Assembly, trafficking and function of $\alpha 1\beta 2\gamma 2$ GABA(A) receptors are regulated by N-terminal regions, in a subunit-specific manner

Wong, Lik Wei; Tae, Han Shen; Cromer, Brett

https://researchrepository.rmit.edu.au/esploro/outputs/9921862805801341/filesAndLinks?institution=61RMIT_INST&index=null

Wong, L. W., Tae, H. S., & Cromer, B. (2015). Assembly, trafficking and function of $\alpha 1\beta 2\gamma 2$ GABA(A) receptors are regulated by N-terminal regions, in a subunit-specific manner. *Journal of Neurochemistry*, 134(5), 819–832. <https://doi.org/10.1111/jnc.13175>
Document Version: Accepted Manuscript

Published Version: <https://doi.org/10.1111/jnc.13175>

Thank you for downloading this document from the RMIT Research Repository.

The RMIT Research Repository is an open access database showcasing the research outputs of RMIT University researchers.

RMIT Research Repository: <http://researchbank.rmit.edu.au/>

Citation:

Wong, L, Tae, H and Cromer, B 2015, 'Assembly, trafficking and function of $\alpha 1\beta 2\gamma 2$ GABA (A) receptors are regulated by N-terminal regions, in a subunit-specific manner', *Journal of Neurochemistry*, vol. 134, no. 5, pp. 819-832.

See this record in the RMIT Research Repository at:

<https://researchbank.rmit.edu.au/view/rmit:33593>

Version: Accepted Manuscript

Copyright Statement: © 2015 International Society for Neurochemistry

Link to Published Version:

<http://dx.doi.org/10.1111/jnc.13175>

PLEASE DO NOT REMOVE THIS PAGE

Assembly, trafficking and function of $\alpha 1\beta 2\gamma 2$ GABA_A receptors are regulated by N-terminal regions, in a subunit-specific manner

Lik-Wei Wong^{1,2}, Han-Shen Tae¹ and Brett A. Cromer^{1¶}

¹Health Innovation Research Institute, School of Medical Sciences, RMIT University, Melbourne VIC

²Department of Pharmacology and Therapeutics, University of Melbourne, Melbourne VIC

[¶]To whom correspondence should be addressed:

Brett A. Cromer, Health Innovation Research Institute, School of Medical Sciences, RMIT University, Bundoora VIC 3082, email: brett.cromer@rmit.edu.au

Abbreviations: GABA, γ -amino butyric acid; pLGIC, pentameric ligand-gated ion channel; GABA_AR, type A GABA-activated pLGIC; GABA_CR, type C GABA-activated pLGIC; GlyR, glycine receptor pLGIC; nAChR, nicotinic acetylcholine receptor pLGIC; 5HT₃R, serotonin receptors pLGIC; GluCl, glutamate-gated chloride channel; AChBP, acetylcholine-binding protein; ELIC, pLGIC from *Erwinia chrysanthemi*; GLIC, pLGIC from *Gloeobacter violaceus*; ECD, extracellular domain; TM trans-membrane region; CDP, chlordiazepoxide; Δ Ex, deletion from the N-terminus to the end of the predicted N-terminal extension; Δ Ex α H, deletion from the N-terminus to just before dileucine motif in putative α -helix; Δ Ex α H+3, deletion from the N-terminus to the end of the putative α -helix (removing dileucine motif)

Abstract

GABA_A receptors are pentameric ligand-gated ion channels (pLGIC) that mediate inhibitory fast synaptic transmission in the central nervous system. Consistent with recent pLGIC structures, sequence analysis predicts an α -helix near the N-terminus of each GABA_A receptor subunit. Preceding each α -helix are 8-36 additional residues, which we term the N-terminal extension. In homomeric GABA_C receptors and nicotinic acetylcholine receptors (nAChR), the N-terminal α -helix is functionally essential. Here we determined the role of the N-terminal extension and putative α -helix in heteromeric $\alpha 1\beta 2\gamma 2$ GABA_A receptors. This role

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/jnc.13175

This article is protected by copyright. All rights reserved.

was most prominent in the $\alpha 1$ subunit, with deletion of the N-terminal extension or further deletion of the putative α -helix both dramatically reduced the number of functional receptors at the cell surface. Conversely, deletion of the $\beta 2$ or $\gamma 2$ N-terminal extension had little effect on the number of functional cell-surface receptors. Additional deletion of the putative α -helix in the $\beta 2$ or $\gamma 2$ subunits did, however, decrease both functional cell surface receptors and incorporation of the $\gamma 2$ subunit into mature receptors. In the $\beta 2$ subunit only, α -helix deletions affected GABA sensitivity and desensitization. Our findings demonstrate that N-terminal extensions and α -helices make key subunit-specific contributions to assembly, consistent with both regions being involved in inter-subunit interactions.

Introduction

GABA_A receptors (GABA_ARs) are anion-selective members of the pentameric ligand-gated ion channel (pLGIC) superfamily. They are the primary mediators of fast inhibitory synaptic transmission in the human central nervous system. The pLGIC superfamily also includes anion-selective GABA_CR and glycine receptors (GlyR) and cation-selective nicotinic acetylcholine receptors (nAChR) and serotonin receptors (5HT₃R) (Barnard et al., 1998; Macdonald and Olsen, 1994; Thompson et al., 2010). GABA_ARs are allosterically modulated by a range of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, neurosteroids, anaesthetics and convulsants (Korpi et al., 2002; Reynolds et al., 2003; Sieghart, 1995). Functional GABA_ARs are thought to be heteropentamers assembled from at least 16 subunit isoforms identified thus far, including $\alpha(1-6)$, $\beta(1-3)$, $\gamma(1-3)$, δ , ϵ , π and θ (Barnard et al., 1998; Macdonald and Olsen, 1994). This gives rise to a great variety of GABA_AR subtypes with different subunit composition, cellular distribution and pharmacology properties. The majority of native receptors are, however, composed of two α , two β and one γ subunit with the $\alpha 1\beta 2\gamma 2$ subunit combination being the most prevalent GABA_ARs in the human brain (Ernst et al., 2005; Olsen and Sieghart, 2009; Sieghart, 1995).

Homopentameric ion channels can be formed from $\beta 1$ or $\beta 3$ subunits alone but they are not GABA-activated (Krishek et al., 1996; Wooltorton et al., 1997b). Unlike $\alpha\gamma$ or $\beta\gamma$, an $\alpha\beta$ subunit composition can form functional GABA-activated GABA_ARs *in vitro*. When the $\gamma 2$ subunit is present, however, $\alpha\beta\gamma$ GABA_ARs are produced preferentially over $\alpha\beta$ GABA_ARs

(Angelotti et al., 1993; Verdoorn et al., 1990). This preference for $\alpha\beta\gamma$ GABA_ARs can be further biased by transfecting with larger amounts of $\gamma 2$ relative to α and β subunit cDNAs (Boileau et al., 2002). As benzodiazepines bind at the interface between the α and $\gamma 2$ subunits, the presence of the $\gamma 2$ subunit, together with α and β subunits, is essential for benzodiazepine sensitivity (Cromer et al., 2002; Pritchett et al., 1989; Sigel et al., 1990; Sigel and Buhr, 1997). This has also been confirmed *in vivo* with $\gamma 2$ subunit knockout mice (Gunther et al., 1995). A markedly lower sensitivity to inhibition by Zn^{2+} is another hallmark of $\alpha 1\beta 2\gamma 2$ GABA_ARs compared to $\alpha 1\beta 2$ GABA_ARs that lack a γ subunit (Draguhn et al., 1990; Saxena and Macdonald, 1994). Two Zn^{2+} binding sites have been identified at the interface between the $\alpha 1$ and β subunits in the extracellular domain and a third, which has the highest affinity for Zn^{2+} , within the channel lumen (Horenstein and Akabas, 1998; Hosie et al., 2003; Wooltorton et al., 1997a). Incorporation of the $\gamma 2$ subunit into GABA_ARs disrupts one of two Zn^{2+} binding sites in the extracellular domain and the high-affinity Zn^{2+} -binding site in the channel lumen, thus decreasing Zn^{2+} sensitivity (Hosie et al., 2003).

The first high resolution structure relevant to pLGICs was of a molluscan acetylcholine-binding protein (AChBP) with homology to the extracellular domain of nAChR (Brejc et al., 2001). Since then, our understanding of pLGIC structure at atomic resolution has advanced dramatically. The first structure of a full length pLGIC at a resolution sufficient to build an atomic model was resolved from the cryo-electron microscopy of *Torpedo* nAChR at 4 Å resolution (Miyazawa et al., 2003; Unwin, 2005). The identification of prokaryotic pLGICs (Tasneem et al., 2005) led to structures, at high resolution, of pLGICs from *Erwinia chrysanthemi* (ELIC) (Hilf and Dutzler, 2008) and *Gloeobacter violaceus* (GLIC) (Bocquet et al., 2009; Hilf and Dutzler, 2009). The first X-ray structure of an anion-selective pLGIC, an invertebrate glutamate-gated chloride channel (GluCl), was resolved in 2011 in an apparently open state (Hibbs and Gouaux, 2011). Recently a GluCl closed state structure was resolved (Althoff et al., 2014) and the first structures of mammalian pLGICs were determined; a homomeric $\beta 3$ GABA_AR (Miller and Aricescu, 2014) and a homomeric 5HT_{3A}R (Hassaine et al., 2014). These structures provide a remarkably consistent picture of pLGIC structure.

