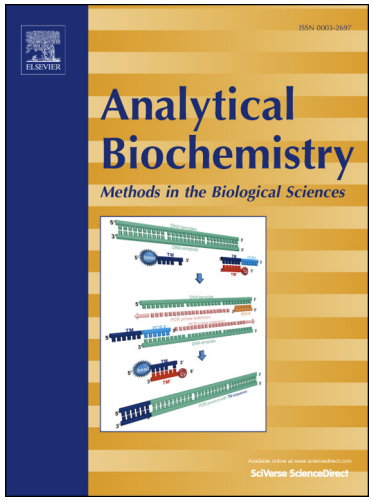

Microbial fluorescence sensing for human neurotensin receptor type 1 using Gα-engineered yeast cells

Jun Ishii, Asami Oda, Shota Togawa, Akira Fukao, Toshinobu Fujiwara, Chiaki Ogino, Akihiko Kondo

PII: S0003-2697(13)00496-X
DOI: http://dx.doi.org/10.1016/j.ab.2013.10.016
Reference: YABIO 11529

To appear in: *Analytical Biochemistry*

Received Date: 6 July 2013
Revised Date: 4 October 2013
Accepted Date: 10 October 2013

Please cite this article as: J. Ishii, A. Oda, S. Togawa, A. Fukao, T. Fujiwara, C. Ogino, A. Kondo, Microbial fluorescence sensing for human neurotensin receptor type 1 using Gα-engineered yeast cells, *Analytical Biochemistry* (2013), doi: http://dx.doi.org/10.1016/j.ab.2013.10.016

Analytical Biochemistry

Manuscript type: **Regular Articles**

Subject category: **Membrane and Receptors**

Microbial fluorescence sensing for human neurotensin receptor type 1 using Gα-engineered yeast cells

Jun Ishii[1], Asami Oda[2], Shota Togawa[2], Akira Fukao[3], Toshinobu Fujiwara[3], Chiaki Ogino[2] and Akihiko Kondo[2]

[1]Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

[2]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

[3]Laboratory of Hygienic Chemistry, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

Short title: Gα-engineered yeast as an NTSR1 signaling biosensor

Address correspondence to: Akihiko Kondo, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University. Tel: +81-78-803-6196, Fax: +81-78-803-6196, E-mail: akondo@kobe-u.ac.jp

Abstract

Neurotensin receptor type-1 (NTSR1) is the member of the G-protein-coupled receptor (GPCR) family. The natural ligand of NTSR1 is neurotensin (NT), a neuromodulator of the central nervous system. Because NT is also involved in many oncogenic actions, the signaling mediator NTSR1 is a significant molecular target in medicinal and therapeutic fields. In the present study, we constructed a fluorescence-based microbial yeast biosensor that can monitor the activation of human NTSR1 signaling responding to its agonist. To increase the sensitivity of the biosensor, a yeast strain with the *GFP* reporter gene was genetically engineered to enhance binding with human NTSR1 expressed on the membrane. Following previous reports, the 5 carboxy-terminal amino acid residues of the guanine nucleotide binding protein α-subunit (Gα) in yeast Gpa1p were substituted with the equivalent human $G\alpha_q$ sequences (Gpa1/$G\alpha_q$ transplant). After optimizing the assay conditions, the Gα-engineered yeast demonstrated significantly improved sensing for NTSR1 signaling. Since detection using a GFP fluorescence reporter considerably simplifies the measurement procedure, this microbial fluorescence sensor holds promise for use in the diagnosis of NTSR1-associated diseases and the development of agonists.

Key words: neurotensin; neurotensin receptor; G-protein-coupled receptor; yeast; green fluorescent protein

Abbreviations used: 5-FOA, 5-fluoroorotic acid; a.a., amino acid residue; CNS, central nervous system; cDNA, complementary DNA; GFP, green fluorescent protein; G-protein, guanine nucleotide binding protein; GPCR, G-protein-coupled receptor; $G_x$tp, Gpa1/Gα_x transplant; *GPA5'*, upstream region of *GPA1* gene; *GPA3'*, downstream region of *GPA1* gene; *hr50*, 50-nucleotides of the homologous region directly upstream of *GPA3'*; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; NT, neurotensin; NTSR, neurotensin receptor; NTSR1, neurotensin receptor type-1; OD_{600}, optical density at 600 nm; SC, synthetic complete; SD, synthetic dextrose; S/N, signal-to-noise; S-14, somatostatin; SSTR, somatostatin receptor; YNB, yeast nitrogen base without amino acids

Introduction

Neurotensin (NT) is a tridecapeptide found throughout the central nervous system (CNS) [1,2]. NT acts as a neuromodulator in the CNS and, in particular, as a modulator of dopamine transmission in the nigrostriatal and mesocorticolimbic systems [3]. Peripherally, NT is also distributed in the gastrointestinal tract, and predominantly exerts hormonal and neurocrine regulation on the digestive process, including the inhibition of small bowel motility and gastric acid secretions, and the facilitation of fatty acid absorption [4]. NT action is mediated by three cloned neurotensin receptors: NTSR1, NTSR2 and NTSR3. NTSR1 (high affinity NT receptor) and NTSR2 (low affinity NT receptor) are members of the G-protein-coupled receptor (GPCR) family with a seven-transmembrane helix topology, whereas NTSR3 (sortilin; encoded by *SORT1*) belongs to the family of single-transmembrane helix receptors [4,5].

In addition to its physiological actions, NT has been reported to exhibit oncogenic action in numerous types of cancer cells and tumors [5]. The cellular events in carcinogenesis are presumably activated by the abnormal expression of high affinity NTSR1 during the early stages of cell transformation in relation with Wnt/β-catenin pathway deregulation [5,6]. Recent clinical data have been essential for identifying NTSR1 expression as an independent pejorative prognosis marker in breast, lung, and head and neck squamous carcinomas [5,7–9]. In addition, it has been recently reported that *NTSR1* SNP polymorphisms are significantly associated with variance in working memory performance among healthy adults [10]. Thus, NTSR1 is a significant

molecular target in various research fields including medicine, therapeutics and pharmaceutics.

The eukaryotic unicellular yeast *Saccharomyces cerevisiae* is a useful microbial host organism for studying GPCRs as monomolecular models [11,12]. All eukaryotes conserve heterotrimer guanine nucleotide binding proteins (G-proteins) that comprise Gα-, Gβ- and Gγ-subunits. Mammalian cells possess several types of G-proteins on the inner leaflets of plasma membranes. These G-proteins enable the regulation of diverse physiological responses through coupling with a variety of transmembrane GPCRs [2]. However, this diversity makes it difficult to identify which G-protein is responsible for controlling which specific signals [11,13]. Because haploid *S. cerevisiae* expresses one type of GPCR (either the **a**-cell specific pheromone receptor, Ste2p, or the α-cell specific pheromone receptor, Ste3p) and one type of heterotrimeric complex of G-proteins (yeast Gα, Gβ and Gγ; Gpa1p, Ste4p and Ste18p) (Fig. 1A), it can offer a simple way to transduce the signal promoted by the agonistic ligand [11–17]. Moreover, the microbial yeast *S. cerevisiase* reproduces rapidly and has a flexible genome. Introduction of reporter genes, such as green fluorescent protein (*GFP*) [17,18], β-galactosidase (*lacZ*) [19,20], luciferase (*luc*) [21,22] and growth selection marker (*HIS3*) [23,24] downstream of the pheromone signaling (G-protein signaling) pathway further facilitates the detection of the agonist-promoted signal.

