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High and low GABA sensitivity α4β2δ $GABA_A$ receptors are expressed in *Xenopus laevis* oocytes with divergent stoichiometries

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ABSTRACT

$GABA_A$ receptors that contain the α4 and δ subunits are thought to be located extrasynaptically, mediating tonic currents elicited by low concentrations of GABA. These α4βδ receptors are modulated by neurosteroids and certain anesthetics, identifying them as important drug targets in research. However, pharmacological studies on these receptors have often yielded variable results, possibly due to the expression of receptors in different stoichiometries or arrangements.

In this study, we injected different ratios of α4, β2 and δ cRNA into *Xenopus* oocytes and measured the sensitivity to GABA and DS2 activation of the resulting receptor populations. By creating a matrix of RNA injection ratios from stock RNA concentrations, we were able to compare the changes in pharmacology between injection ratios where the ratio of only one subunit was altered.

We identified two distinct populations of receptors, the first with an EC_{50} value of approximately 100 nM to GABA, a low Hill slope of approximately 0.3 and substantial direct activation by DS2. The second population had an EC_{50} value of approximately 1 μM to GABA, a steeper Hill slope of 1 and little direct activation, but substantial potentiation, by DS2. The second population was formed with high α4 ratios and low β2 ratios, but altering the ratio of δ subunit injected had little effect. We propose that receptors with high sensitivity to GABA and direct activation by DS2 are the result of a greater number of β2 subunits being incorporated into the receptor.

1. Introduction

Activation of $GABA_A$ receptors ($GABA_A$Rs) in the brain generally leads to cellular inhibition by allowing influx of Cl^- ions. These receptors can be located at both the synapse, where they contribute to phasic inhibition controlled by presynaptic GABA release, and at extrasynaptic and perisynaptic sites where they generate tonic inhibition in response to low concentrations of GABA to fine-tune the inhibitory response of neurons (reviewed in [1]). To perform these different cellular roles, different $GABA_A$Rs subtypes are formed from the large number of distinct receptor subunits. In recent years there has been a growing interest in understanding how $GABA_A$R subtypes, especially receptors containing α4, β and δ subunits, are activated at low GABA concentrations to mediate tonic inhibition [2]. The pharmacology of α4βδ receptors has also been of considerable interest in research, with these receptors identified as important targets for neurosteroids, alcohol, and certain anesthetics [1,3–5]. A deeper understanding of the pharmacology of these receptors is required to fulfill their potential as drug targets for various neurological and psychiatric disorders, including pain, epilepsy, sleep disorders, depression, and schizophrenia [2,6,7].

Despite considerable research efforts in the field, it is striking how the functional and pharmacological studies of δ-containing receptors have yielded variable results. The sensitivity of α4βδ receptors to ethanol is a classic example whereby some researchers report that low concentrations of ethanol potentiate GABA on δ-containing receptors [8–11], while others do not observe this potentiation [12]. Additionally, there are a number of reports that describe α4β3δ receptors forming two distinct receptor populations: one receptor population that is sensitive to nanomolar concentrations of GABA and THIP (gaboxadol) and another population that is sensitive to micromolar concentrations of both compounds. A similar difference in GABA potency was also observed at α4β2δ receptors in one study [13]. While most reports describe micromolar effects of GABA at α4β2δ receptors [14–17],

Abbreviations: $GABA_A$ receptor, γ-amino butyric acid receptor type A; DS, Delta Selective; THIP, tetrahydroisoxazolopyridinol; CRR, concentration–response relationship.

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Eaton et al. report that GABA acts at nanomolar concentrations using concatenated constructs [13].

The subunit composition of $GABA_A$Rs and their pentameric arrangement around a central, ion-conducting pore are crucial determinants of the pharmacological properties of the receptor, determining the putative ligand-binding interfaces and intrinsic activation properties [18]. To date, the exact stoichiometry of α4βδ receptors expressed both natively, and in heterologous systems, remains equivocal. While a number of studies attempt to address this question [19–22], firm conclusions have been difficult to draw over what stoichiometrie(s) or arrangement(s) are formed, and under what conditions these occur. In the abundant α1β2γ2 receptor, the γ2 subunit take the position as a fifth "auxiliary" subunit that does not participate in GABA binding, and it is generally assumed that the δ subunit substitutes for the γ2 subunit in α4βδ receptors. This has been questioned by reports that the level of δ subunit incorporation varies by varying the ratio of cRNA or cDNA in recombinant systems [20], while the expression of concatenated constructs suggest that the δ subunit can occupy various positions within the receptor complex [18,21]. Differences in functional and pharmacological properties of these receptors are thought to accompany these differences in both subunit stoichiometry and arrangement [10,22]. However, a caveat of some of these studies is that while measuring the responses of concatenated constructs can potentially yield information about a specific arrangement and stoichiometry of a single receptor, the technique itself may alter intrinsic receptor properties and force the assembly of receptors that would not occur otherwise. Along this, the use of concatenated constructs was recently shown to significantly alter the sensitivity to GABA and etomidate [23].

