Functional Human 5-HT_6 Receptor Assay for High Throughput Screening of Chemical Ligands and Binding Proteins

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Abstract: Continuous identification and validation of novel drug targets require the development of rapid, reliable, and sensitive cell-based high-throughput screening (HTS) methods for proposed targets. Recently, the 5-HT_6 receptor (5-HT_6R), a member of the class of recently discovered 5-HT receptors, has received considerable attention for its possible implications in depression, cognition, and anxiety. However, the cellular signaling mechanisms of 5-HT_6R are poorly understood due to the lack of selective 5-HT_6R ligands. In the present study, we examined functional coupling of the human 5-HT_6R, 5-HT_{7A}R, or 5-HT_{7B}R with various Gα-proteins (Gα_{15}, Gα_{qs5}, or G$\alpha_{qG66Ds5}$) to develop a reliable cell-based HTS method for 5-HT receptors. Among variable couplings between 5-HT receptors and G-proteins, we found that functional coupling of human 5-HT_6R with G$\alpha_{qG66Ds5}$ produced the highest levels of Ca^{2+} signaling in HEK293 cells as measured by the fluorescence-based HTS plate reader, FDSS6000. After validation of this new 5-HT_6R HTS system (Z´-factor = 0.56) in 96-well plates and characterization of the pharmacological profile of the 5-HT_6R, we screened ~500 synthetic chemical compounds including butanamide and benzenesulfonamide derivatives. Based on this preliminary screening, we found that the butanamide derivative LSG11104 produced an IC_{50} value of 6.3 μM. This compound will serve as a lead structure for further chemical modification to develop novel 5-HT_6R ligands. Furthermore, we demonstrated that this HTS method can be utilized to identify proteins that modulate 5-HT_6R function and present Fyn tyrosine kinase as an example, which is already known as a 5-HT_6R interacting protein. Taken together, these results suggest that the 5-HT_6R/G$\alpha_{qG66Ds5}$ FDSS6000 system can be utilized to screen for selective 5-HT_6R ligands and to examine any functional relationships between 5-HT_6R and its binding proteins.

Keywords: 5-hyrdoxytryptamine (5-HT), 5-HT_6 receptor, HTS, FDSS6000, chimeric G-protein, fluoresce Ca^{2+} imaging, Z´-factor, Fyn.

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is an important neurotransmitter found in both the central and peripheral nervous systems. 5-HT mediates its diverse physiological responses through at least 16 different receptors, which are subdivided into seven distinct subfamilies consisting of 5-HT_{1-7} receptors [1]. Among them, the 5-HT_6 receptor (5-HT_6R) is a recently discovered 5-HT receptor and has been shown to be positively coupled to adenylate cyclase *via* Gα_s-proteins, which is similar to 5-HT_4 and 5-HT_7 receptors [2, 3]. The 5-HT_6R has received considerable attention not only because it is broadly expressed in the brain, especially in the striatum, nucleus accumbens, hippocampus, and cortex, but also because of its high affinity to antipsychotic compounds such as clozapine, as well as some other psychotropic agents [4, 5]. Several functional studies also indicate that 5-HT_6R has been implicated in learning and memory disorders, depression, Alzheimer's disease, and anxiety [6-9]. However, the cellular mechanisms responsible for 5-HT_6R-mediated physiological responses are poorly characterized due to a lack of selective agonists or antagonists. Therefore, it is necessary to set up a reliable high-throughput screening (HTS) method to screen synthetic chemical compounds to develop selective modulators of 5-HT_6R and elucidate the cellular function of 5-HT_6R in the central nervous system (CNS).

Initially a pharmacological profile of 5-HT_6R was assayed based on radioligand binding affinity and/or 5-HT_6R-mediated stimulation of adenylate cyclase activity. While radioligand binding assays remain in use, non-radioactive cell-based functional assays that measure changes in cell signaling in response to G-protein-coupled receptor (GPCR) activation have been widely adopted to HTS assay systems. As the 5-HT_6R normally couples through Gα_s to stimulate adenylate cyclase and further increases the intracellular concentration of cAMP, the activation of 5-HT_6R would not normally cause a change in the intracellular Ca^{2+} level. However, detection of changes in the intracellular concentration of Ca^{2+} is easily amenable to HTS technology using fluorescence imaging plate readers, and Ca^{2+}-based HTS assays have been increasingly used for the identification of endogenous or synthetic ligands of neurotransmitters or orphan GPCRs. The use of promiscuous G proteins such as Gα_{15} or Gα_{16} which allow coupling to a wide range of GPCRs to phospholipase C is an attractive tool to link non-Gα_q-coupled receptors for Ca^{2+}-based HTS assays for Gα_s- or Gα_i-coupled receptors [10]. In addition, chimeric G-proteins

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based on the backbone of $G\alpha_q$ provide the same Ca^{2+}-based HTS assay option [11]. Recent progress has been made in identifying key roles for specific receptor/G-protein interactions and for the development of various chimeric G-proteins to investigate non-$G\alpha_q$-coupled receptors in $G\alpha_q$-mediated signaling events [12-15]. For example, it was reported that a glycine residue within linker I (glycine 66 in $G\alpha_q$), which is highly conserved among $G\alpha$ subunits across all species, is a key residue for constraining the fidelity of G-protein recognition by ligand-activated GPCRs [14]. In addition, it was reported that receptor/G-protein coupling selectivity involves cooperative interactions between the extreme C terminus and linker I of $G\alpha$ proteins and that distinct determinants of selectivity exist for individual receptors. Furthermore, a selected dually modified $G\alpha_q$-protein ($G\alpha_{qG66Ds5}$) produced superiority over wild-type $G\alpha_q$, $G\alpha_{qs5}$, and $G\alpha_{qG66D}$ on functional interaction with selected $G\alpha_s$-coupled receptors [12].

