Human $\alpha 1\beta 3\gamma 2$ L gamma-aminobutyric acid type A receptors: High-level production and purification in a functional state

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Abstract: Gamma-aminobutyric acid type A receptors (GABA_ARs) are the most important inhibitory chloride ion channels in the central nervous system and are major targets for a wide variety of drugs. The subunit compositions of GABA_ARs determine their function and pharmacological profile. GABA_ARs are heteropentamers of subunits, and ($\alpha 1$)₂($\beta 3$)₂($\gamma 2$ L)₁ is a common subtype. Biochemical and biophysical studies of GABA_ARs require larger quantities of receptors of defined subunit composition than are currently available. We previously reported high-level production of active human $\alpha 1\beta 3$ GABA_AR using tetracycline-inducible stable HEK293 cells. Here we extend the strategy to receptors containing three different subunits. We constructed a stable tetracycline-inducible HEK293-TetR cell line expressing human (N)-FLAG- $\alpha 1\beta 3\gamma 2$ L-(C)-(GGG)₃GK-1D4 GABA_AR. These cells achieved expression levels of 70–90 pmol [³H]muscimol binding sites/15-cm plate at a specific activity of 15–30 pmol/mg of membrane protein. Incorporation of the $\gamma 2$ subunit was confirmed by the ratio of [³H]flunitrazepam to [³H]muscimol binding sites and sensitivity of GABA-induced currents to benzodiazepines and zinc. The $\alpha 1\beta 3\gamma 2$ L GABA_ARs were solubilized in dodecyl- β -maltoside, purified by anti-FLAG affinity chromatography and reconstituted in CHAPS/asolectin at an overall yield of ~30%. Typical purifications yielded 1.0–1.5 nmoles of [³H]muscimol binding sites/60 plates. Receptors with similar properties could be purified by 1D4 affinity chromatography with lower overall yield. The composition of the purified, reconstituted receptors was confirmed by ligand binding, Western blot, and proteomics. Allosteric interactions between etomidate and [³H]muscimol binding were maintained in the purified state.

Keywords: human $\alpha 1\beta 3\gamma 2$ GABA_AR; HEK293 TetR cells; purification; reconstitution

Abbreviations: $\alpha 1\beta 3$ GABA_AR, GABA_AR with $\alpha 1$ and $\beta 3$ subunits; $\alpha 1\beta 3\gamma 2$ GABA_AR, GABA_AR with $\alpha 1$, $\beta 3$, and $\gamma 2$ subunits; 5HT_{3A}R, 5-hydroxytryptamine-3A receptor; DDM, *n*-dodecyl- β -D-maltopyranoside; GABA_AR, gamma-aminobutyric acid type A receptor; K_d , equilibrium dissociation constant of the ligand.

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Introduction

Gamma-aminobutyric acid type A receptors (GABA_ARs) are the major inhibitory neurotransmitter-gated chloride-conducting ion channels in the central nervous system.¹ Naturally occurring mutations in these receptors lead to disease states such as epilepsy.² GABA_ARs are the target of neuropharmaceuticals including general anesthetics, benzodiazepines, anticonvulsants, sedative-hypnotics, and anxiolytics (reviewed in Refs. 3–6) as well as ethanol.⁷ The GABA_AR is a member of the Cys-loop superfamily of ligand-gated ion channels, a family characterized by a conserved disulfide bond-linked loop in the extracellular domain of each subunit and an assembly of five homologous subunits around a central transmembrane ion conducting pore. Each subunit has a large extracellular domain containing over 200 amino acid residues, a transmembrane domain composed of four membrane-spanning α -helices, and a highly variable intracellular domain formed by a loop between the third and fourth transmembrane helices. The function, pharmacological properties, and temporospatial distribution of GABA_ARs are highly dependent on their subunit composition. There are eight classes of GABA_AR subunits (alpha, beta, gamma, delta, epsilon, theta, pi, and rho), and some subunits have several subtypes, leading to a total of 19 subunit genes known to date.⁸ Most native GABA_ARs contain two α , two β , and either one γ or one δ subunit; in particular, γ 2-containing GABA_ARs are predominantly located in synapses and represent 75–80% of the GABA_AR population.⁸ The γ 2 subunit is especially important therapeutically because the α 1– γ 2 interface in the extracellular domain is the binding site for benzodiazepines, a major class of sedative and antiepileptic drugs currently used in clinical practice.¹ Furthermore, the general anesthetic etomidate binds between the β 3 and α 1 subunit in the transmembrane domain, and GABA binds between the same subunits in the extracellular domain.⁹ Thus, the interfaces between two adjacent subunits are important for both drug action and gating. However, the mechanisms underlying these subunit-specific properties remain unclear. Several x-ray crystallography structures of ligand-gated ion channels were recently reported,^{10–12} but they are all homomeric and lack an intracellular domain.

