Expression and purification of a functional heteromeric GABA\textsubscript{A} receptor for structural studies

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Abstract

The GABA-gated chloride channels of the Cys-loop receptor family, known as GABA\textsubscript{A} receptors, function as the primary gatekeepers of fast inhibitory neurotransmission in the central nervous system. Formed by the pentameric arrangement of five identical or homologous subunits, GABA\textsubscript{A} receptor subtypes are defined by the subunit composition that shape ion channel properties. An understanding of the structural basis of distinct receptor properties has been hindered by the absence of high resolution structural information for heteromeric assemblies. Robust heterologous expression and purification protocols of high expressing receptor constructs are vital for structural studies. Here, we describe a unique approach to screen for well-behaving and functional GABA\textsubscript{A} receptor subunit assemblies by using the \textit{Xenopus} oocyte as an expression host in combination with fluorescence detection size exclusion chromatography (FSEC). To detect receptor expression, GFP fusions were introduced into the \(\alpha_1\) subunit isoform. In contrast to expression of \(\alpha_1\) alone, co-expression with the \(\beta\) subunit promoted formation of monodisperse assemblies. Mutagenesis experiments suggest that the \(\alpha\) and \(\beta\) subunits can tolerate large truncations in the non-conserved M3/M4 cytoplasmic loop without compromising oligomeric assembly or GABA-gated channel activity, although removal of N-linked glycosylation sites is negatively correlated with expression level. Additionally, we report methods to improve GABA\textsubscript{A} receptor expression in mammalian cell culture that employ recombinant baculovirus transduction. From these methods we have identified a well-behaving minimal functional construct for the \(\alpha_1/\beta_1\) GABA\textsubscript{A} receptor subtype that can be purified in milligram quantities while retaining high affinity agonist binding activity.

Introduction

The magnitude and duration of excitatory signaling events in the central nervous system is controlled by inhibitory neurotransmission mediated primarily by the opening of pentameric
ligand-gated ion channels of the Cys-loop receptor family. GABA (γ-aminobutyric acid) functions as the primary inhibitory chemical messenger and elicits membrane hyperpolarization through rapid diffusion of chloride on the millisecond timescale upon binding to cognate GABA$_A$ receptors [1, 2]. These ion channels are also the targets of endogenous neurosteroids [3, 4] and pharmacological compounds that possess sedative and calming properties through receptor modulation, which defines the clinical basis of general anesthetics and anxiolytics [5–7]. The critical role of these receptors in suppressing excitatory inputs is emphasized further by the strong correlation between reduced GABA$_A$ receptor activity and seizure disorders that vary in severity, including the most debilitating epileptic encephalopathies [8–10].

The potential to alter channel gating by allosteric modulators suggests that GABA$_A$ receptors are excellent candidates for targeted therapies. As an example, the benzodiazepine drug class has been employed as an important therapy for controlling epilepsy [11], anxiety [12] and sleep disorders [13] by directly potentiating GABA$_A$ receptor activity. However, parallel to the broad therapeutic benefits of benzodiazepines is the presentation of adverse side effects, such as amnesia and addiction [7], as a result of long term use. Importantly, the explicit molecular mechanism of drug modulation on GABA$_A$ receptors is not known, although the serendipitous discovery of benzodiazepines was made more than 50 years ago [14].

The lack of atomic resolution structural models that define ligand binding sites and conformational states contributes to an incomplete understanding of GABA$_A$ receptor structure and function relationships. Presently, structural and functional studies are interpreted from homology models generated from bacterial homologs [15–18] or related Cys-loop receptors, such as the nicotinic acetylcholine receptor [19–21] or the glutamate-gated chloride channel [22–24]. In general, all Cys-loop receptors share a common core architecture defined by association of five homologous subunits arranged about a central ion-conducting pore as observed in structures of channels formed from unitary subunits, or homo-pentamers [23, 25, 26]. Each subunit consists of a large extracellular amino-terminal domain connected to a four-helix bundle transmembrane domain that contributes residues for ion selectivity. The orthosteric binding site is formed at the interface of adjacent subunits in the amino-terminal domain. Coupling between agonist binding and channel gating is facilitated by an interaction network composed of residues in transmembrane and extracellular domain loops, including the characteristic disulfide [27].

Despite these similarities, most GABA$_A$ receptors exist as heteromers of unique subunit combinations with discrete ion channel and pharmacological properties [28]. So far 19 GABA$_A$ receptor subunits have been identified and separated into classes and isoforms depending on the primary sequence: α(1–6), β(1–3), γ(1–3), δ, ε, θ, π and ρ(1–3). Although formation of homo-pentamers has been reported from in vitro studies [25], recapitulation of in vivo gating and pharmacology requires two or more subunits [29, 30]. Even though random assembly is unlikely [31], the molecular determinants that drive assembly of specific combinations in defined subunit stoichiometries and organization are not entirely known. Assembly signals encoded within the subunit primary sequence may play a role in limiting receptor subtype diversity [32]. Notably, subunit expression patterns suggest that unique properties of specific subunit combinations are fine tuned to regulate either phasic or tonic inhibition [28]. The most widely distributed receptor combination in adult neurons is the α1/β2/γ2 subtype in a predicted 2α:2β:1γ subunit ratio [33–36]. However, receptors composed of only α/β subunits, which form channels that are strongly antagonized by Zn$^{2+}$ [37–40] with a proposed 2α:3β stoichiometry [41], may play a role during embryonic development [42]. Importantly, incorporation of the γ subunit confers sensitivity to benzodiazepines and the derived clinical benefit is tied to the adjacent α subunit isoform [7, 43].
High resolution models of heteromeric assemblies will support efforts to delineate the structural basis of GABA\textsubscript{A} receptor properties by providing a framework to interpret a wealth of functional data. Indeed, recent structures of the cation-selective \(\alpha 4\beta 2\) nicotinic receptor arranged with two different subunit stoichiometries provide clues toward their distinct ion channel properties [44]. As a prerequisite to these studies, identification of efficient heterologous expression and isolation methodologies are required to generate sufficient quantities of receptor for structural interrogation via X-ray diffraction or single particle cryo-electron microscopy. Development of such methods is not trivial for eukaryotic membrane proteins because functional expression may be dependent on the host system, require co-factors or posttranslational modifications [45, 46]. Here, we outline an approach for identifying and characterizing a well-behaving heteromeric GABA\textsubscript{A} receptor composed of \(\alpha/\beta\) subunits. This approach combines the robust translation efficiency of Xenopus oocytes for screening subunit constructs with technological advances for large-scale expression in mammalian cell culture using a modified baculovirus delivery system [47]. We show that the \(\alpha 1/\beta 1\) receptor can be expressed and purified in milligram quantities while retaining ligand binding activity. These results set the stage for detailed structural investigation of heteromeric GABA\textsubscript{A} receptors and provide a foundation for pursuing other multi-subunit Cys-loop receptor assemblies.

