Characterizing Ligand-Gated Ion Channel Receptors with Genetically Encoded Ca\textsuperscript{2+} Sensors

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Abstract

We present a cell-based system and experimental approach to characterize agonist and antagonist selectivity for ligand-gated ion channels (LGIC) by developing sensor cells stably expressing a Ca\textsuperscript{2+} permeable LGIC and a genetically encoded Förster (or fluorescence) resonance energy transfer (FRET)-based calcium sensor. In particular, we describe separate lines with human α\textsubscript{7} and human α\textsubscript{4}β\textsubscript{2} nicotinic acetylcholine receptors, mouse S-HT\textsubscript{3A} serotonin receptors and a chimera of human α\textsubscript{7/mouse} 5-HT\textsubscript{3A} receptors. Complete concentration-response curves for agonists and Schild plots of antagonists were generated from these sensors and the results validate known pharmacology of the receptors tested. Concentration-response relations can be generated from either the initial rate or maximal amplitudes of FRET-signal. Although assaying at a medium throughput level, this pharmacological fluorescence detection technique employs a clonal line for stability and has versatility for screening laboratory generated congeners as agonists or antagonists on multiple subtypes of ligand-gated ion channels. The clonal sensor lines are also compatible with in vivo usage to measure indirectly receptor activation by endogenous neurotransmitters.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels (LGIC) belonging to the sub-family of Cys-loop receptors. They are found in the central and peripheral nervous systems of both vertebrate and invertebrate species. Specific nAChR subtypes are recognized pharmaceutical targets for many central nervous system (CNS) diseases and conditions [1]. For example: the α\textsubscript{7}-nAChR is targeted for schizophrenia [2], Alzheimer’s disease [3], and cognition enhancement [4], and the α\textsubscript{4}β\textsubscript{2}-nAChR is also targeted for Alzheimer’s disease [5,6], and for tobacco addiction [7,8]. Therefore, a particular challenge in therapeutic considerations is achieving subtype selectivity among the nAChRs. Sorting out the desirable actions within the subunit diversity of the nAChR family from the largely unwanted responses, via off-target receptors, is an important facet of the therapeutic approach [9].