To locate drug-binding sites by photolabeling and to undertake spectroscopic studies of structural changes induced by endogenous ligands and drugs in heteromeric GABA_ARs requires an efficient expression, purification, and reconstitution method to produce sufficient quantities of pure functional protein at high concentrations. Previously, heteromeric GABA_ARs have been expressed in mammalian and insect cell lines, but with relatively low yields (~4 pmol muscimol binding sites/mg membrane pro-

tein).^{13–15} High expression yield for a single-subunit G protein-coupled receptor (GPCR) was achieved by developing a tetracycline-inducible HEK293 cell line containing a constitutive tetracycline repressor (HEK293-TetR) that separates the cell growth and protein expression steps.¹⁶ This HEK293-TetR cell line also enabled the development of stable cells that expressed homomeric 5-HT_{3A}Rs and heteromeric α 1 β 3 GABA_ARs at higher levels than those reported in previous studies.¹⁷ The α 1 β 3 GABA_ARs reconstituted therein has allowed the location of etomidate binding sites by photolabeling and sequencing by Edman-degradation.⁹ However, when the 5-HT_{3A}R was compared to the α 1 β 3 GABA_AR, it was found that addition of a second subunit to the pentamer reduced the specific activity twofold, raising the challenge of whether similar cell lines with more subunits could be developed. Here, we report the high-level expression, purification, and reconstitution of α 1 β 3 γ 2L GABA_ARs in the same HEK293-TetR cell line. Specific activity of agonist binding was maintained, but introduction of the γ 2L-subunit lowered the yield per plate and made solubilization more difficult.

Results and Discussions

Development of stable HEK293-TetR for α 1 β 3 γ 2L GABA_AR

Because there were reports that the γ 2 subunit might be difficult to incorporate during assembly,¹⁸ we first investigated adding an affinity tag to this subunit. The 1D4 epitope (TETSQVAPA) is originally from bovine rhodopsin's C-terminus, and direct addition of the 1D4 tag to the exposed C-terminus of other GPCRs has led to successful purifications.¹⁹ Our previous study with 5HT_{3A}R–1D4 suggested the need for a linker between the C-terminus and the 1D4 sequence to ensure accessibility to the antibody.¹⁷ Therefore, we initially searched for suitable linkers using transient transfection of the readily expressed homomeric 5HT_{3A}R.^{17,20} Five linkers (X) were compared in 5HT_{3A}R–(C)–X–1D4: (1) His₁₂; (2) His₆; (3) VLYKSGGSPG, a 10-residue linker previously applied in sugar porters with extracellular C-termini²¹; (4) (GGG)₃GK, a flexible 11-residue linker extensively utilized in protein conjugates²²; (5) GDDEASATVSK, the 11 C-terminal residues preceding 1D4 epitope in bovine rhodopsin. Construct 1 expressed 5HT_{3A}R–X–1D4 poorly but could indeed be purified, constructs 2–5 expressed equally well, yielding 2.4–4.9 pmol of specific [³H]GR65630 binding sites/mg of membrane protein and 3.5–6.0 pmol/plate. All five linkers increased the binding efficiency to anti-1D4 columns from less than 5% with no linker to 83–94%. Therefore, a linker of 6–12 residues is essential but its exact sequence is less important, so we chose to add the most flexible linker

Figure 1. FLAG- $\alpha 1\beta 3\gamma 2L$ -L3-1D4 GABA_ARs in plasma membranes contain γ -subunits. Whole-cell patch-clamp recordings of GABA-induced chloride currents after induction of GABA_AR expression. (A) Resistance to inhibition by Zn²⁺ demonstrated in paired pulses with and without Zn²⁺. Right panel, statistics of n determinations compared to control when Zn²⁺ was omitted from the second pulse. (B) Enhancement of GABA currents. Upper panel shows a representative trace; lower panel, the statistics relative to control without diazepam in the second pulse. (C) GABA concentration-response curve. Peak currents elicited with varying GABA concentrations were normalized to the second pulse peak elicited with 10 mM GABA.

(GGG)₃GK (called L3 herein) between the C-terminus of the GABA_AR and the 1D4 sequence (Supporting Information Fig. S1).

A stably transfected HEK293-TetR cell line expressing (N)-FLAG- $\alpha 1\beta 3\gamma 2$ -(C)-L3-1D4 GABA_AR was then created as described in Materials and Methods. Four out of ten clones that had good growth rates also had the expected two to one stoichiometry of agonist to benzodiazepine sites, and the highest yielding clone was chosen for further use.