### Materials and methods

#### Genes and construct design

**Construction of vectors.** The full-length rat GABA\textsubscript{A} receptor subunit isoforms \(\alpha 1\) (GeneID 29705), \(\beta 2\) (GeneID 25451) and \(\gamma 2S\) (GeneID 29709) with the native signal sequence were obtained in the pGEM vector as a gift from Dr. David S. Weiss. GluCl (Gene ID 180086) was also inserted into the pGEM vector. This vector contains a T7 RNA polymerase promoter site for \textit{in vitro} RNA transcription. For expression in insect cells, individual subunits with a leading Kozak sequence were cloned into the pFastBac1 vector where expression is driven by the polyhedron (PH) promoter, or into the pFastBac Dual vector in which case a single virus particle contains both \(\alpha 1\) (PH promoter) and \(\beta 2\) (p10 promoter) subunit genes. For expression in mammalian cells, all genes were cloned individually into a novel vector, pEG BacMam [47], where expression is controlled by the human cytomegalovirus (CMV) promoter.

**Generation of fluorescent protein fusions and mutagenesis.** Identification of the signal peptide for each subunit was made using the SignalP 4.1 server [48]. The location of fluorescent protein insertions and sequence deletions in the M3/M4 loop is shown in S1 Fig. In summary, enhanced green fluorescent protein (EGFP) was inserted into GluCl in the M3/M4 loop as described previously [49]. EGFP was ligated to the M3/M4 loop of the \(\alpha 1\) subunit between V399 and K400 using an in-frame non-native Asc1 restriction site (GGGCGCGCC), which adds a three residue linker (G–R–A) on each side of the fluorescent protein to the native receptor sequence. Likewise, mKalama was inserted into the \(\beta 2\) subunit M3/M4 loop between H421 and V422 also using an Asc1 restriction site. To screen M3/M4 loop truncations (“LT”) in \(\alpha 1\), an amino-terminal fluorescent protein fusion of the \(\alpha 1\) subunit was made by inserting GFPuv after the signal peptide (between G27 and Q28) with a 24 residue polypeptide linker (S–S–S–N–N–N–N–N–N–L–G–T–S–G–L–V–P–R–G–S) containing a thrombin protease site. The \(\alpha 1\)-LT construct was generated by replacing M3/M4 cytoplasmic loop residues Y341-P408 with Thr for a final predicted M3/M4 linker of R–G–T. Likewise, \(\beta 2\)-LT was generated by replacing R333-I438 with Gly for a final predicted M3/M4 linker of G–G–T. The \(\beta 1\)-LT subunit gene (GeneID 25450) was synthesized and cloned into pEG BacMam by Bio Basic Inc with residues K334-K439 replaced with Gly-Thr for a final predicted M3/M4 linker of R–G–T.

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linker of G–G–T. Incorporation of fluorescent protein fusions and receptor mutations were made using standard PCR procedures and confirmed by DNA sequencing.

**Construct screening in oocytes**

**RNA synthesis.** Circular plasmid DNA in the pGEM vector was linearized with NheI restriction endonuclease followed by *in vitro* synthesis of capped mRNA using the mMESSAGE mACHINE T7 Ultra kit from Ambion. After polyadenylation, mRNA was precipitated with LiCl, washed with 70% ethanol and re-suspended with DEPC-treated water. Concentration of mRNA was determined by absorbance at 260 nm and the quality was judged by gel electrophoresis.

**FSEC analysis and microscopy.** For FSEC analysis, defolliculated *Xenopus* oocytes (stage V–VI) were injected with 25–50 ng of total mRNA mixed in defined ratios to obtain expression of specific receptor subtypes. Oocytes were incubated at 16–18°C in ND-96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Hepes pH 7.5) supplemented with 250 μg/mL Amikacin for three to five days. Eight to 12 oocytes were mechanically disrupted by vigorous pipetting in 150 μL TBS pH 7.4 (20 mM Tris-HCl, 150 mM NaCl) followed by detergent solubilization with 40 mM n-dodecyl-β-D-maltopyranoside (C₁₂M) in the presence of 2 mM PMSF for 45 minutes with gentle mixing in 1.5 mL Eppendorf tubes. The sample was then centrifuged at 86,000 rcf for 40 minutes and 100 μL of the supernatant injected onto a Superose6 10/300 GL column (GE Healthcare) equilibrated in TBS pH 8.0 buffer containing 1 mM C₁₂M and 1 mM EDTA for analysis by fluorescence detection size exclusion chromatography (FSEC) monitoring EGFP fluorescence (excitation 488 nm, emission 510 nm) as previously described [50]. Ferritin (440 kDa) and EGFP (27 kDa) were used as molecular weight standards to assess the oligomeric integrity of detergent-solubilized receptor. To compare FSEC data of distinct receptor constructs and minimize batch variations in oocyte quality, FSEC traces for each set of experiments shown in the figures are acquired from the same batch and number of oocytes injected with normalized amounts of synthetic mRNA as described in the text. Receptor expression to the oocyte surface was observed using a Zeiss LSM710 laser scanning confocal microscope. Resonance energy transfer studies employed a high intensity laser (488 nm) to photobleach EGFP and subsequently monitor mKalama fluorescence from 410–486 nm.

**Electrophysiology.** For two electrode voltage clamp electrophysiology (TEVC) experiments, 5 ng of total mRNA was injected into defolliculated oocytes. After one to three days incubation at 16–18°C in ND-96 solution, oocytes were impaled with recording pipettes (0.7–2 MΩ) filled with 3 M KCl. Oocytes were voltage clamped to either -30 mV or -60 mV and perfused with 0.1 or 1 mM GABA in the absence or presence of 10 μM zinc in ND-96 buffer. Analog data were filtered at 50 Hz and digitized at ≥1 kHz. The Axoclamp 2B amplifier (Axon Instruments) and pClamp 10 software (Molecular Devices) were used for data acquisition.

