TOWARDS FUNCTIONAL SELECTIVITY FOR $\alpha_6\beta_3\gamma_2$ GABA$_A$ RECEPTORS: A SERIES OF NOVEL PYRAZOLOQUINOLINONES

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BACKGROUND AND PURPOSE
The GABA$_A$ receptors are ligand-gated ion channels, which play an important role in neurotransmission. Their variety of binding sites serves as an appealing target for many clinically relevant drugs. Here, we explored the functional selectivity of modulatory effects at specific extracellular $\alpha^+/\beta^−$ interfaces, using a systematically varied series of pyrazoloquinolinones.

EXPERIMENTAL APPROACH
Recombinant GABA$_A$ receptors were expressed in Xenopus laevis oocytes and modulatory effects on GABA-elicited currents by the newly synthesized and reference compounds were investigated by the two-electrode voltage clamp method.

KEY RESULTS
We identified a new compound which, to the best of our knowledge, shows the highest functional selectivity for positive modulation at $\alpha_6\beta_3\gamma_2$ GABA$_A$ receptors with nearly no residual activity at the other $\alpha_x\beta_3\gamma_2$ ($x = 1−5$) subtypes. This modulation was independent of affinity for $\alpha^+/\gamma^−$ interfaces. Furthermore, we demonstrated for the first time a compound that elicits a negative modulation at specific extracellular $\alpha^+/\beta^−$ interfaces.

CONCLUSION AND IMPLICATIONS
These results constitute a major step towards a potential selective positive modulation of certain $\alpha_6$-containing GABA$_A$ receptors, which might be useful to elicit their physiological role. Furthermore, these studies pave the way towards insights into molecular principles that drive positive versus negative allosteric modulation of specific GABA$_A$ receptor isoforms.

Abbreviations
BZD, benzodiazepine; CGC, cerebellar granule cells; PAM, positive allosteric modulation; PQ, pyrazoloquinolinone
Introduction

Pyrazoloquinolinones (PQs) have been studied since the 1980s for their benzodiazepine (BZD) receptor affinity (Yokoyama et al., 1982). The prototypical PQ, CGS8216 (all residues = H; see scaffold in Figure 1), displaced [3H]-flunitrazepam and [3H]-diazepam binding at subnanomolar concentrations (Czernik et al., 1982), was devoid of in vivo BZD-like activity (Gee and Yamamura, 1982) and showed proconvulsant actions in mice (File, 1983). Residue modifications led to varying neurochemical and behavioural properties. For example, compound 1 (CGS9895; R4’ = methoxy; see Figure 1) (Ramerstorfer et al., 2011; Varagic et al., 2013b) not only antagonized the effects of diazepam but also showed anxiolytic and weak anticonvulsant activity while being devoid of sedation, muscle relaxation or motor impairments (Bennett et al., 1985; Bennett, 1987). These findings encouraged the possibility of separately eliciting only specific desired BZD-like effects (Kolata, 1982). Early on, researchers noted a dissociation between BZD receptor activity and the in vivo pharmacology of PQs (Bennett, 1987; Cooper et al., 1987).

The most abundant GABA\textsubscript{A} receptor types are composed of one \( \gamma \)2, two \( \alpha \) and two \( \beta \) subunits, forming pentameric GABA-gated chloride channels (Olsen and Sieghart, 2008). They are modulated by several allosteric ligand binding sites (Puthenkalam et al., 2016), including the BZD binding site at extracellular \( \alpha+\gamma \)/C0 interfaces (Sigel, 2002; Ernst et al., 2003), and a modulatory PQ binding site at \( \alpha+\beta \)/C0 interfaces (Ramerstorfer et al., 2011). The demonstration that PQs are high affinity null modulators at BZD binding sites and low potency allosteric modulators at modulatory PQ binding sites (Ramerstorfer et al., 2011) helped explain their complex pharmacology and inspired a new search for GABA\textsubscript{A} receptor subtype-specific modulators (Sieghart et al., 2012). Importantly, compound 1 did not require BZD-site binding for its modulatory effects, as GABA current enhancements were almost identical in \( \alpha1\beta2 \) versus \( \alpha1\beta3\gamma2 \) receptors (Ramerstorfer et al., 2011), as well as in \( \alpha1\beta2 \) versus \( \alpha1\beta2\gamma2 \) receptors (Maldifassi et al., 2016), and obstructing the BZD-site with a steric hindrance approach did not diminish compound 1 current modulation in \( \alpha1\beta3\gamma2 \) receptors (Ramerstorfer et al., 2011). A recent study employing \( \alpha1\gamma209 \) point mutations confirmed the modulatory PQ binding site (Maldifassi et al., 2016).

GABA\textsubscript{A} receptor \( \alpha6 \) subunits are highly expressed in cerebellar granule cells (CGCs) (Hörtnagl et al., 2013) and have long been studied for their presumed contribution to the intoxicating effects of ethanol (Valenzuela and Jotty, 2015). Recently, they gained additional interest as potential targets in pain, motor tic and depressive disorders (Puri et al., 2012; Kramer and Bellinger, 2013; Liao et al., 2016; Yang et al., 2016).