Therefore, we examined the functional coupling of human 5-HT$_6$R and other recently cloned serotonin receptors of the $G\alpha_s$ family, 5-HT$_{7A}$R and 5-HT$_{7B}$R, with various $G\alpha$-proteins such as $G\alpha_{15}$, $G\alpha_{qs5}$, and $G\alpha_{qG66Ds5}$. Among variable couplings between 5-HT receptors and G-proteins, we found that functional coupling of human 5-HT$_6$R with $G\alpha_{qG66Ds5}$ produced the highest Ca^{2+} signals as measured using the fluorescence-based HTS plate reader, FDSS6000 (Functional Drug Screening System, Hamamatsu Photonics). In addition, we verified this new 5-HT$_6$R HTS system by measuring the Z´-factor and examined the effects of several established 5-HT$_6$R agonists and antagonists. The Z´-factor is a characteristic parameter for the quality of the assay itself without intervention of test compounds and reflects the assay signal dynamic range [16]. In the present study, we demonstrate that the FDSS6000-based Ca^{2+} assay method using 5-HT$_6$R and $G\alpha_{qG66Ds5}$ chimeric G-protein is a fast and reliable HTS assay method for screening ligands of the 5-HT$_6$R and for examining the functional effects of its binding proteins on the activity of the 5-HT$_6$R.

EXPERIMENTAL PROCEDURES

Plasmid Constructs – Full-length human 5-HT$_6$R, 5HT$_{7A}$R, and 5HT$_{7A}$R cDNAs in pcDNA3.1 were purchased from UMR cDNA Resource Center (Miner Circle Rolla, MO). Full-length Fyn was subcloned (primer; Fw: 5'-CCG GAA TTC ACC ATG GGC TGT GTG C-3', Rv: 5'-CGC GGA TCC TTA CAG GTT TTC ACC-3') into EcoRI/BamHI of pcDNA3.1 (Invitrogen, Carlsbad, CA) from a human brain cDNA library. Plasmid constructs were generated by standard PCR amplification methods, and the subcloned DNA fragments were systematically checked by sequencing. Chimeric G protein $G\alpha_{qs5}$ was kindly provided by Dr. Conklin (University of California, San Francisco, CA).

Cell Culture and Transfection – Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated at a density of 5 x 10^6 cells in 100-mm dishes and transiently transfected with human 5-HT$_6$R in the absence or presence of $G\alpha_{15}$, $G\alpha_{qs5}$, or $G\alpha_{qG66Ds5}$ using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, the cells were transferred to 96-well black wall clear bottom plates at a density of 60,000 - 80,000 cells/well and used within 18 ~ 24 h for the FDSS6000 assay. For efficient expression of both GPCRs and G-proteins, a ratio of 1:1 between GPCRs and G-proteins was used for the respective cDNAs. The amount of total DNAs for each transfection was kept constant at 5 μg of DNA/plate by adding the pcDNA3.1 vector.

FDSS6000 Ca^{2+} Assay – A functional assay of human 5-HT$_6$R was performed based on fluorescence following intracellular Ca^{2+} release using the FDSS6000 Functional Drug Screening System (Hamamatsu Photonics, Japan). Transfected HEK293 cells were loaded with the Ca^{2+} indicator dye Fluo-4-AM (5 μM) and 0.001% Pluronic F-127 (Molecular Probes, Eugene, OR) and incubated in a HEPES-buffered solution (115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM HEPES, and 13.8 mM glucose, pH 7.4) for 1 h at 37 °C. Then, the cells were washed three times with a HEPES-buffered solution and maintained with a volume of 80 μL/well in 96-well plates. During the FDSS6000 assays, cells were washed using the BIO-TEK 96-well washer (BIO-TEK instruments, Winooski, VT). For agonist experiments, a final concentration of 10 μM or indicated various doses of agonists were added to HEK293 cells after a short baseline (< 20 s), and the Ca^{2+} response was observed at 480 nm. For antagonist experiments, the cells were preincubated with compounds for 1.5 min before the addition of an agonist. F is the fluorescent intensity and F_0 is the initial fluorescent intensity at 480 nm. All data were collected and analyzed using the FDSS6000 and related software (Hamamatsu Photonics, Japan).

Drugs – 5-Hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT), 5-methoxytrtptamine, tryptamine, and clozapine were purchased from Sigma (St. Louis, MO). SB258585 (4-indo-N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]benzenesulfonamide) was purchased from Tocris (Bristol, UK). Drugs were dissolved in dimethylsulfoxide (DMSO) as a concentrated stock (10-100 mM) and then serially diluted to its final concentration. In control experiments, maximum concentrations of DMSO up to 0.1-0.3% showed no effect on FDSS6000 Ca^{2+} assay.

Data Analysis – For all concentration-response curves, best-fit lines were analyzed with Prism (GraphPad Software Inc., San Diego, CA) using the logistic equation

$$y/y_{max} = 1/(1+(k_{1/2}/[A])^{nH})$$

where y_{max} is the maximum response, $k_{1/2}$ is the concentration for half-maximum response (EC_{50} or IC_{50}), [A] is the concentration of drugs, and n_H is the Hill coefficient. The percentage inhibition by synthetic chemical compounds tested was calculated as 100 x [($IV_{5\text{-}HT}$ - $IV_{compound}$)/$IV_{5\text{-}HT}$] where IV represents the relative integrated value of fluorescent ratio (F/F_0). All numerical values are represented as mean ± S.E. Statistical significance was performed using an unpaired Student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Functional Interaction of 5-HT$_6$R or 5-HT$_7$R with G-Proteins in Ca^{2+} Mobilization Assays

The activation of $G\alpha_s$-coupled receptors produces intracellular Ca^{2+} increases by using promiscuous $G\alpha_{15/16}$ or chi-

meric G-proteins which allow the functional coupling of non-$G\alpha_q$ coupled receptors to phospholipase C and consequent intracellular Ca^{2+} release [10, 11, 13, 15]. To set up reliable Ca^{2+}-based HTS assay methods for $G\alpha_s$-coupled serotonin receptors, we examined functional coupling of human 5-HT_6R, 5-HT_{7A}R, and 5-HT_{7B}R with various $G\alpha$-proteins such as $G\alpha_{15}$, $G\alpha_{qs5}$, and $G\alpha_{qG66Ds5}$ using a FDSS6000 96-well fluorescence plate reader. After HEK293 cells were transiently transfected with 5-HT_6R, 5-HT_{7A}R, or 5-HT_{7B}R in the absence or presence of various $G\alpha$ proteins for 24 hr, 5-HT-induced intracellular Ca^{2+} release was measured using the Fluo-4-AM fluorescence dye on the FDSS6000 system. As shown in Fig. **1**, co-expression of human 5-HT_6R with $G\alpha_{qG66Ds5}$ (a glycine-to-aspartate mutation within linker I and five amino acids of $G\alpha_s$ sequence at its extreme C terminus) or a promiscuous $G\alpha_{15}$ produced a considerable increase in intracellular Ca^{2+} in response to stimulation with 10 μM 5-HT. On the contrary, no detectable Ca^{2+} mobilization was visible with a $G\alpha_{qs5}$, a chimeric protein in which the five C-terminal amino acids of $G\alpha_q$ were replaced with the corresponding $G\alpha_s$ residues, or a control vector (pcDNA3.1). When human 5-HT_{7A}R and 5-HT_{7B}R were examined under the same conditions, we found that functional coupling of 5-HT_{7A}R and 5-HT_{7B}R with $G\alpha_{15}$ produced the highest Ca^{2+} signals, but signal amplitude was much smaller than the one for 5-HT_6R with $G\alpha_{qG66Ds5}$ whereas 5-HT_{7A}R or 5-HT_{7B}R with $G\alpha_{qG66Ds5}$ produced smaller signals. Based on these results, we found that functional coupling of 5-HT_6R with $G\alpha_{qG66Ds5}$ produced the highest Ca^{2+} signals.