**Insect and mammalian cell expression**

**Recombinant baculovirus production.** Baculovirus was generated as described previously [47, 51, 52]. Briefly, subunit constructs harboring a C-terminal His₈ tag in pFastBac1, pFastBac Dual or pEG BacMam vectors were transformed into DH10Bac competent cells for site-specific transposition of the expression cassette into bacmid. Purified bacmid was transfected into 1x10⁶ cells SF9 cells (Invitrogen™) in a 35 mm dish cultured in serum free SF-900 III SFM medium using Cellfectin II reagent and incubated at 27°C. The medium containing P1 virus was harvested four days later. To generate P2 virus, P1 virus was diluted 1000 fold into SF9 suspension cells at 1x10⁶ cells/mL density and allowed to incubate with shaking (115
rpm) at 27˚C for four days. Afterwards, the cells were removed by centrifugation and the supernatant, containing P2 virus, was filter sterilized. Viruses were stored at 4˚C in the dark supplemented with 2% FBS. P2 virus titer was determined using the Sf9 Easy Titer cell line and the end-point dilution assay [53] or flow cytometry by using the ViroCyt Virus Counter 2100 [54].

**Insect cell expression.** P2 virus derived from pFastBac1 or pFastBac Dual constructs was used to infect Sf9 or High Five cells (Invitrogen™) at a density of 1.5-3x10^6 cells/mL cultured in serum free Sf-900 III SFM or Express Five medium using different absolute and relative multiplicity-of-infection (MOI). Absolute MOI is defined as total virus relative to the total number of cells. Relative MOI is defined by the ratio of subunit viruses added to the cells. For transduction of cells with two viruses harboring individual α and β subunits (pFastBac1 constructs), the absolute MOI = 5 while varying the relative MOI between subunits as noted. An absolute MOI = 2 was used for transduction of virus obtained from pFastBac Dual constructs, which contain both α and β subunits. Infected cells were incubated with shaking (115 rpm) at either 27˚C for the duration of the expression trial, or shifted to 20˚C after an initial 18 hours at 27˚C. Each day for five days, 1 mL of cells were pelleted by centrifugation and frozen at -80˚C. The cells were solubilized in TBS pH 7.4 with detergent supplemented with a protease inhibitor cocktail (5 μg/mL aprotinin, 2 μg/mL leupeptin, 1.5 μg/mL pepstatin A, 0.3 mM PMSF) for FSEC analysis as described for oocytes.

**Expression and purification from mammalian cells.** Virus transduction in mammalian cells followed the protocol described by Goehring et al [47]. In summary, P2 virus was used to infect HEK293 GnTI- suspension cells (ATCC) at a density of 1.5-3x10^6 cells/mL (absolute MOI = 1.5 while varying relative MOI between subunits as noted) cultured in Gibco Freestyle 293 Expression medium supplemented with 2% FBS and placed in a humidity- and CO₂-controlled incubator on an orbital shaker (130 rpm). The total volume of virus added was less than 10% of the culture volume in all cases. Optimized expression parameters were determined by FSEC analysis of C₁₂M detergent-solubilized cells from 1mL aliquots 48 hours post infection. For large-scale expression and purification of α₁/β₁ receptor, infected cells were grown for 12 hours at 37˚C followed by 10 mM sodium butyrate addition and shifting culture temperature to 30˚C. After 60–72 hours post-infection, cultures were harvested by centrifugation and processed by sonication (10 min cycle, 5 sec on/off) while stirring on ice in the presence of protease inhibitor cocktail. After clearing the lysate of cell debris by centrifugation, membranes were isolated by ultracentrifugation at 186,000 rcf for 1.5 hours. The membranes were mechanically homogenized using a Dounce tissue grinder and solubilized with 40 mM C₁₂M in TBS pH 8.0 for 1 hour. Insoluble material was removed by ultracentrifugation at 186,000 rcf for 1 hour and the supernatant mixed with ClonTech Talon Co²⁺ affinity resin for three to four hours at 4˚C. The resin was washed with 30mM imidazole and then eluted with 250 mM imidazole buffer including 1 mM C₁₂M or 0.3 mM lauryl maltose neopentyl glycol (L-MNG) detergent. Fractions containing the receptor were pooled and concentrated using a 100 kDa MWCO filter for size exclusion chromatography (SEC) over a Superose6 10/300 GL column. Receptor purity was assessed by SDS-PAGE and coomassie blue staining using a 12.5% Tris-HCl gel. LC-MS was employed to confirm the identity of subunit bands on the gel. Receptor concentration was determined by absorbance at 280 nm assuming an average A₀°₂₈₀nm determined by the following equation:

\[
\left[ \frac{2 \left( \frac{εa}{MWa} \right) + \left( 3 \left( \frac{εb}{MWb} \right) \right)}{5} \right]
\]

Here we assume a subunit ratio of 2:3, α:β, and theoretical molecular weights (MW) and molar...
absorption coefficients ($\epsilon$) for each mature subunit. Theoretical MW ($\alpha_{1\text{-LT}} = 43.1 \text{kDa}, \beta_{1\text{-LT}} = 41.6 \text{kDa}$) and $\epsilon$ ($\alpha_{1\text{-LT}} = 55600 \text{M}^{-1} \text{cm}^{-1}, \beta_{1\text{-LT}} = 72895 \text{M}^{-1} \text{cm}^{-1}$) was calculated from the primary sequence using the ExPASy SIB Bioinformatics Resource Portal [55].

Radioligand binding to purified $\alpha_1/\beta_1$ receptor

Titration of purified receptor using 500 nM muscimol (10% hot, Perkin Elmer) and 1 mg/mL YSi Copper His tag SPA beads suggested a capacity of 100 nM receptor (~100 pmol) per mg of beads. For saturation binding and competition assays, 5 nM (0.5 pmol) purified receptor was mixed with $^3$H-muscimol (1:10 dilution with cold muscimol, 2 Ci/mmol specific activity) and 1 mg/mL SPA beads in 20 mM Hepes pH 7.35, 150 mM NaCl, 0.3 mM L-MNG in 100 uL final reaction volume. Muscimol was titrated from 3–400 nM to determine $K_D$, or held constant at 100 nM to determine $K_i$ (GABA). Counts were recorded on a MicroBeta TriLux luminescence counter from Perkin Elmer. Specific binding was measured with 1 mM GABA as a cold competitor. Each data point was repeated in triplicate for two separate trials and the standard deviations determined. Muscimol $K_D$ was determined from nonlinear least squares fit of specific binding assuming a single site binding model. $B_{\text{max}}$ was used to estimate the total number of $^3$H-muscimol binding sites assuming that all receptor was bound to the SPA beads. The $IC_{50}$ of GABA was determined from fitting the data points of the competition experiment with the Hill equation, which was then used to determine $K_i$ using the following equation:

$$K_i = IC_{50}/\left(1 + \frac{[\text{muscimol}]}{K_D}\right)$$

All fits were performed with the program Origin (OriginLab Corp).

