

表 題 アゴニスト依存的または非依存的な二量体受容体とβ-アレクチン2の相互作用の解析
(Determination of agonist-dependent and -independent interaction between dimerized receptor and β-arrestin 2)

論文の区分 博士課程

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2021年1月8日申請の学位論文

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Introduction

GPCR

G-protein coupled receptors (GPCRs) are seven transmembrane (TM) and heptahelical receptors, which are mainly present in the plasma membrane. This receptor consists of seven transmembrane helices, intracellular loops, extracellular loops, extracellular amino- (N-) terminus, and intracellular carboxyl- (C-) terminus (Figure. 1) ¹. GPCRs are the largest class of membrane-bound receptors in the human genome and are the targets of approximately 35% of current therapeutics ². Their major role in the human body is to maintain normal physiologies. Therefore, a mutation in GPCR causes many diseases. GPCRs in vertebrates are divided into five families depending on their structures: rhodopsin family A, (701 members), adhesion family (24 members), frizzled/taste family (24 members), glutamate family C (15 members), and secretin family B (15 members) ³.

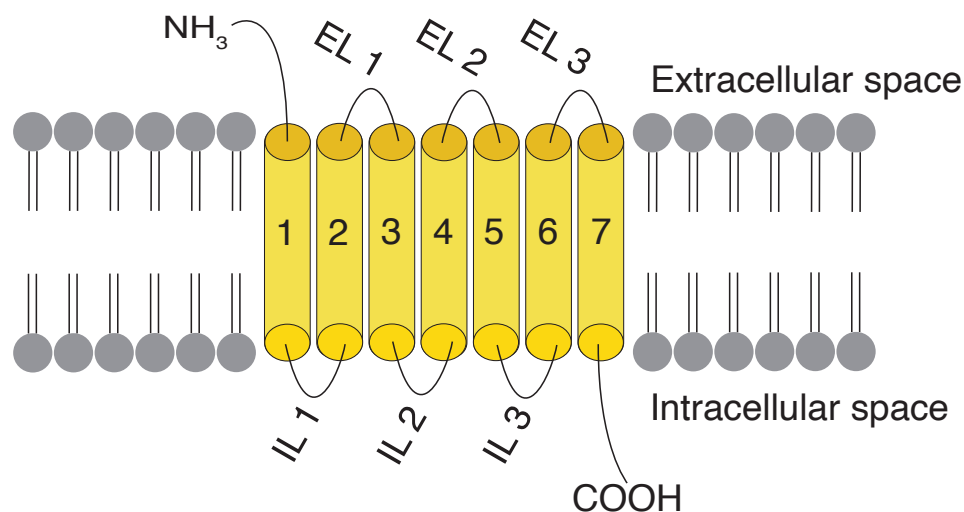


Figure 1. General structure of the GPCRs.

GPCRs are located in the plasma membrane and contain seven transmembrane helices, three extracellular loops, three intracellular loops, amino- (N-) terminus, and intracellular carboxyl- (C-) terminus. EL and IL represent Extracellular loop and Intracellular loop, respectively.

GPCRs generate G-protein-dependent and G-protein-independent signaling pathways

Upon ligand stimulation, GPCR is activated and binds with heterotrimeric G-proteins. Heterotrimeric G-proteins are composed of three subunits: α , β , and γ ⁴. Activated GPCRs function as guanine nucleotide exchange factors for the $G\alpha$ subunits, which release GDP and bind with GTP for G-protein activation ⁵. The $G\alpha$ subunit transduces intracellular signals from different types of active GPCRs to a variety of intracellular processes involving second messenger molecules, such as cAMP, inositol triphosphate, and diacylglycerol. These intracellular signals are called the G-protein dependent signaling pathway. Thereafter, GPCRs are phosphorylated by G-protein receptor kinases (GRKs). The phosphorylated receptors interact with β -arrestin. Binding of β -arrestin to GPCR usually requires both ligand-induced conformational changes in receptor and receptor phosphorylation. The receptor- β -arrestin complex internalizes into the cytosol, resulting in the inhibition of the G-protein dependent signal pathway (Figure 2). This process, called desensitization, leads to a significant deactivation of the G-protein-dependent signaling pathway ⁶. The desensitization process is necessary for regulating the G-protein dependent signal to avoid exaggerated physiological responses. Endocytosis of GPCRs involves β -arrestin, clathrin ⁷, adaptor protein 2 ⁸ and phosphoinositides ⁹. Internalization of GPCRs stimulates the G-protein independent signaling pathway, such as the mitogen-activated protein kinase (MAPKs), extracellular signal-regulated kinase (ERK1/2), and Jun amino terminal kinase (JNK). A number of other kinases can be stimulated by β -arrestin ¹⁰. After internalization, GPCRs can be transferred to lysosomes, where they are degraded or recycled back to the plasma membrane, and this process is called resensitization ⁵. In addition, GPCRs can be divided into two classes, class A and class B, based on their interaction with β -arrestin. Class A receptors, such as the β -adrenergic receptor, temporally bind with β -arrestin 2 with greater affinity than β -arrestin 1 ^{6,11}. Class B receptors, such as the V2 vasopressin receptor (V2R), bind with β -arrestin 2 and β -arrestin 1 at equal affinities with GPCRs remaining bound to β -arrestin for a longer period of time ⁶.