Each pLGIC subunit comprises a large N-terminal extracellular domain (ECD), followed by four transmembrane α -helices (TM1-4) that form the channel domain and a variable intracellular domain between TM3 and TM4. The intracellular domain is basically absent in prokaryotic pLGICs, with a short loop linking TM3 and TM4. All available structural information shows the core of the ECD is a β -sandwich of ten β -strands. The agonist-binding site is formed near the mid-point of the ECD at the interface between two subunits, involving the principal or (+) face of one subunit and the complimentary or (-) face of the other (Figure 1A). The so-called “loop C”, between β strands 9 and 10 of the (+) side subunit, forms a lid over the agonist-binding site. An N-terminal α -helix precedes the β -sandwich in all eukaryotic structures but is absent in prokaryotic structures, raising the question of whether this helix is essential in eukaryotic pLGICs.

In addressing this question, the N-terminal α -helix has been shown to be essential in homomeric $\alpha 7$ nAChRs (Bar-Lev et al., 2011; Castillo et al., 2009), largely due to an important role in receptor assembly and trafficking to the cell surface. We have recently reported similar findings for the putative N-terminal α -helix in $\rho 1$ GABA_CR (Wong et al., 2014). We also found that 46 residues (we term the “N-terminal extension”) preceding this putative α -helix were not functionally essential but contributed to the efficiency of receptor assembly and trafficking and to agonist cooperativity (Wong et al., 2014). Here we investigate the role of the N-terminal extension and putative N-terminal α -helix in heteromeric GABA_AR and whether this role is subunit dependent. We used the natively common $\alpha 1\beta 2\gamma 2$ GABA_AR as a model. In each subunit, deletions of the N-terminal extension and the N-terminal α -helix were created. Additional variants included or removed a conserved di-leucine motif that is important in nAChR assembly (Castillo et al., 2009). We demonstrate that the role of these segments is strongly subunit specific. The $\alpha 1$ N-terminal extension is a key determinant of receptor assembly. Conversely, the $\beta 2$ and $\gamma 2$ α -helices are important for incorporation of the $\gamma 2$ subunit.

Materials and methods

Construction of $\alpha 1\beta 2\gamma 2$ GABA_AR Mutants

The cDNAs encoding human $\alpha 1$, $\beta 2$ and $\gamma 2$ GABA_AR subunit subtypes were individually subcloned into the pCDNA3.1 expression vector. Mutations of GABA_AR subunits were incorporated using the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to manufacturers instructions. All constructs were verified by sequencing (Australian Genome Research Facility Ltd).

Cell Culture and Transfection

Human embryonic kidney (HEK-293T, from ATCC CRL-3216) cells were incubated at 37 °C in a humidified 5% CO₂ incubator and grown in Dulbecco's Modified Eagle Medium (Invitrogen, Life Technologies), supplemented with 10% fetal bovine serum (SAFC Biosciences) and penicillin/streptomycin (100 IU/ml). For expression of wild-type (WT) or mutant $\alpha 1\beta 2\gamma 2$ GABA_ARs, HEK-293T, cells in 6-well tissue culture-treated plates (Corning, NY) were transfected with 4 µg of subunit cDNAs at a ratio of 1:1:3 ($\alpha 1:\beta 2:\gamma 2$), using a modified calcium phosphate transfection method (Jordan and Wurm, 2004). Medium was removed and replaced with fresh growth medium 4 h after transfection. The cells were then incubated at 37 °C for 36-48 h prior to experiments.

Flow Cytometry Analysis

Flow cytometry was performed to examine the surface and total expression of GABA_AR subunits. Transiently transfected cells were collected in phosphate-buffered saline (PBS) and washed twice with PBS on ice. Cells were kept on ice for all subsequent steps. For detection of total $\alpha 1\beta 2\gamma 2$ expression, cells were fixed with 4% paraformaldehyde for 20 min, then permeabilised with 0.2% Triton X-100 (Sigma-Aldrich, USA) for 15 min and washed again with PBS. For detection of cell-surface $\alpha 1\beta 2\gamma 2$ receptors, cells were not fixed or permeabilised prior to antibody staining. All cells were blocked with 10% horse serum (Gibco, Life Technologies) in PBS for 1 h, then incubated with a 1/200 dilution of mouse monoclonal anti-GABA_AR α chain, MAB339 (Millipore) or rabbit polyclonal anti-GABA_AR $\gamma 2$, ab82970 (Abcam) for 1 h, followed by two 15 min washes in PBS. Cells were then

Accepted Article

incubated in a 1/200 dilution of secondary goat anti-mouse antibody Alexafluor® 633 (Invitrogen, Life Technologies) or goat anti-rabbit antibody Alexafluor® 488 (Invitrogen, Life Technologies) in the dark for 1 h, followed by two 15 min washes in PBS. Cell-surface stained cells were then fixed with 4% paraformaldehyde for 20 min and washed with PBS for 15 min. The fluorescence intensity (FI) of 10,000 cells from each sample was measured using a BD FACS Canto II (BD Biosciences) flow cytometer. Each experiment included negative control untransfected cells and positive control $\alpha 1\beta 2\gamma 2$ WT transfected cells stained in the same manner as mutant samples. The FI of untransfected cells was subtracted from the mean FI of all other samples and the resulting mean fluorescence of each mutant expressed as a percentage of $\alpha 1\beta 2\gamma 2$ WT within each experiment. These percentages were averaged across multiple experiments.

Electrophysiology

Electrophysiological recordings were performed on transiently-transfected HEK-293T cells expressing $\alpha 1\beta 2\gamma 2$ GABA_ARs, using ensemble microfluidic plates in a 96-well plate automated electrophysiology platform (IonFlux-16TM System) developed by Fluxion Biosciences, USA (Chen et al., 2012; Golden et al., 2011; Spencer et al., 2012). Cells were washed with PBS and then incubated with TrypLE (Invitrogen, Life Technologies) for 2-5 min, resuspended in serum free media (Invitrogen, Life Technologies), harvested by centrifugation at 1000 rpm for 2 min and washed three times with extracellular solution (in mM: 138 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, 5.6 glucose, pH 7.45 with NaOH). Finally, cells were resuspended in extracellular solution at a concentration of 2–5 million cells/ml for electrophysiology. The intracellular solution was (in mM): 15 NaCl, 60 KCl, 70 KF, 5 EGTA, 5 HEPES, pH 7.25 with KOH. GABA dilutions in extracellular solution were prepared on the day of experiment from a frozen 1M stock solution. Whole-cell currents were recorded from populations of up to 20 cells voltage-clamped at -80 mV. Each GABA application lasted for 3 s, with a 2 min wash between applications to allow the receptors to recover from desensitization. Recordings were performed at room temperature (22-25°C).

The kinetics of macroscopic desensitization were determined by applying a saturating GABA concentration (1 mM) for 10 s to HEK-293T cells expressing WT or mutant $\alpha 1\beta 2\gamma 2$ GABA_ARs. For analysis of desensitization, the time of onset of desensitization was set to zero and the time course of desensitization was fitted by exponential components with a simplex algorithm (Axograph Software).

To determine $\gamma 2$ subunit incorporation into $\alpha 1\beta 2\gamma 2$ GABA_ARs, we tested the inhibitory effect of 30 μ M ZnCl₂ on saturating 1mM GABA-elicited currents in HEK-293T cells expressing WT or mutant GABA_AR. Cells were exposed twice to 1 mM GABA then pre-incubated with extracellular solution containing Zn²⁺ for 2 min, before being exposed to 30 μ M Zn²⁺ together with 1 mM GABA for 3 s. Cells were then washed back into extracellular solution to recover from Zn²⁺ inhibition and retested. Percentage inhibition by Zn²⁺ was calculated from peak currents in the presence ($I_{\text{GABA+ZINC}}$) and absence (I_{GABA}) of Zn²⁺, using the equation $(I_{\text{GABA}} - I_{\text{GABA+ZINC}}) / I_{\text{GABA}} \times 100\%$. We also determined benzodiazepine sensitivity by measuring potentiation by chlordiazepoxide (CDP) of 6 μ M GABA-elicited currents in HEK-293T cells expressing WT or mutant GABA_ARs. Cells were exposed three times to 6 μ M GABA for 3 s with a 2 min wash between each application to ensure a stable response. Cells were then exposed to 1 μ M CDP together with 6 μ M GABA for 3 s. Percentage of CDP potentiation (CDP potentiation %) was calculated from peak currents in the presence ($I_{\text{GABA+CDP}}$) or absence (I_{GABA}) of CDP, using the equation $(I_{\text{GABA+CDP}} - I_{\text{GABA}}) / I_{\text{GABA}} \times 100\%$.

Data analysis and statistics

Secondary structure predictions were carried out with Jpred3 (Cole et al., 2008) (<http://www.compbio.dundee.ac.uk/www-jpred/>). Electrophysiology data were analysed with integrated Fluxion software and Axograph X (<http://www.axograph.com/>). For each experiment, complete GABA dose response data were recorded and peak currents fitted to the Hill equation. EC₅₀ and Hill coefficients from these fits were averaged from several experiments. Prism 6 (GraphPad Software Inc. La Jolla, CA) was used for curve fitting to data and statistical analysis. Numerical data are presented as means $\pm$ SEM. Statistical differences to WT $\alpha 1\beta 2\gamma 2$ GABA_ARs were estimated using one-way ANOVA and Dunnett's

post-hoc multiple comparisons test, with $p < 0.05$ considered indicative of a significant difference (GraphPad Prism Software).