Evaluation of the Quality of the 5-HT_6R/$G\alpha_{qG66Ds5}$ FDSS6000 Assay

Because functional coupling between 5-HT_6R with $G\alpha_{qG66Ds5}$ in Ca^{2+} mobilization assays was clearly superior to other combinations of 5-HT_6R or 5-HT_7R with the G-proteins tested, we next measured Z´-factors to verify our new 5-HT_6R HTS system before screening synthetic chemical compounds. For the analysis of well-to-well and plate-to-plate variability, seven 96-well plates wee screened in the absence or presence of 10 μM 5-HT in HEK293 cells transfected with 5-HT_6R and $G\alpha_{qG66Ds5}$ using a FDSS6000. As shown in Fig. **2A**, integrated values of each well treated with 10 μM 5-HT were significantly higher than the ones treated with no-drug containing buffer. Fig. **2B** shows plate-to-plate variability of the mean value of each plate in 10 μM 5-HT treated and control plates. When the Z´ factor was calculated as described previously [16], the average value of the Z´ factor was 0.56 for 96-well plates (n = 7). The range of the Z´-factor was from 0.5 to 0.69. The Z´-factor ($0.5 \leq Z < 1$) obtained in multi-plate screening experiments suggested that the 5-HT_6R/$G\alpha_{qG66Ds5}$ FDSS6000 can be well suited as an HTS assay.

Pharmacology of the 5-HT_6 Receptor (5-HT_6R) activity on the FDSS6000

Further characterization of the 5-HT_6R/$G\alpha_{qG66Ds5}$ FDSS6000 assay was undertaken using well known selective agonists and antagonists of 5-HT_6R. Fig. **3A** shows the concentration-response curves generated for a series of 5-HT_6R agonists. 5-HT, 5-carboxamidotryptamine (5-CT), 5-methoxytryptamine, and triptamine were tested. All of the agonists tested produced increases in intracellular Ca^{2+} in HEK293 cells transiently co-transfected with 5-HT_6R and $G\alpha_{qG66Ds5}$. The following rank order of agonist potency was observed: 5-

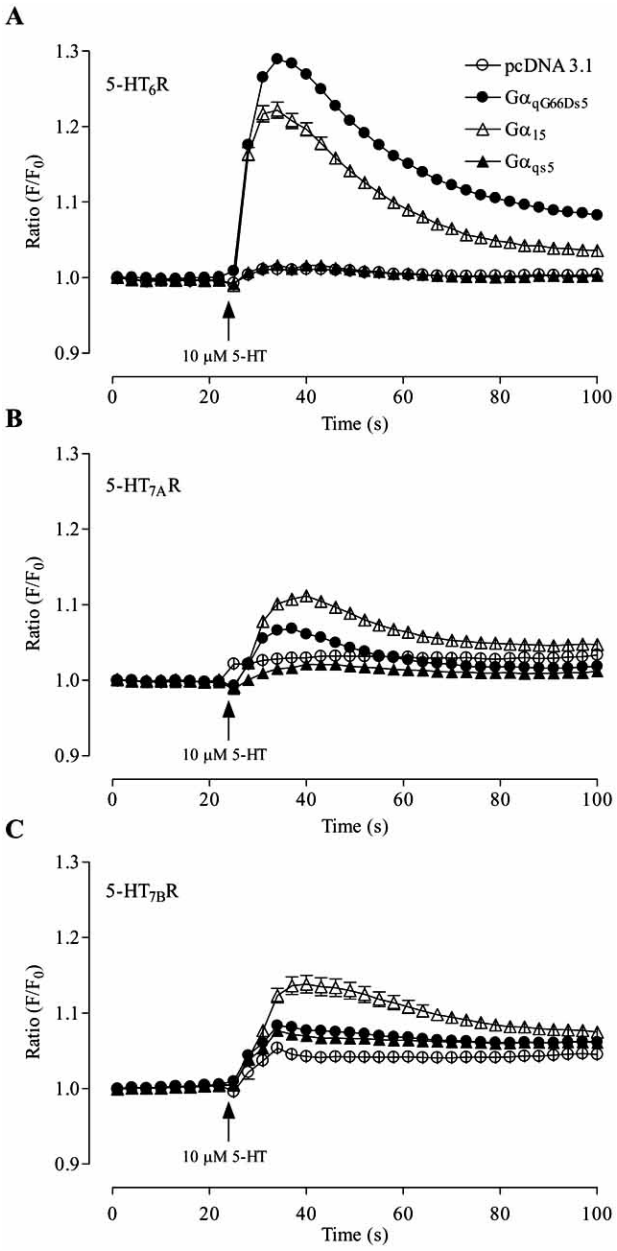


Fig. (1). Functional coupling of 5-HT_6R or 5-HT_7R with $G\alpha_{15}$, $G\alpha_{qs5}$, and $G\alpha_{qG66Ds5}$. HEK293 cells were transiently transfected with 5-HT_6R (**A**), 5-HT_{7A}R (**B**), or 5-HT_{7B}R (**C**) in the presence of control pcDNA 3.1 vector (open circles) or the indicated $G\alpha$ proteins ($G\alpha_{qG66Ds5}$, closed circles; $G\alpha_{15}$, open triangles; $G\alpha_{qs5}$, closed triangles) for 24 hr. Ca^{2+} response was measured using a FDSS6000 96-well fluorescence plate reader after loading with 5 μM Fluo-4-AM and represented as a fluorescent ratio F/F_0 where F and F_0 are the fluorescent intensities and initial fluorescent intensity at 480 nm, respectively. Data points of graphs represent the pooled results obtained from 16 wells of three independent experiments.