Results

Screening methodology of Cys-loop receptor subunit constructs

Historically, *Xenopus* oocytes have proven a convenient vehicle for heterologous gene expression to assess exogenous ion channel properties by electrophysiological methods because they possess the machinery for gene expression but have low endogenous receptor activities [56, 57]. We have taken advantage of the robust gene expression in oocytes as a tractable platform for screening DNA constructs of the multi-subunit Cys-loop receptors. For our studies, genes were designed as fusions with GFP variants and delivered into the cytoplasm by microinjecting mature oocytes with up to 50 ng of total capped RNA transcripts synthesized *in vitro*. After three to five days at 16˚C, a small batch of oocytes (8–12) expressing the same construct was solubilized in detergent and injected onto a Superose6 column for analysis by fluorescence detection size exclusion chromatography (FSEC) monitoring GFP fluorescence [50]. Elution profiles were interpreted in terms of absolute fluorescence intensity as reflective of expression level and peak symmetry (monodispersity) as indicative of uniformity in oligomeric assembly.

As a benchmark, oocytes were injected with either 20 ng or 50 ng of RNA encoding the well-behaved $\alpha$ subunit of the glutamate-gated chloride channel (GluCl$\alpha$) from *C. elegans*, a homo-pentameric Cys-loop receptor that was crystallized previously [23]. The GluCl$\alpha$ construct was designed as a fusion with EGFP within the large cytoplasmic M3/M4 loop (Fig 1A and S1 Fig), which has been shown to not disrupt channel function [49]. After four days, EGFP fluorescence could be observed at the oocyte surface relative to an uninjected control using a laser scanning confocal microscope, consistent with expression of the receptor (Fig 1B). GluCl$\alpha$-EGFP was extracted from the oocytes with n-dodecyl-$\beta$-D-maltopyranoside (C$_{12}$M), a mild detergent used for purification and crystallization of GluCl$\alpha$ [23, 58]. FSEC
analysis detected the receptor peak eluting at a position consistent with an intact detergent-solubilized pentamer (~450 kDa theoretical) based on molecular weight standards (Fig 1C). The GluCl\textsubscript{α}-EGFP elution profile was similar to that observed from detergent-solubilized mammalian cells [47]. The fluorescence intensity of this peak demonstrated sensitivity to the dosage of RNA injected (Fig 1C). Solubilization of uninjected oocytes outlined peaks that are likely to arise from the oocyte itself.

**Oocyte expression of GABA\textsubscript{A} receptor subunits**

The propensity for GABA\textsubscript{A} receptor subunits to form homo- or heteromeric channels was investigated by injecting oocytes with the full-length rat \(\alpha\) isoform as a unitary subunit or in combination with full-length \(\beta\) or \(\gamma\) subunits, or with all three subunits. Analogous to GluCl\textsubscript{α} in Fig 1A, the \(\alpha\) subunit was expressed initially as an EGFP fluorescent protein fusion within the non-conserved M3/M4 loop (\(\alpha\)-EGFP), whereas the other subunits were expressed...
as wild type sequences. For expression of heteromeric assemblies, synthetic RNA transcripts for each subunit were combined in 1:1 ratios while keeping the total RNA injected constant (25–50 ng).

Three days post-injection, oocytes were solubilized in C_{12}M and analyzed by EGFP fluorescence for expression and profile homogeneity by FSEC. Although the α1 subunit alone demonstrated oligomerization near the elution position of GluClα-EGFP, peak shape is consistent with a heterogeneous entity (Fig 2A). Other peaks in the FSEC trace may suggest the presence of lower order oligomeric species (arrow, Fig 2A). Interestingly, co-injection with the β2 subunit increased overall expression and monodispersity of the primary peak and reduced the population of other oligomeric states, suggesting that incorporation of the β2 subunit coincides with oligomeric stabilization (Fig 2A). In contrast, co-injection with the γ2S (short splice variant) subunit did not alter the heterogeneous profile relative to the α1 subunit alone (Fig 2B), which may indicate that α1/γ2S receptors are unlikely to form stable assemblies in oocytes. However, we know that addition of the γ2S subunit in the presence of α1/β2 enables formation of the most common tri-heteromeric receptor in the central nervous system [34, 35]. Relative to the α1/β2 subunit combination, injection of all three subunits (α1-EGFP/β2/γ2S, 12ng each) resulted in substantially reduced fluorescence intensity (Fig 2C), suggesting either lower expression levels for the tri-heteromeric receptor relative to α1-EGFP/β2 or a reduced number of incorporated α1-EGFP subunits. Given the more robust expression and favorable chromatographic behavior, we continued to pursue characterization of the α1/β2 subtype.

We further investigated the putative association of α1 and β2 subunits by fluorescence resonance energy transfer (FRET) [59] between mKalama, a blue-shifted fluorescent protein, fused to the β2 subunit M3/M4 loop and α1-EGFP using a laser confocal microscope. If FRET occurs, donor (mKalama) fluorescence will be quenched in the presence of the acceptor (EGFP). As shown in Fig 3, photobleaching EGFP at the oocyte surface resulted in an increase in mKalama fluorescence relative to an unbleached control region, suggesting that the fluorescent protein pair is in close enough proximity to undergo FRET and is consistent with the formation of a heteromeric GABA_A receptor composed of α1/β2 subunits.

Based on preliminary screening of a restricted pool of subunits, the α/β GABA_A receptor demonstrated the most promising expression profiles relative to the other tested combinations. To determine a functional α/β construct suitable for large-scale expression, purification and downstream structural studies, we employed systematic mutagenesis to trim potentially unstructured polypeptide regions and remove sites of putative chemical modification in both subunits. The mutagenesis was guided by secondary structure prediction algorithms from the PredictProtein server [60] in conjunction with sequence alignments (S1 Fig) with the homologous GluClα in which a high resolution structure has been solved [23].