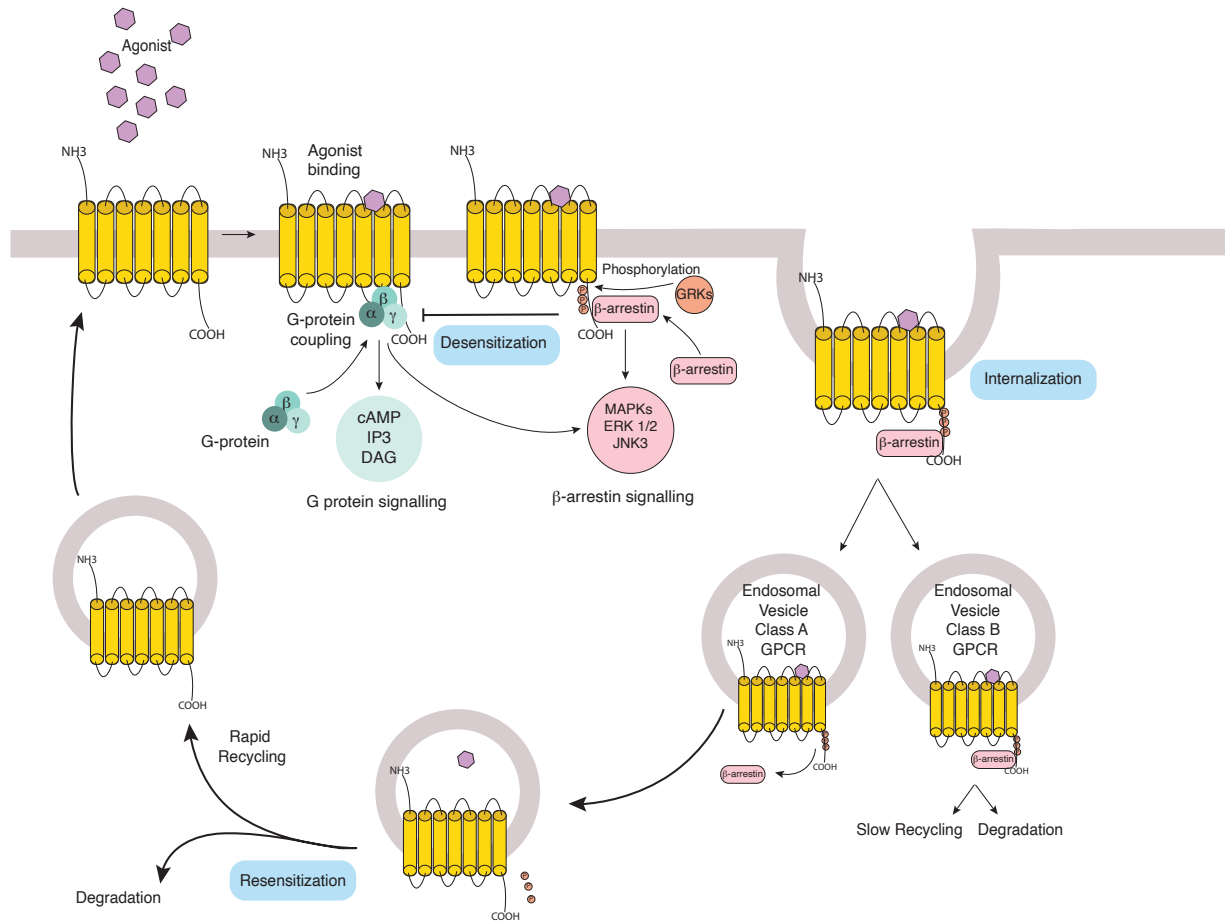


Figure 2. G-protein dependent and independent signaling pathways

After agonist stimulation, GPCRs change conformation and couple with heterotrimeric G-protein¹². Activated G-proteins stimulate the G-protein-dependent signaling pathway. Subsequently, the active receptor is phosphorylated by GRKs and interacts with β -arrestin. This complex generates G-protein-independent signaling pathways. Thereafter, β -arrestin and receptor complex undergo endocytosis. Finally, the receptor is degraded or recycled back to the cell membrane¹³.