For selection of pharmacologic leads, a facilitated and rapid assay of nAChR subtype selective agents is required early on in the screening process [10]. While electrophysiological methods through whole cell recording and patch clamp analysis can subsequently be used to uncover mechanism, they lack the scalability, cost reduction and automation that can be attained through the use of photon generated signals from multi-well plates [11,12]. The scalability and automation derived from fluorometric imaging plate readers (FLIPR) have encouraged the use of fluorescence-based dyes in screening therapeutics on nAChRs [13,14]. Yet conventional fluorescent dyes to detect depolarization or intracellular calcium, are limited by cost, variations in dye administration, dye shelf life, and cell perturbations during injection. Hence a stable, clonal cell line containing a light detection sensor and a receptor, which is generated from incorporating the respective genetic material, become critical considerations in developing a medium throughput assay for selective receptor activation. Bioluminescent Ca\textsuperscript{2+} reporters, such as GFP-aequorin, have been shown to be an applicable method in creating cell lines that contain a genetically encoded light detection method [15,16]. Although bioluminescent reporters address the issues of variations in dye administration, shelf life, and cost; they are still susceptible to cell perturbations during injection, interference from fluorescent ligands, and are limited in time resolution due to the length of their decay. Applications that take advantage of fusing fluorescent protein Förster (or fluorescence) resonance energy transfer (FRET) pairs with Ca\textsuperscript{2+} binding proteins, such that changes in intracellular Ca\textsuperscript{2+} levels can be visualized through a combination of donor quenching and
acceptor sensitization, have become a prevalent method for enabling investigators to monitor signal transduction pathways in varying cells [17]. The use of FRET pairs over Ca$^{2+}$ sensing bioluminescent reporters minimizes the perturbations of ligand fluorescence and cell perturbation by solvent during the injection phase. Although many genetically encoded Ca$^{2+}$ sensors exist [18,19], the development of the Ca$^{2+}$ sensor TN-XXL allowed for a highly sensitive and effective FRET based sensor [20]. Its incorporation with over-expressed G-protein-coupled receptors (GPCR) to produce cell-based neurotransmitter fluorescent engineered reporters (CNiFERS) offered the potential to overcome most of the limitations of the Ca$^{2+}$ bioluminescent reporters [21]. In our previous studies, the generation of a genetically encoded sensitive Ca$^{2+}$ sensor with a stably over-expressed GPCR allowed for in vivo validation of a compound’s activity in situ in brain [21]. High-throughput fluorescence methodology [14], which has been previously utilized, affords a dual application to the stable, clonal cell line sensors we generated for transplanted cells. Here we report on generating and utilizing nAChR CNiFERS to measure nAChR activation via Ca$^{2+}$ flux through ion channels in the cell membrane as opposed to GPCR mediated release primarily from intracellular stores described previously (Figure 1). Our studies extend the application of CNiFERS to prevalent and targeted CNS nAChR subtypes, the $\alpha 7$ and $\alpha 4\beta 2$, and also the 5-HT$\alpha$A serotonin receptor. A chimaeric receptor CNiFER composed of $\alpha 7$/5-HT$\alpha$A was also generated to gain additional insight into activation of the wildtype $\alpha 7$-nAChR. The chimaeric receptor has been shown to have a desensitization rate comparable to 5-HT$\alpha$A receptors, and more recently a higher conducting variant has been created [22,23,24]. This chimaeric receptor still maintains ligand affinities for that of the full-length $\alpha 7$-nAChR, which allows for a possible allosteric modulator (PAM) free study of the $\alpha 7$-nAChR [23]. We report on a cell-based assay incorporating the use of nAChR CNiFERS to identify and characterize nAChR agonists and antagonists with a low cost, medium throughput fluorescence assay. The system is capable of detection on a monolayer of cells in a 7 mm diameter well of a 96-well plate and has the potential to be extended the application of CNiFERS to prevalent and targeted CNS nAChR activation via Ca$^{2+}$ reporter on generating and utilizing nAChR CNiFERs to measure nAChR activation via Ca$^{2+}$ flux through ion channels in the cell membrane as opposed to GPCR mediated release primarily from intracellular stores described previously (Figure 1). Our studies extend the application of CNiFERS to prevalent and targeted CNS nAChR subtypes, the $\alpha 7$ and $\alpha 4\beta 2$, and also the 5-HT$\alpha$A serotonin receptor. A chimaeric receptor CNiFER composed of $\alpha 7$/5-HT$\alpha$A was also generated to gain additional insight into activation of the wildtype $\alpha 7$-nAChR. The chimaeric receptor has been shown to have a desensitization rate comparable to 5-HT$\alpha$A receptors, and more recently a higher conducting variant has been created [22,23,24]. This chimaeric receptor still maintains ligand affinities for that of the full-length $\alpha 7$-nAChR, which allows for a possible allosteric modulator (PAM) free study of the $\alpha 7$-nAChR [23]. We report on a cell-based assay incorporating the use of nAChR CNiFERS to identify and characterize nAChR agonists and antagonists with a low cost, medium throughput fluorescence assay. The system is capable of detection on a monolayer of cells in a 7 mm diameter well of a 96-well plate and has the potential to be scaled to a high throughput platform.