**M3/M4 loop truncations**

The greatest sequence divergence in alignments of GABA_A receptor subunits, and of Cys-loop receptors in general, is found in the large cytoplasmic loop connecting transmembrane helices three and four (M3/M4 loop). Electron microscopy of the related nicotinic acetylcholine receptor has revealed some secondary structure for this loop, but the majority of the peptide chain remains structurally undefined [19]. Although this loop contains a protein-protein interaction motif [61] and charged residues that contribute to the ion permeation pathway [62], agonist activation and ion selectivity persist in Cys-loop receptors when the native loop has been removed [63]. To facilitate crystallization in GluClα, the
Fig 2. Expression of GABA<sub>A</sub> receptors in oocytes. (A) The α1/β2 receptor demonstrates increased expression levels and homogeneity relative to the α1 subunit alone. The arrow indicates a population reduction of smaller oligomers in the presence of the β2 subunit. (B) Co-injection of γ2S mRNA with the α1 subunit does not improve receptor homogeneity. (C) Injection of all three subunits results in attenuated expression levels relative to α1/β2. All FSEC traces were acquired from the same batch of oocytes. Absolute fluorescence intensities are plotted on the same scale.

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Fig 3. Investigation of α1 and β2 subunit association in oocytes through FRET. Photobleaching of EGFP fused to the α1 subunit results in increased β2-mKalam fluorescence at the oocyte surface relative to a control region, indicating that the subunits are in close enough proximity for subunit fluorescent proteins to undergo FRET.

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Fig 4. Truncation of the M3/M4 loop does not perturb receptor assembly or function in oocytes. (A) Cartoon of subunit construct design for α1 and β2 subunits shows locations for GFPuv fusion and loop truncation. A polypeptide linker (red line) connects GFPuv to the N-terminus of the α1 subunit. (B) Expression of GFPuv-α1/β2 increases receptor homogeneity relative to α1-EGFP/β2. However, an increase in cleaved GFPuv fluorescence signal is observed (arrow). (C) Replacement of the native M3/M4 loop in both subunits with a tri-peptide induces a right shift in the elution profile, consistent with a smaller hydrodynamic radius. Absolute fluorescence intensities are shown on the same scale. The FSEC traces shown in (B) and (C) were obtained from the same batch of oocytes. (D) Two-electrode voltage clamp of GFPuv-α1-LT/β2-LT demonstrating that the receptor retains gating activity and sensitivity to zinc.

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predicted 58-residue M3/M4 loop was replaced with the tri-peptide linker Ala-Gly-Thr without altering pentamer formation or channel function [23].

In order to screen M3/M4 loop modifications of the α1 subunit, a new fluorescent protein fusion was generated with the cycle 3 GFP variant GFPuv [64] attached to the N-terminus with a 24 amino acid linker following the predicted signal peptide, designated GFPuv-α1 (Fig 4A). Previous work with the nicotinic acetylcholine receptor indicated that N- or C-terminal GFP fusions did not support proper folding of the chromophore, necessitating placement of GFP in the M3/M4 loop [65]. However, GFPuv is more stable [66] and has been used to study the secretory pathway of class C GPCRs [67]. RNA injection of this fusion construct in oocytes and subsequent FSEC analysis indicated that the GFPuv-α1/β2 receptor expressed with reduced aggregation propensity relative to α1-EGFP/β2 (Fig 4B). Of note, this construct demonstrated higher levels of free fluorescent protein following solubilization as indicated by the arrow in Fig 4B, which is likely a consequence of proteolytic cleavage along the polypeptide linker between GFPuv and the α1 N-terminus. Nevertheless, the FSEC profile is consistent with a well-behaved receptor, suggesting that the construct is useful for subsequent mutagenesis.

A large truncation of the M3/M4 loop was designed for both subunits (S1 Fig), reminiscent of the optimized GluClα construct [23], to investigate changes to the receptor elution profile. In the α1 and β2 subunits, the 70- and 108-residue M3/M4 loops were replaced with Arg-Gly-Thr and Gly-Gly-Thr tri-peptide linkers, respectively (defined as “LT” for loop truncation). Combining the loop truncations in both subunits reduced the molecular weight of the receptor by nearly 51 kDa assuming a subunit assembly ratio of 2:3, α1 to β2. This change in mass is reflected in a 100 second right shift in the FSEC elution peak of GFPuv-α1-LT/β2-LT (Fig 4C). We confirmed that this receptor construct retained gating activity by TEVC experiments. A large amplitude current (μA) was elicited from oocyte perfusion with 0.1 mM GABA that was also antagonized by Zn2+ (Fig 4D).

**N- and C-terminal deletions and removal of glycosylation sites**

Construct optimization of GluClα further required deletion of a long stretch of 41 residues in the amino-terminus following the signal peptide and a short carboxy-terminal tail [23] (Fig 5A and 5B). In sequence alignments, the α1 carboxy-terminus extends beyond both GluClα and β2 subunits. The last eleven residues in the α1 subunit were removed by mutagenesis. Oocyte expression of this mutant in the GFPuv-α1-LT/β2-LT context did not perturb receptor assembly (Fig 5C). Both GABA_A receptor α1 and β2 subunits are predicted to have longer signal peptides relative to GluClα, but shorter amino-termini that precede the predicted α-helix in the extracellular domain (Fig 5A). In striking contrast to deletion of the α1 carboxy-terminus, deletion of nine residues from the amino-terminus in the α1-EGFP subunit and seven residues in the full length β2 subunit demonstrated substantially reduced expression levels relative to wild type as indicated by lower absolute fluorescence intensity in the FSEC trace (Fig 5D). Combining the deletion constructs nearly abolished expression of the receptor.

Both subunit amino-terminal deletions contain a consensus site for N-linked glycosylation (Asn-X-Ser/Thr), which may play a role in surface expression and function of GABA_A receptors [68, 69]. To test this possibility, each of these sites (α1-N37, β2-N32 in the mature receptor) was mutated to either Asp or Ala in the wild type subunit background and then each subunit was individually examined for changes in receptor expression. As shown in Fig 5E, expression levels were attenuated to similar levels as the amino-terminal deletion constructs with mutation of either Asn residue, although mutation in the β2 subunit was more tolerated.
This result suggested that prevention of post-translational modification with N-glycans contributes to diminished expression levels. However, based on these experiments alone, we cannot rule out the potential negative contribution of other deleted residues in the N-terminus to overall expression.