If the expression of human GPCR on the plasma membrane of *ste2*Δ yeast **a**-cell permits coupling with yeast monopolistic G-proteins, the promoted signaling by the

cognate ligand or analog agonist can be easily monitored with reporter gene assays [11,17,18]. The use of an established fluorescence-based reporter gene assay provides the most convenient measurement procedure: the cell culture is simply diluted into buffers and the fluorescence is read using fluorometric instruments [17,18]. A flow cytometer is an especially powerful tool for comparative quantification and quantitative screening (cell sorting) [17,18,25]. However, signaling assays using fluorescent reporter genes have to date been applied to only a few GPCRs, including yeast endogenous Ste2p [15,25], murine olfactory receptor (OR226) [21,22] and human somatostatin receptors (SSTR5 and SSTR2) [16–18,26].

It has been reported that human neurotensin NTSR1 receptor was functionally expressed on the membrane of *S. cerevisiae* and successfully transduced the signal inside the cells [19]. In that report, a β-galactosidase assay with the *lacZ* reporter gene was adopted to detect the signal responding to the stimuli from NT peptide, confirming that the signal could be transmitted through mediation of human NTSR1 and yeast endogenous G-proteins. This report is the sole example to date of a biosensor for NTSR1 constructed using *S. cerevisiae*. There is therefore interest in improving and simplifying a yeast-based detection system to meet the growing needs for treating NTSR1-associated diseases.

In this study, we generated a fluorescence-based microbial yeast biosensor to monitor agonist-promoted signaling for human NTSR1. The sensitivity of the biosensor was improved by using a chimeric Gα. Substitution (transplantation) of the 5

carboxy-terminal amino acid residues of the human Gα subunit into yeast Gpa1p (Gpa1/$G\alpha_x$ transplant; $G_x$tp) [20] aided the coupling of human GPCR and G-proteins expressed in the yeast cells (Fig. 1B). When combined with optimized assay conditions, this approach permitted the convenient and satisfactory flow cytometric sensing of NT peptide using yeast cells.

Materials and methods

Media

Synthetic dextrose (SD) medium contained 6.7 g/L yeast nitrogen base without amino acids (YNB) (BD-Diagnostic Systems, Sparks, MD, USA) and 20 g/L glucose. For SDM medium, 200 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) (Nacalai Tesque, Kyoto, Japan) was added to SD medium and the pH was adjusted from 6.2 to 7.1 (SDM62 – SDM71). Amino acids and nucleotides (20 mg/L histidine, 60 mg/L leucine, and 20 mg/L methionine or 20 mg/L uracil) were added to each medium to provide the relevant auxotrophic components. For synthetic complete (SC) medium, several nutrients including adenine, uracil, inositol and *p*-aminobenzoic acid, in addition to a complete range of amino acids, were added to SD medium. To produce solid medium, 2% (w/v) agar was added.

Plasmid constructions

All plasmids used in this study are listed in **Table 1**. All primers used for plasmid construction are listed in **Supplementary Table 1**. A flow diagram for plasmid construction by the replacement of Gpa1p with $G_s$tp and $G_q$tp is presented in **Supplementary Figure 1**.

A DNA fragment was generated encoding the *$G_s$tp* gene. The *$G_s$tp* gene is a Gpa1/Gα_s transplant in which the coding sequence for the 5 carboxy-terminal a.a. of Gpa1p (KIGII) are replaced with the equivalent human Gα_s residues (QYELL) [20]). *$G_s$tp* was amplified from pSL-GPA1 [27] and digested with XhoI and BglII. The digested fragment was then inserted into the same sites of pGK415 [28] to create the pSL-Gstp plasmid. Then, the DNA fragment encoding the *$G_s$tp* gene with XhoI sites at both ends was amplified from pSL-Gstp and digested with XhoI. The digested fragment was then inserted into the XhoI site of alkaline phosphatase (AP)-treated pUI-GPA5.3 [18] to connect to the start codon of the *$G_s$tp* gene at the *GPA5'* (upstream region of *GPA1* gene) side, creating the pUI2-Gstp plasmid.

A second DNA fragment encoding the *$G_q$tp* gene was generated. The *$G_q$tp* gene is a Gpa1/Gα_q transplant, in which the coding sequence for the 5 carboxy-terminal a.a. of Gpa1p are replaced by the equivalent human Gα_q residues (EYNLV) [20]). *$G_q$tp* was amplified from pSL-GPA1 and digested with XhoI and BglII. The digested fragment was then inserted into the same sites of pGK415, creating the pSL-Gqtp plasmid. Then, the DNA fragment encoding the *$G_q$tp* gene with XhoI sites at both ends was amplified from pSL-Gqtp and digested with XhoI. The digested fragment was then inserted into

167 the XhoI site of AP-treated pUI-GPA5.3 to connect to the start codon of the *$G_q$tp* gene
168 at the *GPA5'* side, creating the pUI2-Gqtp plasmid.

169 A DNA fragment encoding the human *NTSR1* gene was amplified from Human
170 TrueClone for NTSR1 (OriGene Technologies, Rockville, MD, USA) and digested
171 with NheI and BglII. The digested fragment was then inserted into the same sites of
172 pGK421 [29], yielding the pGK421-NTSR1 plasmid.

173 A DNA fragment encoding the human *SSTR2* gene was amplified from
174 complementary DNA (cDNA) (synthesized by reverse-transcription from human brain
175 total RNA (Life Technologies, Gaithersburg, MD, USA)) and digested with NheI and
176 BglII. The digested fragment was then inserted into the same sites of pGK421, yielding
177 the pGK421-SSTR2 plasmid.