Background of the study

GPCRs function as monomers as well as dimers. Recent findings indicated not only homodimerization or heterodimerization of GPCR, but also, higher-order oligomerization¹⁴. Several studies have shown that dimerization occurs soon after biosynthesis. Ligand binding can be accelerated, inhibited, or not modulated¹⁵. Family C of GPCRs has been known to undergo dimerization and for members of this family to function effectively, they must undergo either homodimerization or heterodimerization¹⁶. There is evidence that family A of GPCRs undergoes dimerization. However, information on three molecules composed of a dimerized receptor and β -arrestin is limited. Therefore, we aimed to develop a method to determine the interactions between these three molecules. We focused on family A of GPCR and chose vasopressin V1a receptor (V1aR) and V1b receptor (V1bR) as a model because they have similar structures but different characteristics.

Vasopressin and V1 subtype receptors

Arginine vasopressin (AVP) is a neurohypophyseal peptide known as antidiuretic hormone¹⁷. It is secreted by the hypothalamus and released from the posterior pituitary gland into the blood circulation. AVP plays roles in the central nervous system, such as enhancing learning and memory, social recognition, and anxiety-like behavior¹⁸. In the peripheral system, AVP regulates body fluid homeostasis, hormone secretion, and metabolism¹⁹. Previous studies have demonstrated that AVP is involved in morphine-induced anti-nociception^{20,21}. The vasopressin receptors belong to the A family of GPCRs. Three subtypes of vasopressin receptors are V1aR, V1bR, and V2R. V1aR is mainly expressed in the vascular wall to regulate vasoconstriction. The V1bR is localized in the anterior pituitary to control the secretion of adrenocorticotrophic hormone. V1aR and V1bR couple to G_q heterotrimeric GTP-binding protein and stimulate the phospholipase C pathway. V2R is expressed in the kidney to regulate body water homeostasis. V2R couples to G_s protein and stimulates the adenylate cyclase pathway²². A previous study showed that V1bR and μ -type opioid receptors (MOR) are co-localized in the rostral ventromedial medulla and enhance morphine tolerance. Protein–protein interactions

were studied among V1bR, MOR, and β -arrestin 2. However, the evidence of a complex made from these three molecules was not directly presented ²¹.

Analysis of protein-protein interactions

In the protein-protein interaction assay, fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) are used to transfer energy from fluorescence and luminescence donors to acceptors. In addition to FRET and BRET, protein complementation assays (PCAs) have been developed, in which fluorescent or luminescent protein fragments are used for complementation. These assays are known as bimolecular fluorescence/luminescence complementation. Each of these depends on the ability of protein fragments to reconstitute functional fluorescent/luminescent proteins when brought into close contact by fusion partners ²³.

These methods are commonly used to investigate the interaction between two proteins. Using these methods, information is accumulated on the dimerized GPCRs and the interaction between a receptor monomer and β -arrestin. However, the interaction between the dimerized receptor and β -arrestin has not been assessed. Hence, we assessed the interaction between homodimer or heterodimer receptors and β -arrestin.

NanoBit

NanoLuc® Binary Technology (NanoBit) is a method for the detection of protein-protein interactions. It is composed of two parts: a large bit (LgBit; 18 kDa) and a small bit (SmBit; 11 amino acid). SmBit has a low but consistent affinity for LgBit (190 μ M). LgBit or SmBit were ligated to genes of interest by DNA recombinant technology, and the genes were expressed in cells. When the two proteins interacted, LgBit and SmBit were merged with the whole luciferase protein (Figure 3). Subsequently, a substrate was added to generate the luminescence and measured using a plate reader.

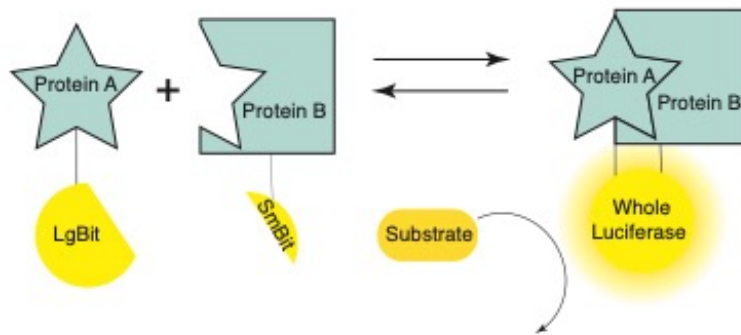


Figure 3. NanoBit method

Two proteins (Protein A and B) were connected to NanoBit. It consists of asymmetrical fragments: LgBit and SmBit. Protein–protein interaction makes the whole luciferase. The instantaneous luciferase intensities were produced by adding the substrate.