Subunit expression profile in HEK293-TetR characterized by electrophysiology

The subunit composition of (N)-FLAG- $\alpha 1\beta 3\gamma 2$ -(C)-L3-1D4 GABA_AR overexpressed in the HEK293-TetR cells was characterized by electrophysiology. Three criteria were used to characterize the presence of the γ -subunit; zinc sensitivity, modulation by a benzodiazepine and the agonist EC₅₀. First, GABA_ARs consisting of $\alpha 1\beta 3$ subunits are inhibited by Zn²⁺, but incorporation of a γ subunit ($\alpha 1\beta 3\gamma 2L$) renders GABA_ARs insensitive to 10 μ M Zn²⁺.^{23–26}

Whole-cell patch-clamp currents elicited by high GABA concentrations were insensitive to Zn²⁺ in our cell line [Fig. 1(A), left panel]. To provide a more sensitive test for the presence of $\alpha 1\beta 3$ containing channels, low concentrations of GABA were used because $\alpha 1\beta 3$ containing channels have a lower GABA EC₅₀ than $\alpha 1\beta 3\gamma 2$ -containing channels [7.4 vs. 36 μ M respectively; see Fig. 1(C)]. Thus, 5 μ M GABA [Fig. 1(A), middle panel] will open ~33% of $\alpha 1\beta 3$ channels and only ~7% of $\alpha 1\beta 3\gamma 2$ channels. Under these conditions, zinc inhibited currents by 33 ± 7% [standard deviations are given throughout; n = 4; Fig. 1(A), right panel], revealing a small fraction of $\alpha 1\beta 3$ subunit-containing GABA channels. Second, in $\alpha 1\beta 3\gamma 2$ containing channels activated with 1 μ M GABA, 1 μ M diazepam enhanced currents by 221 ± 107% [n = 11; Fig. 1(B)]. Third, the GABA concentration current response curve had an EC₅₀ of 36 ± 2 μ M and Hill coefficient of 1.7 ± 0.1 [Fig. 1(C)], comparable to reported values for wild-type $\alpha 1\beta 3\gamma 2$ channels.²³ Based on these results, we estimate that the $\gamma 2$ subunit is present in over 90% of the

Figure 2. FLAG- $\alpha 1\beta 3\gamma 2$ -L3-1D4 GABA_ARs in cell membranes contain γ -subunits. Binding curves of [³H]muscimol and [³H]flunitrazepam determined by filtration assays using cell membranes. Binding curves were fitted to the Hill equation by nonlinear least squares (see Table I and text for parameters).

expressed GABA-activated channels in this stable cell line. Cells expressing only $\alpha 1\beta 3$ receptors were not observed.

Biochemical characterization of the subunit expression profile in HEK293-TetR cells

The ligands [³H]muscimol (a GABA-mimetic agonist binding at the two $\beta 3$ - $\alpha 1$ interfaces) and [³H]flunitrazepam (a benzodiazepine binding at the single $\alpha 1$ - $\gamma 2$ interface) are expected to bind $\alpha 1\beta 3\gamma 2$ GABA_ARs with a stoichiometry of 2:1,¹⁵ and thus the ratio of saturated specific binding sites of [³H]muscimol and [³H]flunitrazepam was used to measure the relative level of subunit expression. Because of the higher GABA_AR expression levels in this cell line, much higher muscimol concentrations ($\leq 1 \mu M$) can be used here than in most previous studies before nonspecific binding became too high. For muscimol binding (Table I), we found a $B_{\max}$ of

Table I. Ligand Binding Properties of Cell Membrane and Reconstituted AntiFLAG-Purified (N)-FLAG- $\alpha 1\beta 3\gamma 2$ -(C)-L3-1D4 GABA_A Receptors^a

Ligand	Membrane		Reconstituted receptors	
	K_d (nM)	n_{Hill}	K_d (nM)	n_{Hill}
[³ H]Muscimol	49 ± 5	1.3 ± 0.1	79 ± 13	1.2 ± 0.3
[³ H]Flunitrazepam	10 ± 1	1.2 ± 0.2	71 ± 18	1.1 ± 0.2

^aData in membranes are mean of three independent determinations and in purified receptors from a single determination.

30 pmol/mg of membrane protein, a Hill coefficient of 1.3, and a dissociation constant of 50 nM compared to literature values for heterologously expressed receptors of $B_{\max} \sim 4$ pmol/mg and K_d s of 5–21 nM.^{13,14,27} A binding curve for [³H]flunitrazepam performed on the same membranes yielded a $B_{\max}$ of 14 ± 0.4 pmol/mg of membrane protein (see Table I for other parameters), yielding muscimol/flunitrazepam site stoichiometry of 2.2 ± 0.1, consistent with most oligomers containing one γ -subunit. Etomidate (10 μM), a general anesthetic that binds GABA_ARs in the transmembrane domain at the $\beta 3$ - $\alpha 1$ subunit interfaces,⁹ decreased the dissociation constant of [³H]muscimol twofold (27 ± 2 nM), suggesting that allosteric interactions between etomidate binding and muscimol binding are retained.