178

179 **Yeast strains**

180 All yeast strains used in this study were generated from the BY4741 parental
181 strain [30] and are listed in **Table 1**. A flow diagram showing the construction of the
182 yeast strain by substituting $G_s$tp (or $G_q$tp) for Gpa1p is presented in **Supplementary**
183 **Figure 2**. Transformation with linear DNA fragments was performed using the lithium
184 acetate method [31]. The DNA fragment containing *GPA5'-$G_s$tp-hr50-URA3-GPA3'*
185 (or *GPA5'-$G_q$tp-hr50-URA3-GPA3'*) was prepared by digestion of pUI2-Gstp (or
186 pUI2-Gqtp) at the SpeI and AseI sites located in the *GPA5'* and *GPA3'* regions,
187 respectively (*GPA3'*, downstream region of *GPA1* gene; *hr50*, 50-nucleotides of the

188 homologous region directly upstream of *GPA3'*). The prepared liner DNA fragment
189 was used to transform the IMFD-70 strain [29]; the transformant was selected on solid
190 SD medium lacking uracil. After confirming integration of the DNA in the correct
191 orientation, the cells were maintained on SC medium containing 1 mg/ml
192 5-fluoroorotic acid (5-FOA) (Fluorochem, Derbyshire, UK) to eliminate the *URA3*
193 selectable marker between the *hr50* and *GPA3'* sequences by homologous
194 recombination with counter selection [32]. The strain substituted with the G_stp (or
195 G_qtp) gene for the *GPA1* gene was designated as IMFD-73 (or IMFD-74).
196 Transformation with receptor expression plasmids was also performed using the
197 lithium acetate method to obtain all transformants used for the assays.
198
199 **NTSR1, SSTR2 and SSTR5 signaling assays**
200 To assay signal transduction mediated by human NTSR1, SSTR2 and SSTR5
201 receptors, the transformants were grown in SD medium at 30°C. Cells were then
202 inoculated into 20 mL of SD medium to give an initial optical density of 0.03 at 600
203 nm (OD_{600}=0.03). Cultures were grown at 30°C with shaking at 150 rpm overnight and
204 then harvested. After washing, the cells were adjusted to an OD_{600}=10 with sterile
205 water. The cell suspensions (10 µL; to give a final OD_{600}=1 for carrying out facile and
206 rapid signaling assays) were added to the wells of 96-well cluster dishes containing
207 fresh SD or pH-adjusted SDM medium (80 µL), and 100 µM or 0 µM neurotensin
208 (NT) (Calbiochem – Merck Millipore, Billerica, MA, USA) or somatostatin (S-14)

(Calbiochem – Merck Millipore) were supplemented for the NTSR1 assays or for the SSTR2 and SSTR5 assays (10 μL; to give a final concentration of 10 μM or 0 μM). Then the plates were incubated at 30°C with shaking at 150 rpm for 4 h. For the NTSR1 assays, the incubation time was extended for up to 10 h using a range of NT concentrations.

Flow cytometric measurement of GFP reporter expression

Flow cytometric measurements of GFP expressing yeast cells used previously reported protocols, as described below [17,18]. Following the signaling assays, the total cell suspensions (100 μL) were directly diluted into test tubes containing sheath solution and GFP fluorescence was measured using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The green fluorescence signal from 10,000 cells was obtained by exciting with a blue laser and the emission was collected through a 530/30 nm band-pass (GFP) filter. The data were analyzed using BD FACSDiva software (BD Biosciences).

Results and discussion

Optimization of medium pH for the NTSR1 assay

To determine the optimum pH for flow cytometric sensing of NT peptide using yeast cells, pH-adjusted SDM media (SD containing MOPSO buffer; pH 6.2 – 7.1) and

pH-unadjusted (plain) SD medium were prepared. Previously constructed IMFD-70 with three deletion alleles (*ste2*Δ, *sst2*Δ and *far1*Δ) [29] was adopted as the prototypic recombinant yeast strain (Table 1 and Fig. 1B). *Ste2*Δ prevents the competitive expression of endogenous GPCR, *sst2*Δ provides hypersensitivity to the agonistic ligand, and *far1*Δ prevents cell cycle arrest [11,17]. In addition to the deletion alleles, IMFD-70 has two genomic copies of the *GFP* reporter gene, whose expression is controlled by the signal-responsive *FIG1* promoter (Fig. 1B) [29]. The IMFD-70 harboring NTSR1 expression plasmid was used in NTSR1 assays together with 10 μM NT as the agonistic ligand, and the fluorescence levels of the yeast cells were evaluated by flow cytometry (Fig. 2A and B). For comparison, the IMFD-70 harboring SSTR2 and SSTR5 expression plasmids were also used in SSTR assays together with 10 μM S-14 ligand (Fig. 2C–F).

IMFD-70 strains expressing SSTR5 incubated in the pH-adjusted SDM media displayed clear expression of the *GFP* reporter gene in response to S-14, whereas the same strains incubated in plain SD medium did not show fluorescence (Fig. 2E and F). An increase in pH provided better results from the assays, with a pH of between 6.8 and 7.1 providing the highest fluorescence and signal-to-noise (S/N) ratio. IMFD-70 expressing SSTR2 showed similar trends for pH dependence, although expression of the reporter was lower than with SSTR5 (Fig. 2C and D).

On the other hand, the IMFD-70 strain expressing NTSR1 in response to NT presented much lower levels of reporter expression, although its pH dependency was

similar to that of the SSTRs (Fig. 2A and B). These results for the three receptors suggested that the amount of NTSR1 receptor, or the coupling efficiency of NTSR1 with the yeast endogenous Gpa1p, was lower than that of the SSTRs. However, an earlier study with the β-galactosidase assay using a multi-copy (20–50 copies) plasmid harboring the *lacZ* reporter gene clearly detected NT-promoted NTSR1 signaling in yeast [19]. The difficulty in monitoring NTSR1 signaling in the present study is probably due to the use of two copies of the genomic insertion and a *GFP* fluorescence reporter gene that cannot amplify the output in response to the reaction time as *lacZ*. However, the constructs used did allow retention of the stable reporter gene and provided a convenient measurement procedure. Thus, improved detection sensitivity was needed to establish a yeast-based fluorescence sensing technique for NTSR1 signaling.

Engineering of yeast Gα by transplanting the human Gα sequences provides an improved NTSR1 assay

To improve the sensitivity of the NTSR1 assay, enhanced coupling of human GPCR and G-proteins in yeast was attempted. Yeast/human chimeric Gα was expressed in which the 5 carboxy-terminal a.a. of yeast Gpa1p were replaced with the equivalent human $G\alpha_x$ (Gpa1/$G\alpha_x$ transplant; $G_x$tp) (Fig. 1B) [20].

The recombinant yeast, IMFD-72, that expressed G_{i3}tp (Gpa1/$G\alpha_{i3}$ transplant; –ECGLY) in place of Gpa1p (–KIGII) [18] was a derivative of the parental IMFD-70

(Table 1). IMFD-73 and IMFD-74 strains were respectively constructed in a similar way by substituting $G_s$tp (Gpa1/$G\alpha_s$ transplant; –QYELL) and $G_q$tp (Gpa1/$G\alpha_q$ transplant; –EYNLV) for Gpa1p in the parental IMFD-70 (Table 1). After transforming these four strains with NTSR1, SSTR2 and SSTR5 expression plasmids, fluorescence was evaluated using a flow cytometer (Fig. 3).

