RESEARCH ARTICLE

Yeast Biosensor

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Modifying Expression Modes of Human Neurotensin Receptor Type 1 Alters Sensing Capabilities for Agonists in Yeast Signaling Biosensor

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Neurotensin receptor type 1 (NTSR1), a member of the G-protein-coupled receptor (GPCR) family, is naturally activated by binding of a neurotensin peptide, leading to a variety of physiological effects. The budding yeast *Saccharomyces cerevisiae* is a proven host organism for assaying the agonistic activation of human GPCRs. Previous studies showed that yeast cells can functionally express human NTSR1 receptor, permitting the detection of neurotensin-promoted signaling using a *ZsGreen* fluorescent reporter gene. However, the fluorescence intensity (sensitivity) of NTSR1-expressing yeast cells is low compared to that of yeast cells expressing other human GPCRs (e.g., human somatostatin receptors). The present study sought to increase the sensitivity of the NTSR1-expressing yeast for use as a fluorescent biosensor, including modification of the expression of human NTSR1 in yeast. Changes in the transcription, translation, and transport of the receptor are attempted by altering the promoter, consensus Kozak-like sequence, and secretion signal sequences of the NTSR1-encoding gene. The resulting yeast cells exhibited increased sensitivity to exogenously added peptide. The cells are further engineered by using cell-surface display technology to ensure that the agonistic peptides are secreted and tethered to the yeast cell wall, yielding cells with enhanced NTSR1 activation. This yeast biosensor holds promise for the identification of agonists to treat NTSR1-related diseases.

1. Introduction

G-protein-coupled receptors (GPCRs) constitute a large and diverse family of transmembrane receptors. These receptors bind to extracellular ligands such as neurotransmitters and hormones, transducing the resulting signals as intracellular responses via guanine nucleotide binding proteins (G-proteins).[1,2] GPCRs are involved in a wide variety of physiological responses and serve as the most important pharmaceutical and therapeutic targets for drug discovery.[3]

Neurotensin (NTS) is a 13-amino-acid peptide found throughout the central nervous system and digestive tract.[4] NTS's action is mediated by three neurotensin receptors: neurotensin receptor types 1, 2, and 3 (NTSR1, NTSR2, and NTSR3). NTSR1 (high-affinity neurotensin receptor) and NTSR2 (low-affinity neurotensin receptor) are members of the seven-transmembrane GPCR family, whereas, NTSR3 belongs to the family of single-transmembrane-helix receptors.[4,5] NTS functions as a neurotransmitter and a hormone in the nervous system and peripheral tissues.[6] In the brain, NTS modulates the activity of dopaminergic systems, opioid-independent analgesia, and inhibition of food intake; in the gut, NTS regulates a range of digestive processes.[4] Moreover, NTS and NTSRs are densely distributed in the entorhinal cortex, a structure that is physiologically crucial for learning and memory and undergoes the earliest pathological alterations in Alzheimer's disease.[7] Decreases in NTSRs have been found in the putamen in Parkinson's disease patients.[8] During the early stages of cell transformation in relation to Wnt/β-catenin pathway deregulation, abnormal expression of NTSR1 is presumed to induce carcinogenesis.[9,10] Thus, NTSR1 is a significant molecular target in various research fields, including medicine, therapeutics, and pharmaceutics.

The eukaryotic, unicellular, budding yeast, *Saccharomyces cerevisiae*, is a useful microbial host organism for making GPCR biosensors.[11–14] Compared with mammalian cell lines, *S. cerevisiae* provides a simple and predictable system for studying GPCR signaling 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, respectively). Expressing human GPCR on the plasma membrane of *ste2*Δ **a**-cells harboring reporter genes facilitates the monitoring of agonist-promoted signaling.[5,11,15,16] Several kinds of reporter genes, including the

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gene encoding green fluorescent protein (GFP) (detected via fluorescence), *HIS3* (detected by complementation of auxotrophy), *lacZ* (detected by colorimetry), and *luc* (detected by luminometry), have been used to detect the activation of GPCR signaling in yeast cells.[17–19] The *GFP* reporter gene provides what may be the simplest and most convenient measurement procedure, requiring only washing the cells and placing them in a relevant instrument (e.g., flow cytometer).[11,12,15,16,20,21]

Yeast cell-surface display technology is a powerful platform that enables proteins expressed in yeast to be tethered to the cell surface.[22–26] Yeast cell-surface display of peptides can form the basis of powerful ligand screening systems.[16,20] Displaying peptides by fusing them to an anchor protein in a yeast strain already expressing a cognate GPCR can enable the occurrence of a series of biological processes from peptide synthesis to agonist detection within a single cell. In such a system, a population of GPCR-producing cells is used to express a library of peptides individually tethered to the plasma membrane via attachment to a glycosyl-phosphatidylinositol (GPI) anchor; upon phosphatidylinositol-specific phospholipase C (PI-PLC) cleavage of the GPI, the peptides, which are fused to the anchor protein, are released from the membrane and trapped in the cell wall.[20] In principle, the host cells automatically detect the ligand binding of peptides to relevant receptors and the resulting agonistic activation on the membrane; in this way, the system can preclude cross-binding of the peptides to other cells because the trapped peptides are unable to diffuse out of the individual producer cells. Thus, this technique facilitates concomitant library synthesis and target peptide screening at the single-cell level.[27,28]

Previous studies showed that expression of the human NTSR1 receptor in yeast cells yielded functional protein, enabling fluorescent detection of signaling in response to the binding of an agonistic peptide (NTS).[5,16] Using this system, heterologously expressed NTSR1 was able to activate the signaling with endogenous yeast Gα (Gpa1p), although NTSR1 exhibited stronger activation with a chimeric yeast/human Gα. However, the fluorescence intensity (sensitivity) of the NTSR1-expressing yeast cells was lower than that of yeast strains expressing other typical human GPCRs such as the human somatostatin receptors (SSTRs).[16] To improve single-cell screening for NTSR1 agonists, it was important to increase the sensitivity of the NTSR1-expressing yeast cells.

In this study, we therefore sought to increase the sensitivity of the NTSR1-expressing yeast cells for use as a fluorescent biosensor; specifically, we attempted to modify the expression of human NTSR1 in yeast cells. To change the level of transcription, translation, and transport of the NTSR1 receptor, we made changes in the gene promoter, in yeast consensus Kozak-like sequence, and in secretion signal sequences, respectively. We then tested the utility of the newly constructed yeast biosensor strain by introducing NTS peptides using cell-surface display technology.

2. Experimental Section

2.1. Plasmid Construction

Plasmids used in this study are listed in **Table 1**. Primers used for plasmid construction are listed in Table S1, Supporting Information.

A DNA fragment containing the *TDH3* promoter (P_{TDH3}) was PCR-amplified from pBTD3-C[29] using primers #1 and #2. The resulting amplicon was digested with *Xho*I + *Nhe*I, and then ligated in similarly digested pGK416-ZsGreen[16] and pGK421,[12] yielding the plasmids pTDH416-ZsGreen and pTDH421, respectively. A DNA fragment encoding the human NTSR1 protein was prepared by digesting pGK421-NTSR1[5] with *Nhe*I + *Bgl*II, and then ligated between the P_{TDH3} and the *PGK1* terminator (T_{PGK1}) in a similarly digested pTDH421, yielding the plasmid pTDH421-NTSR1.

