Yellow Fluorescent Protein-Based Assay to Measure GABA\textsubscript{A} Channel Activation and Allosteric Modulation in CHO-K1 Cells

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Abstract

The \textgamma-aminobutyric acid A (GABA\textsubscript{A}) ion channels are important drug targets for treatment of neurological and psychiatric disorders. Finding GABA\textsubscript{A} channel subtype selective allosteric modulators could lead to new improved treatments. However, the progress in this area has been obstructed by the challenging task of developing functional assays to support screening efforts and the generation of cells expressing functional GABA\textsubscript{A} ion channels with the desired subtype composition. To address these challenges, we developed a yellow fluorescent protein (YFP)-based assay to be able to study allosteric modulation of the GABA\textsubscript{A} ion channel using cryopreserved, transiently transfected, assay-ready cells. We show for the first time how the MaxCyte STX electroporation instrument can be used to generate CHO-K1 cells expressing functional GABA\textsubscript{A} \(\alpha(2)\beta(3)\gamma(2)\) along with a halide sensing YFP-H148Q/I152L (YFP-GABA\textsubscript{A(2)}\textsubscript{β(3)}\textsubscript{γ(2)} cells). As a basis for a cell-based assay capable of detecting allosteric modulators, experiments with antagonist, ion channel blocker and modulators were used to verify GABA\textsubscript{A} subunit composition and functionality. We found that the \(\text{EC}_{50}\) concentration used in the YFP assay affected both basal quench of YFP and potency of GABA\textsubscript{A}. For the first time the assay was used to study modulation of GABA with 7 known modulators where statistical analysis showed that the assay can distinguish modulatory \(\text{pEC}_{50}\) differences of 0.15. In conclusion, the YFP assay proved to be a robust, reproducible and inexpensive assay. These data provide evidence that the assay is suitable for high throughput screening (HTS) and could be used to discover novel modulators acting on GABA\textsubscript{A} ion channels.

Introduction

GABA\textsubscript{A} ion channels mediate inhibitory neurotransmission in the central nervous system. They are members of the pentameric, ligand-gated ion channel family composed of around 18 different subunits which can give rise to a high variability in subunit composition, function and pharmacology [1,2]. The most abundant form in the brain is composed of two \(\alpha\) subunits, two \(\beta\) subunits and one \(\gamma\) subunit [3,4], where the two most widely expressed subtypes are \(\alpha(1)\beta(2)\gamma(2)\) and \(\alpha(2)\beta(3)\gamma(2)\) [5]. In recent studies it has been demonstrated that, in general, \(\alpha(2)\gamma(3)\) containing channels mediate anxiolytic effects, \(\alpha(1)\) containing channels mediate sedative effects and \(\alpha(2)\gamma(3)\) containing channels are involved in memory and learning [6]. The GABA agonist binding site is located at the interface between the \(\alpha\) and \(\beta\) subunits and the benzodiazepine site, one of several modulator sites, is found at the interface between the \(\alpha\) and \(\gamma\) subunits [1]. GABA\textsubscript{A} ion channels are important drug targets for neurological and psychiatric disorders such as sedation, anaesthesia, epilepsy, anxiolysis and muscle relaxation. Nevertheless, there is scope for development of new and improved drugs with subtype selectivity to overcome unwanted side effects of current treatments [7,8]. The aim of this study was to develop a sensitive and robust assay to be able to monitor GABA\textsubscript{A} ion channel modulation and identify compounds that could address this unmet medical need.