In the SSTR2 and SSTR5 assays, the G_{i3}tp-expressing IMFD-72 strain exhibited higher fluorescence and S/N ratios than the parental IMFD-70 in response to 10 μM S-14, while IMFD-73 and IMFD-74 did not activate SSTR signaling (Fig. 3C–F). These results were in agreement with previous reports [19]. In NTSR1 assays with 10 μM NT, both the G_{i3}tp-expressing IMFD-72 and $G_q$tp-expressing IMFD-74 provided increased sensitivity using the *GFP* reporter gene compared to IMFD-70, while the $G_s$tp-expressing IMFD-73 displayed no fluorescence (Fig. 3A and B). This indicated that transplantation of the carboxy-terminal 5 a.a. of $G\alpha_{i3}$ and $G\alpha_q$ into yeast Gpa1p could improve coupling efficiency with the NTSR1 receptor in yeast cells. These features are supported by the fact that human NTSR1 couples with $G\alpha_{i/o}$, $G\alpha_s$ and $G\alpha_{q/11}$ in mammalian cells [5], although it is still unclear why transplantation of the equivalent $G\alpha_s$ showed no coupling with NTSR1. Nevertheless, we demonstrated that the engineering of Gα is a convincing strategy to increase coupling efficiency with NTSR1 in yeast cells (Fig. 1B).

Effect of incubation time for improving the NTSR1 assay

293 Incubation time was investigated to further improve the sensitivity of NTSR1
294 signaling. The regular incubation time (4 h; Fig. 3A and B) was increased to up to 10 h
295 (Fig. 4). As a consequence, three of the yeast strains (but not IMFD-73) showed an
296 increase in fluorescence response with increased incubation time. After exposing the
297 cells to NT for 10 h, G_{i3}tp-expressing IMFD-72 and $G_q$tp-expressing IMFD-74
298 exhibited significantly higher sensitivity than if incubated for 4 h (Fig. 4E and F). The
299 fluorescence of IMFD-72 and IMFD-74 expressing NTSR1 in response to NT for 10 h
300 was elevated to levels comparable to IMFD-70 and IMFD-72 expressing SSTR5 (4 h
301 of incubation; Fig. 3E and F). Thus, we successfully enabled the fluorescence-based
302 yeast sensing for NTSR1 signaling to endure the actual use in the detection of NT
303 peptides.
304
305 **Concentration-response curves of Gα-engineered yeasts as NTSR1 sensors**
306 Finally, to evaluate the utility of Gα-engineered yeasts as NTSR1 signaling
307 sensors, the fluorescence responses of IMFD-70, IMFD-72 and IMFD-74 to various
308 concentrations of NT were examined (Fig. 5). As expected, the Gpa1-intact IMFD-70
309 strain showed lower fluorescence and dynamic range than the two Gα-engineered yeast
310 strains (Fig. 5A). The G_{i3}tp-expressing IMFD-72 provided comparatively lower
311 fluorescence at NT concentrations above 1 μM and higher fluorescence below 10 nM
312 NT (Fig. 5B). Conversely, the $G_q$tp-expressing IMFD-74 displayed higher fluorescence
313 above 1 μM NT and relatively lower fluorescence below 10 nM NT (Fig. 5C). These

results indicated that IMFD-74 yeast harboring $G_q$tp were the best NTSR1 signaling sensors among the tested strains. Since NTSR1 has been reported to bind preferentially to the $G\alpha_{q/11}$ subunit rather than $G\alpha_{i/o}$ or $G\alpha_s$ [5], our findings are consistent with these earlier reports.

Conclusions

In the present study, we established a fluorescence-based microbial yeast biosensor to monitor human NTSR1 signaling responding to its agonist. This biosensor holds promise for use as a simple and convenient measurement procedure. The biosensor was achieved by constructing a Gα-engineered yeast strain, IMFD-74. IMFD-74 was generated from the Gpa1-intact IMFD-70 strain by introducing a *GFP* reporter gene which harbored a chimeric Gα. In this Gα, the 5 carboxy-terminal a.a. were transplanted with the human $G\alpha_q$ (Gpa1/$G\alpha_q$ transplant: $G_q$tp). Small modifications to the assay conditions allowed considerably improved sensitivity of IMFD-74 for NTSR1 signaling. This microbial fluorescence sensor holds promise for the diagnosis of NTSR1-associated diseases and the development of NTSR-1 agonists.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas

(Life surveyor) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. It was also funded in part by the Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe; iBioK) from the MEXT, and by the Naito Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.xxxx.xx.xxx.

References

1. P. McGonigle, Peptide therapeutics for CNS indications, Biochem Pharmacol. 83 (2012) 559-566.
2. D. Pelaprat, Interactions between neurotensin receptors and G proteins, Peptides 27 (2006) 2476-2487.
3. D. Gully, M. Canton, R. Boigegrain, F. Jeanjean, J.C. Molimard, M. Poncelet, C. Gueudet, M. Heaulme, R. Leyris, A. Brouard, et al., Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 65-69.
4. J.I. Hwang, D.K. Kim, H.B. Kwon, H. Vaudry, J.Y. Seong, Phylogenetic history, pharmacological features, and signal transduction of neurotensin receptors in vertebrates, Ann. N.Y. Acad. Sci. 1163 (2009) 169-178.
5. S. Dupouy, N. Mourra, V.K. Doan, A. Gompel, M. Alifano, P. Forgez, The potential use of the neurotensin high affinity receptor 1 as a biomarker for cancer progression and as a component of personalized medicine in selective cancers, Biochimie 93 (2011) 1369-1378.
6. F. Souazé, V. Viardot-Foucault, N. Roullet, M. Toy-Miou-Leong, A. Gompel, E. Bruyneel, E. Comperat, M.C. Faux, M. Mareel, W. Rostène, J.F. Fléjou, C. Gespach, P. Forgez, Neurotensin receptor 1 gene activation by the Tcf/beta-catenin pathway is an early event in human colonic adenomas, Carcinogenesis 27 (2006) 708-716.
7. M. Alifano, F. Souazé, S. Dupouy, S. Camilleri-Broët, M. Younes, S.M. Ahmed-Zaïd, T. Takahashi, A. Cancellieri, S. Damiani, M. Boaron, P. Broët, L.D. Miller, C. Gespach, J.F. Regnard, P. Forgez, Neurotensin receptor 1 determines the outcome of non-small cell lung cancer, Clin. Cancer Res. 16 (2010) 4401-4410.
8. S. Dupouy, V. Viardot-Foucault, M. Alifano, F. Souazé, G. Plu-Bureau, M. Chaouat, A. Lavaur, D. Hugol, C. Gespach, A. Gompel, P. Forgez, The neurotensin receptor-1 pathway contributes to human ductal breast cancer progression, PLoS One 4 (2009) e4223.
9. S. Shimizu, J. Tsukada, T. Sugimoto, N. Kikkawa, K. Sasaki, H. Chazono, T. Hanazawa, Y. Okamoto, N. Seki, Identification of a novel therapeutic target for head and neck squamous cell carcinomas: a role for the neurotensin-neurotensin receptor 1 oncogenic signaling pathway, Int. J. Cancer 123 (2008) 1816-1823.