The plasmids for expressing human NTSR1 proteins with a yeast consensus Kozak-like sequence and secretion signal sequences were constructed as follows. A DNA fragment encoding NTSR1 with a Kozak-like sequence was PCR-amplified from pTDH421-NTSR1 using primers #3 and #4. A DNA fragment encoding the human NTSR1 protein with Kozak-like and Ste2 signal sequences was PCR-amplified from pTDH421-NTSR1 using primers #5 and #4. A DNA fragment encoding NTSR1 with Kozak-like and Suc2 signal sequences was PCR-amplified from pTDH421-NTSR1 using primers #6 and #4. The resulting amplicons were digested with *Nhe*I + *Bgl*II, and then ligated between P_{TDH3} and the T_{PGK1} in a similarly digested pTDH421, yielding the plasmids pTDH421-ykNTSR1, pTDH421-st-NTSR1, and pTDH421-su-NTSR1, respectively.

The plasmids for expressing mNeptune-fused NTSR1 proteins were constructed as follows. A codon-optimized *mNeptune* far-red fluorescence protein gene was ordered from GeneArt Gene Synthesis (Thermo Fisher Scientific, Waltham, MA). A DNA fragment containing the codon-optimized *mNeptune* gene (without the start codon) was PCR-amplified using primers #7 and #8. The resulting amplicon was digested with *Sal*I + *Bam*HI, and then ligated into similarly digested pGK421[12] and pTDH421, yielding the plasmids pGK421-mN and pTDH421-mN, respectively. A DNA fragment containing the *NTSR1* gene (without a stop codon) was PCR-amplified from pTDH421-NTSR1 using primers #9 and #10. The resulting amplicon was digested with *Nhe*I + *Sal*I, and then ligated between the *PGK1* promoter (P_{PGK1}) or P_{TDH3} and the *mNeptune* gene in similarly digested pGK421-mN or pTDH421-mN, yielding the plasmids pGK421-NTSR1-mN and pTDH421-NTSR1-mN, respectively.

A DNA fragment containing the *NTSR1* gene (without a stop codon) with Kozak-like sequence was PCR-amplified from pTDH421-ykNTSR1 using primers #3 and #10. A DNA fragment containing the *NTSR1* gene (without a stop codon) with Kozak-like and Ste2 signal sequences was PCR-amplified from pTDH421-st-NTSR1 using primers #5 and #10. A DNA fragment containing the *NTSR1* gene (without a stop codon) with Kozak-like and Suc2 signal sequences was PCR-amplified from pTDH421-su-NTSR1 using primers #6 and #10. The resulting amplicons were digested with *Nhe*I + *Sal*I, and then ligated between the P_{TDH3} and the *mNeptune* gene in a similarly digested pTDH421-mN, yielding the plasmids pTDH421-ykNTSR1-mN, pTDH421-st-NTSR1-mN, and pTDH421-su-NTSR1-mN, respectively.

The plasmids for expressing mUkG-fused NTSR1 proteins were constructed as follows. A DNA fragment containing the codon-optimized *mUkG1* green fluorescent protein gene (without a start codon) was PCR-amplified from pGK416-ymUkG1[30] using primers #11 and #12. The resulting amplicon

Table 1. Yeast strains and plasmids used in this study.

Strain or plasmid	Specific features	Source
Strain		
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	[43]
IMFD-72ZsD	BY4741 *sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::ZsGreen his3Δ::*P_{FIG1}*-ZsGreen far1Δ gpa1Δ::*G_{i3}*tp*	[16]
Plasmid		
pGK416-ZsGreen	ZsGreen expression, *PGK1* promoter, *CEN/ARS* origin, and *URA3* marker	[16]
pTDH416-ZsGreen	ZsGreen expression, *TDH3* promoter, *CEN/ARS* origin, and *URA3* marker	This study
pGK421	Yeast expression vector containing *PGK1* promoter, 2μ origin, and *MET15* marker	[12]
pGK421-NTSR1	Human NTSR1 receptor expression in pGK421	[5]
pTDH421	Yeast expression vector containing *TDH3* promoter, 2μ origin, and *MET15* marker	This study
pTDH421-NTSR1	Human NTSR1 receptor expression in pTDH421	This study
pTDH421-ykNTSR1	Human NTSR1 receptor expression with consensus Kozak-like sequence in pTDH421	This study
pTDH421-st-NTSR1	Human NTSR1 receptor expression with consensus Kozak-like sequence and Ste2 signal sequence in pTDH421	This study
pTDH421-su-NTSR1	Human NTSR1 receptor expression with consensus Kozak-like sequence and Suc2 signal sequence in pTDH421	This study
pGK426-NTS42	NTS-Flag-Flo42 expression, *PGK1* promoter, 2μ origin, and *URA3* marker	[16]
pGK426-NTS(8-13)42	$NTS_{8\text{-}13}$-Flag-Flo42 expression, *PGK1* promoter, 2μ origin, and *URA3* marker	[16]
pGK426-alpha42	α-factor-Flag-Flo42 expression, *PGK1* promoter, 2μ origin, and *URA3* marker	[20]
pGK421-NTSR1-mN	mNeptune-fused human NTSR1 receptor expression in pGK421	This study
pTDH421-NTSR1-mN	mNeptune-fused human NTSR1 receptor expression in pTDH421	This study
pTDH421-ykNTSR1-mN	mNeptune-fused human NTSR1 receptor expression with consensus Kozak-like sequence in pTDH421	This study
pTDH421-st-NTSR1-mN	mNeptune-fused human NTSR1 receptor expression with consensus Kozak-like sequence and Ste2 signal sequence in pTDH421	This study
pTDH421-su-NTSR1-mN	mNeptune-fused human NTSR1 receptor expression with consensus Kozak-like sequence and Suc2 signal sequence in pTDH421	This study
pGK421-NTSR1-mUkG	mUkG-fused human NTSR1 receptor expression in pGK421	This study
pTDH421-NTSR1-mUkG	mUkG-fused human NTSR1 receptor expression in pTDH421	This study
pTDH421-ykNTSR1-mUkG	mUkG-fused human NTSR1 receptor expression with consensus Kozak-like sequence in pTDH421	This study
pTDH421-st-NTSR1-mUkG	mUkG-fused human NTSR1 receptor expression with consensus Kozak-like sequence and Ste2 signal sequence in pTDH421	This study
pTDH421-su-NTSR1-mUkG	mUkG-fused human NTSR1 receptor expression with consensus Kozak-like sequence and Suc2 signal sequence in pTDH421	This study

was digested with *Sal*I + *Bam*HI, and then ligated between the *NTSR1* gene and T_{PGK1} in similarly digested pGK421-NTSR1-mN, pTDH421-NTSR1-mN, pTDH421-ykNTSR1-mN, pTDH421-st-NTSR1-mN, or pTDH421-su-NTSR1-mN, yielding the plasmids pGK421-NTSR1-mUkG, pTDH421-NTSR1-mUkG, pTDH421-ykNTSR1-mUkG, pTDH421-st-NTSR1-mUkG, and pTDH421-su-NTSR1-mUkG, respectively.

