed at α1β3γ2 receptors [% inhibition at α1: 72 ± 3% ($P < 0.001$); α2: 58 ± 25% ($P < 0.05$); α3: 23 ± 5% ($P < 0.05$); α5: 27 ± 2%

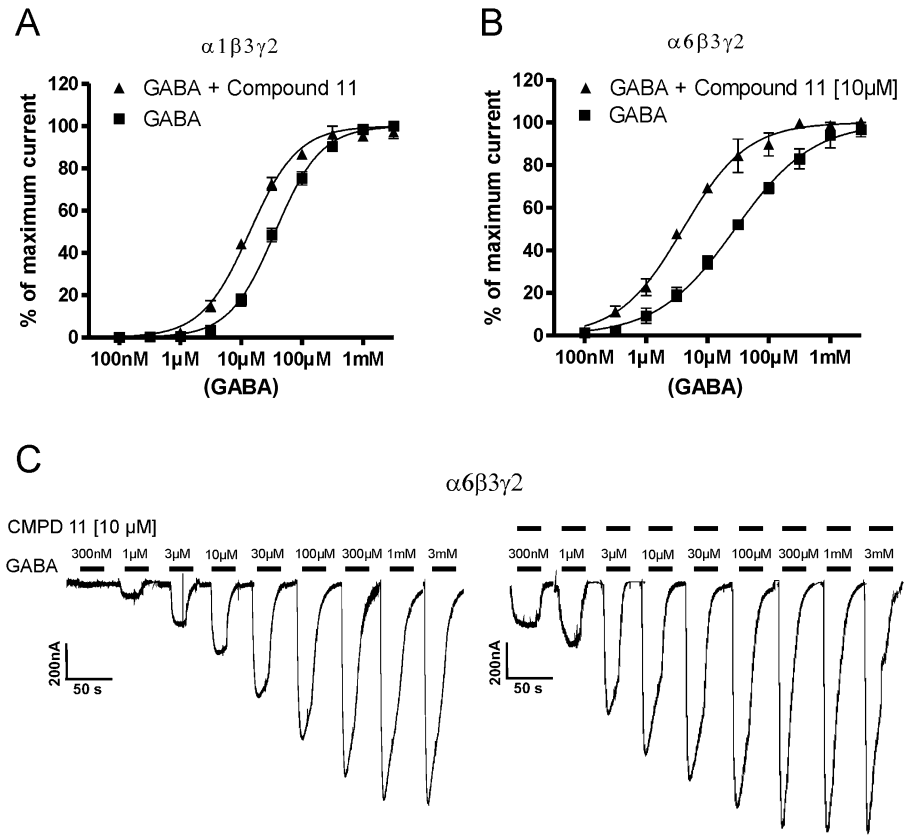


Figure 6

GABA concentration-response curves in the absence (■) or presence (▲) of 10 μM compound 11 at α1β3γ2 (A) and at α6β3γ2 receptors (B); effects are normalized to the maximum evoked GABA current. Compound 11 (10 μM) evokes a left shift of the GABA EC50 value from 36 to 14 μM ($P < 0.001$) at α1β3γ2 receptors (A), and from 28 to 4 μM ($P < 0.001$) at α6β3γ2 receptors; (C) individual current traces of the increasing GABA concentration response in the absence or presence of 10 μM compound 11 at α6β3γ2 receptors. The experiments were performed four to six times in different oocytes.