There are several techniques for monitoring activity of Cl\textsuperscript{-} channels such as GABA\textsubscript{A}. For a functional assay to be suitable for HTS of large compound libraries it should preferably be sensitive, fast, inexpensive and adaptable to high-density format. Patch-clamp electrophysiology is considered the “gold standard” for studying ion channels due to its sensitivity, however, it is costly and often limited in throughput. Binding assays are simple and fast but do not necessarily correlate with functional activity. Membrane potential assays using fast responding fluorescent dyes show medium to high sensitivity, give a robust signal under HTS conditions, have a good dynamic range and are high throughput [9], however, they require dye-loading steps and are relatively expensive. Moreover, there is evidence that some membrane potential dyes directly modulate the GABA\textsubscript{A} ion channel, [10] thereby introducing a risk of generating false positive hits in screening. Considering limitations with existing screening techniques and the difficulty in finding novel GABA\textsubscript{A} modulators we established a YFP assay to study GABA\textsubscript{A} activity and modulation. The method was developed by Galietta and colleagues [11,12].
where YFP was used as an intracellular sensor for monitoring anion flux through the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The assay was later adapted to HTS for CFTR modulators by Sui et al. [13]. YFP is an engineered variant of green fluorescent protein (GFP) carrying four point mutations (S65G, V68L, S72A, T203Y). Random mutation approaches have identified mutations, H148Q and I152L, that further increase the YFP halide sensitivity [12,14]. Upon ion channel activation anions such as I$, NO$_3$, Br$^−$ and Cl$^−$ enter the cell, bind to YFP and quench its fluorescence. Agonist-dependent quench of YFP fluorescence can then be measured with a fluorescent reader and used to determine channel activation, inhibition and modulation. The YFP assay has the benefit of being a noninvasive technique with the ability to measure fast responses. In addition, ions flux in the natural direction of the GABA$_A$ ion channel in comparison to a membrane potential assay where the electrochemical gradient is artificially polarized and a chloride efflux is measured. Furthermore, with no external addition of fluorescent dye the cost is reduced and the risk of GABA modulatory effects from the dye is avoided. A significant advantage of the YFP assay is that it does not require stable cell lines to obtain full dynamic range as only transfected cells produce a signal and simultaneous transfection of YFP-H148Q/I152L and ion channel subunits show high degree of co-expression [15]. Exogenous dyes, on the other hand, are taken up by all cells irrespective of whether they express the target ion channel, thereby reducing the dynamic range.

Difficulties in establishing stable cell lines along with the large investment in time and resources have made large scale transient transfection an attractive way of generating cells for assays. Among transfection technologies electroporation is an efficient way of introducing DNA into cells without the need for chemical transfection reagents. The mechanism for DNA uptake into the cell involves electrophoretic association of DNA with the cell membrane followed by DNA insertion in the membrane and entry into the cell [16]. Due to limitations in scaling up it has previously been difficult to transfect large enough cell batches for screening. In this study we overcome this problem by using MaxCyte STX (MaxCyte, Inc.). The instrument enables small to medium scale static electroporation of 0.5×10$^6$–3.5×10$^9$ cells for minimization work and large scale flow electroporation of up to 10$^{10}$ cells with high transfection efficiency and reproducibility.

The YFP assay has previously been developed for CFTR [11,13]. Glycine [15] and rat GABA$_A_1$ [15,17]. The goal of our work was to develop a YFP assay for human GABA$_A_2$, GABA$_A_3$, and rat GABA$_A_4$ expressed transiently in Chinese Hamster Ovary (CHO-K1) cells, to confirm the applicability of cryopreserved transiently transfected cells, and to test the assay’s suitability for screening of modulators. We found that cryopreserved transiently transfected assay-ready cells showed robust performance in the YFP assay and enabled easy handling and scale-up. Our results show that the YFP assay can be established to study agonist activation and modulation of the GABA$_A$ channel.

**Materials and Methods**

**Chemicals and Reagents**

Diazepam, Alpidem, Lorazepam, Clobazam, Desmethyl-clobazam, TPA-023, L-838417 and Bicuculline were synthesized at AstraZeneca. Ham’s F-12 medium, Picrotoxin, GABA and all other chemicals were purchased from Sigma.

**Cloning**

The cDNA of YFP (Genbank accession number AY818378) with H148Q and I152L mutations was inserted into the pcDNA3.1 vector using 5' BamHI and 3' NotI sites. Human GABA$_A_1$ subunits $\alpha_2$, $\beta_3$ and $\gamma_2$ (Genbank accession numbers; $\alpha_2$ NM_000807, $\beta_3$ NM_000814, $\gamma_2$ NM_000816) were inserted into pGen-IRES-neo2 vectors [18] using NotI 5' and 3' sites.