NanoBRET

This method uses Förster resonance energy transfer from a donor of Nanoluciferase protein to an acceptor of fluorescent protein. NanoBRET is similar to FRET, in which the resonance energy is transferred from donor to acceptor fluorescent proteins. When the two proteins interacted in proximity at about 10–100 Å (approximately 10 nm), the energy of luciferase light, which peaks at 480 nm, was transferred to the acceptor protein, with a peak at 530 nm. Consequently, the acceptor protein emitted fluorescent light at 530 nm, which was measured using by a plate reader. In our study, we focused on the NanoBRET method.

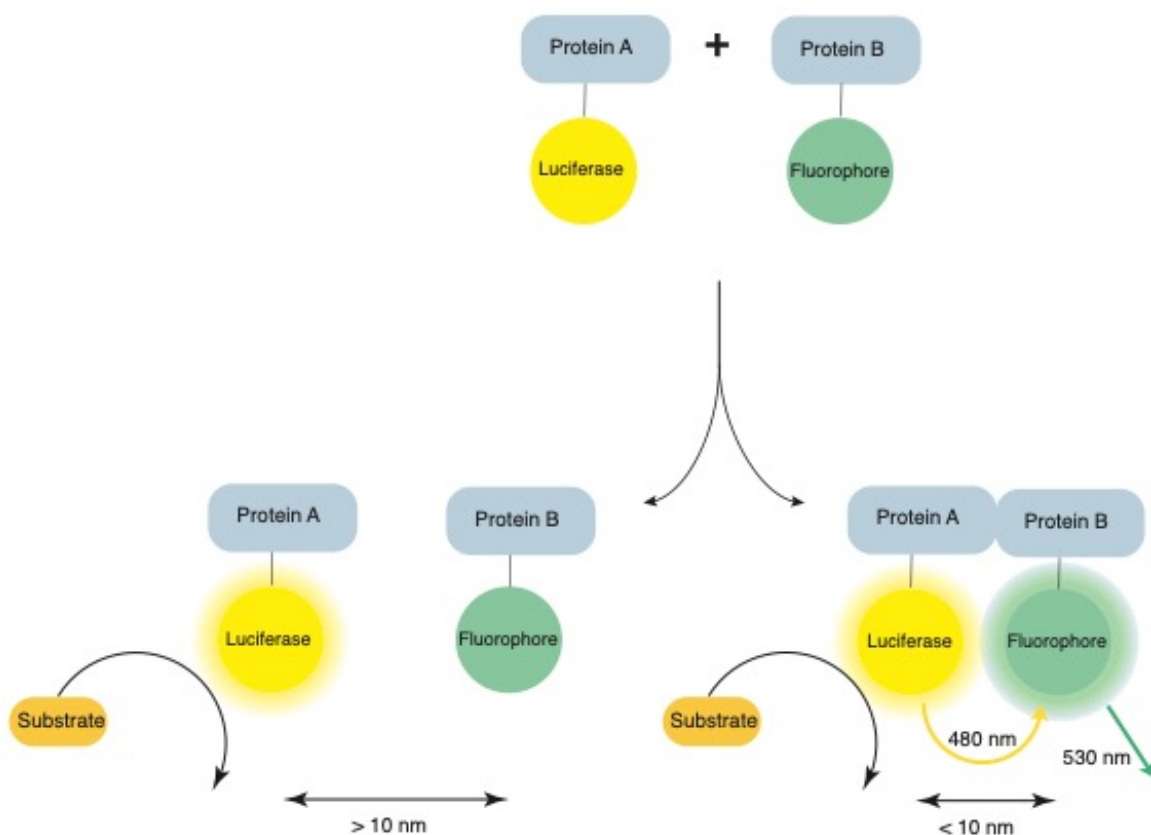


Figure 4. NanoBRET method

Two proteins (Protein A and B) were bound with luciferase or fluorescent protein, respectively. Protein A interacted with protein B. When the two proteins were in proximity within 10 nm, the luciferase light transferred its energy to the fluorophore. Ultimately, it emitted the fluorescent light.

Combination of NanoBit and NanoBRET

To investigate the interaction of the three molecules, we employed the NanoBit and NanoBRET methods. The receptors were tagged with LgBit or SmBit at the C-terminus (NanoBit). β -arrestin 2 was bound with Venus at the N-terminus. When a receptor dimer was formed, luminescence light was generated. When the receptor dimer interacted with β -arrestin 2, NanoBRET occurred between NanoBit Nanoluciferase and Venus (Figure 5).