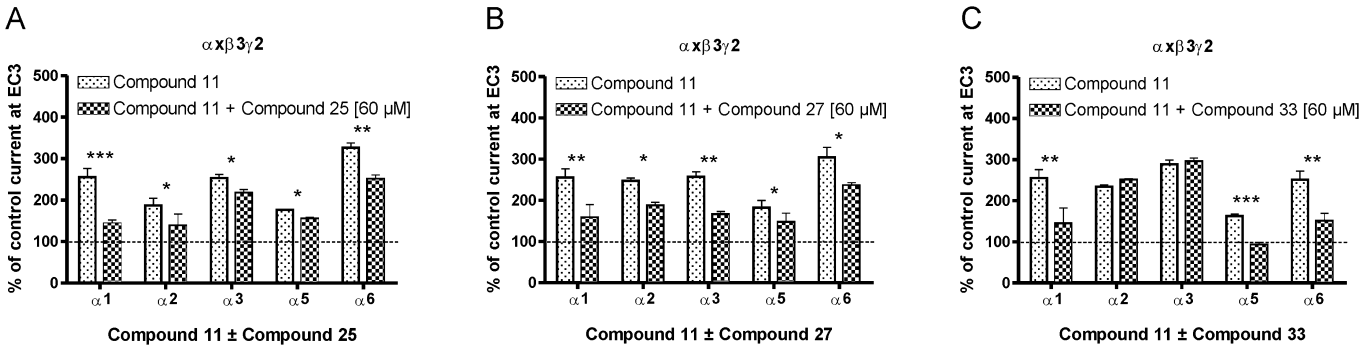


Figure 7

Several pyrazoloquinolinones are null modulators at the αx+β3– binding site of αxβ3γ2 receptors. (A) The positive modulatory effect of compound 11 at 300 nM (α1 and α6) or at 1 μM (α2, α3, α4 and α5) at αxβ3γ2 receptors was significantly inhibited (*$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$) by the co-application of 60 μM compound 25, (B) compound 27, or (C) compound 33. Compound 33 failed to inhibit the positive modulatory effects of 1 μM compound 11 at α2β3γ2 and α3β3γ2 receptors. Data are expressed as means ± SEM (n = 4–6).

($P < 0.05$); α6: 44 ± 11% ($P < 0.01$; Figure 7A)]. Similarly, compound 27 significantly inhibited the effects of compound 11 at all receptors investigated, although to a slightly different extent [% inhibition at α1: 64 ± 8% ($P < 0.01$); α2: 41 ± 8% ($P < 0.05$); α3: 56 ± 7% ($P < 0.01$); α5: 45 ± 14% ($P < 0.05$); α6: 32 ± 12% ($P < 0.05$;Figure 7B)]. Compound 33 strongly inhibited the effects of compound 11 at subtypes α(1, 5, 6)β3γ2 (% inhibition at α1: 72 ± 10% ($P < 0.01$); α5: 98 ± 1%

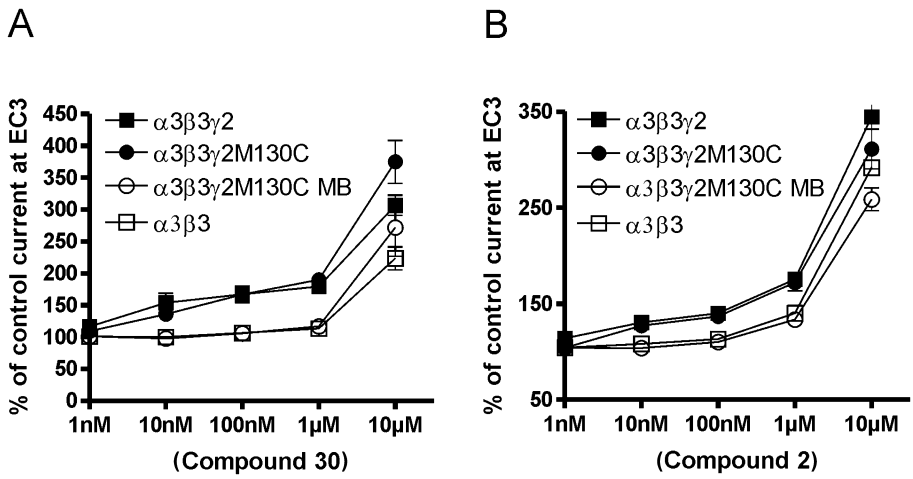


Figure 8

Compound 30 and compound 2 exhibit some high potency action via the benzodiazepine-binding site at α3-containing receptors. (A) Concentration-dependent modulation by compound 30 and (B) compound 2 of GABA EC3 at α3β3γ2 (■), α3β3γ2M130C (●) and MTSEA-biotin-labelled α3β3γ2M130C (○) receptors. The effect of both compounds at α3β3γ2 receptors were reduced significantly in MTSEA-biotin labeled α3β3γ2M130C receptors (10 nM–1 μM, $P < 0.05$) (○) to the effects observed at α3β3 (□) receptors. Data represent means ± SEM (n = 4–5).