Based on Table I, 500 nM [³H]muscimol was chosen for routine assays of agonist binding sites (95% saturation of sites assuming the Hill coefficient is 1.25). Specific activities varied but ~20 pmol/mg of membrane protein was routinely obtained (Table II), about fivefold higher than previously reported for $\gamma 2$ -containing human GABA_ARs, and slightly lower than $\alpha 1\beta 3$ GABA_ARs in the same cell line.¹⁷ Nonetheless, the comparison with published work in Table II demonstrates that each additional subunit type included in the pentamer of a Cys-loop receptor lowers the yield per plate by about a factor of 2. However, the number of subunits forming the oligomer appears to be much less important; the yields of 5HT_{3A}R homo-pentamer are comparable to those obtained with a G-protein receptor.²⁸

Solubilization of $\alpha 1\beta 3\gamma 2$ GABA_AR membrane

Previously 2.5 mM DDM was found sufficient to solubilize 85% of $\alpha 1\beta 3$ GABA_ARs,¹⁷ but the presence

Table II. Yields and Specific Activities of Receptors Expressed on 15-cm Plates^a

	Specific activity (pmol/mg)	Yield (pmol/plate)	Plates to express 10 nmol ^b	Source
(N)-FLAG- $\alpha 1\beta 3\gamma 2$ -(C)-L3-1D4 GABA _A R	15–30	70–90	110–140	This work
FLAG-GABA _A $\alpha 1\beta 3$ R	29–34	140–170	60–70	Dostlova <i>et al.</i> , 2010
FLAG-5-HT _{3A} R-1D4	49–63	245–315	30–40	Dostlova <i>et al.</i> , 2010
$\beta 2$ -Adrenergic Receptor	180–240	190–320	30–50	Chelikani <i>et al.</i> , 2006

Specific activity determined by [³H]muscimol (GABA_AR), [³H]GR65630 (5HT_{3A}R) & [³H]dihydroalprenolol ($\beta 2$ -AR).

^a176 cm² per plate. The figures for this work range from 12 separate preparations.

For comparison, 100 g of cow brain cerebral cortex has 1.3 nmoles of [³H]muscimol sites.³⁵

Table III. Representative Yields of (N)-FLAG- $\alpha 1\beta 3\gamma 2$ -(C)-L3-1D4 GABA_AR Purification Via Anti-FLAG Affinity Chromatography

Purification fractions	Total specific [³ H] muscimol binding sites (pmol) ^a	% Overall yield ^b	% Column efficiency ^b
Starting membranes	4605	100%	–
Solubilized supernatant applied on column	4190	86 ± 15%	100%
Flow through during binding	1127	20 ± 4%	23 ± 6%
First wash flow through	73	2 ± 1%	3 ± 1%
Elution 1 (E1)	678	16 ± 3%	19 ± 5%
E2	344	9 ± 3%	11 ± 3%
E3-E4, pooled	403	8 ± 1%	9 ± 2%
Total elution	1425	31 ± 4%	35 ± 6%

^aTotal amount of GABA_AR, measured as specifically bound [³H]muscimol (500 nM) from one representative purification using 60 plates of cells.

^bMean ± standard deviation from four independent purifications.

of the $\gamma 2$ subunit made solubilization much more difficult. Solubilization efficiency increased rapidly with DDM concentration in the range of 0–20 mM and slowly reached a plateau by 80 mM (Supporting Information Fig. S1). A similar trend was seen with C₁₂E₉. When detergent concentrations were normalized by individual cmc (critical micelle concentration), the two curves superimposed (Supporting Information Fig. S1). Although either detergent was acceptable, we chose 30 mM DDM for large-scale purification, slightly lower than the 40 mM DDM used to solubilize GLIC.²⁹ Further development with 30 mM DDM improved solubilization from ~40 (Supporting Information Fig. S1) to ~86% (Table III) of the starting material in membranes. This improvement was achieved by gradual addition of 37.5 mM DDM stock solution to 60 mL of membrane suspension (5 mg/mL) to a final concentration of 30 mM DDM.

Antibody purification of $\alpha 1\beta 3\gamma 2$ L GABA_AR

In a typical experiment (Table III), membrane pellets from 60 plates containing 4.6 nmoles of [³H]muscimol sites yielded 1.4 nmoles of final purified protein, with an overall yield of 31%, when purified by anti-FLAG affinity chromatography. The average yield from solubilized membranes applied to the FLAG column was 31 ± 4% (four purifications, Table III). Of the starting membrane pellets (100%), ~14% was lost in solubilization, ~22% was lost in column loading and washing, and ~33% remained on the column after four elutions with 0.1 mM FLAG peptide (Table III). Only a small fraction of the latter could be eluted by overnight incubation with more FLAG peptide.

The percent of receptors bound to an anti-1D4 affinity column that could be eluted by the peptide was similar to that with FLAG columns, but the capacity of the columns was lower, so that the overall yield with equal ratio of receptor to affinity beads was about half of that with the anti-FLAG beads. Furthermore, the 1D4 column was more difficult to

regenerate and more costly, and thus it was not routinely applied to large-scale purification.