2.2. Yeast Strains and Media

Yeast strains used in this study are listed in Table 1. Transformation with plasmids was performed using the lithium acetate method.[31]

Synthetic dextrose (SD) medium contained 6.7 g L^{-1} yeast nitrogen base without amino acids (YNB) (BD-Diagnostic Systems, Sparks, MD) and 20 g L^{-1} glucose. For SDM71 media, SD medium was adjusted to pH 7.1 with 200 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) (Nacalai Tesque, Kyoto, Japan). YPD medium contained 10 g L^{-1} yeast extract (Nacalai Tesque), 20 g L^{-1} peptone (BD-Diagnostic Systems), and 20 g L^{-1} glucose. SD medium was supplemented with amino acids and/or nucleic-acid base (20 mg L^{-1} histidine, 60 mg L^{-1} leucine, 20 mg L^{-1} methionine, or 20 mg L^{-1} uracil) as needed to compensate for relevant auxotrophies. For solid medium, agar was added at 20 g L^{-1}.

2.3. Measurements of Promoter Activity and NTSR1 Expression Level or Localization

Yeast transformants were grown in SD medium (supplemented with amino acids and/or nucleic-acid base as needed) at 30 °C overnight; the cells then were inoculated into 5 mL of the respective fresh SD medium to give an initial optical

density of 0.03 at 600 nm ($OD_{600} = 0.03$). The cells were incubated at 30 °C on a rotary shaker at 150 rpm for up to 18 h. After incubation, the yeast cells were observed using a fluorescence microscope; cultures then were diluted at 1:20 into 1 mL of sheath fluid, and fluorescence was analyzed by flow cytometry (see below).

2.4. Flow Cytometric Analysis

Flow cytometry measurements of green and far-red fluorescence were performed in accordance with the previously described procedure.[32] Briefly, fluorescent cells were detected using a BD FACSCanto II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ) equipped with both a 488-nm blue laser (for excitation of GFP; ZsGreen and mUkG were used to evaluate promoter activities or signaling levels, and the receptor expression levels, respectively) and a 633-nm red laser (for excitation of mNeptune; used to evaluate receptor expression levels); the data were analyzed using the BD FACSDiva software (v5.0; Becton, Dickinson and Company). The GFP green fluorescence signal was collected through a 530/30 nm band-pass filter and the GFP-A mean of 10 000 cells was defined as "green fluorescence intensity." The mNeptune far-red fluorescence signal was collected through a 660/20 nm band-pass filter and the APC-A mean of 10 000 cells was defined as "far-red fluorescence intensity."

2.5. Fluorescence Microscopy Imaging

The cultured cells were washed and suspended in distilled water. The cell suspensions were observed using a BZ-9000 fluorescence microscope (Figure 4C; for mUkG to observe receptor localization) or a BZ-X700 fluorescence microscope (Figure S2C, Supporting Information; for ZsGreen and mNeptune to observe signal activation and the receptor localization, respectively) (Keyence, Osaka, Japan). For the BZ-9000 microscope, green fluorescence images were acquired with a 470/40 band-pass filter for excitation and a 535/50 band-pass filter for emission. For the BZ-X700 microscope, green fluorescence images were acquired with a 470/40 band-pass filter for excitation and a 525/50 band-pass filter for emission. Far-red fluorescence images were acquired with a 560/40 band-pass filter for excitation and a 630/75 band-pass filter for emission.

2.6. NTSR1 Signaling Assay With Exogenously Added Ligands

NTSR1 signaling assays were performed according to previously described procedures.[16,33] Briefly, the yeast strains transformed with the NTSR1 expression plasmids were grown in SD medium (supplemented with amino acids and/or nucleic-acid base as needed) at 30 °C overnight; the cells then were inoculated into 5 mL of the respective fresh SD medium to give an initial $OD_{600} = 0.03$. The cells were incubated at 30 °C on a rotary shaker at 150 rpm for up to 18 h, then harvested, washed, and resuspended in distilled water to yield an $OD_{600} = 10$. The resulting yeast cell suspensions were added (at 10 μL/well; $OD_{600} = 1$) to the wells of 96-well cluster dishes containing fresh SDM71 medium (80 μL/well) supplemented with NTS (Calbiochem, Darmstadt, Germany) (10 μL/well) at various concentrations or with distilled water (without NTS, control). The plates were incubated at 30 °C with shaking (150 rpm) for 4 h. After incubation, the yeast cells were observed using a fluorescence microscope, and then were diluted at 1:20 into 1 mL of sheath fluid, and fluorescence was analyzed by flow cytometry. Half-maximal effective concentration (EC_{50}) values were determined using KaleidaGraph4.0 to fit the data to a dose-response logistic model.

2.7. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The yeast strains transformed with the NTSR1 expression plasmids were grown in SD medium (supplemented with amino acids and/or nucleic-acid base as needed) at 30 °C overnight; the cells then were inoculated into 5 mL of the respective fresh SD medium to give an initial $OD_{600} = 0.03$. The cells were incubated at 30 °C on a rotary shaker at 150 rpm for up to 18 h. After harvesting 300 μL of each cell culture, the cells were washed and resuspended in 200 μL of phosphate-buffered saline (PBS). Then, 10 μL of Zymolyase-100T (Seikagaku Corporation, Tokyo, Japan) at 0.5 mg mL^{-1} was added to each cell suspension and the mixtures were incubated at 30 °C for 30 min. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics, Basel, Switzerland) and was eluted in 75 μL of the supplied elution buffer. cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). qPCR was performed using the Mx3005P Real-Time QPCR System (Agilent Technologies, Santa Clara, CA).

2.8. NTSR1 Signaling Assay Using Tethered Peptide Ligands

NTSR1 signaling assays using tethered peptide ligands were performed according to previously described procedures, with minor modifications.[16] Briefly, yeast strains harboring the NTSR1 expression plasmids and pGK426-based peptide display plasmids[16] were grown in SD medium (supplemented with amino acids as needed) at 30 °C overnight, then the cells were inoculated into 5 mL of the respective fresh SDM71 medium to give an initial $OD_{600} = 0.1$. These cultures were incubated at 30 °C for 12 or 24 h on a rotary shaker at 150 rpm, and then were diluted at 1:10 into 1 mL of sheath fluid, and fluorescence was analyzed by flow cytometry.

2.9. Data Analysis

Data are presented mean ± standard deviation for $n = 3$ (representing three independent transformants).

3. Results and Discussion

3.1. General Strategy Used for NTSR1 Assay in Yeast

To monitor the signaling activation of the human NTSR1 receptor, the previously engineered yeast strain IMFD-72ZsD[16] was used as the host. This strain harbors two copies of the *GFP* reporter construct, both of which have been integrated into the genome, because it showed higher fluorescence intensity than the strain harboring single-copy of the *GFP* reporter construct. Each of these two reporter constructs places the gene encoding the tetrameric *Zoanthus* sp. green fluorescent protein (ZsGreen) under control of the signal-responsive *FIG1* promoter. Therefore, signaling activation by agonist stimulus of this strain results in the generation of green fluorescence.[16]

The IMFD-72ZsD strain also harbors *ste2*Δ, *sst2*Δ, and *far1*Δ alleles, and therefore is deleted for the genes encoding (respectively) the yeast single GPCR (the yeast pheromone receptor); the yeast principal negative regulator of G-protein signaling (RGS); and the yeast G1-cyclin-dependent kinase inhibitor, which induces G1 arrest in response to signaling.[34,35] These deletions endow the strain with the following properties (respectively): allowing the cells to express human GPCRs in the absence of the native yeast receptor; hypersensitizing agonist stimulation even at low ligand concentrations; and continuing cell growth and plasmid retention despite receptor activation (due to the lack of G1 arrest).

