Optimizing the Expression of Recombinant $\alpha\beta\gamma$ GABA A Receptors in HEK293 Cells for High-Throughput Screening

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Despite being important clinical targets, it is not straightforward to reliably express recombinant trimeric $\alpha\beta\gamma$ GABA A receptors (GABA ARs) for high-throughput screening. This study therefore sought to devise a simple and reliable means of transiently expressing $\alpha_1\beta_1\gamma_1$ and $\alpha_1\beta_1\gamma_2$ GABA ARs in HEK293 cells. Expression efficiencies resulting from 5 different transfection strategies were assessed by flow cytometry and pharmacological analysis using an anion-sensitive yellow fluorescent protein-based assay. PolyFect™ and Effectene™, employed according to the manufacturers’ instructions, conferred the strongest and most reliable expression of trimeric $\alpha\beta\gamma$ GABA ARs. Functional analysis via the yellow fluorescent protein assay revealed dramatic differences in the pharmacological properties of $\gamma_1$- and $\gamma_2$-containing receptors, consistent with previous electrophysiological characterizations. The authors conclude that this method of expressing and screening recombinant GABA ARs provides an effective means of discovering novel GABA AR modulators for use as therapeutic lead compounds and pharmacological probes. (Journal of Biomolecular Screening 2009;86-91)

Key words: chloride channel, drug discovery, yellow fluorescent protein, inhibitory, neurotransmission

INTRODUCTION

GABA TYPE-A RECEPTOR CHLORIDE CHANNELS (GABA ARs) are pentameric Cys-loop receptors that mediate inhibitory neurotransmission in the central nervous system. Because mammalian GABA ARs are constructed from a family of 16 subunits ($\alpha$-6, $\beta$-1-3, $\gamma$-1-3, $\delta$, $\varepsilon$, $\pi$, $\theta$), there is potentially an enormous variability in the stoichiometry of GABA ARs at central nervous system synapses,1,2 and it remains a major challenge to establish which subunit combinations are expressed at particular synapses and why. Isoform-specific GABA AR inhibitors could achieve this by eliminating the contribution of individual isoforms to the complex mix of conductances active at any one time in a neuron. However, currently available GABA AR pharmacological tools are not sufficiently selective for this purpose.

GABA ARs are important drug targets for therapies directed at muscle relaxation, anxiety, epilepsy, sedation, and anesthesia and are emerging as therapeutic targets for a range of other indications.3 There is, however, considerable scope for the development of new therapeutics with improved beneficial effects and fewer side effects.

It is therefore important, from both clinical and basic research standpoints, to discover novel GABA AR isoform-specific modulators. Indeed, several high-throughput screening (HTS) assays have been developed to screen anion-selective channels, with 2 of the most suitable being voltage-sensitive dyes (VSDs) and the anion-sensitive yellow fluorescent protein (YFP) mutant, YFP-I152L.4 As VSDs are applied exogenously and thus taken up by all cells, stably expressing cell lines are essential for optimal assay sensitivity. In contrast, because transiently cotransfecting YFP-I152L and ion channel cDNAs result in a high rate of coexpression in individual cells,5 the YFP-based assay does not require stably expressing cell lines to achieve full dynamic range. This is advantageous for screening compounds against numerous different GABA AR isoforms as it obviates the need to create and maintain a large range of stably expressing cell lines. However, it is not straightforward to reliably transiently express trimeric $\alpha\beta\gamma$ GABA ARs in recombinant expression systems.6 The aim of this study is to compare 5 transfection methods in an attempt to optimize the transient expression of $\alpha\beta\gamma$ GABA ARs in HEK293 cells.
Optimizing the Expression of GABA<sub>4</sub>Rs in HEK293 Cells

MATERIALS AND METHODS

Molecular constructs

Rat α1 and β1 subunit cDNAs were subcloned into the pIRE2-EGFP or pcDNA3.1 plasmid vectors, with each used as explained in the text. Rat γ1 and γ2 subunit cDNAs were subcloned into the pIRE2-EGFP plasmid vector. The pIRE-EGFP vector permits green fluorescent protein (GFP) fluorescence to be used as an indicator of the successful transfection of the inserted subunit, whereas the pcDNA3.1 vector transfects the inserted cDNA alone. The YFP-I152L cDNA was subcloned into the pcDNA3.1 vector. Its amino acid sequence was identical to that originally published. YFP-I152L is relatively resistant to quench by Cl<sup>-</sup> ions but is potently quenched by F<sup>−</sup> ions flowing into the cell through anion-permeant ion channels. It thus acts as a fluorescent reporter of GABA<sub>4</sub> activation.