Results

Secondary structure predictions, using Jpred3 (Cole et al., 2008), consistently predict an N-terminal α -helix (highlighted in yellow, Figure 1B) in each subunit of the $\alpha 1\beta 2\gamma 2$ GABA_AR. These predictions aligned closely with each other and with α -helices in resolved structures (highlighted in cyan, Figure 1B), including the recent structure of a homomeric $\beta 3$ GABA_AR (Miller and Aricescu, 2014). Unlike nAChRs, mature subunits of GABA_ARs (and GluCl) have a substantial “N-terminal extension” prior to the putative N-terminal α -helix. To determine the functional role of these N-terminal regions, we created a series of deletion mutants of $\alpha 1$, $\beta 2$ and $\gamma 2$ GABA_AR subunits, using a definition of the putative N-terminal α -helix that is consistent across all subunits (highlighted in yellow, Figure 1C). Firstly, we created deletions of each subunit, defined as ΔEx , that lack residues corresponding to the predicted extension. We created two further deletions that extend into the N-terminal α -helix. The first construct, defined as $\Delta Ex\alpha H$, lacks residues from the N-terminus to the amino acid prior to a relatively conserved di-leucine motif at the C-terminal end of the putative α -helix. The second construct, defined as $\Delta Ex\alpha H+3$, completely removes the predicted N-terminal extension and α -helix, including the di-leucine motif. To ensure fidelity of signal peptide cleavage, the first mature residue (Q1) was retained in all deletion constructs.

Deletions to the N-terminal extension do not affect functional properties of GABA_ARs

GABA-activated channel function of WT and mutant GABA_ARs was measured electrophysiologically using an automated microfluidic system (IonFlux-16) that records total whole-cell current from a population of up to 20 cells. Typical current recordings are shown in Figure 2A and normalised average GABA dose-response curves in Figure 2B. The GABA concentration required to elicit half-maximum response (GABA EC₅₀) for $\alpha 1\beta 2\gamma 2$ GABA_AR was 11.0 ± 1.2 with a Hill coefficient of 1.1 ± 0.1 (Table 1). The GABA EC₅₀ (0.5 ± 0.2 μM) for $\alpha 1\beta 2$ GABA_AR was significantly smaller ($p < 0.0001$) compared to $\alpha 1\beta 2\gamma 2$ GABA_AR. Consistent with previous studies for $\alpha 1\beta 2$ GABA_ARs (Angelotti et al., 1993; Boileau et al., 2003), the Hill coefficient for $\alpha 1\beta 2$ (0.9 ± 0.2) was similar to that of $\alpha 1\beta 2\gamma 2$ GABA_AR (Table

1). Mutant $\alpha 1\beta 2\gamma 2$ GABA_ARs with deletions of the N-terminal extension in each single subunit were functional and showed dose-dependent GABA-gated currents (Figure 2A). The GABA EC₅₀ (μM) for $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$ was 11.1 ± 0.5 , 8.0 ± 0.5 and 8.0 ± 1.0 respectively with Hill coefficients ranging from 1.2 to 1.3 (Figure 2B and Table 1). Neither the GABA EC₅₀ nor the Hill coefficients of any of the N-terminal extension deletion mutants were significantly different to that of $\alpha 1\beta 2\gamma 2$ WT (Table 1).

Most GABA_ARs show substantial desensitization in the continued presence of agonist. The rate of desensitization reflects the relative stability and kinetics of conformational changes between different channel states. To examine whether deletion of the N-terminal extension affected desensitization, 1mM GABA was applied for 10 s and the desensitization phase was well fitted with a single exponential decay. Using the 20-cell IonFlux-16 plate format, the time for 10-90% solution exchange is approximately 50 ms (data not shown), a rate of GABA application that may obscure faster components of desensitization. With this constraint in mind, the desensitization rates for $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ (1.5 ± 0.1 s) and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$ (1.2 ± 0.1 s) were not found to be significantly different to $\alpha 1\beta 2\gamma 2$ WT (1.5 ± 0.1 s) (Supplementary Figure 1). As the current amplitudes were relatively small for the extension deletion of the $\alpha 1$ subunit in $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$, it was difficult to reliably measure the rate of desensitization for this mutant.

As small whole-cell currents may indicate a deficit in GABA_AR function, subunit folding, assembly or trafficking, we next quantified the effect of each deletion on peak whole-cell currents. Although there may be large variability in the magnitude of whole-cell currents from transfected cells, this can be greatly reduced by measurement from a cell population, such as up to twenty cells in the current study. The population whole-cell peak current (I_{MAX}) for $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$ was significantly reduced ($p < 0.0001$) to $16 \pm 5\%$ of $\alpha 1\beta 2\gamma 2$ WT (Table 1). There was, however, no significant difference in peak current for $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ ($81 \pm 8\%$ of $\alpha 1\beta 2\gamma 2$ WT) or $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$ ($89 \pm 14\%$ of $\alpha 1\beta 2\gamma 2$ WT) relative to $\alpha 1\beta 2\gamma 2$ WT (Table 1). Taken together, these data indicate that the N-terminal extension is not important for GABA-activation of ion channel function but is important, specifically in the α subunit, for achieving the maximum level of functional GABA_ARs.

Deletion of the α subunit N-terminal extension decreases cell surface $\alpha 1\beta 2\gamma 2$ GABA_ARs

To determine whether the reduced whole-cell peak current of the $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$ mutant was due to a functional defect, decreased total subunit expression or decreased receptor trafficking to the plasma membrane, we measured cell surface and total expression of WT and N-terminal extension deletions of $\alpha 1\beta 2\gamma 2$ GABA_ARs by indirect immunofluorescent labelling of intact and permeabilised cells, respectively. Two anti-GABA_AR subunit primary antibodies were used, anti- α chain (MAB339) and anti- $\gamma 2$ (ab82970) that bind with high affinity to the extracellular domain of $\alpha 1$ and $\gamma 2$ GABA_AR subunits, respectively (Figure 3). These antibodies recognize epitopes in the N-terminal segments that are deleted in our ΔEx constructs, so cannot be used to detect these subunits directly. Consequently, we detected $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$ GABA_ARs with the anti- $\gamma 2$ antibody and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$ with the anti- α chain antibody. The $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ GABA_AR was detected with both antibodies.

The degree of immunofluorescent labelling of each cell in a transfected population was measured using flow cytometry. Histograms of cell surface (intact cells) and total (permeabilised cells) anti- α subunit immunofluorescence labelling (Figure 3A) for cells expressing $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$ and $\alpha 1\beta 2\gamma 2$ WT were all markedly shifted to the right relative to untransfected cells. The shift appeared less for the permeabilised cells than intact cells, largely due to higher non-specific staining of untransfected cells when permeabilised. There was no clear distinction between untransfected and transfected populations of cells, indicating the rate of transfection was high but with a large variation in receptor expression. Consequently, the mean fluorescence of the whole population of cells was used to compare levels of deletion mutants to that of WT GABA_ARs. The amount of $\alpha 1$ subunit detected at the cell surface relative to $\alpha 1\beta 2\gamma 2$ WT was $80 \pm 6\%$ for $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ and $113 \pm 13\%$ for $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$. Neither was significantly different to $\alpha 1\beta 2\gamma 2$ WT (Figure 3A). There was also no significant difference in total receptor expression relative to $\alpha 1\beta 2\gamma 2$ WT, for either $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ or $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$, being $91 \pm 4\%$ and $129 \pm 2\%$ of $\alpha 1\beta 2\gamma 2$ WT respectively (Figure 3A). These results are consistent with a lack of effect of either of these two deletions on the magnitude of whole-cell currents.

Accepted Article

In agreement with the anti- α chain antibody results, detection of $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ with the anti- $\gamma 2$ antibody showed no significant difference to $\alpha 1\beta 2\gamma 2$ WT at either the cell surface ($91 \pm 11\%$) or total receptor expression ($92 \pm 9\%$) (Figure 3B). However, deletion of the N-terminal extension in the $\alpha 1$ subunit ($\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$) markedly and significantly ($p < 0.0001$) reduced the amount of $\gamma 2$ subunit detected at the cell surface to $19 \pm 4\%$ of $\alpha 1\beta 2\gamma 2$ WT. This finding is consistent with the reduced whole-cell currents reported above. The total expression of the $\gamma 2$ subunit for $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$ ($90 \pm 10\%$ of $\alpha 1\beta 2\gamma 2$ WT) was similar to $\alpha 1\beta 2\gamma 2$ WT (Figure 3B). These data may be explained by an effect of $\alpha 1$ subunit N-terminal extension deletion on the efficiency of receptor assembly and trafficking to the cell surface. Alternatively, as cell-surface receptors for this mutant were only detected with the anti- $\gamma 2$ antibody the incorporation of the $\gamma 2$ subunit into cell surface receptors may be reduced. Indeed, $\alpha 1\beta 2$ GABA_ARs that lack the $\gamma 2$ subunit are fully functional GABA-activated channels but have altered pharmacology relative to $\alpha 1\beta 2\gamma 2$ GABA_ARs (Draguhn et al., 1990; Pritchett et al., 1989).

Deletions of the N-terminal extension do not affect $\gamma 2$ subunit incorporation into GABA_ARs

To examine whether deletions of the N-terminal extension alter $\gamma 2$ subunit incorporation into GABA_ARs, we tested their effect on sensitivity to Zn^{2+} inhibition and benzodiazepine potentiation (Figure 4). We recapitulated here that presence of the $\gamma 2$ subunit in GABA_ARs confers sensitivity to benzodiazepine potentiation and reduced sensitivity to inhibition by Zn^{2+} (Draguhn et al., 1990; Pritchett et al., 1989). Addition of $30 \mu\text{M}$ Zn^{2+} inhibited peak whole cell currents in response to 1 mM GABA by $18 \pm 0.2\%$ in HEK-293T cells expressing $\alpha 1\beta 2\gamma 2$ GABA_ARs, significantly less ($p < 0.0001$) than the $79 \pm 0.2\%$ inhibition observed in cells expressing $\alpha 1\beta 2$ GABA_ARs (Figure 4, Table 1). Correspondingly, the benzodiazepine chlordiazepoxide (CDP), at $1 \mu\text{M}$, potentiated peak whole-cell currents in response to $6 \mu\text{M}$ GABA by $54 \pm 5\%$ in cells expressing $\alpha 1\beta 2\gamma 2$ GABA_ARs, significantly greater ($p < 0.0001$) than the absence of potentiation ($1 \pm 1\%$) in cells expressing $\alpha 1\beta 2$ GABA_ARs (Figure 4, Table 1).