10. J. Li, C. Chen, C. Chen, Q. He, H. Li, J. Li, R.K. Moyzis, G. Xue, Q. Dong, Neurotensin receptor 1 gene (NTSR1) polymorphism is associated with working memory, PLoS One 6 (2011) e17365.
11. J. Ishii, N. Fukuda, T. Tanaka, C. Ogino, A. Kondo, Protein-protein interactions and selection: yeast-based approaches that exploit guanine nucleotide-binding protein signaling. FEBS J. 277 (2010) 1982–1995.
12. J. Minic, M. Sautel, R. Salesse, E. Pajot-Augy, Yeast system as a screening tool for pharmacological assessment of G protein coupled receptors, Curr. Med. Chem. 12 (2005) 961-969.
13. Y. Nakamura, J. Ishii, A. Kondo, Rapid, facile detection of heterodimer partners for target human G-protein-coupled receptors using a modified split-ubiquitin membrane yeast two-hybrid system, PLoS One (in press)
14. S. Ryo, J. Ishii, Y. Iguchi, N. Fukuda, A. Kondo, Transplantation of the GAL regulon into G-protein signaling circuitry in yeast, Anal. Biochem. 424 (2012) 27-31.
15. J. Ishii, S. Matsumura, S. Kimura, K. Tatematsu, S. Kuroda, H. Fukuda, A. Kondo, Quantitative and dynamic analyses of G protein-coupled receptor signaling in yeast using Fus1, enhanced green fluorescence protein (EGFP), and His3 fusion protein, Biotechnol. Prog. 22 (2006) 954–960.
16. N. Fukuda, J. Ishii, M. Kaishima, A. Kondo, Amplification of agonist stimulation of human G-protein-coupled receptor signaling in yeast, Anal. Biochem. 417 (2011) 182-187.
17. J. Ishii, N. Yoshimoto, K. Tatematsu, S. Kuroda, C. Ogino, H. Fukuda, A. Kondo, Cell wall trapping of autocrine peptides for human G-protein-coupled receptors on the yeast cell surface, PLoS One 7 (2012) e37136.
18. J. Ishii, M. Moriguchi, K.Y. Hara, S. Shibasaki, H. Fukuda, A. Kondo, Improved identification of agonist-mediated $G\alpha_i$-specific human G-protein-coupled receptor signaling in yeast cells by flow cytometry, Anal. Biochem. 426 (2012) 129-133.
19. P. Leplatois, A. Josse, M. Guillemot, M. Febvre, N. Vita, P. Ferrara, G. Loison, Neurotensin induces mating in *Saccharomyces cerevisiae* cells that express human neurotensin receptor type 1 in place of the endogenous pheromone receptor, Eur. J. Biochem. 268 (2001) 4860-4867.

20. A.J. Brown, S.L. Dyos, M.S. Whiteway, J.H. White, M.A. Watson, M. Marzioch, J.J. Clare, D.J. Cousens, C. Paddon, C. Plumpton, M.A. Romanos, S.J. Dowell, Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein α-subunit chimeras, Yeast 16 (2000) 11-22.

21. Y. Fukutani, J. Ishii, K. Noguchi, A. Kondo, M. Yohda, An improved bioluminescence-based signaling assay for odor sensing with a yeast expressing a chimeric olfactory receptor, Biotechnol. Bioeng. 109 (2012) 3143-3151.

22. Y. Fukutani, T. Nakamura, M. Yorozu, J. Ishii, A. Kondo, M. Yohda, The N-terminal replacement of an olfactory receptor for the development of a yeast-based biomimetic odor sensor, Biotechnol. Bioeng. 109 (2012) 205-212.

23. C. Klein, J.I. Paul, K. Sauvé, M.M. Schmidt, L. Arcangeli, J. Ransom, J. Trueheart, J.P. Manfredi, J.R. Broach, A.J. Murphy, Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast, Nat. Biotechnol. 16 (1998) 1334-1337.

24. T.J. Baranski, P. Herzmark, O. Lichtarge, B.O. Gerber, J. Trueheart, E.C. Meng, T. Iiri, S.P. Sheikh, H.R. Bourne, C5a receptor activation. Genetic identification of critical residues in four transmembrane helices, J. Biol. Chem. 274 (1999) 15757-15765.

25. J. Ishii, T. Tanaka, S. Matsumura, K. Tatematsu, S. Kuroda, C. Ogino, H. Fukuda, A. Kondo, Yeast-based fluorescence reporter assay of G protein-coupled receptor signalling for flow cytometric screening: *FAR1*-disruption recovers loss of episomal plasmid caused by signalling in yeast, J. Biochem. 143 (2008) 667–674.

26. K. Hara, T. Shigemori, K. Kuroda, M. Ueda, Membrane-displayed somatostatin activates somatostatin receptor subtype-2 heterologously produced in *Saccharomyces cerevisiae*, AMB Express 2 (2012) 63.

27. Y. Iguchi, J. Ishii, H. Nakayama, A. Ishikura, K. Izawa, T. Tanaka, C. Ogino, A. Kondo, Control of signalling properties of human somatostatin receptor subtype-5 by additional signal sequences on its amino-terminus in yeast, J. Biochem. 147 (2010) 875–884.

28. J. Ishii, K. Izawa, S. Matsumura, K. Wakamura, T. Tanino, T. Tanaka, C. Ogino, H. Fukuda, A. Kondo, A simple and immediate method for simultaneously evaluating expression level and plasmid maintenance in yeast, J. Biochem. 145 (2009) 701-708.

443 29. S. Togawa, J. Ishii, A. Ishikura, T. Tanaka, C. Ogino, A. Kondo, Importance of
444 asparagine residues at positions 13 and 26 on the amino-terminal domain of human
445 somatostatin receptor subtype-5 in signalling. J. Biochem. 147 (2010) 867–873.
446 30. C.B. Brachmann, A. Davies, G.J. Cost, E. Caputo, J. Li, P. Hieter, J.D. Boeke,
447 Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful
448 set of strains and plasmids for PCR-mediated gene disruption and other applications,
449 Yeast 14 (1998) 115–132.
450 31. D. Gietz, A. St Jean, R.A. Woods, R.H. Schiestl, Improved method for high
451 efficiency transformation of intact yeast cells, Nucleic Acids Res. 20 (1992) 1425.
452 32. R. Akada, T. Kitagawa, S. Kaneko, D. Toyonaga, S. Ito, Y. Kakihara, H. Hoshida, S.
453 Morimura, A. Kondo, K. Kida, PCR-mediated seamless gene deletion and marker
454 recycling in *Saccharomyces cerevisiae*, Yeast 23 (2006) 399–405.
455
456