Here, we expand on previously identified \( \alpha6\beta3\gamma2 \) preferring PQs (Varagic et al., 2013a) and present, to the best of our knowledge, the most effective selective compound for this subtype discovered so far, compound 3 (LAU159). A substituent variation to the o-methoxy [compound 4 (LAU165), see Figure 1 for nomenclature] completely abolishes the positive modulation at all \( \alpha x\beta3\gamma2 \) (\( x = 1–6 \))

Figure 1

PQ structures. Top left: PQ scaffold with labels for rings A to D and residue numbering (R7 and R8 on ring A; R2’ (o), R3’ (m), R4’ (p) on ring D). Top row: 1 is a p-methoxy compound with unsubstituted ring A. R2 = chloro compounds are derived from varying the position of the methoxy group at ring D. Bottom row: compound variants with different residues in R3 m-position.
receptors, providing a potential negative control compound for future in vivo studies. Variation of the functional group in the m-position on ring D led to the first negative modulator (9) at the modulatory PQ site in some receptor isoforms.

**Methods**

**Two-electrode voltage clamp electrophysiology**

Preparation of mRNA for rat α1, α2, α3, α4, α5, α6, β3 and γ2 subunits and electrophysiological experiments with *Xenopus laevis* oocytes were performed as described previously (Forkuo et al., 2016). Mature female *X. laevis* (Nasco, Fort Atkinson, WI, USA) were anaesthetized in a bath of ice-cold 0.17% Tricain (Ethyl-m-aminobenzoate, Sigma-Aldrich, St. Louis, MO, USA) before decapitation and transfer of the frog’s ovary to ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES; pH 7.5). From a total of at most 35 toads, cells were used for this and other parallel studies, reflecting the total number of weeks of data collection. Tissue was obtained in accordance with the rules of the Austrian animal protection law and Austrian animal experiment by-laws, which implement European Directive 2010/63/EU and complies with the ARRIVE guidelines. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015). Following incubation in 1 mg·mL−1 collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, stage 5 to 6 oocytes were dissected out of the ovary and defolliculated using a platinum wire loop or glass Pasteur pipette. Oocytes were stored and incubated at 18°C in NDE medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, 1.8 mM CaCl2; pH 7.5) that was supplemented with 100 U·mL−1 penicillin, 100 μg·mL−1 streptomycin and 2.5 mM pyruvate. Oocytes were injected with an aqueous solution of mRNA. A total of 2.5 ng of mRNA per oocyte was injected. The subunit ratio was 1:1:5 for α1:α2:α6 for 2 receptors. In some cases, concatenated constructs were used (Minier and Sigel, 2004), which was a kind gift of E. Sigel. Specifically, cRNA coding for one triple concatamer γ2-β3-α6 was co-injected in oocytes with cRNA coding for one double concatamer β3-α6 in a 1:1 ratio, and currents confirmed to be equally modulated by PZ-II-029 as nonconcatenated α6β3γ2. Injected oocytes were incubated for at least 3 h before electrophysiological recordings. Oocytes were placed on a nylon-grid in a bath of NDE medium. For current measurements, oocytes were impaled with two microelectrodes which were filled with 2 M KCl and had a resistance of 2–3 MΩ. The oocytes were constantly washed by a flow of 6 mL·min−1 NDE that could be switched to NDE containing GABA and/or drugs. Drugs were diluted in the NDE from DMSO solutions resulting in a final concentration of 0.1% DMSO. To test the effects of the compounds on the GABA-induced currents, a GABA concentration was titrated to trigger 3–5% of the respective maximum GABA-elicited current of the individual oocyte (EC50,3) and was applied to the cell together with various concentrations of test compounds. All recordings were performed at room temperature at a holding potential of −60 mV using a Warner OC-725C two-electrode voltage clamp (TEV) (Warner Instrument, Hamden, CT, USA) or a Dagan CA-1B Oocyte Clamp or a Dagan TEV-200A TEV amplifier (Dagan Corporation, Minneaplis, MN, USA). Data were digitized using a Digidata 1322A or 1550 data acquisition system (Axon Instruments, Union City, CA, USA), recorded using Clampfit 10.5 and GraphPad Prism 6.0 (La Jolla, CA, USA) software. Concentration-response data were fitted using the Hill equation. Data are given as mean ± SEM from at least three oocytes from two batches.