The human NTSR1 receptor is known to transduce signals in yeast cells through the endogenous yeast Gα-subunit (Gpa1p).[36] A previous study generated the yeast/human chimeric Gα (also referred to as the Gpa1/$G\alpha_{i3}$ transplant and G_{i3}tp), in which the carboxyl-terminal 5 amino acid residues of yeast Gpa1p (amino acids KIGII) were replaced with the equivalent residues from human $G\alpha_{i3}$ (ECGLY).[36] The yeast/human chimeric G_{i3}tp interacted with human NTSR1 more effectively than did Gpa1p, resulting in increased human NTSR1-mediated signal transmission in yeast cells.[5] However, the sensitivity of the NTSR1-expressing yeast cells was lower than that of SSTR-expressing yeast cells. Therefore, to optimize single-cell screening for NTSR1 agonists, we sought to increase the sensitivity of the NTSR1-expressing yeast cells as a fluorescent biosensor by modifying the expression modes of human NTSR1 in yeast (**Figure 1**).

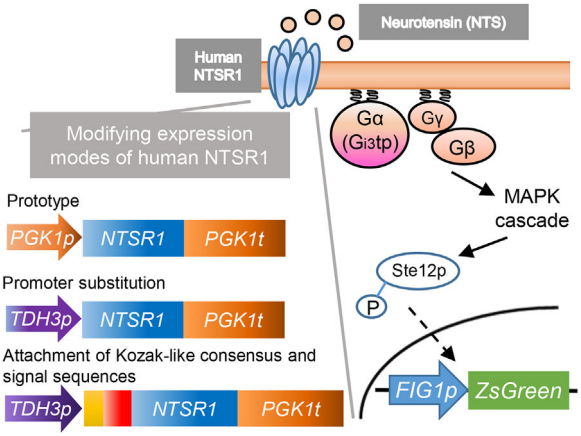

Figure 1. Overview of the NTSR1-expressing yeast signaling biosensor and the genetic modification strategies. Neurotensin (NTS) binding to the human NTSR1 leads to the activation of heterotrimeric G-proteins, the mitogen-activated protein kinase (MAPK) cascade and transcription factor Ste12p. In response to the signaling by NTS binding, Ste12p is phosphorylated and induces transcription of the ZsGreen reporter under the control of pheromone-responsive *FIG1* promoter. To modify the expression modes of the NTSR1 receptor, changes in the promoter, yeast consensus Kozak-like sequences, and secretion signal sequences of the NTSR1-encoding gene were tested.

3.2. Expression of the Human NTSR1 Receptor Using Strong *TDH3* Promoter Improves the *ZsGreen* Reporter Gene Expression to Monitor the Signaling Activation Levels

In previous studies, we used the *PGK1* constitutive promoter to express the human NTSR1 receptor in yeast.[5,16] To change the level of *NTSR1* transcription, we placed the gene under control of the strong, constitutive *TDH3* promoter.[37]

To compare the strength of transcription between the *TDH3* promoter and the *PGK1* promoter, we used each promoter to drive expression of ZsGreen in yeast cells. Single-copy autonomously replicating plasmids pGK416-ZsGreen and pTDH416-ZsGreen were constructed to permit comparison of the expression of the *ZsGreen* gene under the control of the constitutive *PGK1* and *TDH3* promoters, respectively (Table 1). Yeast BY4741 wild-type strains harboring these plasmids were generated and their green fluorescence (the average green fluorescence intensity per cell) was measured by flow cytometry. As expected, cells expressing *ZsGreen* under the control of the *TDH3* promoter exhibited increased (twofold) fluorescence intensity when compared to cells expressing *ZsGreen* under the control of the *PGK1* promoter (Figure S1, Supporting Information). This result was consistent with a previous report comparing the strength of these two promoters.[37]

To evaluate the fluorescence intensity of NTSR1-expressing yeast cells using *ZsGreen* as a reporter gene, we quantified the induction of green fluorescence in response to exogenously added NTS peptide. Multi-copy autonomously replicating plasmids pGK421-NTSR1 and pTDH421-NTSR1 were constructed to express the human NTSR1 receptor under the control of the constitutive *PGK1* and *TDH3* promoters, respectively (**Figure 2**A; Table 1). Yeast IMFD-72ZsD strains harboring these plasmids were generated and used to assess NTSR1-signaling in response to agonistic NTS stimulation. As shown in Figure 2B, the cells expressing NTSR1 under control of the *TDH3* promoter exhibited a 50% higher green fluorescence intensity upon NTS stimulation compared to the degree of induction in the strain harboring the P_{PGK1}-NTSR1 construct. At the level of individual cells, a smaller fraction of the cell population was non-fluorescent (i.e., expressing little or no ZsGreen) with the *TDH3* promoter construct compared to cells harboring the *PGK1* promoter construct (Figure 2C). This difference might indicate that the plasmid with more potent *TDH3* promoter generated a reduced range of fluorescence intensities in cell ensembles, or that plasmid partitioning is more stable in signaling-activated cells. In any event, these results suggested that expression of NTSR1 via the *TDH3* promoter instead of the