Figure 1. Simplistic representation of LGIC-CNiFERS and their response. Upon activation of a calcium permeable receptor, Ca$^{2+}$ enters the cell and upon binding to TN-XXL produces a conformational change eliciting a fluorescence change reflected in quenching of the donor fluorescence and excitation of the acceptor. doi:10.1371/journal.pone.0016519.g001

Results and Discussion

Cell Based Neurotransmitter Fluorescent Engineered Reporters (CNiFERS)

The first CNiFERS were developed for applications of monitoring cortical acetylcholine release in the cerebral cortex of rats using the M1-CNiFER [21]. The CNiFERS presented here were developed to facilitate pharmacological screening, potency rank ordering and characterization of compounds for calcium permeable ligand-gated ion channels (LGICs), with a particular focus on the homomeric $\alpha 7$ and heteromeric $\alpha 4\beta 2$ nAChRs. The 5-HT$\alpha$A serotoninergic LGIC receptor exists in its simplest form as a homopentamer similar to the $\alpha 7$-nAChR [25]. Although the 5-HT$\alpha$A receptor has a large calcium conductance, the 5-HT$\alpha$A heteromeric receptors do not and therefore may have limited applicability with this technique [26]. Since antagonists, such as tropisetron, for the 5-HT$\alpha$A receptor are known to activate $\alpha 7$-nAChRs, 5-HT$\alpha$A CNiFERS not only expand the neurotransmitter target base, but also become a model system for testing opposing receptor responses [27]. LGIC agonist responses were measured with CNiFERS by monitoring TN-XXL FRET ratios, emissions of citrine cp174 (327 nm) to cEFP (465 nm), over 120 seconds with agonist injections occurring at the 30 second time-point. Figure 2a is an example of the fluorescent signals generated from a 5-HT$\alpha$A CNiFER single concentration response to 3 μM 5-Hydroxytryptamine (5-HT) injected at 30 seconds. FRET ratios of the emissions for a concentration range of 0.3–3 μM 5-HT is shown in Figure 2b. Concentration response curves can then be generated from the peak FRET ratio values for each concentration.

$\alpha 7$-nAChR CNiFERS

Due to the rapid rate of desensitization of the $\alpha 7$-nAChR in the time frame of milliseconds [28], monitoring agonist-elicited fluorescence responses were not possible without including a PAM [29]. In our case we used the type II PAM, N-5-Chloro-2,4-dimethoxyphenyl-[N’-(5-methyl-3-isoxazolyl)-urea (PNU-120596), and found that concentrations of 10 μM and above incubated on cells for at least 30 min (data not shown) yielded a maximal agonist-elicited fluorescence response (Figure 3a). Characteristic of a type II PAM, PNU-120596 will increase ion flux through the $\alpha 7$-nChRs by decreasing its desensitization rate [30]. PNU-120596 likely interacts with a region in the transmembrane helices spanning TM1, TM2, and TM4 to prevent an activated $\alpha 7$-nChR from switching to its desensitized state once activated [31]. PNU-120596 modulating activity results in an increase in the number of receptors remaining in an open or activated state. Accordingly, not only is a larger response produced, but also a lower concentration of agonist is likely needed to observe a response. Agonist EC$_{50}$ values measured with the $\alpha 7$-nAChR CNiFER in the presence of PNU-120596 are less than those reported in the literature for $\alpha 7$-nAChR agonists without PNU-120596, as observed in Table 1. Nevertheless, these considerations are not likely to affect the rank ordering for agonist potencies. Assay of antagonists is conducted as a null method and should be independent of agonist used, provided a single receptor subtype is being analyzed. When (2)-epibatidine responses from $\alpha 7$-nAChR CNiFERS were blocked with three concentrations (3, 10, 30 nM) of methyllycaconitine (MLA) (Figure 3b). Schild analysis of the parallel concentration versus fluorescence readout showed MLA to block $\alpha 7$-nAChR CNiFERS competitively with a slope of $-1.2\pm0.3$ and with an observed $K_a$ value of $3.2\pm1.4$ nM for the antagonist (Figure 3c, Table 2).
Figure 2. Direct FRET measurement of response. A. Measured time-course emission wavelengths for eCFP (485 nm) and Citrine cp174 (527 nm) on 5-HT3A CNiFERs. 5-HT was injected at 30 s at 3 μM concentration to produce a Ca2+ influx causing a concentration dependent FRET response from the TN-XXL reporter. Measurements were taken every 3.52 s with excitation at 436 nm. B. FRET-Ratio (Citrine cp147/eCFP) time-course responses from 5-HT3A CNiFERs to varying concentrations of 5-HT. Peak values or initial rates may be used to plot a concentration dependent curve to estimate EC50 values. Peak values were used to generate concentration response curves.
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Figure 3. α7-nAChR CNiFER: PNU-120596 activation and methyllycaconitine antagonism. A. Concentration dependent response of PNU-120596 incubated for 30 minutes on α7-nAChR CNiFERs and stimulated with 0.1 μM (±)-epibatidine. The response curve shows that concentrations greater than 10 μM PNU-120596 would produce a maximal response. B. Competitive blockade of (±)-epibatidine elicited responses on α7-nAChR CNiFERs by MLA. The EC50 values for each curve shifted from 3.6 nM to 17 nM, 29 nM, and 160 nM for 3 nM, 10 nM, 30 nM MLA respectively. C. Schild plot of concentration dependent shifts. A slope of -1.2±0.3 confirmed MLA to block competitively.
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**Table 1. Comparison of Agonist EC50 using CNiFER and non-CNiFER detection**