In addition to these sites, the α1 and β2 subunits contain one (N137) and two (N104, N173) other putative glycosylation sites, respectively. The role of post-translational modifications at these sites was investigated using the GFPuv-α1-LT/β2-LT construct. Site-directed mutagenesis experiments indicated that mutation of both β2 subunit sites lowered expression levels and increased receptor heterogeneity (Fig 6), suggesting that all three glycosylation sites in the β2 subunit are needed for proper folding and/or targeting. This result is consistent with the observation that mutation of each of the three sites in β2 reduced peak amplitudes and altered gating properties in cultured mammalian cells [69]. Furthermore, all three sites were found to be modified with N-glycans in the β3 homo-pentamer [25]. However, mutation of N137 in the α1 subunit does not appear to impede receptor expression in oocytes or substantially change monodispersity, which may indicate that glycosylation at this site is not crucial for α/β receptor assembly. Although previous studies suggested that mutation of N37, N137 or both to Gln in α1 does not impair channel gating in oocytes [68], bulk expression levels appear to be sensitive to the removal of the N37 glycosylation site based on these results. Overall, the data is consistent with the notion that glycosylation plays a crucial role in receptor maturation.
Expression of the α/β GABA<sub>A</sub> receptor in suspension culture

Encouraged by the positive screening results for the rat α1/β2 receptor in oocytes, we investigated suspension cell culture conditions to increase receptor yields for subsequent purification. Preliminary studies were performed in insect cells because homomeric GluCl<sup>α1</sup> [23, 58] and heteromeric GABA<sub>A</sub> receptors [29, 70] have been expressed previously in Sf9 cells. For these experiments, recombinant baculoviruses were created for the α1-EGFP and β2 (full length) subunits by shuttling the genes into separate pFastBac1 vectors, or combining the subunits into a single bicistronic pFastBac Dual vector (Fig 7A). Visible EGFP expression localized to the membrane was observed as early as one day post infection of Sf9 cells (Fig 7B). However, FSEC analysis indicated that a homogeneous assembly either failed to form or was not maintained after whole-cell solubilization with C<sub>12</sub>M at all tested time points (Fig 7C and 7D).

Although we attempted to modify infection parameters, expression temperature, and detergent solubilization conditions, the α1/β2 subtype could not be isolated from Sf9 or High Five insect cells (S2 Fig).

We reasoned that mammalian cells may provide a more natural and stabilizing environment for heteromeric assemblies leading to successful membrane extraction. Therefore, the full length α1-EGFP and β2 subunits were cloned individually into a modified baculovirus...
vector, pEG BacMam [47], specifically designed to increase protein yields in adherent or suspension mammalian cells (Fig 8A). Efficacious virus was used to co-infect HEK293 suspension cells engineered to be deficient of \(N\)-acetylglucosaminyltransferase I (GnTI) and thus eliminate heterogeneous \(N\)-glycan post-translational modifications [71].

In contrast to insect cells, the \(\alpha_1\)-EGFP/\(\beta_2\) receptor was observed by FSEC analysis after whole cell solubilization with \(C_{12}\)M 48 hours post infection (Fig 8B). Subsequently, expression of the \(\alpha_1\)-EGFP/\(\beta_2\) receptor was optimized by screening a variety of parameters to improve yields and peak symmetry reported by the FSEC profile. Importantly, several of these parameters were found to be crucial to boosting receptor expression levels and behavior, as illustrated in Fig 8B–8D. First, inclusion of 10 mM sodium butyrate, a histone deacetylase inhibitor, 12 hours post infection increased expression levels ~five-fold relative to virus alone at the cost of increased propensity for receptor aggregation suggested by a strong leading shoulder in the elution peak (Fig 8B). Second, addition of virus with a relative MOI ratio greater than one (\(\beta\) to \(\alpha\)) increased receptor monodispersity (Fig 8C). Notably, shifting culture temperature to 30˚C following sodium butyrate supplementation reduced aggregation propensity in addition to a concomitant three-fold increase in fluorescence intensity observed at the 48 hour time point (Fig 8D).

To assess the broad application of these expression conditions, other GABA\(_A\) receptor constructs were screened for changes in expression levels in mammalian cells. Indeed, these expression conditions worked well for substantially boosting expression of homomeric (\(\rho_1\)-EGFP) and mutant heteromeric (\(\alpha_1\)-EGFP/\(\beta_2\)-LT) ion channels (Fig 9A and 9B). Interestingly, exchanging the \(\beta_2\)-LT subunit for the \(\beta_1\)-LT isoform (similar M3/M4 loop truncation, S1 Fig) further improved receptor homogeneity and enhanced expression by an additional
three fold (Fig 9B). The increased expression levels allowed for an unambiguous evaluation of the importance of the \( \beta \) subunit to formation of monodisperse oligomers in mammalian cells. Consistent with observations made in oocytes, expression of \( \alpha \)1-EGFP in the absence of either \( \beta \)1 or \( \beta \)2 yields a polydisperse profile, indicating that assembly of a homogeneous entity requires at least \( \alpha \) and \( \beta \) subunits. Additionally, in the presence of either \( \beta \) subunit isoform, introduction of the \( \gamma \)2S subunit to produce tri-heteromeric receptors reduced apparent expression by more than 50% with these methods (S3 Fig). A time course of \( \alpha \)1-EGFP/\( \beta \)1-LT receptor expression indicated that the receptor saturated 60–72 hours post infection (Fig 9C). Similar to \( \alpha \)1-LT/\( \beta \)2-LT, the \( \alpha \)1-LT/\( \beta \)1-LT receptor demonstrated agonist-driven channel activity in oocytes (Fig 9D).

**Purification and characterization of the \( \alpha \)1/\( \beta \)1 receptor**

Based on the expression studies outlined above, we pursued purification of the \( \alpha \)1/\( \beta \)1 receptor combination from mammalian cell membranes. Preliminary experiments focused on the \( \alpha \)1-EGFP/\( \beta \)1-LT receptor to determine optimal purification strategies (S4 Fig). These experiments suggested that 50–55% of the expressed receptor was isolated from the membrane fraction based on EGFP-FSEC analysis. Subsequently, other receptor constructs containing \( \alpha \)1 subunits devoid of EGFP or with truncated M3/M4 loop were isolated and characterized through ligand binding assays. In general, a simple two-step protocol was chosen that combines immobilized metal affinity chromatography (IMAC) followed by gel filtration to remove aggregates and degradation products. An octa-histidine tag was fused to the C-terminus following the thrombin protease recognition sequence in either one or both subunits for IMAC purification and ligand binding analysis via scintillation proximity assay (SPA). Other
elements of primary sequence, such as glycosylation sites, were retained in both subunits to maximize yields.