We measured NanoBRET in cells stimulated with or without various concentrations of agonists. The NanoBRET value was analyzed from light intensities at 530/480 nm.

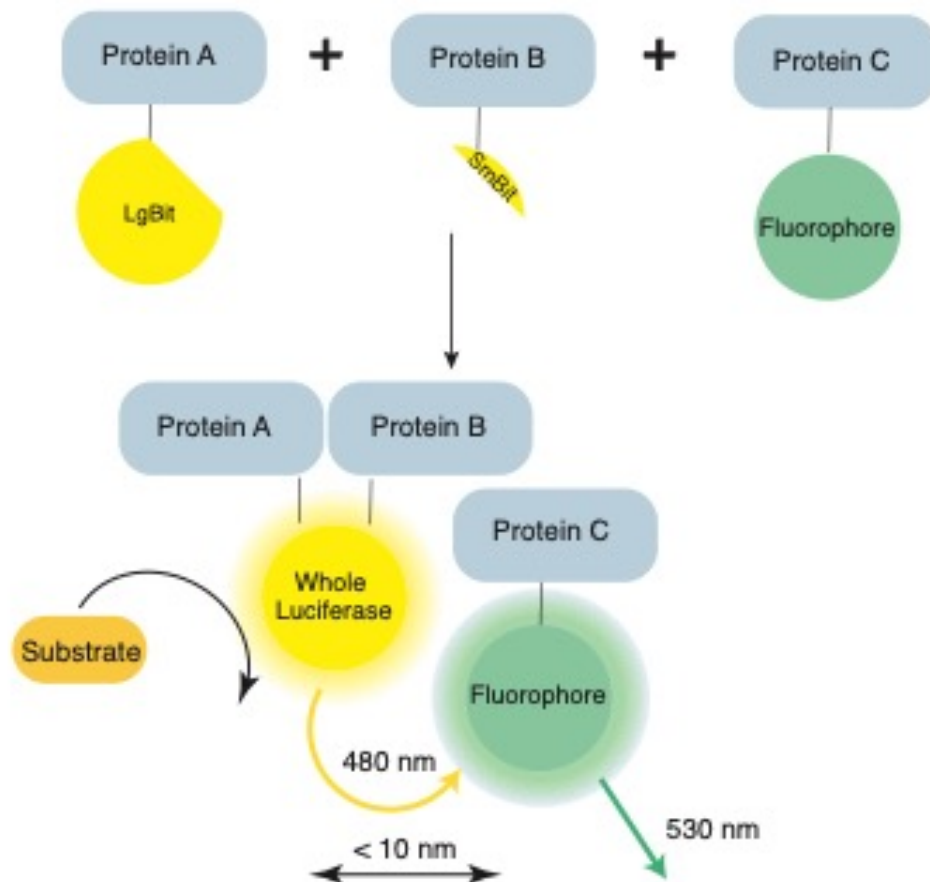


Figure 5. NanoBit and NanoBRET system.

This system was used to investigate the interaction of the three-molecules. The three proteins of interest (Protein A, B, and C) were bound with LgBit, SmBit, and fluorophore protein, respectively. Interaction of proteins A and B generated luciferase light in the presence of the substrate. When the fluorophore was close to the luciferase within 10 nm, NanoBRET generated fluorescent light. Finally, the interactions of the three molecules were determined from light intensities at 530/480 nm.

The purpose of this study

Analgesia by morphine is mainly mediated by MOR. However, repeated or chronic use of morphine causes dependence and analgesic tolerance. In morphine tolerance, analgesic efficacy is reduced and morphine dose needs to be increased in order to obtain the same effect. Although receptor desensitization and internalization are general mechanism of reducing agonist effect in GPCR, morphine tolerance also involves adaptation in cellular and neuronal circuit levels. We recently found that neurohypophyseal hormone vasopressin functionally interact with opioid signal to enhance morphine tolerance through V1bR and MOR ²¹. Genetic deletion of V1bR gene or V1b antagonist treatment into ventral medulla delayed development of morphine tolerance and reduced superactivation of adenylate cyclase, a biochemical hallmark of morphine tolerance and dependence. Using BRET, it was shown that β -arrestin 2-Venus binds to V1bR -Nluc at basal state through leucine-rich amino acid segment of V1bR C-terminus. β -arrestin 2-Venus accessed to MOR-Nluc at basal only in the presence of V1bR possibly through receptor-receptor interaction between V1bR and MOR ²¹. However, in many cases, BRET or fluorescence resonance energy transfer analysis were limited to different combinations of interacting two proteins. Thus, overall relationship among three molecules, MOR, V1b and β -arrestin 2, remained to be determined. In this study, two parts of split Nanoluciferase were connected to receptor C-termini and Venus to β -arrestin 2. This combination allowed us to monitor access of β -arrestin 2 specifically to the dimerized receptors.