**Radioligand displacement assays**

Radioligand displacement experiments were performed using 12- to 16-week-old female Sprague–Dawley rats (Institute for Biomedical Research, Medical University of Vienna, Himberg, Austria). In total, 10 rats were used. All animals were group housed (six animals per cage) at an ambient temperature of 21°C with a light : dark regime of 10:14 h, with ad libitum access to standard food and water. Rats were anaesthetized under CO2 and decapitated in accordance with the Guidelines of the Animal Care Committee of the Medical University of Vienna. The brains were removed immediately, and cerebellar membranes were prepared as described previously (Sieghart and Schuster, 1984). Displacement assays using [3H]-flunitrazepam were performed as described by Simeone et al. (2017). Displacement assays using [3H]-Ro15-4513 were performed as follows: In brief, membrane pellets were incubated for 90 min at 4°C in a total of 500 μL of a solution containing 50 mM Tris/citrate buffer, pH = 7.1, 150 mM NaCl and 5 nM [3H]Ro15-4513 in the presence of 50 μM diazepam (to saturate α1-containing receptors and target α6-containing receptors only) and in the absence or presence of either 100 μM Ro15-1788 (to determine non-specific binding) or various concentrations of receptor ligands (dissolved in DMSO, final DMSO-concentration 0.5%). Membranes were filtered through Whatman GF/B filters and washed twice with 4 mL of ice-cold 50 mM Tris/citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after the addition of 3 mL Rotiszint Eco plus liquid scintillation cocktail. Nonlinear regression analysis of the displacement curves used the equation: log(inhibitor) versus response (variable slope with Top = 100% and Bottom = 0%) Y = 100/(1 + 10^((logIC50-x)*Hill/slope)).

Saturation binding experiments were performed by incubating the membranes with various concentrations of [3H]-Ro15-4513 in the absence or presence of 50 μM diazepam and analysed using the equation Y = Bmax*X/(KD + X), and an equilibrium binding constant KD for rat cerebellum was determined (KD ± SEM n = 3 independent experiments): 1.4 ± 0.1 nM.

IC50 values were converted to Ki values using the Cheng–Prusoff relationship Ki = IC50/(1 + (S/KD)) with S being the concentration of the radioligand (5 nM) and the KD value described above (1.4 nM). All analyses were performed using GraphPad Prism version 7 for PC, GraphPad Software, La Jolla, California, USA, www.graphpad.com.

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**Data and statistical analysis**

All results are expressed as mean ± SEM. The n number stated represents the number of single oocyte experiments. In cases of inactivity, a number of n = 2 was considered as adequate, whereas statistical analysis was performed with a number of n ≥ 5 only. Data were analysed with GraphPad Prism 6.0 and evaluated with one-way ANOVA with Dunnnett’s multiple comparison test. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

**Materials**

Conformation analysis. Conformational analysis of pyrazoloquinolinoine structures was performed using the Conformation Search application integrated in the MOE software [Molecular Operating Environment (MOE), Chemical Computing Group Inc., Montreal, QC, Canada].

Compound synthesis. Compounds 1 (CGS9895) and 2 (PZ-II-028) have been synthesized and published, recently. Synthesis of compounds 3 (LAU159), 4 (LAU165), 5 (DCBS142), 6 (DCBS32), 7 (DCBS146), 8 (DCBS120) and 9 (DCBS125A) was conducted in analogy to previously outlined synthetic routes (Fryer et al., 1993; Savini et al., 1993; Varagic et al., 2001; Varagic et al., 2013b) (see Supporting Information).

Compounds investigated. The following compounds were used: 3 (LAU159): 8-chloro-2-(3-methoxyphenyl)-2H,3H,5H-pyrazolo[4,3-c]quinolin-3-one; 4 (LAU165): 8-chloro-2-(2-methoxyphenyl)-2H,3H,5H-pyrazolo[4,3-c]quinolin-3-one; 5 (DCBS142): 8-chloro-2-(3-methylphenyl)-2H,3H,5H-pyrazolo[4,3-c]quinolin-3-one; 6 (DCBS32): 2-(3-bromophenyl)-8-chloro-2H,3H,5H-pyrazolo[4,3-c]quinolin-3-one; 7 (DCBS146): 3-(8-chloro-3-oxo-2H,3H,5H-pyrazolo[4,3-c]quinolin-2-yl)benzonitrile; 8 (DCBS120): 2-(3-aminophenyl)-8-chloro-2H,3H,5H-pyrazolo[4,3-c]quinolin-3-one; 9 (DCBS125A): 3-(8-chloro-3-oxo-2H,3H,5H-pyrazolo[4,3-c]quinolin-2-yl)benzoic acid.

**Nomenclature of targets and ligands**

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

**Results**

**Impact of R8 = chloro and of variations in the position of the ring D methoxy substitution on GABA_A receptor subtype modulatory profile**

Compound 1 positively modulates EC\textsubscript{3.5} GABA currents (effective concentration eliciting 3–5% of maximum GABA currents) in all αβ3γ2 (x = 1–6) subunit combinations (Ramerstorfer et al., 2011) (see Figure 2A). Compound 1 shows higher modulatory efficacy for αβ3γ2 receptor currents, which are enhanced to roughly 700% at 10 μM compound concentration. The remaining αγβ3γ2 (x = 1–5) subunit combinations are modulated to a lesser extent (see Figure 2A). Interestingly, upon introduction of an R8 = chloro residue, resulting in compound 2, maximum modulations are enhanced by about threefold, while preserving relative modulatory strength between the tested subunit combinations (see Figure 2B). Compound 2 maximum modulation amounts to ~2000% in the case of αβ3γ2 receptor currents and reaches ~1000% at α1β3γ2 and α2β3γ2 receptors (see Figure 2B). The fitted curves suggest that the presence of R8 = chloro does not induce a substantial left shift of the compound dose–response curves at any of the αβ3γ2 (x = 1–6) subunit combinations. Systematic structural variants of compound 2 were subsequently synthesized (see Figure 1), in order to explore how chemically diverse ring D substitutions would affect efficacy and potency in different subtypes.