Cell culture and transfection

Experiments were performed on HEK293 cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Approximately 5 × 10<sup>5</sup> cells suspended in 5 mL DMEM were plated into 60-mm culture dishes and were transfected when 40% to 80% confluent. YFP, α1, and β1 cDNAs were transfected in a 1:1:1 ratio, whereas YFP, α1, β1, and γ cDNAs were cotransfected in a 1:1:1:3 ratio. Four of the transfection procedures followed the manufacturers’ instructions. The reagent quantities used for each of these procedures for transfection into 60-mm dishes are listed as follows:

- FuGENE 6<sup>TM</sup> (Roche Diagnostics, Castle Hill, NSW, Australia): 2 μg cDNA total; 6 μL FuGENE<sup>TM</sup> reagent
- Escort II<sup>TM</sup> (Sigma, St. Louis, MO): 2 μg cDNA total; 120 μL transfection buffer total; 10.5 μL Escort II<sup>TM</sup> Reagent (cDNA/Escort reagent ratio was 1:3)
- Effectene<sup>TM</sup> (Qiagen, Valencia, California): 2 μg cDNA total; 16 μL Enhancer buffer; 20 μL Effectene<sup>TM</sup> Reagent
- PolyFect<sup>TM</sup> (Qiagen): 2 μg cDNA total; 40 μL PolyFect<sup>TM</sup>

We also used a calcium phosphate precipitation transfection technique, as described in Sambrook et al., using a total of 1 μg cDNA for each 60-mm dish.

Preparation of cells for experiments

Following termination of transfection, cells were trypsinized by adding 0.7 mL of 0.25% trypsin–EDTA solution (Gibco BRL), resuspended into DMEM, and 2.5 × 10<sup>5</sup> cells, suspended in 40 μL DMEM, were plated into each well of a transparent 384-well plate for fluorescence imaging experiments. Cells were used in experiments 24 to 36 h later. Individual wells typically contained 2 × 10<sup>4</sup> cells at the time of the experiment. Approximately 1 h prior to commencement of experiments, culture media in 384-well plates were removed and replaced by 25 μL standard control solution, which contained (in mM) NaCl 140, KCl 5, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1, HEPES 10, and glucose 10 (pH 7.4) using NaOH. The NaI test solution was similar in composition to NaCl control solution except the NaCl was replaced by equimolar NaI. The effects of drugs were quantitated in the presence of an EC<sub>50</sub> GABA concentration listed as follows for each subunit combination (in μM): α1β1, 0.6; α1β1γ1, 1.5; and α1β1γ2, 2.5.

For flow cytometry experiments, cells were maintained in a 5% CO<sub>2</sub> incubator for 24 to 36 h following termination of transfection. Approximately 1 h before experiments, cells were trypsinized as described above, resuspended in 5 mL DMEM, and centrifuged at 300 g for 5 min. The cell pellet was resuspended in 500 μL control solution maintained at 4 °C for up to 30 min until used in experiments. Each flow cytometry experiment employed approximately 5 × 10<sup>6</sup> cells.

Imaging experiments

The 384-well plates were placed onto a motorized stage (Prior ProScan II, Prior Scientific Instruments, Cambridge, UK) of an Olympus IX51 inverted microscope, and cells were imaged with a 10x objective (UPPlanFLN, N.A. 0.30, Olympus, Tokyo, Japan). Illumination from a 100-W mercury arc lamp (HBO 103/2, Osram, Germany), passing through a YFP dichroic mirror (86002V2 JP4 C76444, Olympus), was used to excite YFP fluorescence. Fluorescence was imaged by a CCD camera (CoolSNAP monochrome cf /OL, Olympus) and digitized to disk onto a personal computer. The primary resolution of the camera was 1392 × 1040 pixels, although images were binned (2 × 2), resulting in a resolution of 696 × 520 pixels. The maximum image acquisition rate was 1.25 Hz. Liquid handling was performed with an LC PAL autosampler (CTC Analytics, Zwingen, Switzerland) using a 100-μL syringe. A suite of LabView 7.1 and 8.2.1 (National Instruments, Ireland) software routines written in the laboratory was used for hardware control, image acquisition data storage, and image analysis. The standard experimental protocol involved imaging each well twice: once in 25 μL control solution and once again 8 s after the injection of 50 μL NaI test solution. Individual concentration responses were constructed by pooling results from 2 wells exposed to NaI solution containing the same GABA or drug concentration. Each image typically contained 400 to 600 cells with sufficient fluorescence to be used for analysis. Experiments were replicated at least 3 times using cells transfected on different days. GABA concentration-response relationships were fitted with the following equation:

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F = F_{\text{init}}/(1 + (EC_{50}/[\text{GABA}])^{nH})
\]

where F is the fluorescence corresponding to a particular GABA concentration, [GABA]; F<sub>init</sub> is the initial (or control) fluorescence value; EC<sub>50</sub> is the concentration that elicits half-maximal...
activation; and nH is the Hill coefficient. A similar equation was used to fit inhibitory concentration-response relationships, with IC50 being the concentration that elicits half-maximal inhibition. Curve fits were performed using a least squares fitting routine (Origin 7, OriginLab Corporation, Northampton, MA). Statistical significance was determined by unpaired Student t-test with p < 0.05 representing significance. All averaged results are expressed as mean ± SEM.