($P < 0.001$); α6: 61 ± 4% ($P < 0.01$), while not inhibiting at all at α(2, 3)β3γ2 receptors (Figure 7C). This compound, thus, is a null modulator at α1β3γ2, α5β3γ2 and α6β3γ2 receptors, but presumably a non-binder at α2β3γ2 and α3β3γ2 receptors. Thus, not surprisingly, binding properties of null modulators are also receptor subtype dependent. In addition, compounds acting as null modulators at one receptor subtype, could also act as positive modulators at another receptor subtype. This is exemplified by compound 23, that is a null modulator at α1β3 receptors (Varagic *et al.*, 2013) but was able to positively modulate α6β3γ2 receptors (Figure 3F).

Some of the compounds exert part of their actions via the benzodiazepine site of α3β3γ2 receptors

Closer inspection of the dose-response curves of compound 30 and compound 2 at α3β3γ2 receptors indicated that enhancement of GABA-induced currents already occurred at low nM concentrations. Using the steric hindrance approach, it was shown previously that CGS 9895 exerts a weak nM stimulation of GABA EC3 current at α3β3γ2 receptor via the benzodiazepine-binding site (Ramerstorfer *et al.*, 2011). Similar experiments were now performed with compound 30 and compound 2. A steric hindrance introduced into the benzodiazepine-binding site by covalent labelling of γ2M130C with MTSEA-biotin could inhibit the nM effects of compound 30 and compound 2 at α3β3γ2M130C receptors (Figure 8A, B). These data indicate that compounds 30 or 2, like CGS 9895, are weak positive allosteric modulators at the benzodiazepine-binding site of receptors composed of α3β3γ2 subunits.

Effects of α+β– binding site ligands also depend on the β subunit type

In previous studies, it was demonstrated that CGS 9895 exhibited a weaker potentiation of GABA-induced current at receptors containing β1 subunits as compared to those containing β2 or β3 subunits (Ramerstorfer *et al.*, 2011). We thus also investigated the influence of the type of β subunit on the stimulation of GABA-induced currents at α(1–6)β(1–3)γ2 receptors by compound 11 and compound 6.

Compound 11 was found to cause weaker stimulation at αxβ2γ2 (Figure 9B) than at αxβ3γ2 receptors (Figure 2F). Especially, the very strong stimulation of α6β3γ2 receptors no longer was observed at the β2 containing receptor. At αxβ1γ2 receptors, compound 11 did not modulate GABA-induced currents for x = 1, 2 or 3, and weakly stimulated receptors containing α4, α5 or α6 subunits (Figure 9A, Supporting Information Table S2).

Compound 6 exhibited a weak stimulation at α(1, 3, 4, 5, 6)β1γ2 at 10 μM concentration, whereas at α2β1γ2 receptors, it was inactive (Figure 9C). At αxβ2γ2 receptors, stimulation by compound 6 was comparable to that observed at αxβ3γ2 receptors (for x = 1, 2, 4, 5; Figures 1H and 9D, Supporting Information Table S2), but was slightly stronger at α3β2γ2 and weaker at α6β2γ2 receptors as compared to the respective receptors containing a β3 subunit. These data suggest that the strong selectivity of compounds 11 and 6 for α6β3γ2 receptors is not only influenced by the unique properties of the α6 subunit but also by determinants of the β minus side of the interface.