**Cell Culture and Transfection**

CHO-K1 cell line (ATCC, CCL-61) was cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum and incubated at 37°C and 5% CO$_2$. Transient transfection was performed using MaxCyte STX electroporation instrument (MaxCyte, Inc.) with CHO cell line specific electroporation protocol. The cells were split one day prior to electroporation to ensure that cells were in the log phase growth at time of transfection. Cells were harvested with Accutase (PAA Laboratories), pelleted at 250 g for 10 minutes, rinsed with MaxCyte Electroporation Buffer (MaxCyte, Inc.), pelleted again and resuspended in MaxCyte Electroporation Buffer at a density of 1×10$^6$ cells/ml. CHO-K1 cells were electroporated simultaneously with four plasmids using a total amount of 500 μg DNA per 1×10$^6$ cells; YFP-H148Q/I152L (150 μg) and GABA$_A_2$ (72.5 μg $\alpha_2$, 5 μg $\beta_3$ and 72.5 μg $\gamma_2$) plasmids. Following electroporation, cells were incubated at 37°C and 5% CO$_2$ for 20 minutes to recover. Cells were then diluted in culture medium and centrifuged 250 g for 10 minutes. Pelleted cells were resuspended to 5–10×10$^6$ cells/ml in Ham’s F12 with 20% fetal bovine serum and 10% DMSO, and cryopreserved using controlled-rate cryo containers (Nalgene, Thermo Scientific). Cryopreserved assay-ready cells were used for all experiments.

**YFP-based Assay**

Cryopreserved transiently transfected cells were thawed and plated in 384-well poly-D-lysine-coated plates at a density of 15000 cells/well using a Multidrop Combi Reagent Dispenser (Thermo Scientific). After 24 h incubation at 37°C and 5% CO$_2$, cells were washed three times on an ELx405 Select Deep Well Microplate Washer (BioTek), exchanging 50 μl culture medium with 30 μl HBSS buffer supplemented with 20 mM Hepes. Plates were incubated with buffer for 1 h at 37°C and 5% CO$_2$. Cell plates were assayed on a FLIPR Tetra (Molecular Devices) using excitation filter of 470–495 nm and emission filter of 515–575 nm. Starting with 30 μl buffer in each well, cells were pre-incubated with 10 μl compound for 15 minutes. 20 μl GABA in NaI buffer pH 7.5 (140 mM NaX, 20 mM Hepes, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$), NaX was a mix of NaCl and NaI) was added to activate the ion channel and induce an agonist-dependent quench of YFP fluorescence. Data was acquired for 120 s with fluorescence recording at 1 s intervals where GABA/NaI solution was added after 10 s of baseline reading. Compounds were initially solubilized to a concentration of 10 mM in DMSO and then diluted in HBSS buffer with 20 mM Hepes. Final DMSO concentration in the assay was 0.02% for modulators and at 1% for Picrotoxin and Bicuculline. DMSO only controls were included in every experiment and there was no detectable effect of DMSO on GABA channel activation. GABA solutions were prepared fresh for every experiment and 3-fold serial dilutions were performed on a 384-well microplate in NaI buffer using a Biomek FX (Beckman Coulter) liquid handling device.
**Data Analysis**
Data at 1–10 s was adjusted to a baseline of 100% and results were then extracted as % quench at 120 s. % quench is defined as \((F_{\text{init}} - F_{\text{final}})/F_{\text{init}}\times100\) where \(F_{\text{init}}\) and \(F_{\text{final}}\) are the initial and final values of fluorescence respectively. GABA concentration-response curves were fitted with the Hill equation, \(F = F_{\text{init}}/(1 + (\text{EC}_{50}/[\text{GABA}])^{nH})\), where \(F\) is the fluorescence corresponding to a GABA concentration \([\text{GABA}]\), \(F_{\text{init}}\) is the initial fluorescence value, \(\text{EC}_{50}\) is the concentration that elicits half-maximal response and \(nH\) is the Hill coefficient. Curve fits were performed using least-squares fitting routine (GraphPad Prism 5, GraphPad Software Inc.).