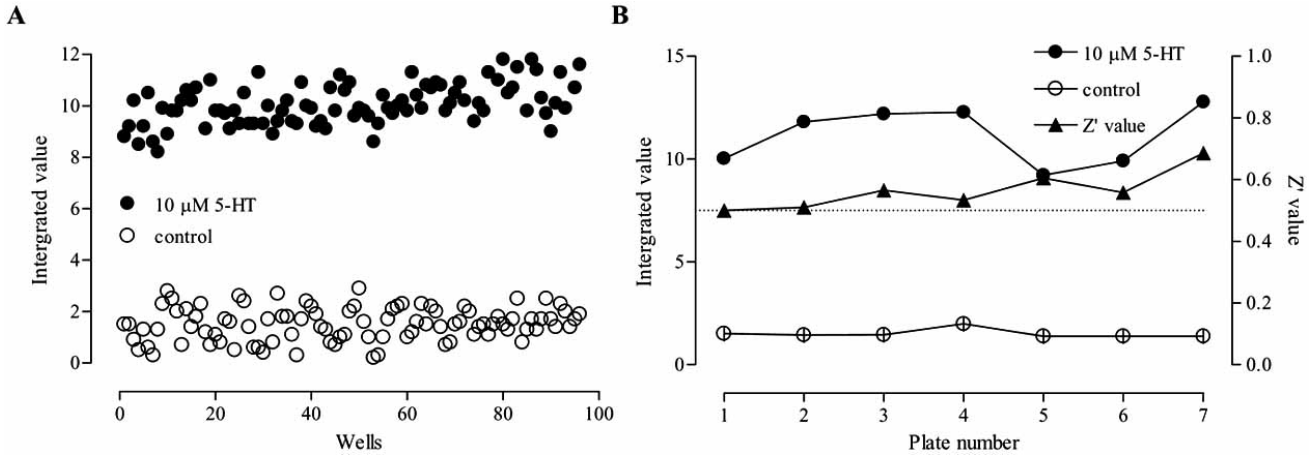


Fig. (2). Evaluation of well-to-well and plate-to-plate variability of the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 assay. (**A**) 96-well plates containing HEK293 cells co-transfected with 5-HT$_6$R and G$\alpha_{qG66Ds5}$ were treated with a HEPES-buffered solution only (control, open circles) or 10 μM concentration of 5-HT (closed circles). Data points represent integrated values of fluorescent ratio of 96 individual wells obtained from examples of control and 5-HT-treated 96-well plates. (**B**) Left Y-axis represents mean integrated values obtained from control and 5-HT-treated 96-well plates. Right axis represents the corresponding Z´-factor (closed triangles). The Z´-factor was calculated as described previously [16]. Most values for the Z´-factor were between 0.5 and 0.7, and an average value of Z´-factors obtained from 7 different plates was 0.56.

HT (EC_{50}, 9 nM) = Triptamine (9.3 nM) = 5-methoxytryptamine (10 nM) > 5-CT (88 nM). In addition, we also examined the ability of clozapine, an atypical antipsychotic drug showing high-affinity to 5-HT$_6$R, and SB258585 (4-iodo-N-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzene-sulphonamide), to function as a selective antagonist of 5-HT$_6$R for blocking 5-HT$_6$R activity. Fig. **3B** shows a concentration-dependent antagonism of clozapine and SB258585 on the Ca^{2+} response to 30 nM 5-HT (the EC_{80} concentration of 5-HT obtained from Fig. **3A**). IC_{50} values for clozapine and SB258585 were 0.55 μM and 66.8 nM, respectively. The summarized results are presented in Table **1** in comparison to previous results [17].

Application of 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 System to Synthetic Chemical Compounds

After verifying the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system by measuring the Z´-factor and pharmacological profile using selective agonists and antagonists, we applied this HTS system to screen selective 5-HT$_6$R ligands to develop novel lead compounds for the 5-HT$_6$R. Herein we assayed 487 compounds including butanamide (LSG & KSG series) and benzenesulfonamide (KKHQ series) derivatives against 5-HT$_6$R using the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system. As a preliminary assay, the activities of the synthetic compounds at 10 μM were determined against 30 nM 5-HT-induced Ca^{2+} response in three independent experiments, and the compounds showing more than ~30% inhibition were further examined to obtain IC_{50} values. For each experiment, the selective 5-HT$_6$R antagonist SB258585 at 10 μM was used as a reference compound. Among the compounds tested, Table **2** shows IC_{50} values and chemical structures of three compounds (LSG11104; 4-(4-(3-chlorobenzyl) piperazin-1-yl)-N-(4-chlorophenyl)butanamide, KSG30034; 4-(4-(3,4-dichlorobenzyl)piperazin-1-yl)-N-(2,4-difluorophenyl)butanamide, and KKHQ10426: N-(2-methoxybenzyl)-N-[2-(dimethylaminoethyl)]-1-naphthylsulfonamide) which produce a moderate inhibitory effect to 5-HT$_6$R along with three structurally related but inactive compounds (LSG11096; 4-(4-(4-fluorophenyl) piperazin-1-yl)-N-(4-chlorophenyl)butanamide, KSG30035; 4-(4-(3-chlorobenzyl)piperazin-1-yl)-N-(3,4,5-trichlorophenyl)butanamide, and KKHQ10501; N-(3-fluorobenzyl)-N-[2-(dimethylaminoethyl)]-4-iodobenzenesulfonamide). Fig. **4** also shows individual dose-response curves of LSG11104, KSG30034, and KKHQ10426 in comparison with SB258585. Among ~500 synthetic chemical compounds, we found that the compound LSG11104 produced the highest inhibitory effect with an IC_{50} of 6.3 μM. Thus, we will perform additional studies to screen chemical derivatives of LSG11104 to develop novel lead compounds of 5-HT$_6$R ligands.