We next tested the effect of each of the three N-terminal extension deletions on Zn^{2+} inhibition and CDP potentiation under the conditions described above. Each of the deletion mutants showed similar sensitivity to both Zn^{2+} inhibition and CDP potentiation as $\alpha 1\beta 2\gamma 2$ WT GABA_ARs. 30 μM Zn^{2+} inhibited peak whole-cell currents activated by 1mM GABA by $15 \pm 4\%$, $13 \pm 4\%$ and $19 \pm 4\%$ for $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$, respectively (Figure 4A). None were significantly different to Zn^{2+} inhibition of $\alpha 1\beta 2\gamma 2$ WT (Table 1). Furthermore, 1 μM CDP potentiated 6 μM GABA-activated currents by $46 \pm 10\%$, $39 \pm 7\%$ and $45 \pm 7\%$ for $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta\text{Ex})\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex})$, respectively (Table 1). Again none were significantly different to CDP potentiation of $\alpha 1\beta 2\gamma 2$ WT. Taken together, these results are clear evidence that incorporation of the $\gamma 2$ subunit into cell surface GABA_ARs was not compromised by the absence of the N-terminal extension in any of the three subunits. In particular, the reduced level of the $\gamma 2$ subunit detected at the cell surface due to the $\alpha 1(\Delta\text{Ex})$ deletion was not due to a decreased incorporation of the $\gamma 2$ subunit. Rather, it must reflect a reduced number of GABA_ARs at the cell surface, consistent with the reduced whole-cell peak current in cells expressing $\alpha 1(\Delta\text{Ex})\beta 2\gamma 2$ GABA_ARs.

N-terminal α -helix deletions, only in the β subunit, affect GABA-activated channel function

We next turned our attention to the role of the putative N-terminal α -helix. Larger deletions from the N-terminus of each subunit to a relatively conserved dileucine motif at the C-terminal end of the putative α -helix, termed $\Delta\text{Ex}\alpha\text{H}$, (Figure 1C), removed most of the helix as well as the N-terminal extension. We chose to retain the dileucine motif based on evidence of it having a key role in nAChRs assembly (Castillo et al., 2009). Each $\Delta\text{Ex}\alpha\text{H}$ deletion was co-transfected with full-length constructs of the other two subunits into HEK-293T cells.

Each of the three $\Delta\text{Ex}\alpha\text{H}$ mutant subunits produced receptors that showed dose-dependent GABA-gated currents (Figure 5A). The GABA EC_{50} was slightly but significantly reduced ($p < 0.05$) to $5.1 \pm 2.3 \mu\text{M}$ for $\alpha 1(\Delta\text{Ex}\alpha\text{H})\beta 2\gamma 2$, whilst the EC_{50} of $7.2 \pm 1.8 \mu\text{M}$ for $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$ was not significantly different to that for WT (Figure 5A and Table 1). Conversely, for $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$, the GABA EC_{50} ($28.1 \pm 4.3 \mu\text{M}$) showed a significant 2.5 fold increase ($p < 0.05$) relative to $\alpha 1\beta 2\gamma 2$ WT. None of the Hill coefficients, 1.0 ± 0.3 for

$\alpha 1(\Delta\text{Ex}\alpha\text{H})\beta 2\gamma 2$, 1.0 ± 0.1 for $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$ and 1.0 ± 0.2 for $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$, were significantly different to that of $\alpha 1\beta 2\gamma 2$ WT (1.1 ± 0.1) (Figure 5A and Table 1).

Kinetic analysis of current desensitization during exposure to 1 mM GABA for 10 s (Supplementary Figure 1) revealed that GABA_ARs with a $\Delta\text{Ex}\alpha\text{H}$ deletion in the $\beta 2$ subunit exhibited a desensitization rate of 2.5 ± 0.1 s that was significantly slower ($p < 0.001$) than that of $\alpha 1\beta 2\gamma 2$ WT (1.5 ± 0.1 s). The plateau of desensitized current for the $\beta 2$ deletion was also significantly increased to more than double that of WT (Supplementary Figure 1). Both kinetic changes suggest a stabilisation of the open state relative to the desensitized state. The desensitization rate (1.6 ± 0.1 s) for $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$ was, however, similar to $\alpha 1\beta 2\gamma 2$ WT (Supplementary Figure 1). As mentioned above, any changes in faster components of desensitization may be hidden by the rate of GABA application. Again, currents for $\alpha 1(\Delta\text{Ex}\alpha\text{H})\beta 2\gamma 2$ were too small to provide reliable measures of desensitization. These results indicate that only in the β subunit does the $\Delta\text{Ex}\alpha\text{H}$ deletion significantly affect GABA-activation of ion channel function.

Deletions to the N-terminal α -helix in any subunit decrease GABA_ARs at the cell surface

Similar to deletion of the N-terminal extension, the $\Delta\text{Ex}\alpha\text{H}$ deletion in the α subunit significantly reduced ($p < 0.0001$), the population whole-cell peak current (I_{MAX}) of $\alpha 1(\Delta\text{Ex}\alpha\text{H})\beta 2\gamma 2$ to $10 \pm 3\%$ of $\alpha 1\beta 2\gamma 2$ WT (Table 1). The larger $\Delta\text{Ex}\alpha\text{H}$ deletion in either the β or γ subunits also significantly reduced peak currents to $40 \pm 3\%$ and $70 \pm 6\%$ of $\alpha 1\beta 2\gamma 2$ WT for $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$, respectively (Table 1).

As above, to investigate the cause of reduced whole-cell currents in deletion mutants we measured cell surface and total subunit expression by flow cytometry using anti- α or anti- $\gamma 2$ antibodies (Supplementary Figure 2). Detection with the anti- α antibody (Figure 6A) showed the level of the α subunit at the cell surface to be significantly decreased to $10 \pm 3\%$ of $\alpha 1\beta 2\gamma 2$ WT for $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$ ($p < 0.0001$) and $54 \pm 5\%$ of $\alpha 1\beta 2\gamma 2$ WT for $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$ ($p < 0.01$). Detection of the γ subunit at the cell surface for $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$ was also significantly reduced to $16 \pm 6\%$ of $\alpha 1\beta 2\gamma 2$ WT, consistent with the decreased detection of

the α subunit. Together, the lower cell surface detection of α and γ subunits provide clear evidence that lower levels of cell surface receptors is the reason for reduced whole-cell currents in $\alpha 1\beta 2(\Delta Ex\alpha H)\gamma 2$ receptors. Indeed, the fractional decrease in subunits detected at the cell surface was more than the decrease in peak currents. Detection of the γ subunit at the cell surface for $\alpha 1(\Delta Ex)\alpha H\beta 2\gamma 2$ was also significantly reduced ($p < 0.0001$) to $11 \pm 2\%$ of $\alpha 1\beta 2\gamma 2$ WT (Figure 6B), correlating closely with the reduction in whole-cell currents for this mutant receptor. Decreased detection of surface γ subunits due to the $\alpha 1(\Delta Ex)\alpha H$ deletion is similar to that caused by the smaller $\alpha 1(\Delta Ex)$ deletion.

For all $\Delta Ex\alpha H$ deletions the total subunits detected from permeabilised cells were similar to $\alpha 1\beta 2\gamma 2$ WT, for both the α subunit ($110 \pm 13\%$ and $105 \pm 7\%$ of $\alpha 1\beta 2\gamma 2$ WT for $\alpha 1\beta 2(\Delta Ex\alpha H)\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta Ex\alpha H)$, respectively (Figure 6A)) and the γ subunit ($107 \pm 16\%$ and $102 \pm 17\%$ of $\alpha 1\beta 2\gamma 2$ WT for $\alpha 1(\Delta Ex\alpha H)\beta 2\gamma 2$ and $\alpha 1\beta 2(\Delta Ex\alpha H)\gamma 2$, respectively (Figure 6B)). Thus a deficiency in trafficking and/or assembly does not appear to result in subunit degradation.

Deletions to the N-terminal α -helix in β or γ subunits decrease $\gamma 2$ incorporation into functional $GABA_A$ Rs

We again used inhibition by $30 \mu M$ Zn^{2+} and potentiation by $1 \mu M$ CDP as measures of $\gamma 2$ subunit incorporation into functional receptors. Currents for the $\alpha 1(\Delta Ex\alpha H)$ deletion were inhibited by Zn^{2+} to $21 \pm 5\%$ of control, similar to $\alpha 1\beta 2\gamma 2$ WT (Figure 6C and Table 1). In agreement with the Zn^{2+} inhibition, CDP potentiation ($48 \pm 6\%$) for $\alpha 1(\Delta Ex\alpha H)\beta 2\gamma 2$ was not significantly different to $\alpha 1\beta 2\gamma 2$ WT (Figure 6D and Table 1). Thus the $\alpha 1(\Delta Ex\alpha H)$ deletion, which had the greatest effect on reducing the number of γ subunits at the cell surface, did not affect incorporation of $\gamma 2$ subunit into mutant receptors.