Discussion

In this study, the effects of 13 pyrazoloquinolinones and three pyrazolopyridinones were functionally investigated at various recombinant GABA$_A$ receptor subtypes. We demonstrated that 10 of the pyrazoloquinolinones and two pyrazolopyridinones were positive allosteric modulators at most of the receptor subtypes investigated, whereas three pyrazoloquinolinones and one pyrazolopyridinone did not modulate

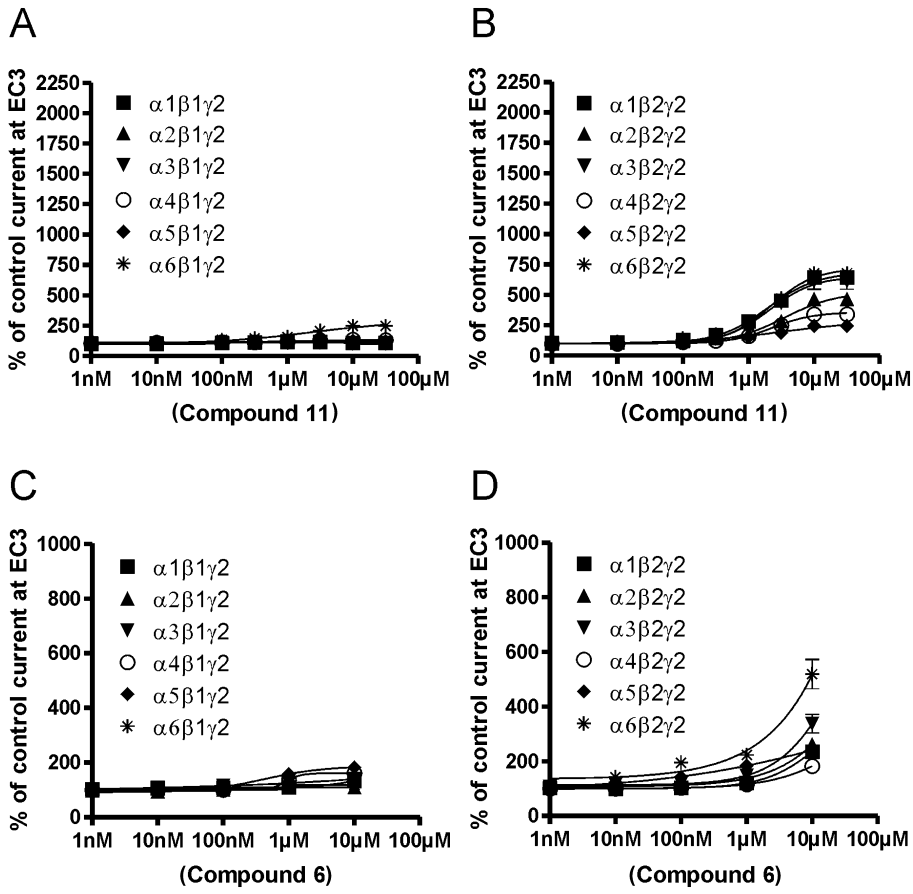


Figure 9

Effects of compound 11 and compound 6 on αxβ1γ2 and αxβ2γ2 receptors: (A) concentration-dependent modulation of GABA EC3 by compound 11 at α1β1γ2 (■), α2β1γ2 (▲), α3β1γ2 (▼), α4β1γ2 (○), α5β1γ2 (◆) and α6β1γ2 (*) receptors; (B) concentration-dependent modulation of GABA EC3 by compound 11 at α1β2γ 2 (■), α2β2γ2 (▲), α3β2γ2 (▼), α4β2γ2 (○), α5β2γ2 (◆) and α6β2γ2 (*) receptors; (C) concentration-dependent modulation of GABA EC3 by compound 6 at α1β1γ2 (■), α2β1γ2 (▲), α3β1γ2 (▼), α4β1γ2 (○), α5β1γ2 (◆) and α6β1γ2 (*) receptors; (D) concentration-dependent modulation of GABA EC3 by compound 6 at α1β2γ2 (■), α2β2γ2 (▲), α3β2γ2 (▼), α4β2γ2 (○), α5β2γ2 (◆) and α6β2γ2 (*) receptors; data represent means ± SEM (n = 4–11).