Changing the methoxy residue on ring D to the m-position (3) completely abolished αβ3γ2 (x = 1–5) modulatory activity, while at αβ3γ2 around 300% modulation with an estimated EC\textsubscript{50} of 3.1 μM remained (see Figure 2C). At 1 μM compound 3, modulation amounted to 148.6 ± 10.5% [mean ± SEM, n = 13, control (GABA) = EC\textsubscript{3.5}]. Compound 3, while not acting as positive modulator in this subtype, still retained some affinity at the modulatory PQ binding site of α1β3γ2, as 10 and 30 μM reduced the modulatory effect of compound 2 (1 μM) at this receptor subtype (see Figure 2F) and thus is a null modulator in this subtype. Further moving the methoxy residue to the o-position (4) abolished any positive allosteric modulation (PAM) activity but led to weak negative modulatory activity at αβ3γ2 (see Figure 2D).

**Compound 4 does not bind at the α6+/β3– interface**

Analogously to compound 3 at α1β3γ2 (see Figure 2F), the lack of modulatory activity of compound 4 at α6β3γ2 (see Figure 2D) could mean that it is either a silent binder or a non-binder at the α6+/β3– modulatory PQ binding site. This cannot be determined using binding assays, because no radioactively labelled ligand with specificity for any of the modulatory α+/β– PQ sites is currently available. We therefore tested whether effects of compound 3 at α6β3γ2 receptors can be inhibited by co-application of compound 4. Up to 30 μM, compound 4 (which is the solubility limit in the assay buffer) was unable to abolish the modulatory effect elicited by compound 3 (3 μM) in this series (see Figure 3A, B).

Based on these results, we asked how the methoxy residue in o-position could possibly interfere with biological activity. The biological effect of a compound may not always arise from the conformation which is the energetic global minimum, but rather from an ensemble of conformations. We therefore performed a conformational analysis using the conformation search application provided by the MOE software package. Since pyrazoloquinolinoines are quite inflexible, we focused on the dihedral angle (φ) between the two plane surfaces of the rings A–C and ring D (see Figure 4). According to this analysis, the p- and m-methoxy substituted compounds possess one main conformation which displays co-planarity between rings A–C and ring D (φ = ~0.003° and φ = ~0.03°), whereas the o-methoxy substitution features 2 possible
conformations (syn and anti, see Supporting Information Figure S3). While the anti-conformation shows already 0 to 200 times stronger rotation between the plane surfaces, the syn-conformation leads to a significant rotation of the ring D up to a dihedral angle of 20° (see Figure 4C and Supporting Information Table S1). This rotation of ring D might be a reasonable explanation for the loss of activity of compound 4.

Since pyrazoloquinolinones are also high-affinity ligands at the benzodiazepine binding site (Ramerstorfer et al., 2011), we next performed radioligand displacement assays for compounds 2, 3 and 4 in cerebellar membranes, to determine their affinities at α1+/γ2− and α6+/γ2− sites (see Supporting Information Table S4). The results show that the tested compounds are high to very high affinity binders at the α1+/γ2− BZ site and moderate to high affinity binders at the diazepam-insensitive α6+/γ2− BZ site. Furthermore, we observed a decrease of affinity when changing the substituent from the p- to the o-position in α1+/γ2− and in α6+/γ2−.

Figure 2

(A–D) GABA<sub>B</sub> receptor subtype activity profile of compounds with systematically varying positions of the methoxy-group on ring D (compound 1, compound 2: p-methoxy; compound 3: m-methoxy; compound 4: o-methoxy) at αβγ<sub>x</sub>2 (x = 1–6) GABA<sub>B</sub> receptors. Y-axis shows % modulation of currents elicited by a GABA concentration amounting to 3−5% of maximum GABA currents per cell. For the purpose of structure–activity comparison, some datasets are reproduced: compound 1 at αβγ<sub>x</sub>2 (x = 1, 2, 3 and 5) reproduced with permission (Ramerstorfer et al., 2011). Compound 2 at αβγ<sub>x</sub>2 (x = 1–6) reproduced with permission (Varagic et al., 2013a). (E) Sample recording of GABA currents and co-application of increasing concentrations of compound 3, from an oocyte injected with α6β3γ2 subunits. (F) Co-application of compound 3 (10 and 30 μM) can inhibit positive GABA current modulation by 1 μM of compound 2.
The remarkable α6β3γ2 selectivity of m-methoxy compound 3 spurred us to screen additional compounds with varying m-substituents (see Figure 1, bottom row), for their modulatory activity at α6β3γ2 receptors (see Figure 5A). Two of these, compound 5 (DCBS142) and compound 9 (DCBS152A), displayed similar or higher modulatory activity at 1 μM concentration at α6β3γ2 compared to compound 3 (see Figure 5A) and were thus investigated further for their α-selectivity profile (see Figure 5B, C). Neither of them was found to be selective for any tested receptor subtype. Selectivity of relevant compounds for α6β3γ2 over α1β3γ2 is quantified in Figure 5D, in comparison to PZ-II-029 (Varagic et al., 2013a), the most α6β3γ2 selective compound until now; 10 μM compound 5 positively modulated α1β3γ2 to 210.7 ± 17% [mean ± SEM, n = 4, control (GABA) = EC3,5] and the other tested subtypes to a lesser extent. Thus, the subtype profile of compound 5 is reminiscent of compound 1. In contrast, m-carboxy compound 9 is a negative modulator at several subtypes, particularly at α1β3γ2, where 10 μM compound 9 reduced GABA responses to 17.9 ± 7.4% [mean ± SEM, n = 4, control (GABA) = EC3,5] (see Figure 5B, C). Thus, compound 9 displays bidirectional modulatory effects depending on the α subunit present. The modulation of α1β3γ2 by compounds 5 and 9 did not require the presence of a γ2 subunit (Ramerstorfer et al., 2011), as modulatory effects in α1β3γ2 and α1β3 were overlapping in both cases (see Figure 5E).