Reconstitution of $\alpha 1\beta 3\gamma 2$ L GABA_AR in CHAPS and asolectin

To replace the low cmc detergent, DDM, with the high cmc detergent, CHAPS, extensive washing with CHAPS/asolectin was employed (see the Methods section). The detergent CHAPS was chosen for reconstitution because experience indicates that it preserves allosteric interactions better than the cholate used previously.¹⁷ CHAPS concentrations of 5–10 mM were equivalent, and 5 mM was routinely used. The asolectin concentration in 5 mM CHAPS could be varied from 0.86 to 0.025 mM without compromising the elution yield, but at 0.01 mM the yield fell by half because more protein was retained on the column after elution. Using the published relationship between lipid concentration and the CHAPS concentrations in aqueous and micelle phases,³⁰ we estimated that increased retention on the column commences when the mixed micelle to (lipid bilayer + mixed micelle) phase boundary is crossed. Eluted column fractions were 25–100 nM in [³H]muscimol sites and contained ~500–2000 lipids per oligomer.

Characterization of affinity purified GABA_AR by SDS-PAGE, mass spectrometry and Western blot

A typical FLAG-purification is shown in the SDS-PAGE denaturing gel in Figure 3(A). The multiple bands present in the solubilized material are reduced to three major bands close to the 56 kDa marker (the expected amino acid molecular weights of the subunits are 52–55 kDa). The eluting peptides are of low MW (~1 kDa) and are not present. Lanes 4 and 5 showed little contamination when up to 45 pmoles was loaded.

All three subunits were identified and shown to be glycosylated by Western blots [Fig. 3(B)]. The α -subunit appeared as a single band, the β -subunit as a double band, and the γ -subunit as a single broad

Figure 3. Purification and subunit composition of FLAG- $\alpha 1\beta 3\gamma 2L$ -L3-1D4 GABA_ARs. Receptors were purified by anti-FLAG Chromatography. (A) Coomassie blue stain on a 14×15 cm SDS-PAGE gel of solubilized (30 mM DDM; lane 1) and purified reconstituted samples (5 mM CHAPS + 25 μ M Asolectin; lane 2, 4, 5, loaded with 4, 25, 45 pmoles respectively). Lane 3 shows purified receptor deglycosylated with PNGase F. (B) Western blots from 8 × 8 cm SDS-PAGE gels of purified reconstituted receptors before (-) and after (+) deglycosylation with PNGase F: Antibodies used for detection where: $\alpha 1$, anti-FLAG; $\beta 3$, anti- $\beta 3$; $\gamma 2$, anti-1D4.

band. Minor γ -subunit bands are associated with dimer and trimer formation (bands at ~ 100 and ~ 160 kDa). Such aggregation was more pronounced after PNGase F treatment, probably caused by the heating step.

A single excised gel piece containing the three major bands from a similar mini gel were digested with trypsin and the peptides identified by HPLC-tandem mass spectrometry. The number of nonoverlapping peptides and the percentage of residues detected respectively were α -subunit, 9 and 21%; β -subunit, 9 and 24%; γ -subunit, 8 and 17%. The

experiment was repeated twice more with comparable results.

The stoichiometry of the α -subunit compared to the γ -subunit in purified receptors was determined by Western blot using the FLAG antibody for the α -subunit and the 1D4 antibody for the γ -subunit. A homomeric 5HT_{3A}R bearing an N-terminal FLAG and a C-terminal 1D4 epitope on each subunit¹⁷ was used for calibration. Three separate experiments gave the stoichiometry as 2.1 ± 0.4 α -subunits for each γ -subunit.

Characterization of purified GABA_AR by radioactive ligand binding assays

Purified (N)-FLAG- $\alpha 1\beta 3\gamma 2$ -(C)-L3-1D4 GABA_ARs bound muscimol and flunitrazepam in a saturable manner (Fig. 4 and Table I). Compared to the same receptors in membranes, the dissociation constants were higher probably because of depletion of the free ligand concentration by dissolution in the micellar phase. The difference for flunitrazepam is much larger than that for muscimol presumably because of its greater lipid solubility. However, we cannot rule out a role for specific detergent-protein interactions.

Purified receptors remained sensitive to etomidate modulation.

The ability of etomidate to interact allosterically with both agonist and benzodiazepine sites in the reconstituted state is retained. Etomidate enhanced [³H]muscimol (2 nM) binding with EC₅₀s of 0.3 ± 0.1 and 1.0 ± 0.5 μ M in membranes and

Figure 4. Purified FLAG- $\alpha 1\beta 3\gamma 2L$ -L3-1D4 GABA_ARs reconstituted in 5 mM CHAPS plus 25 μ M asolectin contain γ -subunits (other details as in Figure 2).