Flow cytometry experiments

Samples were analyzed on a BD Biosciences (San Jose, CA) LSR II using a blue (488-nm) 20-mW laser source and a YFP (530/30BP) fluorescence collection filter. Approximately 10⁴ events per cell population were analyzed using Weasel (WEHI, Parkville, Victoria, Australia) or FCS Express 3 (De Novo Software, Los Angeles, CA).

Pharmacological reagents

All pharmacological reagents, including GABA, bicuculline, diazepam, β-carboline methyl-4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylate (DMCM), zolpidem, and zinc were obtained from Sigma.

RESULTS AND DISCUSSION

Comparison of expression efficiencies by flow cytometry

Flow cytometry was used to quantitate the percentage of cells successfully transfected with α1β1, α1β1γ1, and α1β1γ2 GABAAR subunit cDNAs. GFP fluorescence was used as the transfection readout. The pIRES2-EGFP vector was used when transfecting α1 and β1 subunits without γ subunits. Thus, in these experiments, the fluorescence readout reflects the number of cells expressing either α1 or β1 subunits or both. When expressing trimeric receptors, α1 and β1 subunits were transfected in the pcDNA3.1 vector, whereas γ1 or γ2 subunits were transfected in the pIRES2-EGFP vector. Thus, in this case, the fluorescence readout reflects the number of cells expressing γ subunits. Figure 1A displays the percentage of transfected cells resulting from each of the 5 transfection protocols. Because Effectene™ and PolyFect™ yielded the highest expression efficiencies for all subunits, theoretically they should also produce the highest proportion of cells expressing trimeric α1β1γ1 GABAARs. Experiments designed to investigate this are now described.

Comparison of expression efficiencies by fluorescence imaging

We next compared transfection efficiencies by analyzing the transfected cells by conventional fluorescence microscopy. We first transfected cells with only the bicistronic vector containing γ1 and GFP cDNAs, then measured the number of fluorescent cells per well. Unlike flow cytometry, this technique cannot quantify nonfluorescent cells. The results, presented in Figure 1B, reveal a similar transfection efficiency sequence as in Figure 1A. Next we quantitated the number of fluorescent cells produced by transfection with YFP-II152L and the indicated GABAAR subunit combinations. As shown in Figure 1C, this yielded a similar rank order of transfection efficiencies. The third test involved assaying the number of fluorescent cells that were quenched by at least 20% following application of a saturating concentration (1 mM) of GABA. The result, summarized in Figure 1D, reveals the extent to which YFP-II152L and GABAARs are colocalized in individual cells. The same experiments were reanalyzed to reveal the average maximal quench observed for all fluorescent cells (Fig. 1E). As γ subunits were invariably expressed in the pIRESEGFP vector, it is likely that the magnitude of YFP quench was underestimated in Figure 1E due to contamination by fluorescence from environmentally insensitive GFP. Indeed, this contamination explains why percentage quench is systematically smaller in cells expressing γ subunits than in those expressing α1 and β1 subunits only. In control experiments, the mean GABA-mediated fluorescence quench in cells transfected with YFP-II152L alone was < 5% (not shown). Results from all 5 assays, summarized in Figures 1A-E, indicate that Effectene™ and PolyFect™ provide the most efficient means of transfecting HEK293 cells with YFP and GABAAR subunit cDNAs. However, functional analysis is required to determine whether these methods reliably produce trimeric GABAARs.

To address this, we took advantage of the fact that the γ1 subunit dramatically reduces the GABA sensitivity of αβ GABAARs. Indeed, we recently showed by patch-clamp electrophysiology of identified cells that the mean GABA EC50 values of HEK293 cell-expressed αβ1γ1 and αβ1γ2 GABAARs are 3.0 ± 0.6 and 22.4 ± 2.0 μM, respectively. We, therefore, employed fluorescence quench to quantitate GABA concentration-response relationships of cells transfected with α1 and β1 subunits or α1, β1, and γ1 subunits via each of the 5 transfection conditions. The mean GABA EC50 values, displayed in Figure 1F, strongly suggest that Effectene™ and PolyFect™ produce the highest proportion of cells expressing α1β1γ1 GABAARs.