Conversely, $\Delta Ex\alpha H$ deletions in the $\beta 2$ or $\gamma 2$ subunit showed increased Zn^{2+} inhibition to $43 \pm 3\%$ and $61 \pm 5\%$ for $\alpha 1\beta 2(\Delta Ex\alpha H)\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta Ex\alpha H)$, respectively (Figure 6C and Table 1), both significantly greater ($p < 0.0001$) than $\alpha 1\beta 2\gamma 2$ WT. Furthermore, $\Delta Ex\alpha H$ deletions in either the $\beta 2$ or $\gamma 2$ subunit significantly decreased potentiation by CDP to $15 \pm$

4% for $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$ ($p<0.001$) and $5 \pm 2\%$ for $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$ ($p<0.0001$), relative to $54 \pm 5\%$ for $\alpha 1\beta 2\gamma 2$ WT (Figure 6D and Table 1). Both the higher Zn^{2+} inhibition and lower benzodiazepine potentiation indicate that functional $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$ GABA_ARs had incorporated significantly less $\gamma 2$ subunit. Thus, in both cases, a significant proportion of functional receptors at the cell surface were $\alpha 1\beta 2$ receptors. The effects on both Zn^{2+} inhibition and CDP potentiation were greatest in the $\gamma 2(\Delta\text{Ex}\alpha\text{H})$ deletion, indicating that very few functional receptors would actually contain this deleted $\gamma 2$ subunit.

Complete deletion of the N-terminal helix in $\alpha 1$ or $\beta 2$ subunits does not abolish function

To test the role of the three remaining, LLX, C-terminal residues of the putative N-terminal α -helix, larger deletions were made, termed $\Delta\text{Ex}\alpha\text{H}+3$. These deleted from the N-terminus to the C-terminal end of the putative α -helix, thus removing the di-leucine motif. As the $\gamma 2(\Delta\text{Ex}\alpha\text{H})$ deletion greatly reduced $\gamma 2$ subunit incorporation, we did not make a larger $\Delta\text{Ex}\alpha\text{H}+3$ deletion for the $\gamma 2$ subunit. When coexpressed with the other two WT subunits, a $\Delta\text{Ex}\alpha\text{H}+3$ deletion in either the $\alpha 1$ or $\beta 2$ subunit produced functional GABA_ARs (Table 1). Whole-cell currents from HEK-293T cells expressing $\alpha 1(\Delta\text{Ex}\alpha\text{H}+3)\beta 2\gamma 2$ were very small, with a significant decrease to $9 \pm 1\%$ of $\alpha 1\beta 2\gamma 2$ WT ($p<0.0001$). Although the peak currents were clearly GABA dose-dependent, their small size prevented reliable measurement of a GABA EC_{50} for this mutant receptor. With a similar deletion in the $\beta 2$ subunit of $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H}+3)\gamma 2$, whole-cell peak currents were also significantly reduced ($p<0.0001$), but only to $40 \pm 2\%$ of $\alpha 1\beta 2\gamma 2$ WT (Table 1). The GABA EC_{50} of $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H}+3)\gamma 2$ receptors was 91 ± 19 , significantly greater ($p<0.0001$) than that of $\alpha 1\beta 2\gamma 2$ WT. The Hill coefficient (1.0 ± 0.1) was similar to $\alpha 1\beta 2\gamma 2$ WT (Figure 5B and Table 1). Deleting the three extra residues ($\Delta\text{Ex}\alpha\text{H}+3$) to completely remove the putative α -helix exacerbates the effects of the slightly small deletions ($\Delta\text{Ex}\alpha\text{H}$) in terms of both the $\alpha 1$ subunit effect on smaller peak currents and the $\beta 2$ subunit effect on reduced sensitivity to GABA. In contrast to previous studies of homomeric pLGICs (Bar-Lev et al., 2011; Castillo et al., 2009; Wong et al., 2014), complete removal of the putative α -helix does not abolish GABA-activated channel function.

GABA_ARs with N-terminal deletions in all subunits retain some function

To compare this study more directly with earlier work on monomeric receptors, we next investigated the effect of N-terminal deletions in all expressed subunits. First, HEK-293T cells were transfected with a Δ Ex deletion of α 1, β 2 and γ 2 subunits, denoted as all Δ Ex GABA_ARs. All Δ Ex α 1 β 2 γ 2 GABA_ARs, without any N-terminal extensions, were functional with a GABA EC₅₀ ($3.9 \pm 1.1 \mu\text{M}$), significantly lower ($p < 0.01$) than that of α 1 β 2 γ 2 WT (Figure 5C and Table 1) and a Hill coefficient of 1.1 ± 0.2 that was similar to α 1 β 2 γ 2 WT. The population whole-cell peak current for this triple deleted all Δ Ex mutant was significantly reduced ($p < 0.0001$) to $12 \pm 2\%$ of α 1 β 2 γ 2 WT (Table 1). The magnitude of this reduction is similar to the deletion of α 1 Δ Ex alone, emphasizing the dominant role of the α 1 subunit N-terminal extension.

The larger deletion, denoted all Δ Ex α H, that removed most of the N-terminal α -helix and the N-terminal extension in all subunits of α 1 β 2 γ 2 GABA_ARs also produced functional receptors (Figure 5C). The all Δ Ex α H GABA EC₅₀ was $1.2 \pm 0.6 \mu\text{M}$, significantly lower ($p < 0.05$) than α 1 β 2 γ 2 WT (Figure 5C and Table 1). Further emphasizing the key role of the α 1 subunit N-terminus, similar to the α 1(Δ Ex α H) alone, this triple mutant significantly reduced ($p < 0.0001$) population whole-cell peak current to $10 \pm 2\%$ of α 1 β 2 γ 2 WT (Table 1).

Discussion

The α 1 N-terminal extension is crucial for receptor assembly and trafficking

We found that deletion of the N-terminal extension in α 1 subunit dramatically decreased whole-cell currents to $< 20\%$ of WT due an equivalent decrease in receptors at the cell surface. Equivalent deletions in either β 2 or γ 2 subunits had little effect on either the number of receptors at the cell surface or peak GABA-gated whole-cell currents. None of the deletions affected GABA-activated channel function or incorporation of the γ 2 subunit into functional GABA_ARs, as measured by benzodiazepine sensitivity and reduced sensitivity to Zn^{2+} inhibition. Simultaneous deletion of the N-terminal extension in all subunits caused a marked reduction in whole-cell currents and cell surface receptors but the reduction was similar to that for the deletion in the α 1 subunit alone. Although additional deletions in β 2 and γ 2 subunits had no additional effect on receptor assembly and trafficking, they did cause

a small decrease in GABA EC₅₀. Taken together, these data show that the N-terminal extension is a major determinant of $\alpha 1\beta 2\gamma 2$ GABA_AR assembly and trafficking, specifically in the α subunit.

The basis for this asymmetry in the importance of the N-terminal extension is not immediately clear but may be rationalised by considering what is known about the process of GABA_AR assembly in the context of GABA_AR structural models. There is clear evidence that the assembly of GABA_AR subunits into mature pentameric receptors is a non-random hierarchical process that predominantly produces receptors with a 2 α , 2 β , 1 γ composition (Angelotti et al., 1993; Bolland et al., 2003; Rabow et al., 1995; Sarto Jackson and Sieghart, 2008; Verdoorn et al., 1990; Woollorton et al., 1997b). In the context of recent structural information, the predominant arrangement of subunits can be defined as $-\beta+/-\alpha+/-\gamma+/-\beta+/-\alpha+/-$ with the last $\alpha+$ interfacing with the first $-\beta$ in a pentameric ring (Figure 1A). Agonist- and benzodiazepine-binding sites are formed at $\beta+/-\alpha$ and $\alpha+/-\gamma$ interfaces, respectively, near the middle of the ECD (Cromer et al., 2002). Analysis of assembly intermediates has shown that α , β and γ subunits can all contribute to heterodimers (Klausberger et al., 2001; Tretter et al., 1997), but it has been proposed that assembly may start with a $\beta+/-\alpha$ hetero-dimer (Sarto Jackson and Sieghart, 2008). Indeed, the ability of agonists or antagonists that bind to the $\beta+/-\alpha$ interface to act as pharmacological chaperones and increase functional receptors at the cell surface (Eshaq et al., 2010), demonstrates the importance of this interface in a rate-limiting step in receptor assembly. As all eukaryotic pLGIC structures place the N-terminus of the N-terminal α -helix, and hence the N-terminal extension when present, at the (-) face of each subunit, this places the α subunit N-terminal extension close to the β subunit at the same $\beta+/-\alpha$ interface (Figure 1A). Thus the importance of the α subunit N-terminal extension for receptor assembly and trafficking may be due to an interaction with the β subunit (+) face that enhances $\beta+/-\alpha$ hetero-dimer formation.

N-terminal α -helices are not functionally essential in heteromeric GABA_ARs

In contrast to previous findings for homomeric eukaryotic pLGICs where deletion of the putative N-terminal α -helix abolished functional receptors, we found that some function was retained in heteromeric GABA_ARs lacking the putative α -helix in any single subunit or

lacking the bulk of the helix in all subunits. These data indicate that N-terminal α -helices are not essential for subunit folding or receptor function. We did, however, observe reductions in whole-cell currents, cell-surface receptors and $\gamma 2$ subunit incorporation that further support the contribution of N-terminal α -helices to receptor assembly and trafficking. As all of the α -helix deletions also included deletion of the N-terminal extension, their effects must be compared to the effect of the N-terminal extension alone. Deletion of the $\alpha 1$ subunit from the N-terminus to nearly ($\Delta\text{Ex}\alpha\text{H}$) or completely ($\Delta\text{Ex}\alpha\text{H}+3$) remove the N-terminal α -helix markedly reduced both cell surface receptors and whole currents. Most of this reduction was due to deletion of the N-terminal extension, with only slight additional decreases due to additional deletions of the N-terminal α -helix. Likewise, the decrease in whole-cell currents due to N-terminal α -helix deletions in all subunits ($\text{all}\Delta\text{Ex}\alpha\text{H}$) was similar to that caused by a similar deletion in the $\alpha 1$ subunit only or indeed by the smaller deletion of just the $\alpha 1$ N-terminal extension. The $\Delta\text{Ex}\alpha\text{H}$ deletion in all subunits did not abolish function.