Figure 5. Etomidate modulates muscimol binding to FLAG- $\alpha 1\beta 3\gamma 2\text{L-L3-1D4}$ GABA_ARs in membranes and purified reconstituted (5 mM CHAPS plus 25 μM asolectin) preparations. The [^3H]muscimol concentration was 2 nM. The data were fitted to the Hill equation with fixed slope of 1.25 and normalized to the maximum for display purpose (see text for results).

purified reconstituted receptors, respectively (Fig. 5). Similarly, it enhanced [^3H]flunitrazepam binding with EC₅₀s of 0.3 ± 0.1 and 0.2 ± 0.2 μM respectively. These assays monitor etomidate's ability to modulate the preexisting equilibrium between low and high affinity conformations of the receptor.^{31,32} In reconstituted systems, it has long been known that this ability is detergent dependent.³³ Consistently, we found lower modulation in the reconstituted receptors than in the membranes. Thus, in eight purifications that were reconstituted into 5 mM CHAPS and asolectin from 25–200 μM , etomidate (10 μM) significantly enhanced ($p = 0.0005$) [^3H]muscimol (2 nM) binding by $137 \pm 17\%$, whereas enhancement in the source cell membranes was greater ($222 \pm 24\%$, $p = 0.0001$). There was no dependence on asolectin concentration. Modulation in membranes solubilized in 30 mM DDM was significantly lower ($p = 0.003$) and half that in the reconstituted preparations above (p values from two-tailed t -tests).

Conclusions

The strategy of using HEK293–TetR cells to separate the growth and production phase of GABA_AR expression raises the specific activity of agonist binding sites that may be routinely achieved approx-

imately fivefold even when the heteropentamer contains three different subunits ((N)–FLAG– $\alpha 1\beta 3\gamma 2$ –(C)–L3–1D4). Electrophysiological and ligand binding assays establish the presence of agonist, benzodiazepine, and etomidate binding sites that interact allosterically, suggesting that the pentamers are assembled correctly. These receptors can be purified in good yield and functionally reconstituted in CHAPS/asolectin. Sufficient quantities can be provided for biochemical procedures such as Edman degradation.³⁴ It should be possible to purify and concentrate enough material to undertake structural studies such as EPR, although this may be easier with those pentamers with the fewest number of different subunits.

Materials and Methods

Materials

Synthetic oligonucleotides were purchased from MGH–DNA Core Facility (Boston, MA). Restriction enzymes and buffers and PNGase F were purchased from New England Biolabs (Ipswich, MA). HEK293–TetR cells were a gift from Dr. H. G. Khorana's Laboratory at the Massachusetts Institute of Technology. Anti-FLAG antibody-coupled M2 affinity agarose beads, FLAG peptide bromide, soybean asolectin, γ -aminobutyric acid, muscimol, flurazepam, and flunitrazepam were purchased from Sigma-Aldrich (St. Louis, MO). CNBr-activated sepharose beads were from GE Healthcare Bio-Sciences (Piscataway, NJ). Detergents C₁₂E₉, n-dodecyl- β -D-maltopyranoside (DDM, Anagrade) and CHAPS (Anagrade) were from Anatrace-Affymetrix (Santa Clara, CA). Radioactive [^3H]muscimol (3-Hydroxy-5-Aminomethylisoxazole, [Methylene- ^3H (N)], 22.46 or 25.5 Ci/mmol), and [^3H]flunitrazepam, ([Methyl- ^3H], 75.7 Ci/mmol) were from Perkin Elmer (Waltham, MA). The monoclonal antibody, Rho-1D4, was prepared by the Cell Culture Center (Minneapolis, MN) from a cell line provided by Dr. R.S. Molday (University of British Columbia, Vancouver, Canada). The 1D4 peptide was prepared by the Association of Biomolecular Resource Facilities (Charlestown, MA). Phosphate-buffered saline (PBS, 10 $\times$, final pH 7.4), BCA protein assay kit, and EZ-RUN BP3603 (11–170 kDa) protein molecular weight markers for SDS-PAGE were from Thermo Fisher Scientific (Rockford, IL). Dextran sodium sulfate (M_r 6000–8000) was from MP Biomedicals (Solon, OH), primatone RL/UF was from Kerry Bio-Science (Norwich, NY). D-glucose, glutaMAX, pluronic F-68, penicillin-streptomycin, zeocin, blasticidin, hygromycin B, G418, 293fectin, and MES-SDS running buffer were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS, premium) was from Atlanta Biologicals (Lawrenceville, GA). Scintillation liquid Liquiscint was from National Diagnostics (Atlanta, CA).