Table 1. Yeast strains and plasmids used in this study

Strain or plasmid	Specific features	Source
Yeast strain		
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	[30]
IMFD-70	BY4741 *sst2*Δ::*AUR1-C ste2*Δ::*LEU2 fig1*Δ::*EGFP his3*Δ::P_{FIG1}-*EGFP far1*Δ	[29]
IMFD-72	BY4741 *sst2*Δ::*AUR1-C ste2*Δ::*LEU2 fig1*Δ::*EGFP his3*Δ::P_{FIG1}-*EGFP far1*Δ *gpa1*Δ::G_{i3}*tp*	[18]
IMFD-73	BY4741 *sst2*Δ::*AUR1-C ste2*Δ::*LEU2 fig1*Δ::*EGFP his3*Δ::P_{FIG1}-*EGFP far1*Δ *gpa1*Δ::G_s*tp*	This study
IMFD-74	BY4741 *sst2*Δ::*AUR1-C ste2*Δ::*LEU2 fig1*Δ::*EGFP his3*Δ::P_{FIG1}-*EGFP far1*Δ *gpa1*Δ::G_q*tp*	This study
Plasmid		
pGK415	Yeast expression vector containing *PGK1* promoter, *PGK1* terminator, *CEN/ARS* origin and *LEU2* marker	[28]
pSL-GPA1	Yeast endogenous Gα (Gpa1p) expression, *GPA1* promoter, *CEN/ARS* origin and *LEU2* marker	[27]
pSL-Gstp	Yeast–human chimeric Gα ($G_s$tp) expression, *GPA1* promoter, *CEN/ARS* origin and *LEU2* marker	This study
pSL-Gqtp	Yeast–human chimeric Gα ($G_q$tp) expression, *GPA1* promoter, *CEN/ARS* origin and *LEU2* marker	This study
pUI-GPA5.3	*GPA5'-hr50-URA3-GPA3'* in pUC19	[18]
pUI2-Gstp	*GPA5'-*G_s*tp-hr50-URA3-GPA3'* in pUC19	This study
pUI2-Gqtp	*GPA5'-*G_q*tp-hr50-URA3-GPA3'* in pUC19	This study
pGK421	Yeast expression vector containing *PGK1* promoter, *PGK1* terminator, 2μ origin and *MET15* marker	[29]
pGK421-NTSR1	Human NTSR1 receptor expression in pGK421	This study
pGK421-SSTR2	Human SSTR2 receptor expression in pGK421	This study
pGK421-SSTR5	Human SSTR5 receptor expression in pGK421	[18]

Figure captions

Fig. 1. Outline of the engineering strategy for the NTSR1 signaling assay. (A) Schematic representation of the major proteins involved in pheromone signal transduction in wild-type yeast. Ste2p, yeast endogenous GPCR; Gpa1p, yeast endogenous Gα-subunit; Ste4p, yeast endogenous G-subunit; Ste18p, yeast endogenous G-subunit; Sst2p, Gpa1-specific GTPase-activating protein (a member of regulator of G-protein signaling, RGS, family that stimulates hydrolysis of GTP to GDP); Far1p, cyclin-dependent kinase inhibitor (mediates cell-cycle arrest in response to the pheromone signaling); T.F., transcription factor. (B) Schematic representation of the engineered components of the NTSR1 signaling assays. Yeast Ste2p was deleted to avoid competitive expression by the endogenous yeast GPCR. Alternatively, human NTSR1 was expressed from a multi-copy episomal plasmid under the control of the constitutive *PGK1* promoter. A yeast–human chimeric G-protein in which the carboxyl-terminal 5 amino acid residues of Gpa1p was replaced by the equivalent residues to the human $G\alpha_x$ (Gpa1/$G\alpha_x$ transplant; $G_x$tp) was substituted for Gpa1p. Sst2p was deleted to confer hypersensitivity to agonists. Far1p was deleted not only to promote cell-cycle progression by avoiding G1 arrest even in signal-activated states, but also to allow the recovery of episomal plasmids from signal-activated yeast cells that is important for extensive screening. *GFP* reporter gene under the control of signal-responsive *FIG1* promoter was used to sense the NTSR1 signaling in the presence of neurotensin (NT).

Fig. 2. GFP fluorescence signaling assays using the wild-type G-harboring yeast strain, IMFD-70. Several pH-adjusted SDM media and a non-pH-adjusted SD medium were used for the assays. (A, C, E) Black and white bars present the average GFP intensities of yeast cells incubated in the medium with and without ligands (A, NT; C and E, S-14), respectively. (B, D, F) Gray bars present the S/N ratio of yeast cells incubated in the medium with and without ligands (B, NT; D and F, S-14), respectively. Incubation time was 4 h. Error bars represent the standard deviations (*n*=3).

Fig. 3. GFP fluorescence signaling assays using G engineered yeast strains. Several chimeric G (G transplants) engineered yeast strains were used for the assays. IMFD-70, intact Gpa1; IMFD-72, G_{i3}tp; IMFD-73, $G_s$tp; IMFD-74, $G_q$tp. (A, C, E) Black and white bars present the average GFP intensities of yeast cells incubated in SDM medium with and without ligands (A, NT; C and E, S-14), respectively. (B, D, F) Gray bars present the S/N ratio of yeast cells incubated in SDM medium with and without ligands (B, NT; D and F, S-14), respectively. The pH of the media was adjusted to 7.1. Incubation time was 4 h. Error bars represent the standard deviations (*n*=3).

Fig. 4. NTSR signaling assays following different incubation times. Several

chimeric Gα (Gα transplants) engineered yeast strains were used for the assays. IMFD-70, intact Gpa1; IMFD-72, G_{i3}tp; IMFD-73, $G_s$tp; IMFD-74, $G_q$tp. (A, C, E) Black and white bars present the average GFP intensities of yeast cells incubated for different lengths of time in SDM medium with and without NT, respectively. (B, D, F) Gray bars present the S/N ratio of yeast cells incubated for different lengths of time in SDM medium with and without NT, respectively. The pH of the medium was adjusted to 7.1. Error bars represent the standard deviations (*n*=3).

Fig. 5. Concentration-response curves for NTSR signaling assays. Several chimeric Gα (Gα transplants) engineered yeast strains were used for the assays. (A) IMFD-70, intact Gpa1. (B) IMFD-72, G_{i3}tp. (C) IMFD-74, $G_q$tp. Each data point presents the relative fluorescence unit (RFU) of yeast cells incubated in SDM medium containing various concentrations of NT. The pH of the media was adjusted to 7.1. Incubation time was 10 h. Error bars represent the standard deviations (*n*=3).