In contrast to the $\alpha 1$ subunit, deletion of the N-terminal extension in the $\beta 2$ subunit had no significant effect on cell surface receptors or whole-cell currents. Further deletions of most ($\Delta\text{Ex}\alpha\text{H}$) or all ($\Delta\text{Ex}\alpha\text{H}+3$) of the $\beta 2$ subunit N-terminal α -helix reduced whole-cell currents to 40% of $\alpha 1\beta 2\gamma 2$ WT. Although deletions of the putative α -helix had greater effects on reducing total levels of functional receptors than deletion of just the N-terminal extension, robust whole-cell currents remained, demonstrating that the putative N-terminal α -helices are not essential for functional receptors.

N-terminal α -helices in $\beta 2$ and $\gamma 2$ subunits are important for $\gamma 2$ subunit incorporation

Similar to the $\beta 2$ subunit, deletion to include most of the N-terminal α -helix in the $\gamma 2$ subunit ($\gamma 2\Delta\text{Ex}\alpha\text{H}$) reduced cell surface receptor levels and whole-cell currents to $\leq 70\%$ of $\alpha 1\beta 2\gamma 2$ WT, whereas deletion of just the N-terminal extension had no significant effect. As functional GABA_ARs can be formed from just α and β subunits (Angelotti et al., 1993; Verdoorn et al., 1990), a key question with any $\gamma 2$ subunit mutation is whether it affects $\gamma 2$ incorporation into receptors, producing a mixed population of $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs (Baburin et al., 2008; Baumann et al., 2001; Boileau et al., 2002; Boileau et al., 2003). Indeed, reduced benzodiazepine potentiation and increased Zn^{2+} inhibition, clearly

Accepted Article

demonstrated that the $\gamma 2(\Delta\text{Ex}\alpha\text{H})$ deletion, removing most of the N-terminal α -helix, markedly reduces $\gamma 2$ incorporation so that the majority of functional GABA_ARs would be WT $\alpha 1\beta 2$. In this case, the reduced whole-cell current may be partly due to the deleted $\gamma 2(\Delta\text{Ex}\alpha\text{H})$ subunit competing unproductively for assembly. The failure of $\gamma 2$ incorporation due to deletion of most of the $\gamma 2$ N-terminal α -helix is consistent with assembly and trafficking deficits due to similar deletions in homomeric pLGICs (Wong et al., 2014).

Similar deletion of most of the N-terminal α -helix in the $\beta 2$ subunit ($\beta 2(\Delta\text{Ex}\alpha\text{H})$) also markedly reduced $\gamma 2$ incorporation, as demonstrated by decreased benzodiazepine potentiation and increased Zn^{2+} inhibition, but to a lesser extent than the $\gamma 2(\Delta\text{Ex}\alpha\text{H})$ deletion. Conversely, the $\alpha 1(\Delta\text{Ex}\alpha\text{H})$ deletion did not impair $\gamma 2$ subunit incorporation, despite a dramatic reduction in functional receptors at the cell surface. These differential effects may be explained by noting that structural models place the N-terminus of the putative α -helix at the (-) face of each subunit (Figure 1). Thus, this region in one of the β subunits, but not in the α subunits, could interact with the (+) face of the $\gamma 2$ subunit and contribute to $\gamma 2$ incorporation. Likewise, the importance of the $\gamma 2$ subunit N-terminal α -helix for $\gamma 2$ incorporation may be explained by a similar interaction with the (-) face of the α subunit.

The di-leucine motif in GABA_ARs does not play a critical role relative to the whole helix

Deletions of the complete N-terminal α -helix ($\Delta\text{Ex}\alpha\text{H}+3$), in either $\alpha 1$ or $\beta 2$ subunits, had no greater effect on peak whole-cell currents than deletions retaining the last three helix residues ($\Delta\text{Ex}\alpha\text{H}$), including the di-leucine motif (red in Figure 1B). These data provide no support for a key role for the di-leucine motif above that of the helix as a whole. We designed this comparison based on a previous investigation of $\alpha 7$ nAChRs (Castillo et al., 2009). They found that single mutations within the α -helix, particularly of Leu11 that aligns with the first residue in the di-leucine motif, had similar effects to complete α -helix deletion in abolishing functional cell-surface receptors. In GluCl, this equivalent leucine residue is buried against the top of the β -sandwich (Hibbs and Gouaux, 2011). Interestingly related single mutations also abolished functional cell surface 5HT3Rs but not GlyRs (Castillo et al., 2009). We recently showed that in $\rho 1$ GABA_CRs, retaining six C-terminal residues of the putative α -helix, including the di-leucine motif, partially restored functional receptors that were

abolished by complete deletion of the α -helix (Wong et al., 2014). Thus, whilst the N-terminal α -helix is consistently shown to contribute to the appearance of functional receptors at the cell surface for a variety of pLGICs, the degree of this contribution and the role of particular residues or segments differ between pLGICs.

N-terminal α -helix deletions had minor effects on GABA_AR functional characteristics

Although deletions of the subunit N-terminal regions markedly reduced the number of functional receptors at the cell surface, they had relatively minor effects on the functional characteristics of the remaining receptors, including GABA EC₅₀, Hill coefficient and desensitization rate. It should be noted that in the experimental system used here, the rate of solution exchange will limit detection of changes in faster components of desensitization. None of the deletions investigated here had any significant effect on the Hill coefficient for GABA activation. Furthermore, no single subunit deletion of the N-terminal extension affected the GABA EC₅₀. Larger single-subunit deletions of the N-terminal α -helix did reduce the sensitivity to GABA (2.5 to 8-fold higher EC₅₀), but only when in the β subunit. Conversely N-terminal deletions in all subunits slightly decreased the GABA EC₅₀, although this was on a background of very small currents. Likewise, it was only in the β subunit that deletions in the α -helix showed small but significant effects on the rate of desensitization. The specific role of the β subunit α -helix in these functional characteristics may be linked to the β subunit contributing the principal face of the GABA-binding site. As the N-terminal helix is not close to the GABA-binding site, it is likely to contribute to conformational changes of GABA-activated gating, rather than directly to GABA binding. Indeed, recent structural comparison of GluCl in various open and closed conformations shows distinct movements at the N-terminal α -helix (Althoff et al., 2014).

Conclusions

In conclusion, our results demonstrate a significant contribution of the N-terminal extension, specifically in the α subunit, to the appearance of functional receptors at the cell surface. The N-terminal α -helices in all subunits also contribute to receptor maturation, with those in the β and γ subunits being important for γ subunit incorporation into GABA_ARs. Our data do not define which steps of subunit folding, receptor assembly or trafficking to the cell surface are

particularly affected. As we saw no effect of any deletion on the total expression of the other two subunits, it appears that intracellular subunits that are folded but incompletely assembled are relatively stable and not subject to degradation, consistent with previous findings (Castillo et al., 2009; Wong et al., 2014). The importance of this N-terminal region to GABA_AR function in the brain is highlighted by the identification of epilepsy-linked mutations γ 2R43Q, just after the N-terminal α -helix, and β 3G7R (Gurba et al., 2012), in the N-terminal extension, both in patients with febrile seizures and childhood absence epilepsy. Both mutations alter subunit composition and reduce receptor levels at the plasma membrane (Frugier et al., 2007; Gurba et al., 2012; Kang and Macdonald, 2004; Sancar and Czajkowski, 2004; Tan et al., 2007; Wimmer et al., 2010).

Acknowledgements

We thank the flow cytometry core facility at School of Medical Sciences, RMIT University. Research was approved by RMIT University and supported by the National Health and Medical Research Council (566672 to BAC) and ANZ Trustees. The authors have no conflicts of interest to declare.

References

- Althoff T, Hibbs RE, Banerjee S and Gouaux E (2014) X-ray structures of GluCl in apo states reveal a gating mechanism of Cys-loop receptors. *Nature* **512**:333-337.
- Angelotti TP, Uhler MD and Macdonald RL (1993) Assembly of GABAA receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J Neurosci* **13**:1418-1428.
- Baburin I, Khom S, Timin E, Hohaus A, Sieghart W and Hering S (2008) Estimating the efficiency of benzodiazepines on GABA(A) receptors comprising gamma1 or gamma2 subunits. *Br J Pharmacol* **155**:424-433.
- Bar-Lev DD, Degani-Katzav N, Perelman A and Paas Y (2011) Molecular dissection of Cl⁻-selective Cys-loop receptor points to components that are dispensable or essential for channel activity. *J Biol Chem* **286**:43830-43841.
- Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN and Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* **50**:291-313.
- Baumann SW, Baur R and Sigel E (2001) Subunit arrangement of gamma-aminobutyric acid type A receptors. *J Biol Chem* **276**:36275-36280.
- Bocquet N, Nury H, Baaden M, Le Poupon C, Changeux J-P, Delarue M and Corringer P-J (2009) X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. *Nature* **457**:111-114.