Dulbecco's Modified Eagle Medium containing nutrient mixture F-12 (DMEM/F-12, ATCC, Manassas, VA) was used for adherent cell cultures with 10% (v/v) heat-inactivated FBS and 0.2% (v/v) penicillin-streptomycin liquid. Selection factors included 250 $\mu\text{g/mL}$ Zeocin, 5 $\mu\text{g/mL}$ Blasticidin, 50 $\mu\text{g/mL}$ Hygromycin B, and 200 $\mu\text{g/mL}$ G418. The media for induction contained additional 1 $\mu\text{g/mL}$ tetracycline and 5 mM sodium butyrate. Cell lysis buffer contained 10 mM HEPES (pH 7.4), 1 mM EDTA, and protease inhibitors (10 $\mu\text{g/mL}$ pepstatin, 2 $\mu\text{g/mL}$ aprotinin, 10 $\mu\text{g/mL}$ chymostatin, 10 $\mu\text{g/mL}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Purification base buffer is composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM CaCl_2 , 5 mM KCl, 5 mM MgCl_2 , 4 mM EDTA, and further supplemented with 10% (v/v) glycerol, protease inhibitors or detergents as specified below. Binding assay buffer contained 200 mM KCl, 1 mM EDTA, and $1\times$ PBS (pH 7.4).

Construction and stable cell line generation

The genes encoding GABA_AR $\alpha 1$, $\beta 3$, and $\gamma 2$ subunits were respectively cloned into expression vectors containing independent antibiotic selection. Preparation of the plasmids Flag-bGABA_AR $\alpha 1$ /pcDNA4/TO-Zeocin and hGABA_AR $\beta 3$ /pcDNA3.1/TO-Hygro⁺ was described previously.¹⁷ The plasmid hGABA_AR $\gamma 2$ -(GGG)3GK-1D4/pACMV/TO-blasticidin was made by adding a (GGG)3GK-1D4 tag into the C-terminal of hGABA_AR $\gamma 2$ cDNA, and then cloning into G418 selectable pACMV-TO vector. The identity of open reading frame coding for each gene was confirmed by DNA sequencing. HEK293-TetR cells (Blasticidin resistance) at 50% confluence in a 15-cm culture plate in DMEM medium were transfected with 40 mg of the three constructs at a molar ratio of $2\alpha:2\beta:1\gamma$ using 293fectin (Invitrogen, Grand Island, NY). Transfected cells were suspended and transferred to 96-well plates at 100 and 200 cells/well in regular DMEM medium 24 hours after transfection. DMEM-containing antibiotics (Zeocin, Hygromycin, G418, and Blasticidin) was added to the wells after 18 hours for single colony selection. Clonal colonies were isolated and amplified in the same selection medium. Inducible gene expression and receptor production were verified by quantitative reverse-transcription polymerase chain reaction (RT-PCR) and Western blotting, respectively. The best cell lines were selected based on growth rate, number of agonist sites ($[^3\text{H}]$ muscimol), and the expected 2:1 ratio of agonist to benzodiazepine ($[^3\text{H}]$ flunitrazepam) sites.

Preparation of 1D4 antibody-coupled agarose beads

CNBr-activated sepharose beads (0.6 g; wet volume 2 mL) were quickly rinsed three times with 10 mL of 1 mM HCl, incubated overnight with 2 mL 1D4 antibody solution (5 mg/mL) mixed with 2 mL cou-

pling buffer (0.2M NaHCO₃ and 1.0M NaCl (pH 8.80)). This 2:1 volume ratio gave a coupling efficiency of 98%. The anti-1D4 agarose beads were stored at 4°C for further use.

Immunoaffinity purification of GABA_AR

Stably transfected HEK293-TetR cells were grown at 37°C for 72 hours, induced with tetracycline and 5 mM sodium butyrate for 24 hours, harvested and lysed using an ultrasonic probe and grinder as reported previously.¹⁷ Membrane pellet suspensions at typical protein concentrations of 5–10 mg/mL obtained from sixty 15-cm plates were flash-frozen in liquid nitrogen and stored at -80°C for further use. Protein purification was carried out at 4°C. With continuous moderate stirring, thawed membrane pellets were solubilized by dropwise addition of the purification base buffer supplemented with DDM (final concentration 30 mM, 1.5%, m/v) and protease inhibitors, to a final protein concentration of 1 mg/mL over 30 min, followed by equilibration for 2.5 hours. Unsolubilized material was removed by centrifugation (43,000g, 30 min), and the supernatant was transferred to 3×15 mL columns containing 2 mL of anti-FLAG or anti-1D4 beads pretreated with poly-D-lysine hydrobromide.¹⁷ To replace DDM with CHAPS plus asolectin, the beads were washed twice with six column volumes of the buffer containing 8.5 mM asolectin and 17 mM CHAPS and then equilibrated on a rocker/shaker for 1 hour. This washing buffer was replaced by the elution buffer (asolectin (0.025–0.86 mM as required) and CHAPS (5 or 10 mM)) by washing twice with six column volumes. Elution was then initiated by addition of one column volume of the same buffer containing either 0.15 mM 1D4 peptide or 0.1 mM FLAG peptide, followed by rocking for 90 min. The eluate was collected, and the process was repeated three to four times. Eluted protein fractions were frozen in liquid nitrogen and stored at -80°C .