GABA-induced currents at most of the receptor subtypes. Ten of the 12 positive allosteric modulators (nine pyrazoloquinolinones and one pyrazolopyridinone) had been demonstrated previously to exhibit a high affinity for the benzodiazepine-binding site of $GABA_A$ receptors (He *et al.*, 1999; Yu *et al.*, 1999; He, 2000). Nevertheless, stimulation of GABA-induced currents by these positive modulators was of low potency and more or less comparable at most αxβ3 and αxβ3γ2 receptors containing the same α subunit type, supporting the conclusion that allosteric modulation did not depend on the presence of a benzodiazepine-binding site at the αx+γ2– interface. Steric hindrance experiments performed in the accompanying study (Varagic *et al.*, 2013) and in a previous study (Ramerstorfer *et al.*, 2011) indicated that compounds 11 and CGS 9895, respectively, are mediating their effects via the α1+β3– interface. In the present study, we performed similar steric hindrance experiments with the effects of compounds 11, 6 and 1, at α6β3γ2 receptors (Figure 5). Again, we demonstrated that these compounds mediate their effects at α6β3γ2 receptors via the α6+β3– interface. Finally, we demonstrated that three of the four α1+β3– null modulators (Varagic *et al.*, 2013) that were investigated in the present study were able to inhibit the αx+β3– effects of compound 11 at most αxβ3γ2 receptors investigated (Figure 7). We conclude that all the structurally similar pyrazoloquinolinones and pyrazolopyridinones investigated in this study mediate most of their actions via a binding site at the αx+β3– interface. This interpretation, as well as the absence of a contribution of, or crosstalk with, the benzodiazepine-binding site located at the αx+γ2– interface is further supported by experiments showing that the effects of CGS 9895, and of compounds 11 and 6 on α(1,6)β3γ2 receptors could not be inhibited by the benzodiazepine site null modulator Ro15-1788 or by steric hindrance via the benzodiazepine-binding site (Ramerstorfer *et al.*, 2011; Varagic *et al.*, 2013, and Figure 5A, C in this manuscript). The rather similar modulation by most of the investigated compounds of αxβ3 and αxβ3γ2 receptors containing the same α subunit thus indicates that presumably binding of these compounds to a single αx+β3– site is sufficient to generate the maximal effect that can be elicited by the compound at the respective receptor subtype.

Some additional, much smaller effects cannot be attributed to this interface. For the special cases of compounds 2 and 30, exhibiting a weak stimulation of GABA-induced currents at nM concentrations at α3β3γ2 receptors, steric hindrance experiments indicated that this stimulation was due to an effect mediated via the benzodiazepine-binding site of α3β3γ2 receptors (Figure 8A, B). These data, as well as similar observations for CGS 9895 (Ramerstorfer *et al.*, 2011) indicate that some of the investigated compounds that are null modulators at the benzodiazepine-binding site of most αxβ3γ2 receptor subtypes, can be weak positive allosteric modulators at α3β3γ2 receptors.

Although exhibiting low affinity for the benzodiazepine-binding site, compounds 8 (Figure 2C, D) and 32 (Figure 4E, F; Table 1) are positive allosteric modulators at αxβ3 and αxβ3γ2 receptors. These compounds, thus, seem to generate most of their effects via the αx+β3– interface. Interestingly, compound 8, as well as compound 30, exhibit an effect nearly twice as high at α1β3 as at α1β3γ2 receptors. We did not follow up on this experimentally and thus can only hypothesize that in this special case, either an additional binding site such as the second αx+β3– site, the β+β– site of α1β3 receptors, or the presence of a γ2 subunit might have contributed to the effects of these compounds.