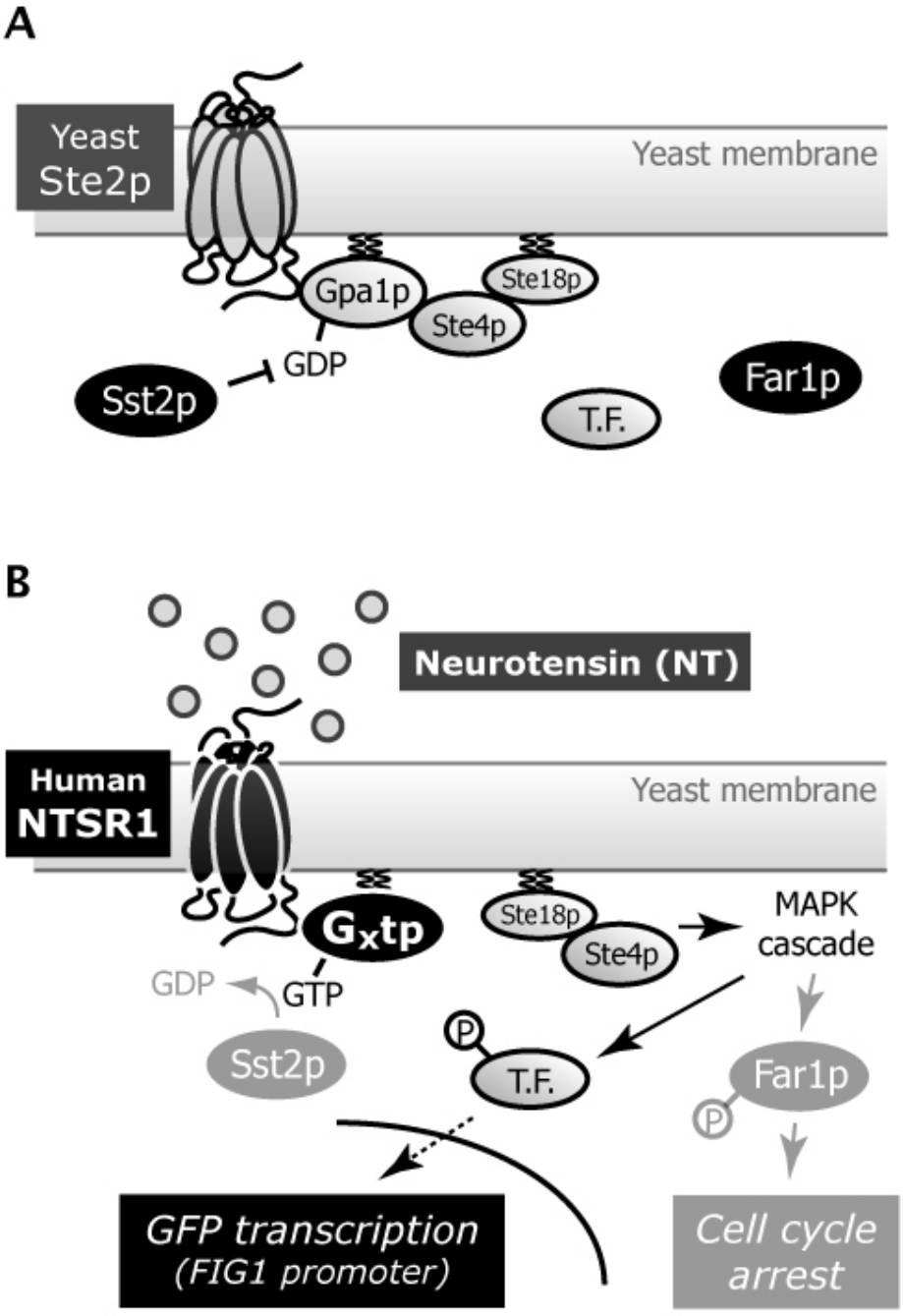
A
Yeast
Ste2p
Yeast membrane
Gpa1p
Ste18p
Ste4p
GDP
Sst2p
T.F.
Far1p
B
Neurotensin (NT)
Human
NTSR1
Yeast membrane
Gxtp
Ste18p
Ste4p
MAPK
cascade
GDP
GTP
Sst2p
P
T.F.
Far1p
P
GFP transcription
(FIG1 promoter)
Cell cycle
arrest