Application of 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 System to 5-HT$_6$R-Binding Proteins

We previously reported the physical interaction between 5-HT$_6$R and Fyn, a member of the Src family of non-receptor protein tyrosine kinases (PTKs), by using glutathione *S*-transferase pulldown and co-immunoprecipitation assays [18]. Therefore we next examined whether the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system could be used for screening functional interactions between 5-HT$_6$R and its binding proteins. If it works, the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system will be a valuable HTS tool to evaluate functional interaction of 5-HT$_6$R-binding proteins. After transfecting HEK293 cells with 5-HT$_6$R and G$\alpha_{qG66Ds5}$ in the absence or presence of Fyn, 10 μM 5-HT-induced Ca^{2+} increases were measured. The 5-HT-induced Ca^{2+} response was significantly increased by the expression of Fyn, compared to cells expressing only the pcDNA3.1 vector (Fig. **5A**). This Fyn-mediated increase of 5-HT$_6$R activity was also examined with a full dose of 5-HT. As shown in Fig. **5B**, concentration-response curves to 5-HT showed increased maximum activity by Fyn expression without any significant change in apparent 5-HT$_6$R affinity. These results suggest that this new HTS assay for 5-HT$_6$R can be utilized to examine any functional interaction between the 5-HT$_6$R and its binding proteins as well as screening synthetic chemical compounds for selective 5-HT$_6$R ligands.

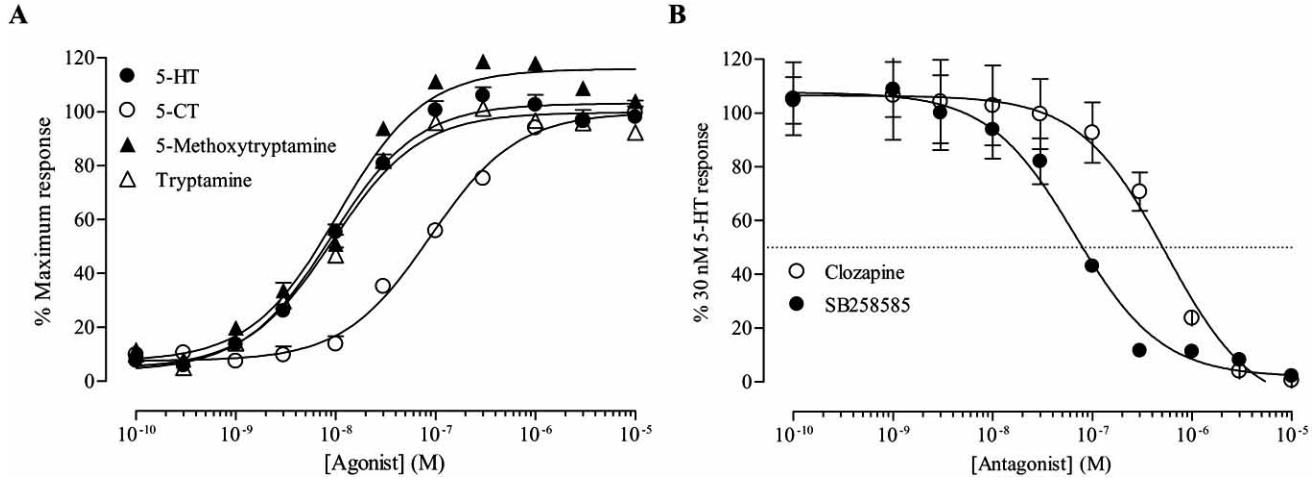


Fig. (3). Pharmacological profile of human 5-HT$_6$R using the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 assay. (**A**) After transfection, HEK293 cells loaded with Fluo-4-AM were stimulated with increasing concentrations of agonists (5-HT; closed circles, 5-CT; open circles, 5-methoxytryptamine; closed triangles, tryptamine; open circles) and changes of intracellular Ca^{2+} responses were measured using a FDSS6000 system. Drug responses were expressed as a percentage of the maximum response observed with a 10 μM concentration of 5-HT. (**B**) Antagonism of 5-HT stimulated Ca^{2+} responses in the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system. For antagonist experiments, the cells were preincubated with an antagonist for 1.5 min before the addition of 5-HT, and a concentration of 5-HT at 30 nM was used. Clozapine and SB258585 are represented by open and closed circles, respectively. All data are represented as means ± S.E. from 24 replicates obtained from three independent experiments.

DISCUSSION

Continuous identification and validation of novel drug targets require the development of rapid, reliable and sensitive cell-based screening assays in HTS settings. Although the 5-HT$_6$R was discovered recently, its almost exclusive distribution in the brain makes it a promising and novel target for CNS diseases such as depression, Alzheimer's disease, obesity, and anxiety. The 5-HT$_6$R is coupled to the Gs-family of G-proteins and has been demonstrated to increase cAMP formation in recombinant expression systems and neurons [19, 20]. Although the 5-HT$_6$R-induced increase in cAMP is recordable in a 96-well HTS format [19, 21], 96-well-based fluorescence detection of Ca^{2+} changes using promiscuous or chimeric G-proteins, which allow coupling of non-Gα_q coupled receptors to mobilization of intracellular Ca^{2+} *via* activation of phospholipase C, provides an easily amenable and fast HTS assay. Zhang *et al.* [17] previously showed the feasible coupling of the 5-HT$_6$R receptor to Ca^{2+} signaling read-out using a chimeric Gα_q/Gα_s G-protein, comprised of Gα_q with five C-terminal amino acids from Gα_s, to facilitate assays on a fluorometric imaging plate reader (FLIPR, Molecular devices).

Using the fluorescence-based HTS plate reader FDSS6000 system (Hamamatsu Photonics), we initially tried to set up a HTS assay method for 5-HT$_6$R and 5-HT$_7$R following the previous study [17]. However, we failed to obtain

Table 1. Pharmacological Profile of Human 5-HT$_6$ Receptors Evaluated Using a FDSS6000 Ca^{2+} Assay

Agonist	**G$\alpha_{qG66Ds5}$/ FDSS6000, EC$_{50}$**	**Gα_{qs5}/ FLIPR, EC$_{50}$ ***
5-methoxytryptamine	10 nM	9 nM
5-HT	9 nM	12 nM
Tryptamine	9.3 nM	86 nM
5-CT	88 nM	119 nM
Antagonist	**G$\alpha_{qG66Ds5}$/ FDSS6000, IC$_{50}$**	**Gα_{qs5}/ FLIPR, IC$_{50}$ ***
Clozapine	0.55 μM	45 nM
SB258585	66.8 nM	NA

After 24-hr transfection of HEK293 cells with 5-HT$_6$R and G$\alpha_{qG66Ds5}$, the cells were loaded with Fluo-4-AM, and 5-HT-induced intracellular Ca^{2+} changes were recorded in a 96-well pate. For agonist experiments, various final doses of agonists (from 0.1 nM to 10 μM) were added to HEK293 cells after a short baseline, and the Ca^{2+} response was observed at 480 nm. For antagonist experiments, the cells were preincubated with an antagonist for 1.5 min before the addition of 30 nM 5-HT. EC$_{50}$ and IC$_{50}$ values are represented as means ± S.E. from 24 replicates obtained from three independent experiments.