The finding that the presence of a γ2 subunit did not affect modulation by compounds 5 and 9 (see Figure 5E) prompted us to investigate the functional selectivity profile of compound 3 with respect to a third subunit. Since Jeclhinger et al. (1998) reported that α6β2 and α6βδ are populations of comparable size, we thus aimed to investigate compound 3 in α1,4,6βδ receptors subtypes (see Supporting Information Table S5). In line with the data for compounds 5 and 9, we noticed for compound 3 that the presence of a γ2 subunit did not affect current modulation. Moreover, we even observed a comparable functional selectivity for the α6-containing αβδ receptor subtype.
Towards functional selectivity for \(\alpha 6\beta 3\gamma 2\) GABA\(_A\) receptors

**Discussion**

Previous work from our labs has identified several PQ compounds with strong preferential modulatory activity at \(\alpha 6\beta 3\gamma 2\) over other \(\alpha x\beta y\gamma\) \(x = 1–5\) GABA\(_A\) receptors, via the modulatory PQ binding site at the \(\alpha x/\beta y\) interface (Varagic \textit{et al.}, 2013a). However, the most \(\alpha 6\)-selective compound characterized in this earlier study, PZ-II-029 (\(R^2 = \text{methoxy, } p\)-methoxy), at 10 \(\mu M\) concentration still displays considerable residual modulatory activity of roughly 200\% of control GABA currents in non-\(\alpha 6\), \(\alpha x\beta y\gamma\) receptors (Varagic \textit{et al.}, 2013a). We took the observation as a starting point for the present study that introducing a chloro substituent in position \(R^2\) of the PQ compound \(1\) (resulting in compound \(2\)) greatly enhances efficacy while preserving relative modulatory activities at \(\alpha x\beta y\gamma\) \(x = 1–5\) receptors and preference for \(\alpha 6\beta 3\gamma 2\). By systematically varying residues on ring \(D\), we explored whether the subtype profile could be steered towards increased separation between \(\alpha 6\)- and other \(\alpha\)-combinations. Indeed, we found that moving the methoxy residue from \(p\)- to \(m\)-position virtually abolished efficacy in all non-\(\alpha 6\), \(\alpha x\beta y\gamma\) \(x = 1–5\) receptors. The resulting compound \(3\), at a concentration of 10 \(\mu M\), retained around 300\% modulation of control GABA currents at \(\alpha 6\beta 3\gamma 2\) receptors and an estimated EC\(_{50}\) of 3.1 \(\mu M\). Hence, compound \(3\), to the best of our knowledge, displays the best \(\alpha 6/\alpha x\) \(x = 1–5\) efficacy ratio in \(\alpha x\beta y\gamma\) \(x = 1–6\) GABA\(_A\) receptors described to date.

Particular interest has recently developed for \(\alpha 6\)-containing GABA\(_A\) receptors and their involvement in various diseases (Kramer and Bellinger, 2013; Liao \textit{et al.}, 2016; Yang \textit{et al.}, 2016). \(\alpha 6\) subunits have a highly specific expression pattern in the central and peripheral nervous system. They are most abundantly found not only in CGCs but also in olfactory bulb, cochlear nucleus, trigeminal ganglion, sensory neurons and dorsal horn (Gutiérrez \textit{et al.}, 1996; Puri \textit{et al.}, 2012; Hörtnerl \textit{et al.}, 2013; Yang \textit{et al.}, 2016). Studies implicating \(\alpha 6\)-containing receptors in tic disorders (Liao \textit{et al.}, 2016), depressive behaviours (Yang \textit{et al.}, 2016) or inflammatory (Puri \textit{et al.}, 2012) and myofascial pain of the trigeminal innervation area (Kramer and Bellinger, 2013) underscore the potential importance of selective compounds for \(\alpha 6\)-containing receptors, ideally exhibiting little to no residual effects via other GABA\(_A\) receptor subtypes.