- Boileau AJ, Baur R, Sharkey LM, Sigel E and Czajkowski C (2002) The relative amount of cRNA coding for gamma2 subunits affects stimulation by benzodiazepines in GABA(A) receptors expressed in *Xenopus* oocytes. *Neuropharmacol* **43**:695-700.
- Boileau AJ, Li T, Benkwitz C, Czajkowski C and Pearce RA (2003) Effects of gamma2S subunit incorporation on GABAA receptor macroscopic kinetics. *Neuropharmacol* **44**:1003-1012.
- Bollan K, Robertson LA, Tang H and Connolly CN (2003) Multiple assembly signals in gamma-aminobutyric acid (type A) receptor subunits combine to drive receptor construction and composition. *Biochem Soc Trans* **31**:875-879.
- Brejck K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB and Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **411**:269-276.
- Castillo M, Mulet J, Aldea M, Gerber S, Sala S, Sala F and Criado M (2009) Role of the N-terminal alpha-helix in biogenesis of alpha7 nicotinic receptors. *J Neurochem* **108**:1399-1409.
- Chen Q, Yim P, Yuan N, Johnson J, Cook J, Smith S, Ionescu Zanetti C, Wang Z-J, Arnold L and Emala C (2012) Comparison of cell expression formats for the characterization of GABA(A) channels using a microfluidic patch clamp system. *Assay Drug Dev Technol* **10**:325-335.
- Cole C, Barber J and Barton G (2008) The Jpred 3 secondary structure prediction server. *Nucleic Acids Res* **36**:W197-W201.
- Cromer BA, Morton CJ and Parker MW (2002) Anxiety over GABA(A) receptor structure relieved by AChBP. *Trends Biochem Sci* **27**:280-287.
- Draguhn A, Verdorn TA, Ewert M, Seeburg PH and Sakmann B (1990) Functional and molecular distinction between recombinant rat GABAA receptor subtypes by Zn²⁺. *Neuron* **5**:781-788.
- Ernst M, Bruckner S, Boresch S and Sieghart W (2005) Comparative models of GABAA receptor extracellular and transmembrane domains: important insights in pharmacology and function. *Mol Pharmacol* **68**:1291-1300.
- Eshaq RS, Stahl LD, Stone R, 2nd, Smith SS, Robinson LC and Leidenheimer NJ (2010) GABA acts as a ligand chaperone in the early secretory pathway to promote cell surface expression of GABAA receptors. *Brain Res* **1346**:1-13.
- Frugier G, Coussen Fo, Giraud M-F, Odessa M-Fo, Emerit M, Boue-Grabot E and Garret M (2007) A gamma 2(R43Q) mutation, linked to epilepsy in humans, alters GABAA receptor assembly and modifies subunit composition on the cell surface. *J Biol Chem* **282**:3819-3828.
- Golden AP, Li N, Chen Q, Lee T, Nevill T, Cao X, Johnson J, Erdemli G, Ionescu-Zanetti C, Urban L and Holmqvist M (2011) IonFlux: a microfluidic patch clamp system evaluated with human Ether-a-go-go related gene channel physiology and pharmacology. *Assay Drug Dev Technol* **9**:608-619.
- Gunther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, Crestani F, Aguzzi A, Arigoni M and Lang Y (1995) Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **92**:7749-7753.
- Gurba K, Hernandez C, Hu N and Macdonald R (2012) GABRB3 mutation, G32R, associated with childhood absence epilepsy alters $\alpha 1\beta 3\gamma 2L$ γ -aminobutyric acid type A (GABAA) receptor expression and channel gating. *J Biol Chem* **287**:12083-12097.
- Hassaine G, Deluz C, Grasso L, Wyss R, Tol MB, Hovius R, Graff A, Stahlberg H, Tomizaki T, Desmyter A, Moreau C, Li XD, Poitevin F, Vogel H and Nury H (2014) X-ray structure of the mouse serotonin 5-HT₃ receptor. *Nature* **512**:276-281.
- Hibbs RE and Gouaux E (2011) Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature* **474**:54-60.
- Hilf RJC and Dutzler R (2008) X-ray structure of a prokaryotic pentameric ligand-gated ion channel. *Nature* **452**:375-379.
- Hilf RJC and Dutzler R (2009) Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. *Nature* **457**:115-118.

- Horenstein J and Akabas MH (1998) Location of a high affinity Zn²⁺ binding site in the channel of alpha1beta1 gamma-aminobutyric acidA receptors. *Mol Pharmacol* **53**:870-877.
- Hosie A, Dunne E, Harvey R and Smart T (2003) Zinc-mediated inhibition of GABA(A) receptors: discrete binding sites underlie subtype specificity. *Nat Neurosci* **6**:362-369.
- Jordan M and Wurm F (2004) Transfection of adherent and suspended cells by calcium phosphate. *Methods* **33**:136-143.
- Kang J-Q and Macdonald R (2004) The GABAA receptor gamma2 subunit R43Q mutation linked to childhood absence epilepsy and febrile seizures causes retention of alpha1beta2gamma2S receptors in the endoplasmic reticulum. *J Neurosci* **24**:8672-8677.
- Klausberger T, Ehya N, Fuchs K, Fuchs T, Ebert V, Sarto I and Sieghart W (2001) Detection and binding properties of GABA(A) receptor assembly intermediates. *J Biol Chem* **276**:16024-16032.
- Korpi ER, Grunder G and Luddens H (2002) Drug interactions at GABA(A) receptors. *Prog Neurobiol* **67**:113-159.
- Krishek BJ, Moss SJ and Smart TG (1996) Homomeric beta 1 gamma-aminobutyric acid A receptor-ion channels: evaluation of pharmacological and physiological properties. *Mol Pharmacol* **49**:494-504.
- Macdonald RL and Olsen RW (1994) GABAA receptor channels. *Annu Rev Neurosci* **17**:569-602.
- Miller PS and Aricescu AR (2014) Crystal structure of a human GABAA receptor. *Nature* **512**:270-275.
- Miyazawa A, Fujiyoshi Y and Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423**:949-955.
- Olsen R and Sieghart W (2009) GABA A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* **56**:141-148.
- Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR and Seeburg PH (1989) Importance of a novel GABAA receptor subunit for benzodiazepine pharmacology. *Nature* **338**:582-585.
- Rabow LE, Russek SJ and Farb DH (1995) From ion currents to genomic analysis: recent advances in GABAA receptor research. *Synapse* **21**:189-274.
- Reynolds D, Rosahl T, Cirone J, O'Meara G, Haythornthwaite A, Newman R, Myers J, Sur C, Howell O, Rutter AR, Atack J, Macaulay A, Hadingham K, Hutson P, Belelli D, Lambert J, Dawson G, McKernan R, Whiting P and Wafford K (2003) Sedation and anesthesia mediated by distinct GABA(A) receptor isoforms. *J Neurosci* **23**:8608-8617.
- Sancar F and Czajkowski C (2004) A GABAA receptor mutation linked to human epilepsy (gamma2R43Q) impairs cell surface expression of alphabeta gamma receptors. *J Biol Chem* **279**:47034-47039.
- Sarto Jackson I and Sieghart W (2008) Assembly of GABA(A) receptors (Review). *Mol Membr Biol* **25**:302-310.
- Saxena NC and Macdonald RL (1994) Assembly of GABAA receptor subunits: role of the delta subunit. *J Neurosci* **14**:7077-7086.
- Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* **47**:181-234.
- Sigel E, Baur R, Trube G, Mohler H and Malherbe P (1990) The effect of subunit composition of rat brain GABAA receptors on channel function. *Neuron* **5**:703-711.
- Sigel E and Buhr A (1997) The benzodiazepine binding site of GABAA receptors. *Trends Pharmacol Sci* **18**:425-429.
- Spencer CI, Li N, Chen Q, Johnson J, Nevill T, Kammonen J and Ionescu Zanetti C (2012) Ion channel pharmacology under flow: automation via well-plate microfluidics. *Assay Drug Dev Technol* **10**:313-324.
- Tan H, Reid C, Single F, Davies P, Chiu C, Murphy S, Clarke A, Dibbens L, Krestel H, Mulley J, Jones M, Seeburg P, Sakmann B, Berkovic S, Sprengel R and Petrou S (2007) Reduced cortical inhibition in a mouse model of familial childhood absence epilepsy. *Proc Natl Acad Sci USA* **104**:17536-17541.

- Tasneem A, Iyer LM, Jakobsson E and Aravind L (2005) Identification of the prokaryotic ligand-gated ion channels and their implications for the mechanisms and origins of animal Cys-loop ion channels. *Genome Biol* **6**:R4.
- Thompson AJ, Lester HA and Lummis SCR (2010) The structural basis of function in Cys-loop receptors. *Q Rev Biophys* **43**:449-499.
- Tretter V, Ehya N, Fuchs K and Sieghart W (1997) Stoichiometry and assembly of a recombinant GABAA receptor subtype. *J Neurosci* **17**:2728-2737.
- Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. *J Mol Biol* **346**:967-989.
- Verdoorn TA, Draguhn A, Ymer S, Seeburg PH and Sakmann B (1990) Functional properties of recombinant rat GABAA receptors depend upon subunit composition. *Neuron* **4**:919-928.
- Wimmer VC, Reid CA, So EY, Berkovic SF and Petrou S (2010) Axon initial segment dysfunction in epilepsy. *J Physiol* **588**:1829-1840.
- Wong LW, Tae HS and Cromer BA (2014) Role of the rho1 GABA Receptor N-Terminus in Assembly, Trafficking and Function. *ACS Chem Neurosci* **5**:1266-1277.
- Wooltorton JR, McDonald BJ, Moss SJ and Smart TG (1997a) Identification of a Zn²⁺ binding site on the murine GABAA receptor complex: dependence on the second transmembrane domain of beta subunits. *J Physiol (Lond)* **505 (Pt 3)**:633-640.
- Wooltorton JR, Moss SJ and Smart TG (1997b) Pharmacological and physiological characterization of murine homomeric beta3 GABA(A) receptors. *Eur J Neurosci* **9**:2225-2235.

Table legends

Table 1: GABA dose-response parameters and pharmacology of WT and mutant GABA_ARs. Parameters are listed for fits to the Hill equation, EC₅₀, Hill coefficient (n_H), and maximum peak current, normalised to WT $\alpha 1\beta 2\gamma 2$ transfected and measured at the same time within each experiment, from n independent measurements. The degree of CDP potentiation (%) and Zn²⁺ inhibition (%) are from a separate set of n independent measurements. All are shown as mean $\pm$ SEM, with asterisks indicating significant differences; * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 compared to $\alpha 1\beta 2\gamma 2$ WT. “ND” indicates not determined.