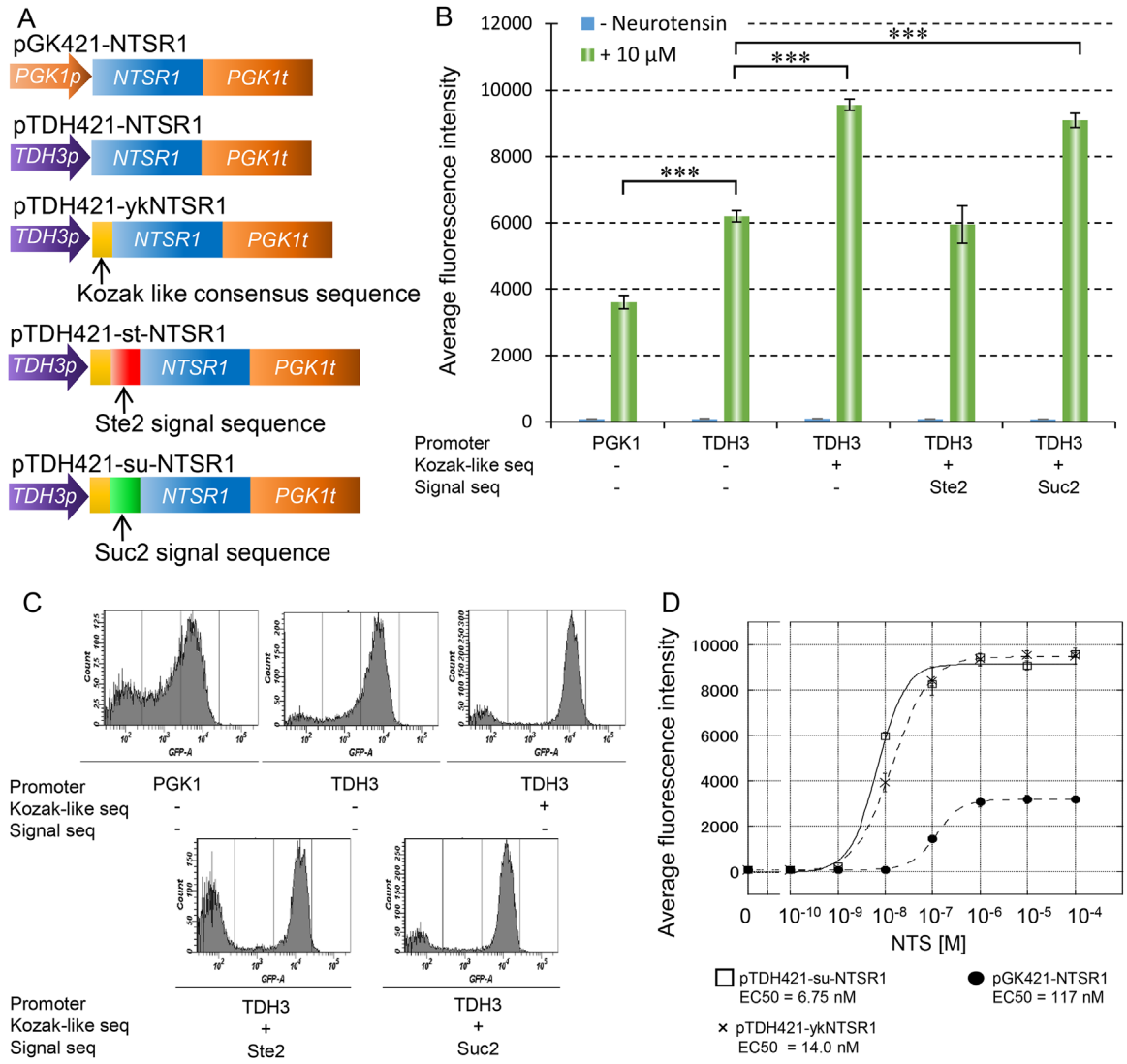

Figure 2. Neurotensin (NTS)-specific NTSR1 signaling activities of recombinant yeast cells. A) Schematic maps of plasmid constructs. B and C) Yeast strain IMFD-72ZsD was transformed with pGK421-NTSR1 (*PGK1* promoter), pTDH421-NTSR1 (*TDH3* promoter), pTDH421-ykNTSR1 (*TDH3* promoter and consensus Kozak-like sequence), pTDH421-st-NTSR1 (*TDH3* promoter, consensus Kozak-like sequence, and Ste2 signal sequence), or pTDH421-su-NTSR1 (*TDH3* promoter, consensus Kozak-like sequence, and Suc2 signal sequence). All transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium with or without 10 µM NTS (13-aa peptide). B) The mean ZsGreen fluorescence of 10 000 cells was measured by flow cytometry. C) Histogram of NTS-stimulated cell populations. D) Dose-response curves for NTS-specific NTSR1 signaling activities in yeast cells. Yeast strains IMFD-72ZsD harboring pGK421-NTSR1, pTDH421-ykNTSR1, or pTDH421-su-NTSR1 were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium with NTS at various concentrations. The mean ZsGreen fluorescence of 10 000 cells was measured by flow cytometry. Error bars represent the standard deviation from three independent transformants ($n = 3$). Statistical significance was assessed by the *t*-test ($^{***}p < 0.001$).

PGK1 promoter yielded improved *ZsGreen* reporter gene expression.

3.3. Incorporation of a Consensus Kozak-Like Sequence and/or Suc2 Signal Sequence Further Improves the NTSR1-Sensing System

To further improve the NTSR1-sensing system, we tested the effect of incorporating a yeast consensus Kozak-like sequence (AAAAAAAUGUCU;[38]) and sequences encoding signal peptides (derived from the yeast pheromone receptor Ste2 or invertase Suc2;[11,28]) upstream of the *NTSR1* open reading frame (ORF). The existence of consensus Kozak-like sequences in eukaryotic mRNAs is thought to facilitate ribosomal recognition of the ATG initiation codon and to enhance the efficiency of translation.[39] Therefore, the multi-copy autonomously replicating plasmid pTDH421-ykNTSR1 was constructed to examine the effect of a consensus Kozak-like sequence on expression of the NTSR1 receptor (Figure 2A; Table 1).

Separately, a previous study on the expression of the human somatostatin receptor subtype-5 (SSTR5) receptor in yeast showed that the inclusion of the Ste2 signal sequence (corresponding to the amino-terminal 20 amino acid residues (a.a.) of the encoded protein) improved the expression level and the signaling activity of heterologously expressed SSTR5.[11] Similarly, inclusion of the Suc2 secretion signal sequence (corresponding to the amino-terminal 19 a.a. of the encoded protein) enhanced the expression of the human epidermal growth factor receptor (EGFR), a single-transmembrane-helix protein.[28] To test the effect of such signal sequences in our system, we constructed multi-copy autonomously replicating plasmids pTDH421-st-NTSR1 and pTDH421-su-NTSR1. These plasmids encoded NTSR1 with amino-terminal Ste2 or Suc2 signal sequences (respectively), while also incorporating consensus Kozak-like sequence into the DNA upstream of the ORFs (Figure 2A; Table 1).

Yeast IMFD-72ZsD strains harboring these plasmids were generated and used to assess NTSR1 signaling in response to agonistic NTS stimulation. As shown in Figure 2B and C, the incorporation of a Kozak-like sequence upstream of the *NTSR1* ORF yielded a 70% higher green fluorescence intensity upon NTS stimulation compared to induction in the strain harboring the construct lacking these sequences. The additional inclusion of the Ste2 signal sequence resulted in decreased fluorescence intensity (Figure 2B). At the level of individual cells, a larger fraction of the cell population was non-fluorescent (i.e., expressing little or no ZsGreen) with the Ste2 signal sequence construct compared to those lacking that sequence (Figure 2C). In contrast, the inclusion of the Suc2 signal sequence did not adversely impact fluorescence intensity compared to cells harboring the equivalent plasmid lacking the signal peptide sequences (Figure 2B and C).

To evaluate the dose-dependency of NTSR1 receptor signaling, the half-maximal effective concentration (EC_{50}) values for NTS were determined (Figure 2D). IMFD-72ZsD strains harboring the plasmids pGK421-NTSR1 (*PGK1* promoter), pTDH421-ykNTSR1 (*TDH3* promoter and yeast consensus Kozak-like sequence), or pTDH421-su-NTSR1 (*TDH3* promoter, yeast consensus Kozak-like sequence, and Suc2 signal sequence) were used to determine the dose-response curves. The EC_{50} values with these constructs were 117, 14.0, and 6.8 nM, respectively (Figure 2D). These results indicated that the NTS sensitivities of NTSR1-expressing yeast strains were increased by the use of *TDH3* promoter and the inclusion of a yeast consensus Kozak-like sequence and/or sequences encoding the Suc2 signal peptide.