|        | a7/5-HT3A CNiFER | a7/5-HT3A | a7-nAChR CNiFER | a7-nAChR | a4β2-nAChR CNiFER | a4β2-nAChR |
|--------|------------------|-----------|-----------------|-----------|------------------|-----------|
| (±)-Epibatidine | 260±50 (n = 3)   | 250±20   | 28±16 (n = 24)  | 17±2.6   | 14±9 (n = 17)  | 27±6 (n = 6) |
| (−)-Nicotine       | 10,000±2,600 (n = 3) | 19,100±7,300 | 410±270 (n = 10) | 1,160±180 | 92±12 | 1,850±810 (n = 4) | 2,700±200 |

a. Measured in aCSF.
b. Measured with Ca2+ dye (Fluo-3-AM) on HEK293 cells expressing human receptors [23]. *Geometric Mean [−SD = 2.4, +SD = 5.1][39].
c. Measured with 10 μM PNU-120596 in aCSF.
d. Measured with potential-sensitive dye against human receptors. *(2x3b) [35].
e. Measured with potential-sensitive dye and 3 μM PNU-120596.
f. To be published Kuryatov A, Mukherjee J, and Lindstrom J.

**α7/5-HT3A Chimaeric Receptor CNiFER**

The chimaeric α7/5-HT3A receptor does not exhibit the rapid desensitization rate seen with the wildtype (wt) α7-nAChR and thus neither requires PNU-120596, nor would PNU-120596 be effective [31], for achieving sufficient fluorescence signals within the time frame of our measurements [33]. Schild MLA is too slow to allow for complete dissociation from the receptor in the timeframe of our measurements [33]. Schild analysis showed a slope of −1.8±0.2, which indicates that MLA is not solely a competitive antagonist at the α7/5-HT3A CNiFERs (Figure 4b). However, the major competitive component yielded a K0 of 2.5±0.1 nM, a value very close to that measured on the α7-nAChR CNiFERs (Table 2). The similarity of the antagonist affinities shows that using a PAM only affects agonist constants and employing the null method for determining Ka values negates the effect of PAMs. The similarity of these values for occupation by the antagonist validates the use of these chimaeric α7/5-HT3A CNiFERs for characterizing ligands for the α7-nAChR without the need to use a PAM. Antagonist binding is more apt to be characterized by a single binding state as shown with the muscle nAChRs [34].