**Figure 5**

(A) Screening of a series of compounds \(5–9\) at \(\alpha 6\beta 3\gamma 2\) GABA\(_A\) receptors (see bottom row in Figure 1). At 1 \(\mu M\) compound concentration, compounds \(3\) (dashed blue line representing the fitted curve of Figure 2c), \(5\) and \(9\) show more than 150\% modulation of GABA EC\(_{5,5}\) currents. At 10 \(\mu M\) compound concentrations, compounds \(5\), \(8\) and \(9\) show strongest modulation of GABA EC\(_{5,5}\) currents, comparable to compound \(3\). (B, C) Subunit profile of compounds \(5\) (B) and \(9\) (C) at \(\alpha x\beta y\gamma\) \(x = 1–6\) GABA\(_A\) subunit combinations. Note that some receptors, particularly \(\alpha 1\beta 3\gamma 2\), are positively modulated by compound \(5\) but negatively modulated by compound \(9\). (D) Effective selectivity of 10 \(\mu M\) compound at \(\alpha 6\beta 3\gamma 2\) over \(\alpha 1\beta 3\gamma 2\). Modulation at \(\alpha 1\beta 3\gamma 2\) was calculated as fraction of the modulation at \(\alpha 6\beta 3\gamma 2\) \([\text{baseline (100\%) } = 0, \text{ efficacy at } \alpha 6\beta 3\gamma 2 = 1; \text{ all signs positive}]\) and subtracted from 1. (E) Separate experiment comparing effects of compound \(5\) and compound \(9\) at \(\alpha 1\beta 3\) versus \(\alpha 1\beta 3\gamma 2\) receptors, demonstrating independence from the \(\gamma\) subunit.
Selective positive modulators for α6-containing GABA_A receptors might thus have future disease indications. The flavone hispidulin is a PAM at α6β2γ2S receptors, among other subtypes (Kavvadias et al., 2004). It has been isolated from a plant extract and successfully used in a patient with intractable motor tic disorders (Huang et al., 2015) and was found to specifically reduce metamphetamine-induced hyperlocomotion via cerebellar α6-GABA_A receptors (Liao et al., 2016). Interestingly, hispidulin did not interfere with spontaneous locomotor activity or motor coordination measured by rotarod performance. Looking beyond the cerebellum, considerable α6 subunit expression has also been found in trigeminal ganglion neurons and the brainstem trigeminal subnucleus caudalis of rats (Hayasaki et al., 2006; Puri et al., 2011, 2012; Kramer and Bellinger, 2013). Knockdown of α6 expression in the trigeminal ganglion using locally infused Gabaα6 siRNA aggravated the hypersensitivity associated with inflammatory temporomandibular joint arthritis (Puri et al., 2012) and likewise increased myofascial nociception triggered by a ligature of the masseter muscle tendon (Kramer and Bellinger, 2013), supporting the notion that α6-containing GABA_A receptors inhibit processing of nociceptive signals in the trigeminal pathway. These findings open up the possibility that positive modulators specific for α6-containing GABA_A receptor might be suited to alleviate pain states of the trigeminal innervation area.

We found o-methoxy compound 4 to be devoid of positive modulatory activity at any receptor subunit combination tested. In fact, co-application with compound 3 suggests that compound 4 is a non-binder, rather than a silent binder, at α6β3γ2 receptors, rendering it a potentially useful negative control compound for future in vivo studies. Conformational analysis of compounds 2, 3 and 4 revealed rotated dihedral angles between the plane surfaces of ring-system A–C and ring D in the case of compound 4, suggesting that this difference in geometry might interfere with binding at the modulatory PQ binding site. In contrast, the binding affinity of compound 4 for the benzodiazepine binding site remained in the submicromolar range, suggesting that the dihedral angle of ring D might have a bigger impact on binding at the α+β– site.

In a series of different m-substituents, we could not identify additional selective compounds with improved efficacy or potency in the α6β3γ2 receptor. However, two of those compounds, 5 and 9, showed a curious pattern where m-methyl versus m-carboxy turns the former into a positive and the latter into a negative modulator of non-α6, α6β3γ2 (x = 1–5) receptors, particularly at α1β3γ2. This finding is also the first demonstration of a negative modulatory effect mediated via the modulatory PQ site, as it is independent from the γ2 subunit and hence the BZD site. Additionally, we demonstrated that compound 3 does not require a γ2 subunit for current modulation, as it remains functionally α6-selective in α6β3 receptor subtypes. Comparing compounds 5 and 9 with 3, we observed three different modulatory effects at α1β3γ2 (3 = PAM, 3 = null (silent) modulator or SAM and 9 = negative allosteric modulation), which seem to be induced by only the chemically different substituents in the m-position. Interestingly, positive allosteric modulation corresponds to an electron donating hydrophobic substituent (5, R^3 = Me), whereas null modulation is observed for an electron withdrawing hydrogen bond acceptor group (3, R^3 = OMe) and negative modulation is induced by an electron withdrawing negatively ionizable group (9, R^3 = COOH). These findings might prove useful for the design of new ligands and further structure–activity studies thereof. A systematic expansion along these lines may ultimately lead to structure–activity insights on efficacy selectivity and thus accelerate the targeted development of compounds with desired subtype profiles tremendously.