**Statistical Analysis**
To evaluate the statistical significance of pEC\(_{50}\) differences seen for different \(\Gamma\) concentrations, Analysis of Variance was used. This allowed for \(\Gamma\) concentration and experimental occasion, to enable t-tests, to use a pooled estimate of variability when comparing \(\Gamma\) concentrations. Two-sided tests were used.

To calculate the pEC\(_{50}\) difference at which two modulators can be considered to have statistically significant differences in GABA potency, 7 known modulators were tested in the YFP assay on 3 different occasions. A pooled estimate of variability was used to calculate the standard error of the difference between two compounds' average of 3 occasions pEC\(_{50}\). The 95% confidence interval was calculated as \(2 \times \text{standard error}\). Taking the antilog converts this to a fold change (ratio) between two EC\(_{50}\) values.

**Results**

**Optimization of Transient Transfection**
To generate cells expressing the GABA\(_A\) ion channel for the YFP assay, four different plasmids (GABA\(_A\) α2, β3, γ2 and YFP-H148Q/I152L) were electroporated simultaneously using MaxiPrep. DNA ratio for subunits of GABA\(_A\) H148Q/I152L) were electroporated simultaneously using MaxiPrep, previously been optimized by Liu et al. [19] (and unpublished channel specific, 40 mM NaI buffer was added to CHO-K1 cells to enable t-tests, to use a pooled estimate of variability when analyzing the pEC\(_{50}\) values. Analyzing the pEC\(_{50}\) values, a trend of increasing pEC\(_{50}\) with increasing \(\Gamma\) concentration was observed (Figure 3). Analysis of variance with two-sided tests was employed to determine the significance of the pEC\(_{50}\) values for the different \(\Gamma\) concentrations. Differences in pEC\(_{50}\) turned out to be statistically significant at \(p<0.01\) (*) when comparing 5 mM and 10 mM \(\Gamma\) and \(p<0.05\) (**) when comparing 10 mM and 20 mM \(\Gamma\) (Figure 3). These results emphasize the importance of careful optimization of \(\Gamma\) concentration in the YFP assay. To reduce the artifact of agonist-independent \(\Gamma\) permeability while maintaining high signal to background and consistent data in GABA concentration-response mode, 10 mM \(\Gamma\) was considered to be the optimal concentration in assay.

**\(\Gamma\) Influences GABA Potency**
The above results indicate that appropriate \(\Gamma\) concentration is important for optimal assay performance. To fully understand its implications, concentration-responses of GABA were determined and EC\(_{50}\) values calculated in the presence of different \(\Gamma\) concentrations. Analyzing the pEC\(_{50}\) values, a trend of increasing pEC\(_{50}\) with increasing \(\Gamma\) concentration was observed (Figure 3). Analysis of variance with two-sided tests was performed to determine the significance of the pEC\(_{50}\) values for the different \(\Gamma\) concentrations. Differences in pEC\(_{50}\) turned out to be statistically significant at \(p<0.01\) (*) when comparing 5 mM and 10 mM \(\Gamma\) and \(p<0.05\) (**) when comparing 10 mM and 20 mM \(\Gamma\) (Figure 3). These results emphasize the importance of careful optimization of \(\Gamma\) concentration in the YFP assay. To reduce the artifact of agonist-independent \(\Gamma\) permeability while maintaining high signal to background and consistent data in GABA concentration-response mode, 10 mM \(\Gamma\) was considered to be the optimal concentration in assay.

**Antagonist Effect of Bicuculline**
To confirm the agonist mediated functionality of transiently transfected cells, a competitive GABA\(_A\) antagonist, Bicuculline, was used. Figure 4 shows Bicuculline inhibition of the ion channel when stimulated with EC\(_{50}\) of GABA. IC\(_{50}\) of Bicuculline was calculated to be 2.14 \(\mu\)M.