**α4β2-nAChR CNiFERS**

To characterize the α4β2-nAChR CNiFERS, concentration-response relationship data were generated with various concentrations of (±)-epibatidine in the absence and presence of a known competitive antagonist, dihydro-beta-erythroidine (DHβE) (0.3, 1, 3 μM). The resulting concentration-response curves showed a parallel shift with little decrease in the maximum revealing competitive antagonism (Figure 5a). Schild analysis confirmed DHβE acts competitively with a slope of −0.9±0.1 and measured a K0 of 136±54 nM (Figure 5b, Table 2).

**5-HT3A Serotonin CNiFERS**

The 5-HT3A receptor was generated from a single transfected subunit to form a homomeric pentamer. Concentration-response curves show serotonin to be an agonist, as expected, with an EC50 of 349±78 nM (n = 16). A near competitive block is achieved with ondansetron as an antagonist (Figure 6, Table 2). Other setrons, such as tropisetron and granisetron appear to show a greater non-competitive component (data not shown), but this observation may result from slow dissociation of the antagonist during the agonist application.

**Table 2. Comparison of Antagonist K0 from CNiFERs to non-CNiFERs detection**

| LGIC-CNIFER | Antagonist | CNiFER K0 (nM) | Schild Slope | IC50 (nM) |
|-------------|------------|----------------|--------------|-----------|
| α7-nAChR    | MLA        | 1.2±1.4 (n = 6) | −1.2±0.3     | 2.9±1.2   |
| α7/5-HT3A   | MLA        | 2.5±0.1 (n = 2) | −1.8±0.2     | 36±13     |
| α4β2-nAChR  | DHβE       | 136±54 (n = 5)  | −0.9±0.1     | 88±24 \*  | 85(K0)  |
| 5-HT3A      | Ondansetron | 1.6±0.9 (n = 7) | −1.3±0.04    | 16.0±5.7(K0) |

a. Measured with 10 μM PNU-120596 in aCSF.
b. Measured in aCSF.
c. Measured with potential-sensitive dye (to be published Kuryatov A, Mukherjee J, and Lindstrom J). *(2x3b) [40].
d. Measured with potential-sensitive dye (Fluo-3-AM) on HEK293 cells expressing human receptors [23]. *(Geometric Mean [−SD = 66, +SD = 109][39].
e. Measured competition against [3H]Ics-205-930 in mouse neurolblastoma-glioma cells (NG-108-15) (5-HT3A)[41,42].
f. Each n is calculated as a Schild regression plot.

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Concluding Analysis

CNiFER detection of agonist responses and blockade of the responses by antagonists, originally shown for a GPCR, muscarinic M1 receptor, are equally applicable to calcium permeable ligand-gated ion channels, as shown here for subunits of the Cys-loop receptor family. The chief advantages stem from the versatility of the system enabling one to measure responses to a variety of receptors in the same family by multi-array analysis. By developing the assay in a clonal cell line in which stable lines were first generated from the receptor subtype followed by plasmid transfection, or transduction with a viral vector containing the recombinant DNA encoded sensor, one can robustly generate previously established receptor subtype clones containing the FRET based sensor. These sensor clones may be used for either a robust medium-throughput assay or for in vivo implantation to determine neurotransmitter release [21]. The Molecular Devices FlexStation III instrument allows for multiple well samples in a 96 well plate to be assayed over 30 min plate intervals. This method could be scaled for use with higher throughput assaying via the FLIPR, or even for integration with high content screening platforms. Comparison of LGIC CNiFERs to their non-CNIFER counterparts shows similar ligand affinities and demonstrates that the TN-XXL sensor does not affect LGIC dissociation constants (Table 2). Our demonstration of (±)-epibatidine and 5-HT-elicited responses with predicted concentration ratio shifts from well known competitive antagonists provides a characterized cell system to distinguish defined receptor responses from indirect actions. We establish the utility of the CNiFER sensor for characterizing agonist and antagonist parameters for new ligand candidates on LGIC nicotinic and serotonin systems.