Following solubilization with 40 mM C\textsubscript{12}M, the α1/β1 receptor was purified successfully into either 1 mM C\textsubscript{12}M or 0.3 mM lauryl maltose neopentyl glycol (L-MNG) detergent micelles (Fig 10). Typical yields from IMAC purification for all constructs were in the range of 0.4–0.7 mg/L (~2.5 nmol/L) of suspension culture based on absorbance at 280 nm assuming an average $A_{280}$ (Materials and Methods). Isolation of monodisperse receptor was obtained from preparative size exclusion chromatography (Fig 10A and 10B). SDS-PAGE analysis indicated that the mobility of each subunit corresponds to the expected molecular weight (α1-LT = 43.1 kDa; β1-LT = 41.6 kDa; Fig 10C). Confirmation of the band identities was determined by LC-MS. In addition, each subunit ran as diffuse band(s) that would collapse upon treatment with an endoglycosidase cocktail (S5 Fig), suggesting that the receptor is glycosylated.

The functional integrity of purified receptor was investigated by measuring $^3$H-muscimol binding activity using SPA. Specific binding of $^3$H-muscimol was observed in both C\textsubscript{12}M and L-MNG (Fig 10D). Titration of 0.5 pmol receptor with $^3$H-muscimol produced a monotonic binding isotherm (Fig 10E). Non-linear least squares fitting assuming a single site binding model revealed a $K_D$ of 24±1.1 nM, consistent with previously reported values for the α1/β1 receptor in intact cells [72]. Assuming that all receptor was bound to the SPA beads, $B_{\text{max}}$ corresponded to ~1.5 $^3$H-muscimol binding sites per receptor. Furthermore, a competition assay in which 100 nM $^3$H-muscimol was displaced by increasing concentrations of GABA
demonstrated an IC\textsubscript{50} of 507±28 nM and a K\textsubscript{i} of 98±6.5 nM (Fig 10F). These results are consistent with the presence of two canonical, high affinity agonist binding sites at the \(\beta/\alpha\) interface [28].

**Discussion**

Here we have shown that construct screening in *Xenopus* oocytes in combination with FSEC is a viable alternative to previously described approaches, such as *in vitro* transient transfection [50], to identify a suitable heteromeric GABA\textsubscript{A} receptor for large-scale expression and purification. This method presents a number of advantages over plasmid DNA transfection, such as straightforward gene delivery, dosage control and ease of manipulation. To enhance plasmid transfection efficiencies, cellular endocytosis is facilitated by cationic “carrier” molecules such as charged lipids or small chemical compounds like calcium phosphate. However, transfection reagents can induce a cytotoxic response and the relative ease of transfection varies with the cell line, which can produce artificially low gene expression levels. Furthermore, protocol optimization becomes more imperative for multi-gene studies, such as with multiple GABA\textsubscript{A} receptor subunits, in which overall transfection efficiency becomes compounded. Although viral transduction is an efficient strategy for delivering genetic cargo, making a library of high titer recombinant viruses for expression studies can be time consuming, costly and counterproductive for the iterative pathway of identifying a lead gene construct for expression and downstream structural studies.

Exploiting the GABA\textsubscript{A} \(\alpha1\) subunit as a fluorescent protein fusion facilitated rapid construct screening in oocytes by monitoring GFP variant fluorescence as a surrogate reporter of
receptor subtype expression levels and oligomeric integrity [50]. This approach not only led to identification of the α/β subunit combination as a viable candidate for further investigation, but also informed gene design since expression levels and homogeneity were modulated by construct mutagenesis. The application of the FSEC technique for construct screening in oocytes adds a new dimension to the process of identifying the best expressing and behaving constructs. Oocytes provide a tratable platform for identifying functional constructs, but investigating electrophysiological properties alone may lead to false positives since large expression levels are not needed to generate ion channel currents in oocytes. Importantly, the oocyte expression profiles of receptor subtypes investigated here were mirrored by mammalian cells, suggesting continuity between disparate expression systems.

Our approach has underscored important determinants for receptor assembly. Studies have suggested that high affinity binding sites in GABA_A receptors are formed only in the presence of the β subunit [73–75]. As shown in oocytes and mammalian cells, incorporation of the β subunit supports formation of a defined monodisperse assembly consistent with a pentamer. Furthermore, the observation that a monodisperse assembly contains the β subunit was supported by FRET and electrophysiology experiments in oocytes. In the absence of the β subunit, a homogeneous α1 assembly fails to form leading to broad, polydisperse gel filtration profiles that are the hallmark of destabilized oligomers. The lack of a homogeneous receptor population composed of α1 only or α1/γ2S correlates with the lack of functional surface expression previously seen in oocytes [76] or in transfected L929 cells [77]. Our experiments also demonstrate that large truncations in the cytoplasmic M3/M4 loop are not detrimental to expression, subunit association or GABA-gated currents, which is consistent with previous studies of homomeric 5-HT3 and p1 receptors [63]. In contrast, interference with subunit glycosylation substantially reduced expression levels.

To our surprise, the most tractable subunit combination identified in oocytes, α1/β2, could not be isolated by detergent from Sf9 or High Five insect cells. A number of studies have shown that di- and tri-heteromeric GABA_A receptors, including α1/β2, can be expressed as functional channels with high affinity ligand binding sites in Sf9 cells using recombinant baculovirus [29, 70, 74]. Notably, these studies examined receptor activity in cell membranes or intact cells. We found that the α1-EGFP/β2 combination expressed to the membrane, which suggested that the receptor is being properly secreted and targeted. However, detergent solubilization resulted in gross breakdown of the receptor as judged by FSEC analysis, which indicated that insect cells are not a practical means of over-expressing and purifying large quantities of intact α1/β2 receptor.

Although the α/β receptor could be expressed in and extracted from mammalian cells, high level expression was only obtained after exploring culture conditions and baculovirus infection parameters. Remarkably, the addition of 10 mM sodium butyrate and the reduction of culture temperature to 30˚C, both of which have been shown to strongly impact protein expression in mammalian cells [51, 78–81], demonstrated a synergistic effect on receptor expression and behavior by increasing the α1/β2 receptor yields approximately 15-fold. Enhancement of protein yields by sodium butyrate has previously been observed to increase rhodopsin 2–3 fold in stable HEK293 cell lines [82], and 10–15 fold in combination with a tetracycline-inducible stable cell line [81].