**Positive Allosteric Modulation of GABA Signal in YFP-GABA\(_A\) Cells**
To investigate the YFP assay’s suitability for screening of positive allosteric modulators, GABA concentration-response curves in the absence and presence of Diazepam, a strong positive allosteric modulator, were generated (Figure 5). Upon Diazepam addition the GABA EC\(_{50}\) decreased from 0.34 \(\mu\)M±0.08 \(\mu\)M (n = 3) to 0.20 \(\mu\)M±0.02 \(\mu\)M (n = 3) which equals a 1.7 fold EC\(_{50}\) shift. To further investigate the YFP assay’s ability to distinguish GABA modulation, concentration-responses of GABA in the presence of 7 known modulators were determined. A pooled estimate of variability was used to calculate the standard error of the difference between two compounds’ pEC\(_{50}\) values (average of 3 occasions). The data show that known modulators yielding a ≥1.40 times shift in GABA EC\(_{50}\), or a difference in pEC\(_{50}\) ≥0.15, compared to GABA, could be considered to have a statistically significant effect on GABA potency with 95% confidence, when averaging across 3 occasions (Table 1). Consequently, Diazepam, Lorazepam, Clozapam and Alprazolam are modulators that have a significant positive allosteric effect on GABA (Table 1). However, we were not able to detect any
significant allosteric modulation using the weak modulators TPA-023 or L-838417 (Table 1 and Figure 6).

Discussion

In this study we establish a YFP assay capable of detecting GABAA ion channel activity and modulation using transiently transfected YFP-GABAA2 cells. We show that transient transfection using MaxCyte STX electroporation instrument can be used to successfully generate functional YFP-GABAA2 cells. In addition, we show that the transfected cells can be cryopreserved, stored long term and subsequently thawed and used as assay-ready cells. The YFP assay proves to be fast and inexpensive with no need for dye loading, commercial kits or costly reagents. Previously, this assay has been used to study GABAA functionality using GABA and the competitive GABAA antagonist Bicuculline [15,17]. We demonstrate for the first time how the YFP assay can be used to study agonist modulation. Studying the characteristics of the assay, we show that I− concentration affects not only basal quench but also GABA potency, something that has not been reported before. Furthermore, it is a robust assay that can be used to distinguish small differences in modulator efficacy and has the potential to be used to discover novel modulators of the GABAA ion channel in an HTS format.

To study ion channel functionality certain basic criteria of the...
cell line need to be considered. These include high transfection efficiency and low endogenous ionic current. CHO-K1 cells were chosen in this study since they are efficiently transfected, are highly viable after electroporation, show good transgene expression and have low expression of endogenous ion channels [20]. Transient transfection offers speed and flexibility which enables rapid assay evaluation and development compared to stable cell line generation. In addition it provides the possibility to titrate target expression. In the case of targets with multiple subunits, e.g. GABA\textsubscript{A}, it simplifies systematic titration of DNA amount to achieve optimal subunit composition. The difficulties associated with generation of functional stable GABA\textsubscript{A} cell lines, the complexity of subunit compositions and the limited diversity of commercial cell lines makes transiently transfected cells an attractive alternative. Small-scale transfections to generate GABA\textsubscript{A} cells using lipid-based reagents are common [15,17,19], although successful generation of transiently transfected GABA\textsubscript{A} cells using large-scale electroporation has previously not been reported. This approach will provide a powerful tool when studying GABA\textsubscript{A} ion channel pharmacology and diversity.