A chief limitation is the slow read time between measurements and the sensitivity of the sensor, resulting in difficulty with fast desensitizing receptor systems; yet with the judicious use of agents that maintain an active state and retard desensitization, this obstacle may be overcome and a suitable rank ordering of potency determined for agonists and antagonists. Distortions that might be achieved with more prolonged measurements of maximal

Figure 4. a7/5-HT3a CNiFER: Methyllycaconitine antagonism. A. Competitive blockade of (±)-epibatidine elicited responses on a7/5-HT3a CNiFERs by MLA. The EC50 values for each curve shifted from 86 nM to 230 nM, 900 nM, and 8.3 μM for 3 nM, 10 nM, 30 nM MLA respectively. B. Schild plot of concentration dependent shifts. A slope of −1.8±0.2 shows MLA to block competitively but with a percentage of receptors still occupied, attributing to a slight non-competitive component that may be an artifactual result of slow dissociation. doi:10.1371/journal.pone.0016519.g004

Figure 5. a4/2-nAChR CNiFER: DHbE antagonism. A. Competitive blockade of (±)-epibatidine elicited responses on a4/2-nAChR CNiFERs by DHbE. The EC50 values for each curve shifted from 8.9 nM to 42 nM, 130 nM, and 270 nM for 300 nM, 1 μM, 3 μM DHbE respectively. B. Schild plot of concentration dependent shifts. A slope of −0.9±0.1 confirmed DHbE to block competitively. doi:10.1371/journal.pone.0016519.g005
The steep Hill coefficient of 2.6 shows the more cooperative nature of the 5-HT3A receptor. The EC50 values for each curve shifted from 220 nM to 1.6 μM, 2.6 μM, and 4.5 μM for 5 nM, 10 nM, 15 nM ondansetron respectively.

**Methods**

**Generation of Stable Ca2+ Sensing nAChR Cell Lines**

Individual HEKtsA201 human α7β2 nicotinic receptor stable cell lines were generated as previously described [35]. Briefly, human cDNA clones encoding α7 (pcDNA3.1/Zeo(−)) and β2 (pRc/CMV) nAChR subunits were transfected in equal amounts into HEKtsA201 cells using the FuGene6 transfection agent (Roche Diagnostics, Indianapolis, IN). Zeocin (0.5 mg/ml) was used for selection of α7β2 nAChR cell lines and their pharmacological properties have been reported by Kuryatov, Mukherjee, and Lindstrom. The basic approach to preparing the line was similar to that used for the α4β2 nAChR cell line and its functional properties were similarly assayed using a potential-sensitive indicator after treatment with chemical chaperones to upregulate the amount of AChR. The stable clones were used to generate individual nAChR CNiFERs. The TN-XXL control CNiFERs (lacking over-expressed receptors) [20,21], and mouse 5-HT3A CNiFERs were developed as described in [21]. TN-XXL gene expression was introduced into each of the α7-Ric3 and α7β2 cells via lentiviral transduction as described [21] and fluorescence-activated cell sorted (FACS) for high levels of eGFP and Citrine cp174 fluorescence. Chimaeric receptor DNA of human α7[1-202]/mouse 5-HT3A was kindly provided by Dr. Steven Sine (Mayo Clinic, Rochester, MN). The α7/5-HT3A chimaeric CNiFER was generated by calcium phosphate transfection of TN-XXL control cells with an α7/5-HT3A gene subcloned into pcDNA3.1+ containing 5-HT3A conductance-enhancing mutations of R132Q, R136D, and R440A [36]. Stable selection was achieved using G418 (0.5 mg/ml) to yield a TN-XXL control cell, as described in [21], containing over-expressed α7/5-HT3A receptors, which were then selected by FACS as noted above.