In addition to culture conditions, co-infection with the β1 subunit drove expression levels even higher than with the β2 subunit. The origin of the difference in expression levels between α1/β1 and α1/β2 subtypes is not clear. Even though the β1 subunit is the most divergent of the three known isoforms, most of the sequence variation in the mature subunit arises from the M3/M4 loop which can be removed without impairing expression or function based on these studies. Furthermore, we note that expression of α/β/γ2S receptors appeared attenuated
relative to α/β in both oocytes and mammalian cells based on a decrease in GFP fluorescence intensity. Since GFP variants have been genetically attached to the α1 subunit, differences in apparent expression levels between α1/β1, α1/β2 and α/β/γ2S receptors may reflect distinct α1 subunit stoichiometries. Alternatively, production of the tri-heteromeric receptor may require an altered expression time course in mammalian cells (Fig 9C). Additionally, lowered expression levels of α/β/γ2S may be a consequence of infection with multiple viruses that is detrimental to cell health. The pliability of pEG-BacMam to combine multiple genes into a single vector could be a possible means of increasing α/β/γ2S expression levels by eliminating the need for infecting with multiple viruses.

The purified rat α1/β1 receptor contains high affinity ligand binding sites, consistent with a defined subunit organization that represents native associations. We estimate that 2.5 nmol/L receptor was isolated from the mammalian cell membrane during purification, which corresponds to ~3.8 nmol/L 3H-muscimol sites according to saturation binding analysis (Fig 10). This yield is comparable with that reported for human α1/β3 with an amino-terminal FLAG tag from an HEK293-derived tetracycline-inducible stable cell line (3.2 nmol/L 3H-muscimol sites) [83]. The benefit of the baculovirus system as described here is the ability to rapidly and efficiently express many different constructs without the time consuming process of generating stable cell lines, which is highly advantageous for studies that seek to unveil the role of specific residues in protein structure and function through site directed mutagenesis.

In conclusion, the methods described here have garnered exceptional results toward efficiently expressing and purifying a functional heteromeric GABA<sub>A</sub> receptor for structural analysis. Although this approach is focused uniquely upon GABA<sub>A</sub> receptors, we anticipate that these methods will be broadly applicable to other complex protein systems that may require multiple protein subunits or binding partners. In general, expression of eukaryotic membrane proteins in our laboratory has benefited immensely using the mammalian system employed here [84–89].

Supporting information
S1 Fig. Sequence alignment and schematic of fluorescent protein fusions and truncations in the M3/M4 loop. Alignment of GABA<sub>A</sub> subunit genes used in this study with GluCl<sub>α</sub> highlights regions of sequence variation found in the N-terminus, C-terminus and the M3/M4 loop. The alignment was used in conjunction with the GluCl<sub>α</sub> crystal structure to guide mutagenesis. The alignment was created in CLC Main Workbench 7. Incorporation of an AscI restriction site (GRA) was used to insert fluorescent proteins into the M3/M4 loop of either the α1 or β2 subunit. Truncation of the M3/M4 loop of GABA<sub>A</sub> subunits were designed to mimic the shortened loop of GluCl<sub>α</sub> (AGT) in the crystal structure. The location of M3 and M4 (red) were derived from alignments with GluCl<sub>α</sub> combined with secondary structure prediction.
(TIF)

S2 Fig. Screening for conditions to express and extract α1-EGFP/β2 from Sf9 or high five insect cells. (A) Infection of Sf9 cells with two baculoviruses harboring the α1-EGFP and β2 subunits at different subunit ratios (relative MOI) does not improve expression as observed 72 hours post infection. (B) Screening a panel of detergents for whole-cell solubilization. (C) Infection of High Five cells or shifting cultures to lower temperatures does not improve receptor recovery. The FSEC traces show absolute fluorescence intensities and are plotted on the same scale as Fig 7C. The arrows point to the expected elution position of the receptor.
(TIF)
S3 Fig. Expression of tri-heteromeric GABA<sub>A</sub> receptors in mammalian cells. Incorporation of the γ2S subunit appears to reduce receptor expression levels relative to α1-EGFP/β1-LT (A) or α1-EGFP/β2-LT (B) at similar time points.

(TIF)

S4 Fig. Purification of α1-EGFP/β1-LT from mammalian cells. (A) The receptor demonstrates a similar FSEC profile whether solubilized from whole cells (black trace) or from the membrane fraction (red trace). The traces are normalized to emphasize similarities. Based on the FSEC analysis, approximately 50–55% (~3.3 mg) of the expressed receptor (6.4 L culture) was extracted from the membrane fraction. Of this material, 90% of the receptor was bound to Talon resin after three hours batch binding as reported by a depletion of receptor from the solution (blue trace). Approximately 3mg of receptor (0.5 mg/L or ~2 nmol/L) eluted from the resin (B), and was concentrated for preparative SEC (C). Peak fractions as identified by the red box in (C) are monodisperse by FSEC analysis (D). (E) SDS-PAGE analysis demonstrates the purity of the receptor as a function of purification steps. The first lane shows contaminants eliminated by a wash with 30 mM imidazole during IMAC purification, followed by two lanes showing elution fractions obtained from 250mM imidazole. The following six lanes show purity in fractions obtained across the SEC elution peak, which correspond to the FSEC analysis shown in (D). Notably, the presence of EGFP in the α1 M3/M4 loop enhanced proteolytic cleavage relative to non-fusion constructs, giving rise to a ~30 kDa band in (E). Confirmation that this band contained EGFP was made by in-gel fluorescence prior to fixing and staining (F). Although we did not explicitly determine the identity of this band, the migration position is consistent with EGFP plus the M4 α-helix. However, cleavage of the loop likely does not disrupt native associations since the purified receptor demonstrated monodispersity by FSEC analysis as shown in panel D.

(TIF)

S5 Fig. Endoglycosidase treatment removes N-glycans from α1 and β1 subunits from purified receptor. Following IMAC purification, the receptor was treated with EndoH (1:1, w/w) and EndoF<sub>3</sub> (1:50, w/w) for two hours at room temperature at pH 6.5. After purification by SEC, the receptor was analyzed by SDS-PAGE and showed that treatment caused the diffuse bands seen in the IMAC fractions to collapse into a single prominent band for both subunits.

(TIF)

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