Not only the α subtype determines compound action, our observations on compounds 11 and 6 give insight into β selective effects. Whereas replacement of β3 by the β2 subunit did not dramatically reduce the stimulation of compound 11 at αxβ2γ2 receptors (x = 1–5), the efficacy of compound 11 for the stimulation of α6β2γ2 was drastically reduced compared to that observed at α1β3γ2 receptors. Compound 11 behaved as a weak allosteric modulator at all αxβ1γ2 receptors stimulating GABA-induced currents only up to 250% of GABA EC3. Similar observations were made with compound 6. Thus, in agreement with previous studies on CGS 9895 (Ramerstorfer *et al.*, 2011), the allosteric modulation of GABA-induced currents is much weaker at receptors containing β1, than at those containing β2, or β3, subunits.

The most interesting compound in terms of future research applications is the highly α6β2/3γ2 selective compound 6. As many of our compounds show some degree of selectivity for α6β3γ2 receptors, it is rewarding to search for structural features that correlate with this functional selectivity. In the accompanying manuscript (Varagic *et al.*, 2013), it was observed that substituents in position R_7 are detrimental for modulation via the α1+β3– site. Compounds 5 and 6 possess OMe in this position – and stimulate α6β3γ2 receptors with very high functional selectivity. Thus, this R_7 substituent seems to be a highly selective match for the α6+/β3– binding pocket's unique shape. Compound 8, also featuring some functional preference for α6β3γ2 receptors, exhibits a tBu group in R_6. Unfortunately, no further analogue for this compound was available in this study. If the whole group of α6β3γ2 receptor preferring compounds 5, 6, 8, 20, 22 are considered, it becomes evident that the overall substitution pattern on ring A must be in a pocket position, where differences between α subtypes can be targeted specifically. Surprisingly, all these compounds show nearly no modulatory effect on α4β3γ2 receptors, although the α4 subunit exhibits the highest homology to the α6 subunit (Olsen and Sieghart, 2008). These data clearly indicate that the structure of α4β3γ2 and α6β3γ2 receptors is sufficiently different to allow the development of compounds, which exhibit distinct interactions with these receptor subtypes. Thus, further exploration of a broader range of analogues of the investigated compounds will be helpful in gaining selective compounds and insight into selective interactions at different receptor subtypes.

Conclusion

The present results confirm and support previous evidence (Ramerstorfer *et al.*, 2011) that pyrazoloquinolinones are predominantly mediating their effects via a binding site at the αx+β3– interface present at αxβ3 and αxβ3γ2 receptors. Although some of them additionally exhibit a high affinity at the benzodiazepine-binding site of $GABA_A$ receptors, these compounds in most cases are acting as null modulators or weak allosteric modulators via this site. Three prototypes of compounds were identified in this study. Compound 11 exhibits the strongest modulation of all receptor subtypes investigated already at a concentration of 300 nM–1 μM. Although the efficacy of this compound for enhancing GABA-induced currents varies in different receptor subtype, it is still strong enough for an efficient modulation of each subtype. Compounds such as compound 11 not only should interact with αβγ, but also with αβ, αβδ, αβε, αβπ or αβθ receptors and should thus be ideally suited as anticonvulsants. By interacting with a larger number of receptor subtypes, such compounds enhance GABAergic inhibition on more targets, and thus might be superior in reducing overexcitation in neurons that cannot be sufficiently modulated by benzodiazepines (Sieghart *et al.*, 2012).

The second prototype of drugs is represented by the null modulators identified in this and the accompanying study (Varagic *et al.*, 2013). Null modulators not only are essential for terminating all effects of positive or negative allosteric modulators in experimental (or clinical) studies, but are also excellent tools for the identification of novel compounds mediating their action via the αx+β3– interface. Finally, they can exclude or confirm a possible interaction of compounds with the αx+β3– interface in cases where the exact site of interaction of compounds is not known. Interestingly, compounds 25, 27 and 33 were found to be null modulators with specific and unique subtype profiles.