Vasopressin V1a and V1b receptors, which share highest amino acid sequence similarity among GPCRs, show largely distinct cellular distribution: V1a receptors are effectively sorted to the plasma membrane, while a large part of V1b receptors is detected in the cytoplasmic space ²⁴. In addition to the V1bR and MOR, we explored how β -arrestin 2 access to the homomeric V1aR or V1bR using split Nanoluciferase system and BRET measurement to directly monitor complex formation among three molecules.

Materials and methods

Materials

Human embryonic kidney (HEK)-derived cells were purchased from the American Type Culture Collection (Rockville, MD, USA). AVP was obtained from the Peptide institute incorporated (Osaka, Japan). [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin acetate salt (DAMGO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-4 acetoxymethyl ester (Fluo-4 AM) dye (MW: 1096.95, Ref F14201) was purchased from Invitrogen™ by Thermo Fisher Scientific. FuGENE®HD transfection reagent (Ref E2312) and Nano-Glo® Luciferase Assay System (Ref. N1138 and Ref. N1120) were purchased from Promega Corporation (Tokyo, Japan). The Mini and Midi kits (Ref 12143 cat.NO. 12143) for plasmid preparation were purchased from QIAGEN company (Tokyo, Japan). *Escherichia coli* (*E. coli*) strain DH5α (code 9057) was purchased from Takara Bio Inc. (Kusatsu, Japan). Restriction enzymes were purchased from Takara Bio Inc. and New England Biolabs Inc. (Tokyo, Japan). All other chemicals were of reagent grade (Wako Pure Chemical Industries, Osaka, Japan).

Plasmid preparation

Construction of the expression plasmid containing a coding sequence of mouse V1aR, V1bR, or MOR was described in a previous study ^{21,25,26}. The C-termini of mouse V1aR, V1bR, and MOR were connected to split luciferase, LgBit or SmBit using a restriction enzyme-based cloning method. The sequences of LgBit and SmBit were added at KpnI/HindIII and stop codons of the receptors were removed in the pcDNA3.1 vector. The N-terminus of β-arrestin 2 was connected to Venus (Venus-β-arrestin 2), a bright version of the yellow fluorescent protein ^{21,27}. The P2X purinergic receptor channels (P2XRs) were connected to Venus, LgBit, and SmBit at the C-terminus. P2X2aR-LgBit and P2X2aR-SmBit were used for measuring positive control and non-specific binding in the NanoBRET assay. *E. coli* strain DH5α was used for plasmid transformation after which the Mini and Midi preparations of the plasmid were performed. Nucleotide sequences of all PCR-derived constructs were verified. Finally, if new constructs were prepared only with restriction enzyme digestion and ligation of the DNA fragment, we confirmed the new

constructs with restriction enzyme digestion and the size of plasmid fragments in agarose gel electrophoresis.

Cell culture and transfection

HEK cells were seeded at a density of 1×10^5 cells in a 35-mm cell culture dish in 2 mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 $\mu\text{g/mL}$ streptomycin, 100 unit/mL penicillin at 37 °C in 5% CO_2 in an air-ventilated humidified incubator. HEK cells were transiently transfected with a pair of plasmids for receptors with or without Venus- β -arrestin 2 for the detection of interaction of three or two molecules. The mixture of 200 μL DMEM, 6–9 μL FuGene® HD transfection reagent, and 2–3 μg of plasmid DNA was incubated for 15 min at ambient temperature of 23°C and was applied to the cells. These cells were cultured for 24–48 h before intracellular calcium measurement and BRET analysis.

Intracellular calcium measurement

Single-cell intracellular calcium ion measurements were performed as described previously, with slight modifications ²⁸. A day after transfection, the medium was removed and cells were incubated at 37 °C for 1 h in 2 μM fluo-4AM in assay buffer containing 137 mM NaCl, 5 mM KCl, 1.2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES (pH 7.4), and 10 mM glucose. The cells were washed once with 1 mL of assay buffer and kept in 0.5 mL assay buffer for measurement of intracellular calcium ion concentration under a fluorescent microscope (Nikon ECLIPSE TI-U). The cells were incubated with the buffer or various concentrations of AVP: 0.01, 0.1, 1, 10, 100, or 300 nM (0.5 mL). After low concentrations of AVP stimulations (0.01, 0.1, and 1 nM), the cells were incubated with 2 mM adenosine-5'-triphosphate dipotassium salt hydrate (ATP). The calcium responses after AVP stimulations were recorded for 300–500 s. Each of these images of intensity changes was measured every 300–500 ms under a 20x objective in the fluorescence microscope. Intracellular calcium responses of 30 cells were averaged to evaluate the basal and peak intensities of fluo-4 fluorescence using the ImageJ program (Version 1.50i, National Institute of Health, USA).