* FDSS6000 data were compared with FLIPR assay data which previously obtained by Zhang *et al.* [17]. Zhang *et al.* recorded 5-HT-induced Ca^{2+} responses in HEK cells expressing 5-HT$_6$R and the chimeric G-protein comprising Gα_q with the C-terminal five amino acids substituted from Gα_s using the Ca^{2+} indicator dye Fluo-3-AM and the FLIPR system (Molecular Devices, Sunnyvale, CA).

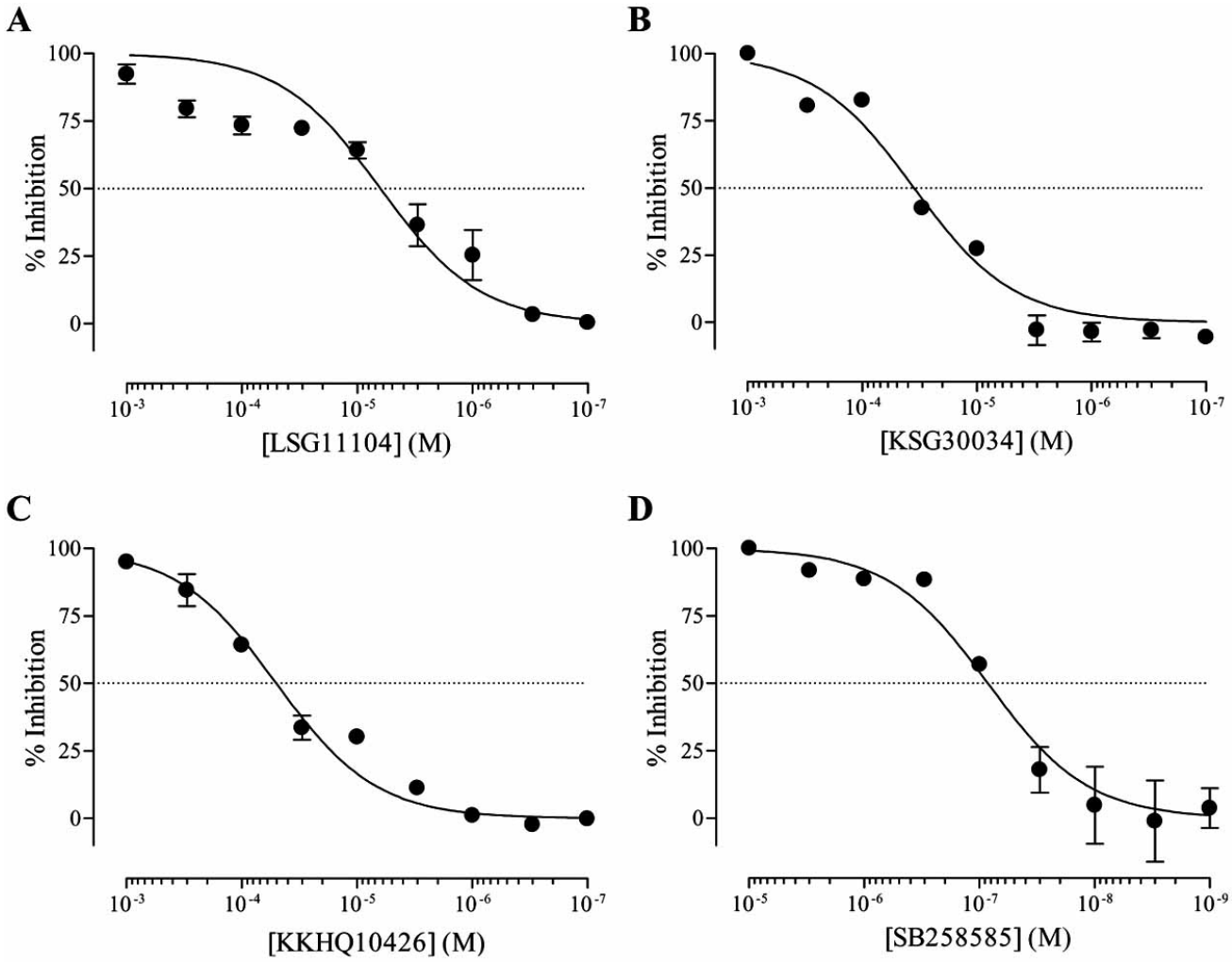
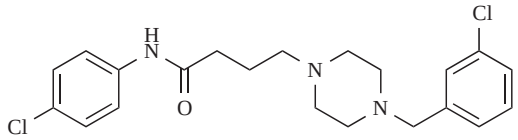
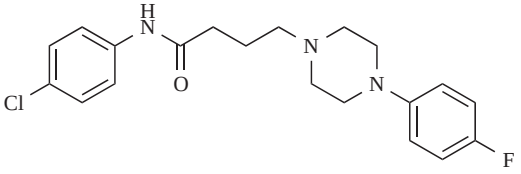
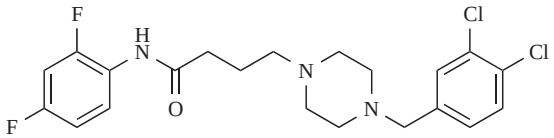
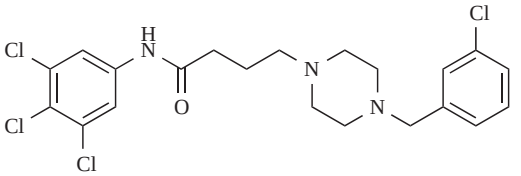
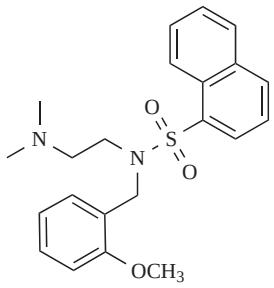
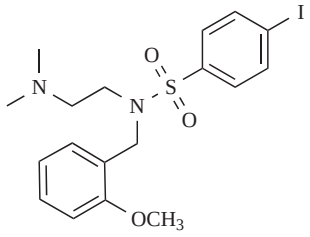


Fig. (4). Examples of dose-response curves of synthetic chemical compounds against 5-HT$_6$R. For dose-response curves of LSG11104 (**A**), KSG30034 (**B**), and KKHQ10426 (**C**), 5-HT-induced Ca^{2+} responses were observed at 480 nm after each drug at a final concentration from 0.1 μM to 1 mM was pretreated for 1.5 min before the addition of 30 nM 5-HT. For SB258585 (**D**), a final concentration from 1 nM to 10 μM was tested. IC_{50} values were obtained from 24 replicates obtained from three independent experiments.