Genetically modified YFP-H148Q/I152L has the capacity to bind small anions resulting in quench of its fluorescence. The relative affinity of anions to YFP-H148Q/I152L are I\textsuperscript{−}>NO\textsubscript{3} \textsuperscript{−}>Br\textsuperscript{−}>Cl\textsuperscript{−} [12]. This characteristic, along with its intracellular expression, photostability and fluorescent properties makes it suitable as an intracellular sensor to study anionic influx through ion channels. Since the reversal potential of Cl\textsuperscript{−} across the cell membrane is close to the resting potential of the cell, the driving force for Cl\textsuperscript{−} flux through the GABA\textsubscript{A} ion channel is small. This low electrochemical driving force across the membrane as well as a fairly low affinity of Cl\textsuperscript{−} for YFP-H148Q/I152L, has led to replacement of Cl\textsuperscript{−} with I\textsuperscript{−} for optimal assay performance when studying GABA\textsubscript{A} in the YFP assay. In addition, I\textsuperscript{−} has been demonstrated to have a higher permeability than Cl\textsuperscript{−} through the GABA\textsubscript{A} ion channel, with a permeability sequence of I\textsuperscript{−}>Br\textsuperscript{−}>Cl\textsuperscript{−}>F\textsuperscript{−} [21]. Basal I\textsuperscript{−} permeability can occur through GABA\textsubscript{A} ion channel or through other ion channels or transporters. This could be due to spontaneous opening of the ion channel with high extracellular I\textsuperscript{−} working as a driving force or due to I\textsuperscript{−} acting as a GABA\textsubscript{A} modulator [21]. Basal I\textsuperscript{−} permeability results in quenching of YFP signal and reduces the maximum relative quench that can be achieved upon agonist activation of the ion channel. Previously, GABA\textsubscript{A} ion channel functionality has not been studied in CHO-K1 cells using the YFP assay. To optimize signal to background different I\textsuperscript{−} concentrations, ranging from 5–40 mM final concentration, were investigated and 10 mM was determined to be optimal in terms of minimal basal quench, maximum assay window and data consistency. Previously de-

**Figure 4. Inhibition of GABA\textsubscript{A} with Bicuculline.** Concentration-response of Bicuculline was added to YFP-GABA\textsubscript{A2} cells 15 min prior to addition of EC\textsubscript{80} of GABA in 10 mM NaI buffer. Data are mean ± SD of triplicate wells. IC\textsubscript{50} = 2.14 μM. doi:10.1371/journal.pone.0059429.g004

**Figure 5. Modulation of GABA with Diazepam.** YFP-GABA\textsubscript{A2} cells were pre-incubated with DMSO or 1 μM Diazepam 15 minutes prior to addition of a concentration-responses of GABA at 10 mM I\textsuperscript{−}. The graph shows representative mean data ± SD of quadruplicate wells. The experiment was repeated 3 times with similar results. doi:10.1371/journal.pone.0059429.g005

**Figure 6. Modulation of GABA signal with Diazepam (Dia) and TPA-023.** YFP-GABA\textsubscript{A2} cells were exposed to EC\textsubscript{20} of GABA alone or in the presence of 1 μM of modulator. Data at 1–10 s was adjusted to a baseline of 100%. Representative time-courses of fluorescent quench at basal (10 mM I\textsuperscript{−}) and maximum GABA response (GABA\textsubscript{max}). A) Modulation of GABA EC\textsubscript{20} quench showing a significant increase in the presence of Diazepam. B) No significant change of GABA EC\textsubscript{20} quench in the presence of TPA-023. The experiment was repeated three times with similar results. doi:10.1371/journal.pone.0059429.g006

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YFP Assay to Measure GABAA Functionality

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experiments are therefore needed to ensure correct subunit assembly and ion channel function [3,22]. The subunit composition determines the GABA sensitivity and the pharmacological properties of the channel. Experiments were performed on cells expressing GABA<sub>A</sub> α2β3γ2 optimized to yield a composition of two α2, two β3 and one γ2 subunit stoichiometry. Functionality of the GABA<sub>A</sub> ion channel was confirmed by agonist activation using GABA as well as inhibition using Bicuculline and Picrotoxin. The benzodiazepine site is located at the interface of the α and γ subunits and a correct subunit composition of the channel is required to yield agonist modulatory effect with a benzodiazepine-class compound. We report modulation of GABA activation using different positive allosteric modulators. The strong positive modulator Diazepam shifted GABA EC<sub>50</sub> 1.7 fold, which corresponds to a pEC<sub>50</sub> difference of 0.23, compared to GABA alone. This may be considered a small shift and can be compared to a pEC<sub>50</sub> difference of 0.41 generated with VSD assay by Liu and coworkers [19]. However, when testing 7 known modulators, statistical analysis showed that our YFP assay can distinguish pEC<sub>50</sub> differences as small as 0.15 when averaging across 3 occasions. A direct comparison of EC<sub>50</sub> values of these 7 different modulators compared to literature values is however not relevant, given the difference in modulator effect observed using different subunit compositions of the GABA channel and the variation in EC<sub>50</sub> values reported from different cell backgrounds and assays. The ability to modulate GABA potency upon modulator addition confirms that our YFP-GABA<sub>A</sub> cells express functional GABA<sub>A</sub> α2β3γ2 ion channels with correct subunit composition containing a benzodiazepine binding site. It further implies that the assay can be used to screen for modulators and rank order modulators with small pEC<sub>50</sub> differences. However, using the current assay protocol, we were not able to detect a statistically significant effect of known weak positive allosteric modulators. In mouse fibroblast L(tk<sup>−</sup>) cells expressing GABA α2β3γ2, relative in vitro efficacy of TPA-023 or L-838417 was reported to be 11% and 34% respectively, measured as the potentiation of GABA EC<sub>50</sub> compared to a 100% response from a classical benzodiazepine full agonist, chlordiazepoxide [23,24]. The inability to measure modulation of the GABA signal with these compounds is a limitation with the YFP assay compared to the VSD assay. Using the VSD assay Liu and coworkers detected modulation of GABA signal using the weak modulator L-838417 [19]. A way to improve the sensitivity of the YFP assay may be to further optimize the I<sup>γ</sup> concentration. Using different I<sup>γ</sup> concentrations we observed a trend of increased positive modulator efficacy with