The third prototype of drugs identified in this study is represented by compound 6, the strongest α6β2/3γ2-selective compound available. This is the first report of a compound that acts selectively at this target. This compound can now be used to investigate the function of α6-containing receptors in brain tissue and in living animals. Such receptors exclusively are located in the granule cells of cerebellum and in the cochlea (Gutiérrez *et al.*, 1996; Pirker *et al.*, 2000). A selective modulation of these receptors, thus, will produce very limited and selective effects in the brain and will allow the study of the function of α6β2/3γ2 receptors in the respective cell types. Granule cells in cerebellum are important for motor coordination and for cerebellar learning and memory (Thompson and Steinmetz, 2009; Gao *et al.*, 2012). The function of the α6β3γ2 receptors in the cochlea to the best of our knowledge is not known (Maison *et al.*, 2006). Compound 6

will thus be invaluable for a further dissection of the neuronal circuits in the cerebellum and cochlea. Although the compounds so far available are not very potent, their potency is sufficient to demonstrate their effectiveness in various animal models of epilepsy and to determine the function of α6β3γ2 receptors in the brain. Nevertheless, future efforts along the lines discussed above have to be undertaken to develop compounds with higher potency and receptor subtype selectivity that possibly could be fundamental for the development of new therapies for various diseases.

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Conflict of interest

The authors state no conflict of interest.

References

Brown C, Martin I, Jones B, Oakley N (1984). *In vivo* determination of efficacy of pyrazoloquinolinones at the benzodiazepine receptor. Eur J Pharmacol 103: 139–143.

Ebert V, Scholze P, Sieghart W (1996). Extensive heterogeneity of recombinant gamma-aminobutyric acid A receptors expressed in alpha 4 beta 3 gamma 2-transfected human embryonic kidney 293 cells. Neuropharmacology 35: 1323–1330.

Gao Z, van Beugen BJ, De Zeeuw CL (2012). Distributed synergistic plasticity and cerebellar learning. Nat Rev Neurosci 13: 619–635.

Gutiérrez A, Khan ZU, De Blas AL (1996). Immunocytochemical localization of the α6 subunit of the γ-aminobutyric acid$_A$ receptor in the rat nervous system. J Comp Neurol 365: 504–510.

Hadley SH, Amin J (2007). Rat alpha6beta2delta GABA$_A$ receptors exhibit two distinct and separable agonist affinities. J Physiol 581: 1001–1018.

He X (2000). Studies of Molecular Pharmacophore/Receptor Models for GABAA/BZR Subtypes: Chemical and Computer Assisted Approach in Search of Selective Ligands for GABAA/BZR Subtypes, PhD Thesis, UW-Milwaukee, USA.

He X, Huang Q, Ma C, Yu S, McKernan R, Cook JM (1999). Pharmacophore/receptor models for GABA$_A$/BzR alpha2beta3gamma2, alpha3beta3gamma2 and alpha4beta3gamma2 recombinant subtypes. Included volume analysis and comparison to alpha1beta3gamma2, alpha5beta3gamma2, and alpha6beta3gamma2 subtypes. Drug Des Discov 17: 131–171.

Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR (1993). Gene splicing by overlap extension. Methods Enzymol 217: 270–279.

Li X, Cao H, Zhang C, Furtmueller R, Fuchs K, Huck S *et al.* (2003). Synthesis, in vitro affinity, and efficacy of a bis 8-ethynyl-4H-imidazo[1,5a] – [1,4]benzodiazepine analogue, the first bivalent alpha5 subtype selective BzR/GABA$_A$ antagonist. J Med Chem 46: 5567–5570.

Loo P, Braunwalder A, Wood PL (1987). Benzodiazepine receptor modulation of [^{35}S]TBPS binding to the chloride channel. Noncompetitive inhibition of classical benzodiazepines and competitive inhibition of the partial agonist, CGS 9895, by CGS 8216. Neuropharmacology 26: 775–778.

Maison SF, Rosahl TW, Homanics GE, Liberman MC (2006). Functional role of GABAergic innervation of the cochlea: phenotypic analysis of mice lacking GABA$_A$ receptor subunits alpha 1, alpha 2, alpha 5, alpha 6, beta 2, beta 3, or delta. J Neurosci 26: 10315–10326.