5-HT$_6$R- or 5-HT$_7$R-mediated Ca^{2+} changes with Gα_{qs5} which is one of the widely used chimeric G-proteins for the Gs-family. Instead, we obtained the highest Ca^{2+} signal using the combination of 5-HT$_6$R and G$\alpha_{qG66Ds5}$. G$\alpha_{qG66Ds5}$ is a chimeric G-protein with a glycine-to-aspartate mutation at glycine 66 within linker I and five amino acids of the Gα_s sequence at its extreme C terminus. Based on the previous study [12], this dually modified Gα_q-protein was superior over wild-type Gα_q, Gα_{qs5}, and Gα_{qG66D} on the functional interaction with selected Gα_s-coupled receptors such as glucagon-like peptide 1 (GLP1), gastric inhibitory peptide (GIP), dopamine 2 and adrenergic β2 receptors. In the present study, we confirmed the superiority of G$\alpha_{qG66Ds5}$ over Gα_{qs5} and Gα_{15} for 5-HT$_6$R-mediated Ca^{2+} signals, and this is the first report showing the functional coupling between 5-HT$_6$R and the G$\alpha_{qG66Ds5}$ chimeric protein to stimulate phospholipase C and further mobilize intracellular Ca^{2+}. Interestingly, although 5-HT$_{7A}$R and 5-HT$_{7B}$R belong to the same Gs-family as the 5-HT$_6$R, a combination of G$\alpha_{qG66Ds5}$ with 5-HT$_{7A}$R or 5-HT$_{7B}$R produced a much smaller signal than 5-HT$_6$R/G$\alpha_{qG66Ds5}$. Among the Gα-proteins tested, a coupling of 5-HT$_{7A}$R or 5-HT$_{7B}$R with Gα_{15} produced the highest Ca^{2+} signals, but the signal intensity was too small to be utilized in a HTS platform assay. Why did we observe much smaller signals with 5-HT$_{7A}$R or 5-HT$_{7B}$R even though they exhibit the same G-protein coupling profile as does the 5-HT$_6$R? As control experiments, we indirectly checked transfection efficiency using co-expression with pEGFP-N1 vector and found that there was no difference in expression level by the types of 5-HT receptors (data not shown). Therefore, we speculate about the reasons for smaller signals with 5-HT$_{7A/B}$R as follows. First, although it is possible to perform Ca^{2+}-based HTS assay for non-Gα_q-coupled receptors, it is necessary to find the best promiscuous or chimeric G proteins for each non-Gα_q-coupled receptors. In the present study, we suggest that functional coupling of 5-HT$_6$R with G$\alpha_{qG66Ds5}$ produced sufficient signals for Ca^{2+}-based HTS assay. However, one needs to identify optimal G-proteins for 5- HT$_{7A/B}$R for Ca^{2+}-based HTS assay. Secondly, it is possible that each GPCR has a different desensitization rate. Ac-

Table 2. Estimated IC_{50} Values of Synthesized Compounds for Human 5-HT_6 Receptors Using a FDSS6000 Ca^{2+} Assay

Compound	IC_{50}	% Inhibition	Compound	IC_{50}	% Inhibition
LSG11104	6.3 μM	64.1 ± 3.1 @ 10 μM	LSG11096	NA	3.4 ± 5.6 @ 10 μM
		73.7 ± 3.5 @ 100 μM			
					
KSG30034	35.5 μM	27.3 ± 1.7 @ 10 μM	KSG30035	NA	8.8 ± 1.5 @ 10 μM
		82.6 ± 2.7 @ 100 μM			
					
KKHQ10426	50.3 μM	30.2 ± 2.3 @ 10 μM	KKHQ10501	426 μM	19.0 ± 3.5 @ 10 μM
		64.2 ± 2.0 @ 100 μM			33.6 ± 5.2 @ 100 μM
					

Compound	IC_{50}	% Inhibition
SB258585	66.8 nM	93.5 ± 1.3 @ 10 μM

Data were analyzed as described in EXPERIMENTAL PROCEDURES to calculate the percentage inhibition and IC_{50} values. Percentage inhibition and IC_{50} determinations were obtained from 24 replicates obtained from three independent experiments. All data were represented as means ± S.E.

cording to the previous studies [4, 22], both 5-HT_6R and 5-HT_7R have been reported to be desensitized in mammalian cells. If the desensitization rate of 5-HT_7R is faster than the one of 5-HT_6R, it possible to have small or non-detectable signals with 5-$HT_{7A/B}$R. Therefore, more trials to find efficient coupling conditions for the 5-HT_7R/Gα-protein for development of a FDSS6000-based 5-HT_7R HTS system are still needed.