Fig. 1

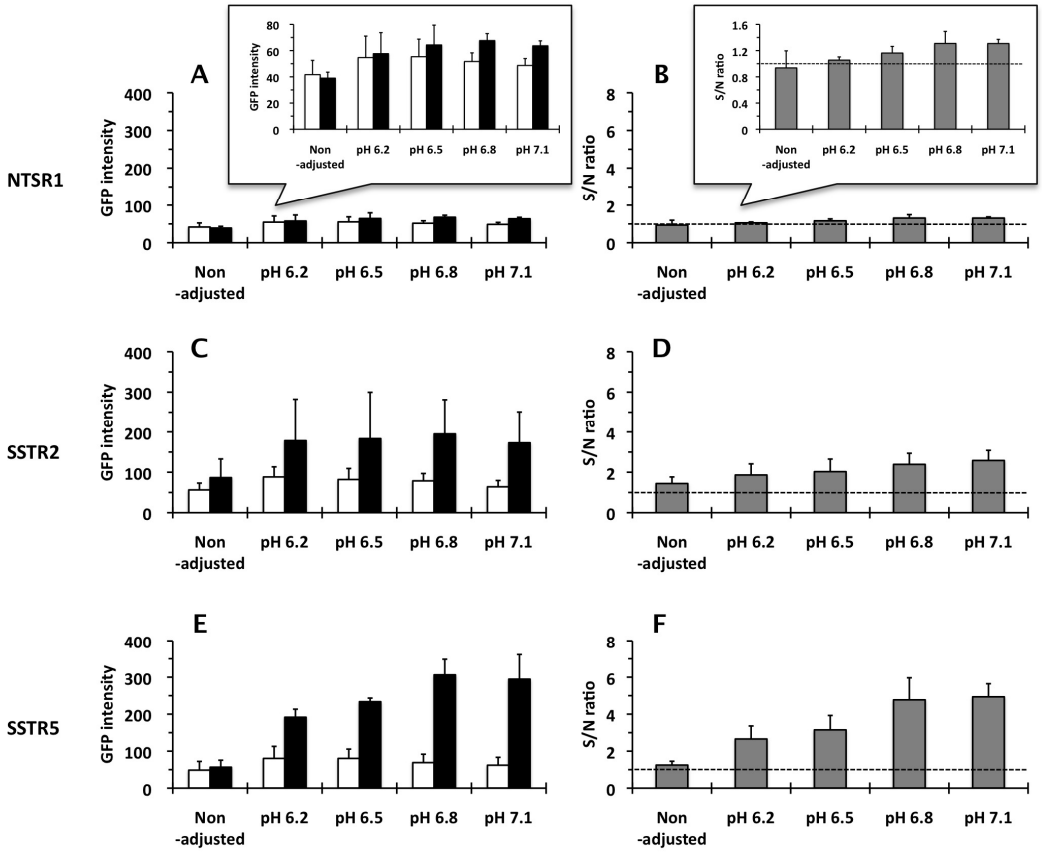

Fig. 2

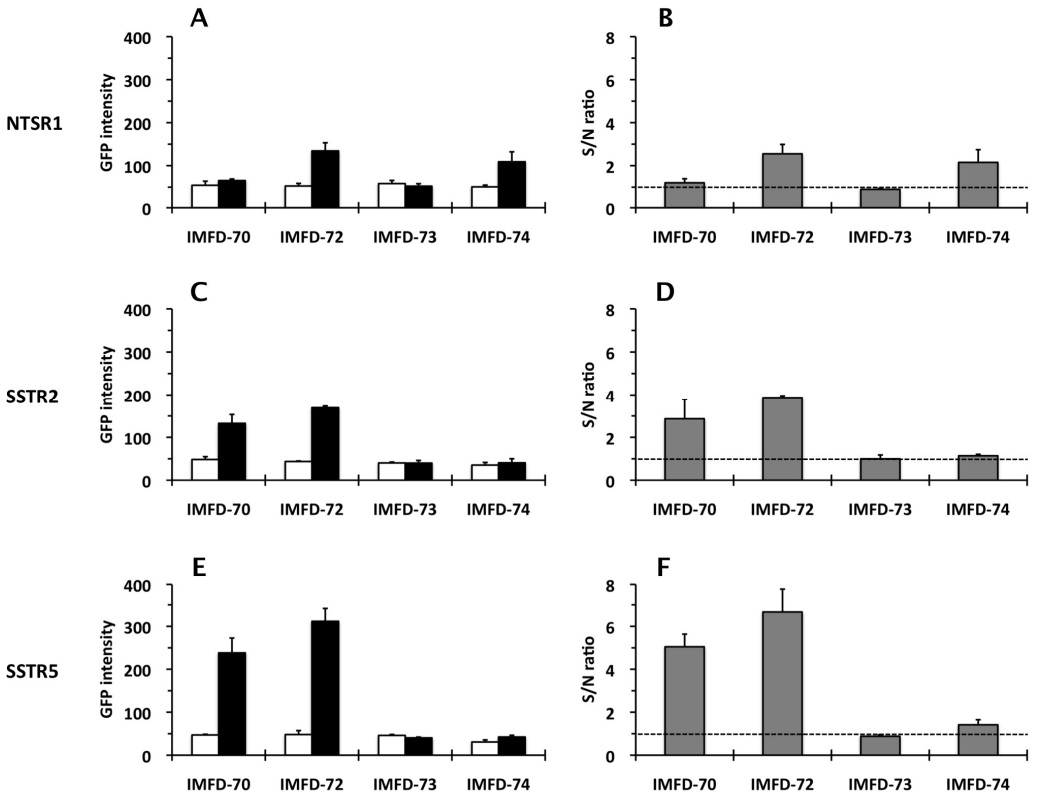

Fig. 3

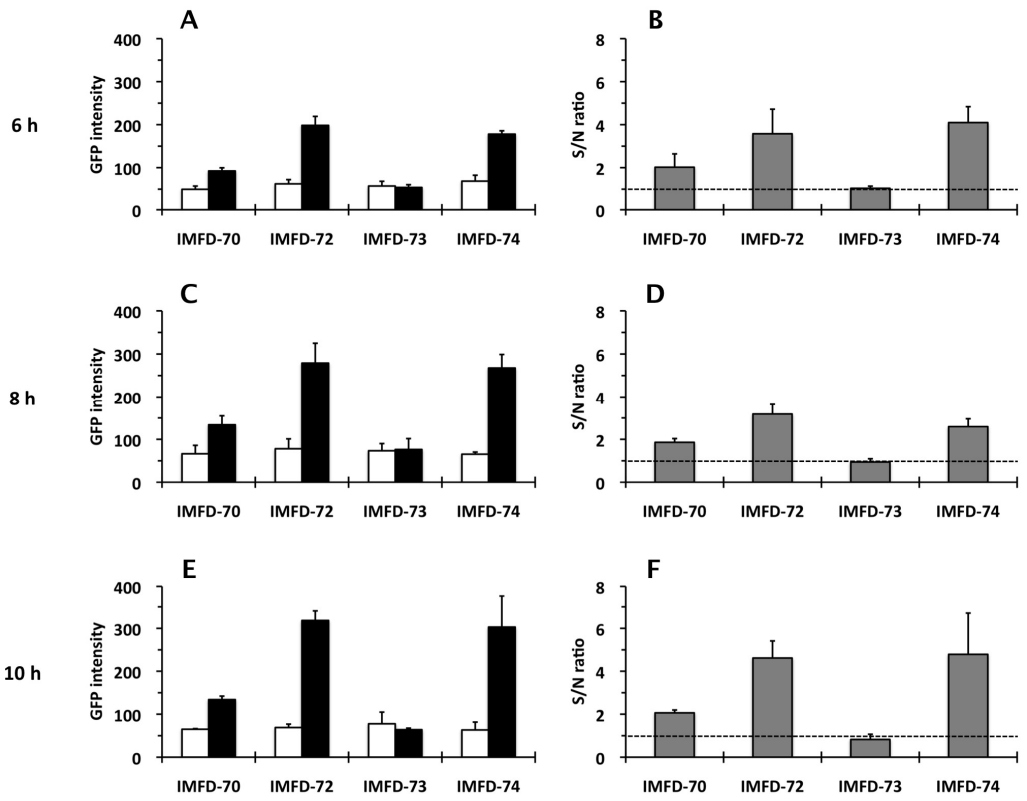

Fig. 4

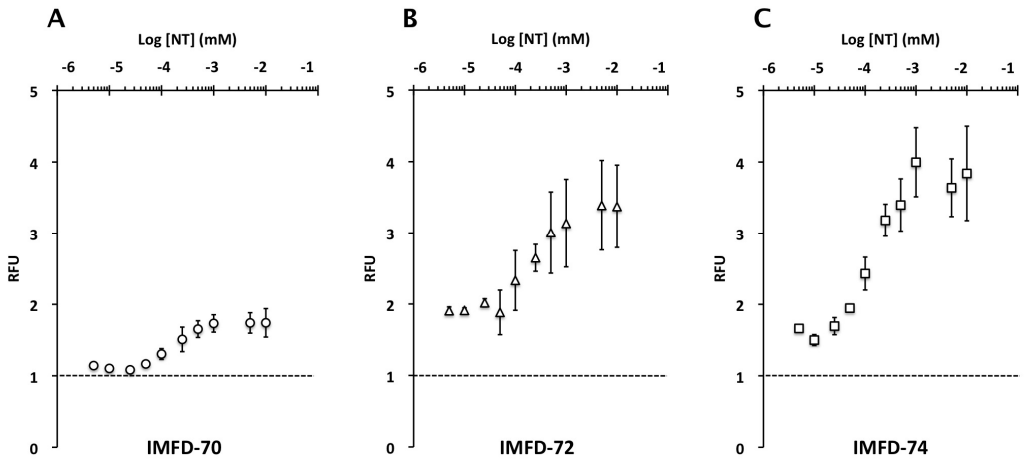

Fig. 5