3.4. Examination of Expression Levels of Transcript and Protein, and Localization of Human NTSR1 Receptor

To investigate the relative *NTSR1* transcript levels in the cells expressing the gene with the different expression systems, RT-PCR was performed using total RNA extracts from the cells in the absence of NTS. As shown in **Figure 3**, expression under control of the *TDH3* promoter yielded a 20% increase in *NTSR1* transcript levels compared to those obtained with the *PGK1* promoter. Addition of a consensus Kozak-like sequence had no apparent effect on *NTSR1* transcript levels, consistent with the expectation that Kozak-like sequence affect regulation at the translational level. In contrast, encoding NTSR1 with an amino-terminal signal sequence (whether from Ste2 and Suc2) resulted in an apparent decrease in *NTSR1* transcript accumulation (Figure 3).

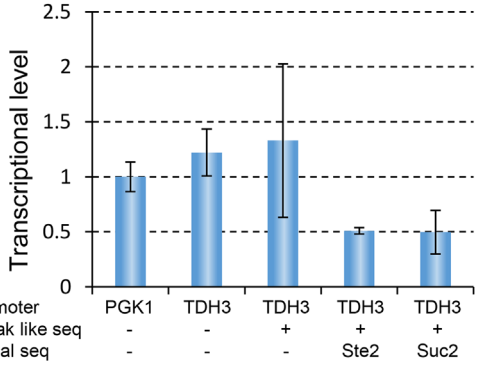

Figure 3. The relative expression ratio of the *NTSR1* gene in engineered yeast strains. Yeast strain IMFD-72ZsD was transformed with pGK421-NTSR1 (*PGK1* promoter), pTDH421-NTSR1 (*TDH3* promoter), pTDH421-ykNTSR1 (*TDH3* promoter and consensus Kozak-like sequence), pTDH421-st-NTSR1 (*TDH3* promoter, consensus Kozak-like sequence, and Ste2 signal sequence), or pTDH421-su-NTSR1 (*TDH3* promoter, consensus Kozak-like sequence, and Suc2 signal sequence). All transformants were grown in SD selective medium without NTS for 18 h. The *NTSR1* transcript levels in total RNA extracts were measured by RT-PCR. Error bars represent the standard deviation from three independent transformants ($n = 3$). Values were normalized to that obtained with pGK421-NTSR1 (*PGK1* promoter).

To measure the relative expression levels and the localizations of the NTSR1 protein, the far-red fluorescence protein mNeptune[40] was fused to carboxyl-terminus of NTSR1. Multi-copy autonomously replicating plasmids pGK421-NTSR1-mN, pTDH421-NTSR1-mN, pTDH421-ykNTSR1-mN, pTDH421-st-NTSR1-mN, and pTDH421-su-NTSR1-mN were constructed and used to transform the IMFD-72ZsD strain (Figure S2A, Supporting Information; Table 1). Flow cytometric measurement of the far-red fluorescence of these cells in the absence of NTS (Figure S2B, Supporting Information) indicated that the rank-order of far-red fluorescence intensities in these strains showed limited correlation with that of the green fluorescence intensities (in strains lacking the mNeptune fusion with NTSR1; Figure 2B). This result suggested that changes in sensitivity to NTS signaling (indicated by green fluorescence) reflected changes in NTSR1 protein levels (indicated by far-red fluorescence). Notably, however, NTSR1 expressed with the Suc2 signal sequence yielded apparently comparatively higher far-red fluorescence, indicating that the Suc2-NTSR1 fusion protein accumulated to higher levels. We additionally noted that the constructed mNeptune-fused NTSR1 receptors were functional in yeast, given that these strains showed green fluorescence in response to NTS exposure (Figure S2C, Supporting Information; green fluorescence images). The cells encoding mNeptune-fused NTSR1 downstream of a yeast consensus Kozak-like sequence showed similar trends for green fluorescence intensities as strains encoding non-mNeptune-fused NTSR1 (Figure

S3, Supporting Information and Figure 2B). Unfortunately, localization (using fluorescence microscopy) of mNeptune-fused NTSR1 within the cells was unsuccessful; far-red fluorescence in these cells was too weak to permit imaging at the needed resolution (Figure S2C, Supporting Information; red fluorescence images).

Instead of mNeptune far-red protein, we used a monomeric green fluorescent protein (mUkG) that has been shown to be readily expressed in yeast cells.[30] Multi-copy autonomously replicating plasmids encoding mUkG-fused NTSR1 protein were constructed and used to transform the IMFD-72ZsD strain (**Figure 4**A; Table 1). When cultured in the absence of ligand (NTS) stimulation, the resulting strains showed higher fluorescence intensities than seen with far-red fluorescence obtained with mNeptune fusion proteins; however, the rank-order of green fluorescence intensities among the transformants was the same as that obtained with the mNeptune-fused NTSR1 constructs (Figure 4B and Figure S2B, Supporting Information). Therefore, the localization of NTSR1-mUkG fusion proteins was observed using fluorescence microscopy (Figure 4C). NTSR1-mUkG expressed from a promoter that included a consensus Kozak-like sequence exhibited strong localization, primarily to the cellular (plasma and endoplasmic reticulum [ER]) membranes. NTSR1-mUkG expressed with the Suc2 signal sequence also localized to these cellular membranes. In contrast, NTSR1-mUkG