Bioluminescence resonance energy transfer (BRET) assay.

Cells grown in a 35-mm dish were transfected with plasmids for receptor-LgBit, receptor-SmBit, and Venus- β -arrestin 2 in a 1:1:2 ratio and 6–9 μ L Fugene[®]HD transfection reagent according to the manufacturer's instructions. After incubation for 48 h, the cells were washed once with 1 mL of assay buffer and collected in 1 mL assay buffer using a cell scraper. Cells in 1.5 mL Eppendorf tubes were mixed 10 times by gentle pipetting. The cells were aliquoted into a 96-well white plate (Lumitrac 600, Greiner bio-one) at 90 μ L/well. Ten microliters of assay buffer or increasing concentrations of AVP (0.1, 1, 10, 30, 100, or 1000 nM) were added to each well and gently mixed by pipetting. The cells were incubated for 5 min at ambient temperature and 90 μ L of Nano-Glo[®] substrate solution was added to the well. A final dilution factor of the substrate was 100 \times . The intensity of luminescence from the samples was measured in a plate reader (SpectraMax M3; Molecular Devices, Sunnyvale, CA, USA) at 480 nm and 530 nm for BRET signal every 30 s for 5 min. Fluorescent light at 530 nm was used to measure expression levels of Venus- β -arrestin-2. The BRET ratio was calculated from luminescence at 530/480 nm (Figure 6).

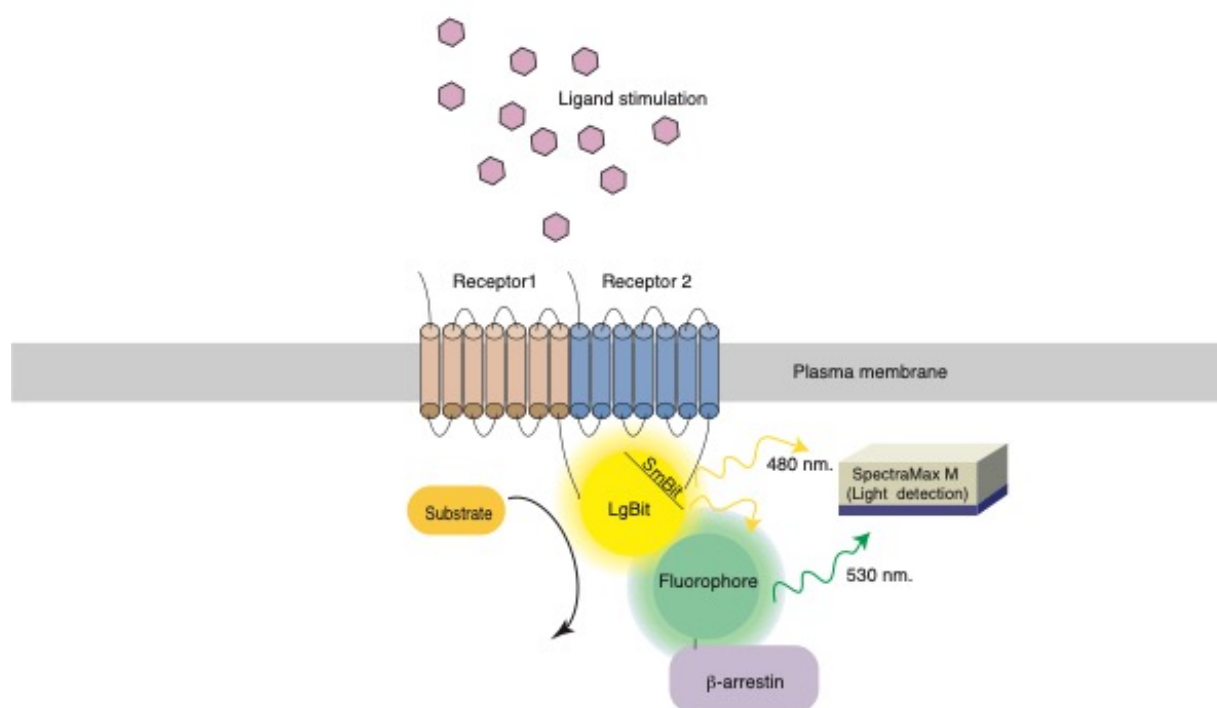


Figure 6. NanoBit-NanoBRET system was applied in dimerized receptors and β -arrestin 2 for assessment of interaction of the three molecules. LgBit or SmBit was tagged with the receptors and the fluorophore was connected to β -arrestin 2. These DNA plasmids were transfected into the HEK cells. The illustration describes the interaction between the dimerized receptors and β -arrestin 2.