In summary, by systematically expanding on previously identified α6-prefering PQ compounds, we identified the highly α6β3γ2-selective compound 3. This compound has a substitution pattern distinct from the so far best published α6β3γ2-selective PZ-II-029 [R^7-methoxy, p-methoxy; published as “compound 6” (Varagic et al., 2013a)]. PZ-II-029 and compound 3 represent two separate prototypes for α6β3γ2-prefering PQs, differing in their ring A and D substitution patterns. Our present data are consistent with ring D substituents additionally determining efficacy selectivity for specific α-subunit-containing receptors. Future computational docking and functional studies are needed to determine the precise binding requirements for α-subunit specificity and efficacy determinants of this compound class. Furthermore, compound 3 could be used as tools to probe the functional role of α6β3γ2 GABA_A receptors in cerebellar neuron cultures and brain slices, to complement existing heterologous data and future in vivo studies in disease models.

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Author contributions

M.T. and D.C.B.S. contributed equally to this study. M.E., M.S. and M.D.M. participated in the design of the research. Experiments were conducted by M.T., D.C.B.S., K.B., R.H., L.W., Z.V., J.F., F.S. and P.S. Data analysis was performed by M.T., D.C.B.S., K.B., J.F., F.S. and P.S. All authors wrote or contributed to the writing of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.
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References

Alexander SPH, Peters JA, Kelly E, Marrion NV, Facenda E, Harding SD et al. (2017). The Concise Guide to PHARMACOLOGY 2017/18: Ligand-gated ion channels. Br J Pharmacol 174: S130–S159.

Bennett, D.A. (1987). Pharmacology of the pyrazolo-type compounds: agonist, antagonist and inverse agonist actions. Physiol Behav 41: 241–245.

Bennett DA, Amrick CL, Wilson DE, Bernard PS, Yokoyama N, Lieberman JM (1985). Behavioral pharmacological profile of CGS 9895: a novel anxiomodulator with selective benzodiazepine agonist and antagonist properties. Drug Dev Res 6: 313–325.

Cooper SJ, Kirkham TC, Estall LB (1987). Pyrazolquinolines: second effects benzodiazepine receptor ligands have heterogeneous effects. Trends Pharmacol Sci 8: 180–184.

Curtis MJ, Bond RA, Spina D, Ahiwualla A, Alexander SP, Giembycz MA et al. (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461–3471.

Czernik AJ, Petrack B, Kalinsky HJ, Psychoyos S, Cash WD, Tsai C et al. (1982). CGS 8216: receptor binding characteristics of a potent benzodiazepine antagonist. Life Sci 30: 2245–2252.

Ernst M, Brauchart D, Boresch S, Sieghart W (2003). Comparative modelling of GABA_A receptors: limits, insights, future developments. Neuroscience 119: 933–943.

File SE (1983). Proconvulsant action of CGS 8216. Neurosci Lett 35: 317–320.

Forkuo GS, Guthrie ML, Yuan NY, Nieman AN, Kodali R, Jahan R et al. (2016). Development of GABA_A receptor subtype-selective imidazobenzodiazepines as novel asthma treatments. Mol Pharm 13: 2026–2038.

Fryer RI, Zhang P, Rios R, Gu ZQ, Basile AS, Skolnick P (1993). Structure-activity relationship studies at the benzodiazepine receptor (BZR): a comparison of the substituent effects of pyrazolquinolinone analogs. J Med Chem 36: 1669–1673.

Gee KW, Yamamura HI (1982). A novel pyrazoloquinoline that interacts with brain benzodiazepine receptors: characterization of some in vitro and in vivo properties of CGS 9896. Life Sci 30: 2245–2252.

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

Hayasaki H, Sohma Y, Kanbara K, Maemura K, Kubota T, Watanabe M (2006). A local GABAergic system within rat trigeminal ganglion cells. Eur J Neurosci 23: 745–757.

Hörnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G (2013). Patterns of mRNA and protein expression for 12 GABA_A receptor subunits in the mouse brain. Neuroscience 236: 345–372.

Huang WJ, Lee HJ, Chen HL, Fan PC, Ku YL, Chiu LC (2015). Hispidulin, a constituent of Clerodendrum inerme that remitted motor tics, alleviated methamphetamine-induced hyperlocomotion without motor impairment in mice. J Ethnopharmacol 166: 18–22.

Jechlinger M, Peiz R, Tretter V, Klausberger T, Sieghart W (1998). Subunit composition and quantitative importance of hetero-oligomeric receptors: GABA_A receptors containing alpha6 subunits. J Neurosci 18: 2449–2457.

Kavvadias D, Sand P, Youdim KA, Quizer MZ, Rice-Evans C, Baur R et al. (2004). The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood-brain barrier and exhibits anticonvulsant effects. Br J Pharmacol 142: 811–820.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kolata G (1982). New valiums and anti-valiums on the horizon. Science 216: 604–605.

Kramer PRR, Bellinger LLL (2013). Reduced GABA_A receptor α6 expression in the trigeminal ganglion enhanced myofascial nociceptive response. Neuroscience 245: 1–11.