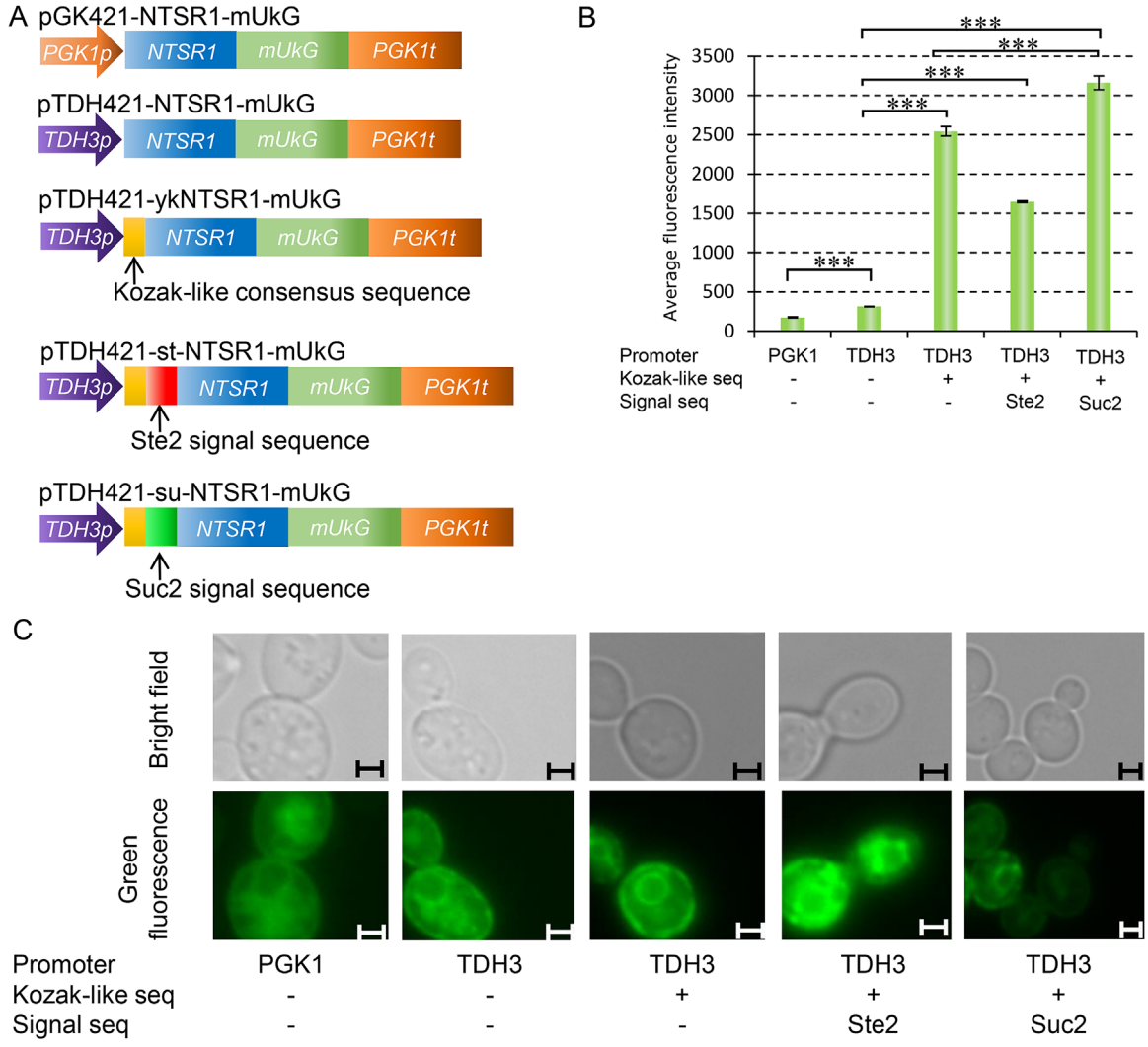

Figure 4. Expression levels and localizations of human NTSR1 receptors fused at the carboxyl-terminus with a green fluorescent protein (mUkG). A) Schematic maps of plasmid constructs. B and C) Yeast strain IMFD-72ZsD was transformed with pGK421-NTSR1-mUkG (*PGK1* promoter), pTDH421-NTSR1-mUkG (*TDH3* promoter), pTDH421-ykNTSR1-mUkG (*TDH3* promoter and consensus Kozak-like sequence), pTDH421-st-NTSR1-mUkG (*TDH3* promoter, consensus Kozak-like sequence, and Ste2 signal sequence), or pTDH421-su-NTSR1-mUkG (*TDH3* promoter, consensus Kozak-like sequence, and Suc2 signal sequence). All transformants were grown in SD selective medium without NTS for 18 h. B) The mean mUkG fluorescence of 10 000 cells was measured by flow cytometry. Error bars represent the standard deviation from three independent transformants ($n = 3$). Statistical significance was assessed by the *t*-test ($^{***}p < 0.001$). C) Cellular fluorescence was observed with a fluorescence microscope. The upper photographs are bright field micrographs, and the lower photographs are fluorescence micrographs. Scale bar: 2 μm.

expressed with the Ste2 signal sequence localized primarily to the ER membrane and only rarely to the plasma membrane (Figure 4C). This result was consistent with previous reports indicating that heterologous proteins expressed with Ste2 signal sequences localized to the ER membrane in yeast,[41,42] and may support our observation that fusion to the Ste2 signal sequence caused a cryptogenic increase in the sub-population of non-fluorescent cells (Figure 2C). In summary, the inclusion of a consensus Kozak-like sequence (as part of the promoter) and/or protein fusion to the Suc2 signal sequence enhanced the sensitivity of NTSR1-expressing yeast cells to agonistic activation. This increased sensitivity reflected increased NTSR1 protein levels and maintenance of the correct membrane localization of the receptor protein.

3.5. Application of Human NTSR1-Sensing Strain to Signaling Assay of Agonistic Peptides Using Yeast Cell-Surface Display Technology

Finally, we tested whether the improved NTSR1 expression system was amenable to a signaling assay using yeast cell-surface display technology. For this assay, we used NTS and its analog, neurotensin-(8-13) (NTS_{8-13}) as agonistic peptides.