Figure legends

Figure 1: N-terminus of GABA_AR subunits; structure predictions and deletion mutants. (A) Homology models of the GABA_AR $\alpha 1\beta 2\gamma 2$ (left) and $\alpha 1\beta 2$ (right) stoichiometry, shown in ribbon representation, built on GluCl template (3RIF) and viewed along the symmetry axis from the extracellular side. The $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits are shown in red, blue and green, respectively, with (+) and (-) faces of each subunit labelled. N-terminal extensions are shown as random coil in thicker tubes of the same colour as the main subunit, with that of $\gamma 2$ subunit labelled. Putative N-terminal α -helices are internally highlighted in yellow for all subunits. (B) ClustalW alignment of pLGIC N-terminal amino acid sequences (excluding signal peptides) manually adjusted for structural alignment to align a partially conserved di-leucine motif (red text). Highly conserved residues are shown in bold. Protein data bank codes are given for sequences with known structures. Regions highlighted are: start of β sandwich domain (green), N-terminal α -helices from crystal structures (cyan) and Jpred3 α -helix predictions (yellow). (C) Amino acid sequences of the N-terminal region of WT and deletion mutants of $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits using a consistent colour definition of the putative N-terminal α -helix as in (B). Each deletion is named at left, with deleted residues in brackets.

Δ Ex represents deletion of the N-terminal extension. Δ Ex α H represents deletion of the N-terminal extension and α -helix, while maintaining the di-leucine motif (red text). Δ Ex α H+3 represents deletion of the N-terminal extension and α -helix, including the di-leucine motif.

Figure 2: Effect of N-terminal extension deletions on GABA sensitivity. (A) Representative population whole-cell currents from HEK-293T cells expressing $\alpha 1\beta 2\gamma 2$, $\alpha 1(\Delta$ Ex) $\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta$ Ex) $\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta$ Ex) GABA_ARs. The indicated concentrations of GABA (μ M) were applied for 3 s to HEK cells voltage-clamped at -80 mV. (B) Normalised GABA dose-response curves for cells expressing $\alpha 1\beta 2\gamma 2$ (dashed curve fit) and $\alpha 1\beta 2$ (dotted curve fit), $\alpha 1(\Delta$ Ex) $\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta$ Ex) $\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta$ Ex) GABA_ARs. Error bars indicate $\pm$ SEM. Curves are Hill equation fits of each data set.

Figure 3: Effect of N-terminal extension deletions on cell-surface and total α and γ subunits detected using flow cytometry. (A) Top: Immuno-fluorescence intensity histograms of cell surface or total anti- α detection for intact or permeabilised HEK-293T cells, respectively. Cells were either untransfected (black) or transfected with $\alpha 1\beta 2\gamma 2$ (red), $\alpha 1\beta 2\Delta$ Ex $\gamma 2$ (green) or $\alpha 1\beta 2\gamma 2\Delta$ Ex (blue) subunits. Below: average population mean fluorescence relative to $\alpha 1\beta 2\gamma 2$ (as %) from 4-5 separate experiments. Error bars indicate $\pm$ SEM. (B) Top: Immuno-fluorescence intensity histograms of cell surface and total anti- $\gamma 2$ detection, as for (A), with untransfected (black) or transfected with $\alpha 1\beta 2\gamma 2$ (red), $\alpha 1(\Delta$ Ex) $\beta 2\gamma 2$ (green) or $\alpha 1\beta 2(\Delta$ Ex) $\gamma 2$ (blue) subunits. Below: average population mean fluorescence relative to $\alpha 1\beta 2\gamma 2$ (as %) from 4-5 separate experiments. Error bars indicate $\pm$ SEM. **** $p < 0.0001$ compared to $\alpha 1\beta 2\gamma 2$.

Figure 4: Effect of N-terminal extension deletions on $\gamma 2$ incorporation into functional GABA_ARs. (A) Representative population whole-cell currents (black) in response to 1 mM GABA (black line) from HEK-293T cells expressing $\alpha 1\beta 2\gamma 2$, $\alpha 1(\Delta$ Ex) $\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta$ Ex) $\gamma 2$, $\alpha 1\beta 2\gamma 2(\Delta$ Ex) or $\alpha 1\beta 2$ subunits (left to right). Overlaid are whole-cell currents (grey) in response to 1 mM GABA in the presence of and pre-incubated with 30 μ M Zn²⁺ (grey line). (B) Representative population whole-cell currents from HEK-293T cells expressing $\alpha 1\beta 2\gamma 2$, $\alpha 1(\Delta$ Ex) $\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta$ Ex) $\gamma 2$, $\alpha 1\beta 2\gamma 2(\Delta$ Ex) or $\alpha 1\beta 2$ GABA_ARs (from left to right) elicited by 6 μ M GABA (black line) or subsequent application of 1 μ M CDP together with 6 μ M GABA (grey dashed line).

Figure 5: Effect of deletions to the putative α -helix on GABA sensitivity. Normalised GABA dose-response curves for cells expressing $\alpha 1\beta 2\gamma 2$ (dashed curve fit) and $\alpha 1\beta 2$ (dotted curve fit), reproduced in all panels, together with: (A) $\alpha 1(\Delta$ Ex α H) $\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta$ Ex α H) $\gamma 2$ and $\alpha 1\beta 2\gamma 2(\Delta$ Ex α H) (B) $\alpha 1\beta 2(\Delta$ Ex α H+3) $\gamma 2$ and (C) all Δ Ex $\alpha 1\beta 2\gamma 2$ and all Δ Ex α H $\alpha 1\beta 2\gamma 2$ GABA_ARs. Error bars indicate $\pm$ SEM. Curves are fits to the Hill equation of each data set.

Figure 6: Effect of putative α -helix deletions on cell surface receptors and $\gamma 2$ incorporation. Above: immunofluorescent detection of (A) $\alpha 1$ subunits from $\alpha 1\beta 2(\Delta$ Ex α H) $\gamma 2$ - and $\alpha 1\beta 2\gamma 2(\Delta$ Ex α H)-expressing cells, and (B) $\gamma 2$ subunits from $\alpha 1(\Delta$ Ex α H) $\beta 2\gamma 2$ - and $\alpha 1\beta 2(\Delta$ Ex α H) $\gamma 2$ -expressing cells. Data shown are average population mean cell surface or total immunofluorescence from intact or permeabilised HEK-293T cells, respectively, expressed as a % of $\alpha 1\beta 2\gamma 2$ WT, from 3-4 separate experiments in each case. Error bars indicate $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ compared to $\alpha 1\beta 2\gamma 2$.

Representative fluorescence histograms for A and B are shown in Supplementary Figure 2. Below: representative population whole-cell currents from HEK-293T cells expressing $\alpha 1\beta 2\gamma 2$, $\alpha 1(\Delta\text{Ex}\alpha\text{H})\beta 2\gamma 2$, $\alpha 1\beta 2(\Delta\text{Ex}\alpha\text{H})\gamma 2$, $\alpha 1\beta 2\gamma 2(\Delta\text{Ex}\alpha\text{H})$ and $\alpha 1\beta 2$ GABA_ARs (left to right). **(C)** Sensitivity to Zn^{2+} inhibition, with response to 1 mM GABA (black line) or to 1 mM GABA together with, and preincubated in, 30 μM Zn^{2+} (grey line). **(D)** Sensitivity to CDP potentiation, with response to 6 μM GABA (black line) or to 1 μM CDP together with 6 μM GABA (grey dashed line).

Construct	GABA EC ₅₀ (μ M)	Hill Coefficient (n _H)	Maximum current, I _{MAX} (% of α 1 β 2 γ 2)	n	CDP potentiation (%)	Zn ²⁺ inhibition (%)	n
α 1 β 2 γ 2	11.0 $\pm$ 1.2	1.1 $\pm$ 0.1	100	5	54 $\pm$ 5	18 $\pm$ 2	8
α 1 β 2	0.5 $\pm$ 0.2****	0.9 $\pm$ 0.2	86 $\pm$ 6	5	1 $\pm$ 1****	79 $\pm$ 2****	4
α 1(Δ Ex) β 2 γ 2	11.1 $\pm$ 0.5	1.2 $\pm$ 0.1	16 $\pm$ 5****	4	46 $\pm$ 10	15 $\pm$ 4	3
α 1 β 2(Δ Ex) γ 2	8.0 $\pm$ 0.5	1.3 $\pm$ 0.1	81 $\pm$ 8	4	39 $\pm$ 7	13 $\pm$ 4	5
α 1 β 2 γ 2(Δ Ex)	8.0 $\pm$ 1.0	1.2 $\pm$ 0.1	89 $\pm$ 14	4	45 $\pm$ 7	19 $\pm$ 4	6
α 1(Δ Ex α H) β 2 γ 2	5 $\pm$ 2*	1.0 $\pm$ 0.3	10 $\pm$ 3****	4	48 $\pm$ 6	21 $\pm$ 5	4
α 1 β 2(Δ Ex α H) γ 2	28 $\pm$ 4*	1.0 $\pm$ 0.1	40 $\pm$ 3****	4	15 $\pm$ 4****	43 $\pm$ 3****	5
α 1 β 2 γ 2(Δ Ex α H)	7.2 $\pm$ 1.8	1.0 $\pm$ 0.2	70 $\pm$ 6**	5	5 $\pm$ 2****	61 $\pm$ 5****	5
α 1(Δ Ex α H+3) β 2 γ 2	-	-	9 $\pm$ 1****	4	ND	ND	
α 1 β 2(Δ Ex α H+3) γ 2	91 $\pm$ 19****	1.0 $\pm$ 0.1	40 $\pm$ 2****	4	ND	ND	
all Δ Ex	3.9 $\pm$ 1.1**	1.1 $\pm$ 0.2	12 $\pm$ 2****	6	ND	ND	
all Δ Ex α H	1.2 $\pm$ 0.6****	1.7 $\pm$ 0.6	10 $\pm$ 2****	3	ND	ND

Fig 2

A Zn^{2+} Inhibition

B CDP potentiation