Liao Y-H, Lee H-J, Huang W-J, Fan P-C, Chiu L-C (2016). Hispidulin alleviated methamphetamine-induced hyperlocomotion by acting at α6 subunit-containing GABA_A receptors in the cerebellum. Psychopharmacology (Berl) 233: 3187–3199.

Maldifassi MC, Baur R, Sigel E (2016). Molecular mode of action of CGS 9895 at αβ2 γ2 GABA_A receptors. J Neurochem 138: 722–730.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br J Pharmacol 172: 3189–3193.

Minier F, Sigel E (2004). Positioning of the alpha-subunit isoforms confers a functional signature to gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101: 7769–7774.

Olsen RW, Sieghart W (2008). International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid A (GABA_A) receptors: classification on the basis of subunit composition, pharmacology, and function. Pharmacol Rev 60: 243–260.

Puri J, Bellinger LL, Kramer PR (2011). Estrogen in cycling rats alters gene expression in the temporomandibular joint, trigeminal ganglia and trigeminal subnucleus caudalis/upper cervical cord junction. J Cell Physiol 226: 3169–3180.

Puri J, Vinothini P, Reuben J, Bellinger LL, Ailing L, Peng YB et al. (2012). Reduced GABA(A) receptor α6 expression in the trigeminal ganglion alters inflammatory TMJ hypersensitivity. Neuroscience 213: 179–190.

Putthakanal R, Hieckel M, Simeone X, Suwattanasophon C, Feldbauer RV, Ecker GF et al. (2016). Structural studies of GABA_A receptor binding sites: which experimental structure tells us what? Front Mol Neurosci 9: 1–20.

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

Savini L, Chiasserini L, Pellerano C, Biggio G, Maciocco E, Serra M et al. (2017). The Concise Guide to PHARMACOLOGY 2017/18: GABA(A) receptors. Curr Top Med Chem 2: 833–839.

Simeone X, Siebert DCB, Bampali K, Varagic Z, Treven M, Rehman S et al. (2016). Development of GABAA receptor subtype-selective interactions between 1300 protein targets and 6000 ligands. Nucl Acids Res 44: D1054–D1068.
Valenzuela CF, Jotty K (2015). Mini-review: effects of ethanol on GABA<sub>A</sub> receptor-mediated neurotransmission in the cerebellar cortex – recent advances. Cerebellum 14: 438–446.

Varagic Z, Ramerstorfer J, Huang S, Rallapalli S, Sarto-Jackson I, Cook J <em>et al.</em> (2013a). Subtype selectivity of αβ– site ligands of GABA<sub>A</sub> receptors: identification of the first highly specific positive modulators at α6β2/3γ2 receptors. Br J Pharmacol 169: 384–399.

Varagic Z, Wimmer L, Schnürch M, Mihovilovic MD, Huang S, Rallapalli S <em>et al.</em> (2013b). Identification of novel positive allosteric modulators and null modulators at the GABA<sub>A</sub> receptor α+β– interface. Br J Pharmacol 169: 371–383.

Yang L, Xu T, Zhang K, Wei Z, Li X, Huang M <em>et al.</em> (2016). The essential role of hippocampal alpha6 subunit-containing GABA<sub>A</sub> receptors in maternal separation stress-induced adolescent depressive behaviors. Behav Brain Res 313: 135–143.

Yokoyama N, Ritter B, Neubert AD (1982). 2-Arylpyrazolo[4,3-c]quinolin-3-ones: novel agonist, partial agonist, and antagonist of benzodiazepines. J Med Chem 25: 337–339.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Table S1 Calculated number of conformations and their corresponding dihedral angles (φ) of p-, m- or o-methoxy substituted pyrazoloquinolinolones.

Table S2 Increments of Conformation Search application.

Figure S3 “Syn” and “Anti” conformation of compound 4.

Table S4 Radio displacement assay data.

Table S5 Functional data in δ containing receptor subtypes.

Figure S6 ¹H NMR spectrum of compound 3 (LAU159).

Figure S7 APT NMR spectrum of 3 (LAU159).

Figure S8 ¹H NMR spectrum of compound 4 (LAU165).

Figure S9 APT NMR spectrum of compound 4 (LAU165).

Figure S10 ¹H NMR spectrum of compound 5 (DCBS142).

Figure S11 ¹3C NMR spectrum of compound 5 (DCBS142).

Figure S12 ¹H NMR spectrum of compound 6 (DCBS32).

Figure S13 ¹3C NMR spectrum of compound 6 (DCBS32).

Figure S14 ¹H NMR spectrum of compound 7 (DCBS146).

Figure S15 ¹3C NMR spectrum of compound 7 (DCBS146).

Figure S16 ¹H NMR spectrum of the precursor of compound 8.

Figure S17 ¹3C NMR spectrum of the precursor of compound 8.

Figure S18 ¹H NMR spectrum of compound 8 (DCBS120).

Figure S19 ¹3C NMR spectrum of compound 8 (DCBS120).

Figure S20 ¹H NMR spectrum of compound 9 (DCBS152A).

Figure S21 ¹3C NMR spectrum of compound 9 (DCBS152A).