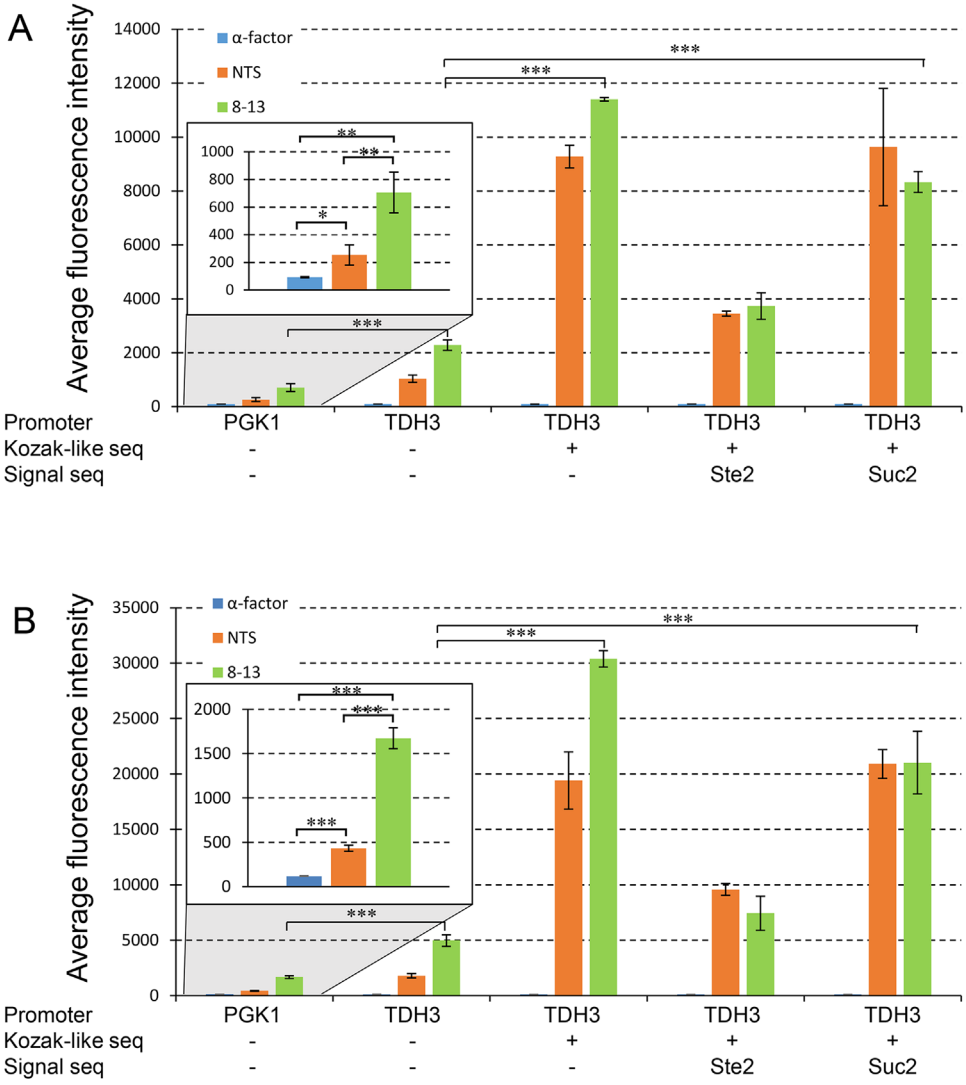

Figure 5. NTSR1 signaling activities of recombinant yeast cells expressing cell surface-displayed neurotensin (NTS) peptides. Yeast strain IMFD-72ZsD was co-transformed with an NTSR1-encoding plasmid (pGK421-NTSR1 [*PGK1* promoter], pTDH421-NTSR1 [*TDH3* promoter], pTDH421-ykNTSR1 [*TDH3* promoter and consensus Kozak-like sequence], pTDH421-st-NTSR1 [*TDH3* promoter, consensus Kozak-like sequence, and Ste2 signal sequence], or pTDH421-su-NTSR1 [*TDH3* promoter, consensus Kozak-like sequence, and Suc2 signal sequence]) and a plasmid encoding a surface-displayed peptide (pGK426-NTS42 [NTS peptide], pGK426-NTS(8-13)42 [NTS_{8-13} peptide], or pGK426-alpha42 [α-factor peptide]). The resulting strains were incubated in pH-adjusted SD selective medium for 12 h (A) or 24 h (B). The mean ZsGreen fluorescence of 10 000 cells was measured by flow cytometry. Error bars represent the standard deviation from three independent transformants ($n = 3$). Statistical significance was assessed by the *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

NTS_{8-13} is a pharmacophore of NTS that is known to exhibit higher potency and efficacy than the full-length peptide.[4] To ensure display on the yeast cell surface, these two agonistic peptides were encoded with an N-terminal secretion signal sequence (the prepro region of the α-factor pheromone) and a C-terminal anchor protein ("Flo42," corresponding to the carboxyl-terminal 42 a.a. of the Flo1p cell-wall protein, which contained the GPI anchor attachment signal sequence). These N-terminal signal sequence and C-terminal anchor protein have been used successfully in previous studies.[16,20]

Yeast strain IMFD-72ZsD was co-transformed with the NTSR1 expression plasmids and either pGK426-NTS42 or pGK426-NTS(8-13)42, which encode NTS or NTS_{8-13} (respectively) for display on the yeast cell-surface. As the negative control, the pGK426-alpha42 for displaying α-factor, the agonistic peptide for the yeast Ste2p receptor, was used to transform IMFD-72ZsD with the NTSR1 expression plasmid. The resulting strains were assessed for NTSR1-mediated signaling in the absence of exogenous NTS peptides. As shown in **Figure 5**A and B, the control strains expressing NTSR1 and α-factor never induced *ZsGreen* reporter gene expression. In contrast, the yeast strains concomitantly expressing NTSR1 and either NTS or NTS_{8-13} showed green fluorescence, indicative of signaling activation. Using either *PGK1* or *TDH3* promoter and/or the Kozak-like consensus sequence for the expression of NTSR1 (without secretion signal sequences), fluorescence with NTS_{8-13} appeared to exceed that obtained with the full length NTS (Figure 5A and B). Additionally, the use of *TDH3* promoter and Kozak-like sequence led to the highest fluorescence intensity among the tested strains. In contrast, the additional insertion of Ste2 or Suc2 signal sequence exhibited no difference in fluorescence intensities between full length NTS and NTS_{8-13}, despite of the high fluorescence responses. This implies that these secretion signal sequences might have a little bit affected the discrimination capabilities for ligand recognition. These results indicated that the improved NTSR1 expression system (using *TDH3* promoter and Kozak-like sequence) was amenable to use in yeast cell-surface display signaling assays for NTS agonists.

The rank-order of green fluorescence intensities, which should reflect signaling levels, was similar when assessed with yeast cell-surface display or with exogenously added NTS (Figures 5B and 2B, respectively). However, the relative levels of fluorescence in strains with cell-surface display in combination with the *ZsGreen* reporter gene (indicating signaling levels) best resembled the relative levels of fluorescence in strains harboring the NTSR1-mUkG fusion proteins (indicating receptor expression levels) (Figures 5B and 4B, respectively). These results suggested that signal induction by the cell-surface display of NTS peptides correlated well with NTSR1 expression levels (Figures 4B and 5B). The EC_{50} values obtained from the dose-response curves (Figure 2D) were also correlated moderately with them. The smaller differences in signaling levels seen with exogenous addition of NTS may reflect "overdosing" of the system due to the inordinately high levels of agonistic peptide (10 μM) used in that experiment (Figure 2B).

4. Conclusions

In the present study, we modified the expression modes of the human NTSR1 receptor in an attempt to improve the sensitivity of a yeast-based fluorescent biosensor strain. Expressing NTSR1 under control of the *TDH3* promoter (in place of the weaker *PGK1* promoter) yielded increased fluorescence intensity (indicating induction of the *ZsGreen* reporter gene) in response to exogenously added agonistic NTS peptide. The additional incorporation of a yeast consensus Kozak-like sequence to the NTSR1 expression cassette further increased the maximum fluorescence intensity and the sensitivity for responding to the NTS peptide. Despite the high fluorescence responses, additional incorporation of sequences encoding the Suc2 signal peptide could not expose the difference in fluorescence intensities responding to full length NTS and NTS_{8-13}. The improved NTSR1 expression system (using *TDH3* promoter and Kozak-like sequence) was successfully applied to the signaling assays for NTS agonists using yeast cell-surface display technology, permitting assessment of a series of biological processes, from peptide synthesis to agonist detection, within the same cell. This improved yeast biosensor is expected to facilitate the screening of lead peptides for use as NTSR1-specific anti-cancer, -Alzheimer, and -Parkinson drugs.

Abbreviations

a.a., amino acid residues; EC_{50}, half-maximal effective concentration; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; Flo42, cell-surface display anchor protein corresponding to the carboxyl-terminal 42 a.a. of the Flo1p; GFP, green fluorescent protein; G_{i3}tp, Gpa1/Gαi3 transplant; GPCR, G-protein-coupled receptor; GPI, glycosylphosphatidylinositol; G-proteins, guanine nucleotide binding proteins; MAPK, mitogen-activated protein kinase; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; mUkG, monomeric umi-kinoko green; NTS, neurotensin; NTS_{8-13}, neurotensin-(8–13); NTSR1, neurotensin receptor type 1; NTSR2, neurotensin receptor type 2; NTSR3, neurotensin receptor type 3; OD, optical density; ORF, open reading frame; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; P_{PGK1}, *PGK1* promoter; P_{TDH3}, *TDH3* promoter; RGS, regulator of G-protein signaling; RT-PCR, reverse transcription-polymerase chain reaction; SD, synthetic dextrose; SDM71, SD medium adjusted to pH 7.1 with 200 mM MOPSO; SSTR, somatostatin receptor; SSTR5, somatostatin receptor subtype-5; T_{PGK1}, *PGK1* terminator; YNB, yeast nitrogen base without amino acids.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the authors.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest.

Keywords

consensus Kozak-like sequence, G-protein-coupled receptor, neurotensin, signal sequence, yeast

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