Our 5-HT_6R/$G\alpha_{qG66Ds5}$ FDSS6000 system as an HTS platform assay provides several advantages over existing intracellular Ca^{2+} and adenylate cyclase assay methods for 5-HT_6R. First, it could be used with a short incubation time of drugs, especially for antagonists, to minimize any chance of receptor desensitization. The prolonged incubation time (typically > 10 min) of drugs is used in adenylate cyclase assay studies [17, 19, 21]. For FLIPR-based intracellular Ca^{2+} assay, antagonists of 5-HT_6R were pretreated with cells for 45 min before stimulation with 5-HT [17]. In the presence study, agonists were added to cells right after obtaining stable baseline measurements (usually < 20 s), and antagonists were pretreated only for 1.5 min before the addition of an agonist. This could be one of the possible reasons why we obtained a different IC_{50} value for clozapine from Zhang *et al.* [17], although there is a good correlation between two studies in terms of the EC_{50} values of agonists. Minimizing drug treatment time to obtain accurate efficiency is very important for the HTS format of GPCRs including 5-HT_6R and 5-HT_7R because drug-induced desensitization of 5-HT_6R and 5-HT_7R were previously reported [4, 22]. Recently, cAMP cell-based assays using homogeneous time resolved fluorescence (HTRF®) technology have been developed and are now commercially available. Compared to our drug incubation time (1.5 min), they still require a longer incubation time (30 min). Secondly, our system provides a reliable and sensitive cell-based HTS assay method for 5-HT_6R. Cell-based HTS assays are essential tools for membrane proteins such as GPCRs and ion channels which should keep their

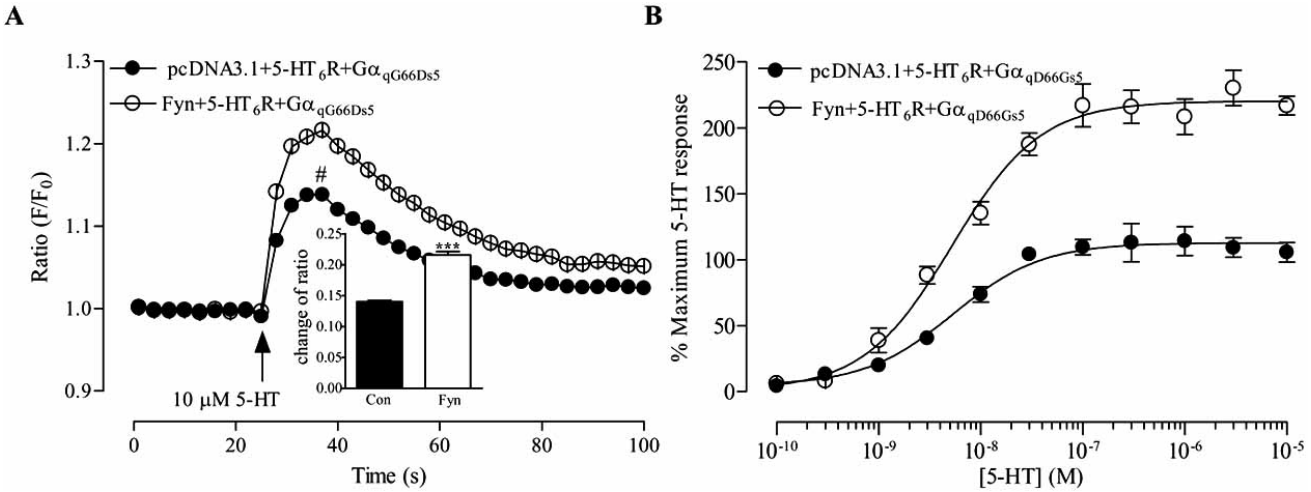


Fig. (5). The modulation of 5-HT$_6$R activities by the 5-HT$_6$R-binding protein, Fyn. (**A**) The changes of intracellular Ca^{2+} by 10 μM 5-HT (arrow) in 5-HT$_6$R and G$\alpha_{qG66Ds5}$ transfected HEK293 cells without (closed) or with Fyn (open circles) recorded using the FDSS6000 system. F is the fluorescent intensity and F_0 is the initial fluorescent intensity at 480 nm. Inset, pooled results showing the mean change of ratio (F/F_0) measured at the indicated time (#). The mean changes of ratio by 10 μM 5-HT were 0.140 ± 0.003 and 0.216 ± 0.006 (n = 4) in the absence and in the presence of Fyn, respectively. (**B**) Dose response relationship of 5-HT$_6$R-mediated Ca^{2+} responses in the presence of pcDNA3.1 (closed) or Fyn (open circles). Best-fit lines were computed for all concentration-response curves using the logistic equation $y/y_{max}=1/(1+(k_{1/2}/[5\text{-HT}])^{nH})$ where y_{max} is the maximum response, $k_{1/2}$ is the concentration for half-maximum response (EC_{50}), and n_H is the Hill coefficient. Ca^{2+} changes were expressed as the percentage of the maximum response obtained at a 10 μM concentration of 5-HT.

three dimensional structural motifs in plasma membranes. The ability to identify hits from a particular HTS assay largely depends on the reliability or quality of the assay used in the screening. The quality of the HTS assay is commonly measured by the *Z'*-factor, a value that takes into consideration the dynamic range of the assay as well as variations within the assay [16]. *Z'* values above 0.5 and values that approach 1 indicate a robust assay with low variability. In our study, the *Z'* value for the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ was 0.56 for 96-well plates, suggesting that 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system is well suitable and reliable HTS assay method. After we evaluated 487 synthetic chemical compounds in an HTS format utilizing the 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system, we found that the butanamide derivative LSG11104 produced an IC_{50} value of 6.3 μM. Further screening processes of compounds modified from LSG11104 are currently under development to obtain a selective antagonist of 5-HT$_6$R. Finally, we demonstrated that our 5-HT$_6$R/G$\alpha_{qG66Ds5}$ FDSS6000 system is a fast and reliable assay system not only for the screening of 5-HT$_6$R ligands but also for examining functional interactions between 5-HT$_6$R and its binding proteins. The number of protein candidates to be investigated in terms of protein-protein interactions have enormously increased due to recent progress of molecular biological approaches such as affinity tagging, the yeast two-hybrid system, and proteomic techniques [23]. However, validation of a protein-protein interaction in terms of physical and functional aspects requires many complicated procedures such as *in vitro* glutathione *S*-transferase pulldown, *in vivo* co-immunoprecipitation, co-localization, and other essential functional assays. Our data provide a possible functional screening method for protein-protein interactions using an example of the interaction between 5-HT$_6$R and Fyn. If an HTS system is available for target proteins, this will facilitate functional interactions and shorten long and tedious validation procedures for protein-protein interactions. Furthermore, there is a possibility to use our system for screening of compounds which could interrupt these protein-protein interactions.

In conclusion, this optimized FDSS6000 assay system using HEK293 cells transiently co-transfected with the Gα_s-coupled 5-HT$_6$ receptor and the chimeric G-protein, G$\alpha_{qG66Ds5}$ will become integrated into assay schemes for drug discovery. These assay tools will be explored for the screening of compounds with high potency and selectivity for human 5-HT$_6$R and for basic research to understand 5-HT$_6$R-mediated mechanisms in the CNS.

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