Table 1. Modulation of GABA Signal with Allosteric Modulators.

| Compound          | pEC<sub>50</sub> | pEC<sub>50</sub> difference | Significance 95% confidence |
|-------------------|-----------------|-----------------------------|-----------------------------|
| GABA              | 6.48±0.11       | 0                           | -                           |
| Diazepam          | 6.71±0.05       | 0.23                        | Significant difference      |
| Lorazepam         | 6.71±0.09       | 0.23                        | Significant difference      |
| Clobazam          | 6.66±0.05       | 0.18                        | Significant difference      |
| Alpidem           | 6.84±0.07       | 0.36                        | Significant difference      |
| L-838417          | 6.47±0.11       | −0.01                      | non-significant             |
| TPA-023           | 6.38±0.10       | −0.10                      | non-significant             |
| Desmethyl-clobazam | 6.53±0.07       | 0.05                        | non-significant             |

Modulator pEC<sub>50</sub> values were calculated from GABA concentration-response curves in the presence of 1 μM modulator. pEC<sub>50</sub> differences were calculated from \( \log(\text{EC}_{50} \text{ GABA alone}) - \log(\text{EC}_{50} \text{ GABA with modulator}) \) and stated as statistically significant or non-significant at \( p < 0.05 \). A pooled estimate of variability was used to calculate the standard error of the difference between two compounds’ average of 3 occasions pEC<sub>50</sub>. The 95% confidence interval was calculated as \( 2 \times \) this standard error. Taking the anti-log converts this to a fold change (ratio) between two EC<sub>50</sub> values.

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decreased I^- concentrations, (data not shown). However, a consequence of decreased I^- concentration is a decreased assay window and an increased variability of the assay. Taken together, increasing the sensitivity of the YFP assay by decreasing the I^- concentration results in reduced assay reproducibility and robustness. Even though there is an obvious challenge of using the YFP assay for screening of positive GABAA modulators in an HTS format, our initial results indicate that the assay produces robust data for moderate to strong modulators.

Although it is beyond the scope of the current study, screening a larger set of compounds would confirm the YFP assay's robustness and provide an answer about the utility of the assay in HTS to identify positive allosteric modulators of GABAA. To find subtype selective modulators of GABAA, cells with different subunit compositions would need to be generated and used in screening. Our work demonstrates how to successfully generate YFP-GABAA32 cells. To generate transiently transfected cells expressing GABAA with a different set of subunits, DNA ratio optimization could be evaluated with functional characterization similar to what has been described here. In conclusion, using the YFP assay with transiently transfected CHO-K1 cells provides a flexible means for discovering novel GABAA modulators.

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Author Contributions

Conceived and designed the experiments: TJ TN HPS. Performed the experiments: TJ TN HPS. Analyzed the data: TJ TN HPS. Contributed reagents/materials/analysis tools: TJ TN HPS. Wrote the paper: TJ TN HPS.

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