The pharmacological tool described as the Delta Selective agent (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide (DS2)) has proven useful at identifying δ-containing $GABA_A$Rs in native systems where it enhances tonic currents. At the molecular level, DS2 can either positively modulate GABA-elicited currents, or directly activate α4β3δ receptors. Similarly, there are reports of various levels of direct activation of α4β2δ receptors by DS2 but it is not well understood what conditions favor direct activation as opposed to positive modulation [13–15,24].

We hypothesized that variations in the pharmacology of α4β2δ receptors arise from the expression of receptors with either different stoichiometries or different subunit arrangements. In this study, we tested this hypothesis by injecting a matrix of different cRNA injection ratios that varied the subunit ratio of α4, β2, and δ cRNA in *Xenopus laevis* oocytes. The pharmacology of the receptors expressed was evaluated with GABA and DS2 to determine functional changes between the receptors expressed under different injection ratios. Our results demonstrate that GABA and DS2 activity were markedly changed depending on the subunit ratio of α4:β2, clearly indicating that receptors with different stoichiometries are expressed. There are at least two discrete receptor populations: one containing high GABA sensitivity and high efficacious DS2 direct activation and another with lower GABA sensitivity and relatively low-efficacy DS2 activation. At the latter receptors, DS2 positively modulated the GABA-elicited current. These data suggest that variability of the pharmacological properties of α4β2δ receptors are likely to be the result of the expression of receptors with different stoichiometries.

2. Materials and methods

2.1. Compounds

GABA, DMSO and all the ingredients for buffers used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA) while DS2 was purchased from Tocris Bioscience (Bristol, UK). THIP (4,5,6,7-tetrahydroisoxazolo (5,4-c)pyridine-3(-ol) (gaboxadol)) was generously provided by Dr. Bjarke Ebert (H. Lundbeck A/S, Valby, Denmark).

2.2. GABA receptor subunit construct

Human cDNA α4 and δ subunits were subcloned into pcDNA1/Amp and the β2 was subcloned into pcDM8. The plasmids containing the α4 and δ subunits were linearized with the restriction enzymes *Hpa*I (Life Technologies) and β2 plasmids were linearized with *Not*I (Life Technologies). cRNAs were transcribed using mMESSAGE mMACHINE® T7 Transcription Kit (Life Technologies). DNAse was added to the cRNA and then polyadenylated using the Poly-A-tailing kit (Life Technologies) and purified using lithium chloride precipitation. The concentrations of cRNAs were measured with a Nanodrop® ND-1000-UV–Vis spectrophotometer and the quality was determined using 1% agarose gel electrophoresis.

2.3. X. laevis surgery and oocyte injection

Surgery procedures on *X. laevis* were approved by Animal Ethics Committee of the University of Sydney (reference number: 2013/5915). Female *X. laevis* was anesthetized by submerging the *X. laevis* in 0.17% tricaine. A small incision on the abdomen was made to obtain several ovarian lobes. The ovarian lobes were cut into smaller lobes and stored in oocytes Ringer's buffer 2 (OR2) (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES hemisodium salt; pH 7.4). The lobes were then treated with collagenase A (Boehringer Mannheim, Indianapolis, IN, USA) diluted in OR2 solution for 1–2 h at 18 °C to separate oocytes from follicle cell layer and connective tissues. After the digestion was completed, the oocytes were rinsed thoroughly with OR2 and ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES; pH 7.4) supplemented with 2.5 mM sodium pyruvate and 0.5 theophylline. Stages V–VI oocytes were collected and injected (Nanoject, Drummond Scientific Co.) with 5–8 ng of cRNA in a final volume of 50.6 μl in the ratios stated in the text. The injected oocytes were incubated in ND96 augmented with 50 μg/ml gentamycin and tetracycline at 18 °C for 4–7 days prior to recording.

2.4. Injection ratio matrix

All cRNA was derived from a single RNA stock of each subunit to allow for a comparison between relative cRNA ratios. An initial ratio of 1:1:1 (α4:β2:δ) RNA was made and injected into *Xenopus* oocytes. As the aim was not to study potential receptor populations arising from extreme cRNA ratios, changes were kept modest, and the lowest and highest percentages a given subunit represented in the cRNA mixture was within the range of approximately 10–70%. A matrix of injection ratios were developed such that the relative α4 ratio was altered by creating a 5:1:1 and 1:5:5 ratio, while the β2 ratio was altered by creating a 1:5:1 and 5:1:5 ratio, and the δ ratio was altered by creating a 1:1:5 and 5:5:1 ratio.

2.5. Electrophysiological recording

Whole-cell current was recorded using the two-electrode voltage clamp technique with a Geneclamp 500B amplifier and digitized with a Powerlab/200 analogue to digital conversion with the Chart version 3.5 software as previously described [25]. The recording microelectrodes were filled with 3 M KCl and had resistance between 0.2 and 1.2 MΩ. Oocytes were impaled and clamped at −60 mV and superfused with ND96 during recording. DS2 was