Pharmacology of γ subunit-containing receptors

To confirm this result and to determine whether this assay is suitable as a drug discovery tool, we characterized the pharmacological profiles of cells transfected via PolyFect™ with α1β1, α1β1γ1, and α1β1γ2 GABAAR subunits. In each experiment, GABA was applied at the EC50 concentration as given in Materials and Methods. The mean IC50 values of the antagonist, bicuculline, at α1β1, α1β1γ1, and α1β1γ2 GABAARs were 2.0 ± 1.1, 1.7 ± 0.7, and 1.3 ± 0.3 μM (n = 3 preparations each), respectively, which were not significantly different from each other by unpaired Student t-test (Fig. 2A). This agrees with a previous study that showed γ subunits do not affect bicuculline sensitivity.¹¹
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**FIG. 1.** Comparison of transfection efficiencies by fluorescence analysis. (A) The percentage of green fluorescent protein (GFP) cells expressing the indicated subunit combinations as determined by flow cytometric analysis. When expressing \(\alpha_1\) and \(\beta_1\) subunits only, both cDNAs were transfected in pIRES-EGFP. When expressing all 3 subunits, \(\alpha_1\) and \(\beta_1\) cDNAs were transfected in the pcDNA3.1 vector, and the \(\gamma\) cDNA was transfected in the pIRES-EGFP vector. Results in B through F were obtained using fluorescence imaging. In all experiments, individual values were averaged from 6 wells each, with all displayed data points averaged from at least 3 different transfections. (B) Number of fluorescent cells/well following transfection of \(\gamma_1\) and GFP cDNAs in pIRES-EGFP vector. (C) Number of fluorescent cells/well following transfection with yellow fluorescent protein (YFP) and indicated GABA\(_A\) subunit cDNAs. The \(\alpha_1\) and \(\beta_1\) subunits were transfected in the pcDNA3.1 vector. (D) Mean percentage of fluorescent cells that displayed a >20% quench following addition of 1 mM GABA. The % quench was defined as \(1 - \frac{(F_{\text{final}}/F_{\text{init}})}{100}\). The \(\alpha_1\) and \(\beta_1\) subunits were transfected in the pcDNA3.1 vector. (E) Average maximal GABA-induced quench of all fluorescent cells, determined by the same equation. The \(\alpha_1\) and \(\beta_1\) subunits were transfected in the pcDNA3.1 vector. (F) Averaged GABA EC\(_{50}\) values curves for HEK293 cells transfected with \(\alpha_1\)\(\beta_1\) and \(\alpha_1\)\(\beta_1\)\(\gamma_1\) GABA\(_A\)Rs with * and ** representing statistical significance by unpaired Student t-test at the \(p < 0.05\) and \(p < 0.001\) levels, respectively. The \(\alpha_1\) and \(\beta_1\) subunits were transfected in the pcDNA3.1 vector.

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Zinc produced a potent concentration-dependent inhibition of the α1β1 GABAAR with a mean Ic50 of 8.6 ± 1.1 µM and an nH of 0.80 ± 0.07 (n = 3; Fig. 2B). However, the zinc Ic50 at both γ subunit-expressing receptors exceeded 1 mM. This agrees with previous studies that demonstrated that γ subunits confer zinc insensitivity to αβ GABAARs.10,12 To distinguish the pharmacological properties of α1β1γ1 and α1β1γ2 GABAARs, we investigated the effects of DMcM and zolpidem, both of which discriminate strongly between γ1- and γ2-containing receptors. DMcM potentely inhibits γ2-containing GABAARs but has either no effect on or modestly potentiates γ1-containing GABAARs.9,10,13,14 Zolpidem potentiates both γ1- and γ2-containing receptors but has much greater efficacy at γ2-containing GABAARs.5,10,13,14 The effects of zolpidem at 0.01- and 1-µM concentrations on α1β1, α1β1γ1, and α1β1γ2 GABAARs, determined using both the fluorescence assay and electrophysiology on identical receptors recombinantly expressed in HEK293 cells (reproduced from Esmaeili et al.10), are presented in Figure 2C. The effects of 0.01 and 1 µM DMcM on all 3 receptors using both assays are shown in Figure 2D. The imaging and electrophysiology results for both assays are clearly highly consistent, indicating that PolyFect™ transfection reliably produces a large proportion of trimeric αβγ GABAARs.

**Conclusions**

We conclude that transfection of HEK293 cells with PolyFect™ or Effectene™ provides an efficient, reliable means of transiently expressing trimeric GABAARs in HEK293 cells. Fluorescence analysis of transfected cells using an anion-quenchable YFP revealed dramatic differences in the pharmacological properties of γ1- and γ2-containing receptors that were consistent with previous electrophysiological results. These results validate these assays as a tool for identifying novel positive and negative GABAAR modulators. This method of expressing and screening recombinant GABAARs should provide an effective means of discovering novel GABAAR subunit-specific compounds for use as therapeutic lead compounds and pharmacological probes.
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REFERENCES


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