Radioactive ligand binding assays

Filtration (membranes) and precipitation/filtration binding assays were as previously described,¹⁷ except that to determine the oligomer concentration 0.15 mg membrane protein/mL were incubated with 500 nM $[^3\text{H}]$ muscimol or with 500 nM $[^3\text{H}]$ flunitrazepam at room temperature (21°C) for 80 min in the binding assay buffer, followed by filtration as previously described. Nonspecific binding for $[^3\text{H}]$ muscimol and $[^3\text{H}]$ flunitrazepam was measured in the presence of 10 mM GABA and 100 μM flurazepam dihydrochloride, respectively.

SDS-PAGE and protein identification of purified receptors

Protein composition of the final anti-FLAG affinity purified $\alpha 1\beta 3\gamma 2$ GABA_AR was examined by SDS-

PAGE using 8% Bis-Tris gels with MES-SDS running buffer and protein molecular weight markers for 26–170 kDa. Gels were either stained with Coomassie blue, or subjected to Western blot using monoclonal anti-GABA_Aβ2,3R extracellular domain (MAB341, Millipore, Billerica, MA), FLAG- or 1D4-antibodies. Stained gel bands at 50–60 kDa were excised for in-gel trypsin digestion followed by proteomic analysis for protein identification.

The ratio of α- to γ-subunit was determined semiquantitatively by Western blot. Increasing amounts of purified (N)-FLAG-α1β3γ2-(C)-L3-1D4 GABA_AR (1, 2, 4, 6, 12 μL of ~40 nM protein) were applied symmetrically to lanes 1–5 and 8–13 of a 15-well 10% NUPAGE Bis-TrisGel (Invitrogen). (N)-FLAG-5HT_{3A}R-(C)-1D4 membranes were added to lanes 6, 7, 14, and 15. After running the SDS-PAGE gel and transferring to a PVDF membrane (Millipore), the latter was cut into two halves, blocked, washed, and one half of the membrane was incubated with Anti-Flag Ab (1:1000 dilution), and the other half with Rho-1D4 Antibody (1:5000) (overnight, 4°C). After three TBST washes, bovine anti-mouse IgG-HRP was added (1 hour at RT), and chemiluminescence of the Pierce ECL 2 Substrate was scanned (Bio-Rad VersaDoc) and processed in ImageJ software taking the ratio of FLAG to 1D4 intensity in the 5HT_{3A}R lanes as one.

Ion channel functions by electrophysiology

Whole-cell patch-clamp current recordings were carried out as previously described for α1β3 containing GABA_A.¹⁷ Cells were seeded on a glass coverslip and protein expression was induced with tetracycline (2 μg/mL) and 10 mM sodium butyrate for 5–26 hours before recordings. Open pipette resistances ranged from 1.6 to 4 MΩ. Cells were voltage clamped at –50 mV using the patch clamp amplifier (Axopatch 200B, Molecular Devices Corp., Sunnyvale, CA). Series resistance ranged from 1.5 to 5 MΩ and cell capacitances from 3.5 to 10.5 pF. Receptors were activated using 500 ms pulses of GABA delivered via a multichannel superfusion pipette coupled to piezo-electric elements that switched solutions in less than 1 ms. Currents were filtered at 5 kHz and digitized at 10 kHz using pCLAMP v8.1 (Molecular Devices Corp., Sunnyvale, CA) for off-line analysis. The sensitivity of receptors to 10 μM ZnCl₂ was tested by applying two 0.5 s pulses of 10 mM GABA 5 s apart and 10 μM ZnCl₂ was included in the second pulse only. Control experiments without zinc in the second pulse were performed to correct for a small amount of desensitization. Similar experiments were also done using 5 μM GABA as a positive control for zinc. Diazepam modulation of GABA_A currents was tested by exposing cells first to 1 μM GABA (500 ms), a wash period followed by application of 1 μM GABA supplemented with 1 μM

diazepam for 500 ms. Control experiments were performed by omitting diazepam in the second pulse. For GABA concentration-response studies, two pulses of GABA were presented to the cell. The first pulse (500 ms) delivered GABA concentrations ranging from 1 μM to 10 mM, and 7 s later a second 500 ms pulse of 10 mM GABA was applied. Peak currents of the first GABA pulse were normalized to those of the second pulse. Pooled normalized data were fitted with logistic (Hill) functions using non-linear least squares in Origin 6.1 (OriginLab, Northampton, MA). Statistical analysis was performed in Graphpad Prism v.4 software (Graphpad Software, Inc., San Diego, CA). All data are expressed as mean ± SD.

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