**Flex Assay on Receptors**

Cells were cultured in 10 cm plates with DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA; Atlanta Biologicals, Lawrenceville, GA) and 1% Glutamine (Invitrogen), and incubated at 37°C with 10% CO2. Cells were selected at ~70% confluence and plated the day before using 100 μl volumes per well into black, transparent flat-bottom, TC treated 96-well plates (Thermo, Waltham, MA; E&K, Greiner Campbell, CA). Plates were removed from the incubator the next day and the media was replaced with 100 μl of artificial cerebral spinal fluid (aCSF) [containing 121 mM NaCl, 2.4 mM Ca2+, 1.3 mM Mg2+, 5 mM KCl, 26 mM NaHCO3, 1.2 mM Na2HPO4, 10 mM glucose, 5 mM Hepes Na+, pH 7.4]. For all assays performed on α7-nAChR CNiFERs 10 μM of the PAM, PNU-120596 (Tocris, Ellisville, MO), was prepared in aCSF. In the case of antagonist measurements, antagonists, at 1.5 times the final desired concentration, were prepared in aCSF. For non-CNiFER cell lines, Blue Membrane Potential dye (Molecular Devices, Sunnyvale, CA) was prepared in aCSF and diluted 2 fold the recommended dilution. Plates were incubated for at least 30 min at 37°C and 10% CO2 for any assay that included antagonist, dye, or PAM modification to the aCSF. Following incubation, plates were removed and placed into the Molecular Devices FlexStation III instrument and run at 37°C. Injection rates varied between 27–48 ml/s, and 50 μl of the specified agonist prepared at 3x final concentration in the aforementioned aCSF solution was injected after 30 s of baseline measurement. Reads of both donor eCFP (485 nm) and acceptor Citrine cp174 (527 nm) emission with eCFP excitation (436 nm) were made at 3.52 s intervals as shown in Figure 2a, or for non-CNiFER experiments with blue membrane dye, 565 nm was read with an excitation of 530 nm, for 90–120 s depending on the experiment. FRET ratios of the two wavelengths were plotted as shown in Figure 2b, and the peak height or initial rate of ion flux for the well was used to determine EC50 values for the injected agonist, acquired using SoftMax Pro 5.2 (Molecular Devices). All wells were replicated (two or three times) in the same plate. Mean peak FRET ratios calculated from

![Image](58x24 to 76x41)

![Image](58x565 to 554x730)
replicates were exported and plotted against agonist concentrations using Graphpad Prism 4 (Carlsbad, CA). A sigmoidal concentration-response (variable slope) regression of the mean peak FRET ratios was fit to generate a concentration-response curve and obtain an EC_{50} value.

To measure potencies of competitive antagonists, three concentration-response curves with different antagonist concentrations were compared to a control curve (without antagonist). Mean FRET responses generated concentration response curves, and antagonist dependent shifts in EC_{50} values were used to calculate dose ratios (DR), as a concentration ratio of EC50'/EC50 for the specified agonist (where EC50' is in the presence of the antagonist at the specified concentration). DRs were used to produce a Schild regression plot for analysis of competitive antagonism and generate a K_a value. Analysis consisted of potting Log (DR-1) against –Log [Antagonist]. The Schild K_a value for competitive antagonists was calculated from the x-intercept at y = 0, as the –Log (K_a) [37,38]. All errors reported are arithmetic standard deviations unless otherwise noted.

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**Author Contributions**

Conceived and designed the experiments: JGY. AN QTN AM LFS JL DK PT. Performed the experiments: JGY AN QTN LFS JL. Analyzed the data: JGY AN TTT JL PT. Contributed reagents/materials/analysis tools: JL DK PT. Wrote the paper: JGY AN PT. a7/5HT3A-CNIFER cell line design: JGY. α7, αh2-CNIFER cell line design: JGY. AN 5-HT3A-CNIFER cell line design: QTN LFS AM. Assay development and optimization: AN LFS. NO-CNIFER experimental work: JGY AN JL. Provided α7, αh2 cell line: JL. Manuscript edits and review: JGY AN QTN AM LFS JL DK PWT.

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