Ogris W, Poltl A, Hauer B, Ernst M, Oberto A, Wulff P *et al.* (2004). Affinity of various benzodiazepine site ligands in mice with a point mutation in the GABA$_A$ receptor gamma2 subunit. Biochem Pharmacol 68: 1621–1629.

Olsen RW, Sieghart W (2008). International Union of Pharmacology. LXX. Subtypes of GABA$_A$ receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev 60: 243–260.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000). GABA$_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101: 815–850.

Ramerstorfer J, Furtmuller R, Vogel E, Huck S, Sieghart W (2010). The point mutation gamma 2F771 changes the potency and efficacy of benzodiazepine site ligands in different GABA$_A$ receptor subtypes. Eur J Pharmacol 636: 18–27.

Ramerstorfer J, Furtmuller R, Sarto-Jackson I, Varagic Z, Sieghart W, Ernst M (2011). The GABA$_A$ receptor alpha+beta-interface: a novel target for subtype selective drugs. J Neurosci 31: 870–877.

Richter L, de Graaf C, Sieghart W, Varagic Z, Morzinger M, de Esch IJ *et al.* (2012). Diazepam-bound GABA$_A$ receptor models identify new benzodiazepine binding-site ligands. Nat Chem Biol 8: 455–464.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM *et al.* (1999). Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature 401: 796–800.

Sieghart W (1995). Structure and pharmacology of gamma-aminobutyric acid$_A$ receptor subtypes. Pharmacol Rev 47: 181–234.

Sieghart W, Ernst M (2005). Heterogeneity of GABA$_A$ receptors: revived interest in the development of subtype-selective drugs. Curr Med Chem 5: 217–242.

Sieghart W, Ramerstorfer J, Sarto-Jackson I, Varagic Z, Ernst M (2012). A novel GABA(A) receptor pharmacology: drugs interacting with the alpha+beta-interface. Br J Pharmacol 166: 476–485.

Sigel E (2002). Mapping of the benzodiazepine recognition site on GABA$_A$ receptors. Curr Top Med Chem 2: 833–839.

Sigel E, Baur R, Trube G, Möhler H, Malherbe P (1990). The effect of subunit composition of rat brain GABA$_A$ receptors on channel function. Neuron 5: 705–711.

Smith AJ, Alder L, Silk J, Adkins C, Fletcher AE, Scales T *et al.* (2001). Effect of alpha subunit on allosteric modulation of ion channel function in stably expressed human recombinant gamma-aminobutyric acid(A) receptors determined using (36)Cl ion flux. Mol Pharmacol 59: 1108–1118.

Thompson RF, Steinmetz JE (2009). The role of the cerebellum in classical conditioning of discrete behavioral responses. Neuroscience 162: 732–755.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997). Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J Neurosci 17: 2728–2737.

Varagic Z, Wimmer L, Schnürch M, Mihovilovic MD, Huang S, Rallapalli S *et al.* (2013). Identification of novel positive allosteric modulators and null modulators at the $GABA_A$ receptor α+β– interface. Br J Pharmacol 169: 371–383.

Williams M, Bennett DA, Loo PS, Braunwalder AF, Amrick CL, Wilson DE *et al.* (1989). CGS 20625, a novel pyrazolopyridine anxiolytic. J Pharmacol Exp Ther 248: 89–96.

Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci 12: 1040–1062.

Yu S, He X, Ma C, McKernan R, Cook JM (1999). Studies in search of α2 selective ligands for $GABA_A$/BzR receptor subtypes. Part 1. Evidence for the conservation of pharmacophoric descriptors for DS subtypes. Med Chem Res 9: 186–202.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Efficacy (% of modulation of control GABA EC3 current = 100%) of pyrazoloquinolinones/ pyrazolopyridinones at recombinant rat αxβ3/αxβ3γ2 receptors expressed in Xenopus laevis oocytes.

Table S2 Efficacy of compound 11 and compound 6 at recombinant rat αxβ1γ2 and αxβ2γ2 receptors expressed in Xenopus laevis oocytes.