Data analysis

All data are reported as the mean and SEM. Graphs were plotted using Igor (WaveMetrics, Lake Oswego, OR, USA) and the ggplot2 in R program. The statistics were calculated using the statistical computer program R (R Core Team, 2018).

Results

1. Receptor-tagged LgBit or SmBit did not alter receptor function

Connecting LgBit or SmBit to the receptor C-terminus did not alter receptor function relative to intracellular calcium levels (Figure 7). The intracellular calcium responses of V1aR-SmBit and V1aR-LgBit were comparable to those of V1aR (Figure 7a). In addition, the intracellular calcium responses of V1bR-LgBit and V1bR-SmBit were similar to those of V1bR (Figure 7b). Therefore, LgBit and SmBit, fused to V1aR or V1bR, did not impede the receptor function.

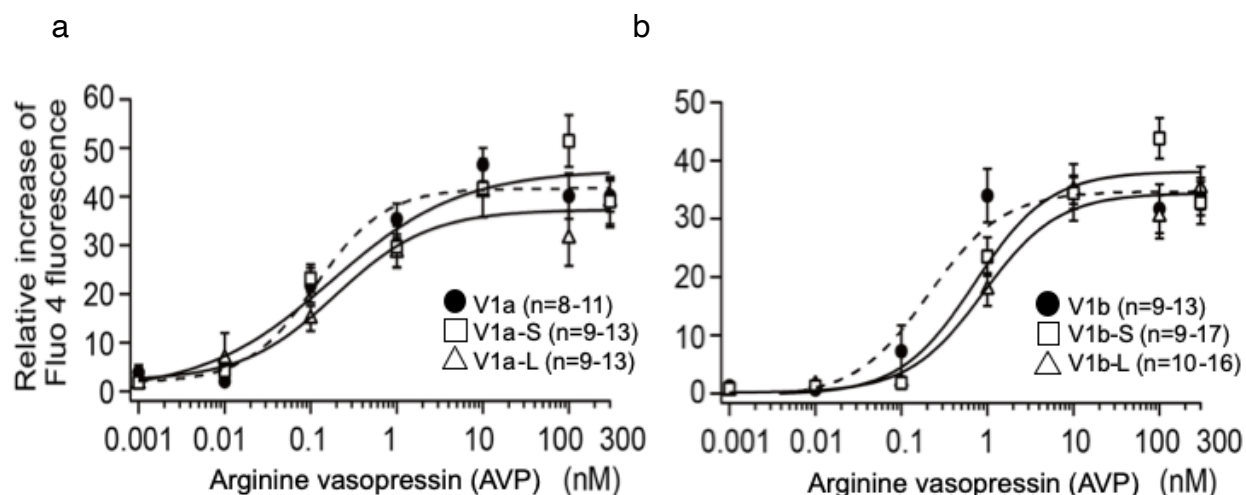


Figure 7. Intracellular calcium responses by the V1a or V1b receptors tagged with LgBit or SmBit. HEK cells were transiently transfected with the plasmids for V1a (a) and V1b (b) receptors connected with each part of split luciferase. The intracellular calcium response was measured at different agonist concentrations. The maximum amplitudes of the calcium responses were calculated and plotted against AVP concentrations.

2. Homodimers of V1aR, V1bR, and MOR were assessed using split luciferase protein

To assess protein-protein interactions between the C-terminal tails of the receptors, we used the split luciferase, LgBit or SmBit, which were tagged with the C-termini of the

receptors. The combination of LgBit and SmBit proteins generated strong luminescence in the presence of the substrate. The plasmids were constructed for V1aR-LgBit, V1aR-SmBit, V1bR-LgBit, V1bR-SmBit, MOR-LgBit, MOR-SmBit, LgBit, and SmBit. The receptors with LgBit and SmBit were co-expressed to check the formation of specific receptor dimerization. Strong signals were observed in cells co-expressing receptor-LgBit and -SmBit. Our results clearly indicated that homodimerization of receptors occurred in our system (Figure 8).

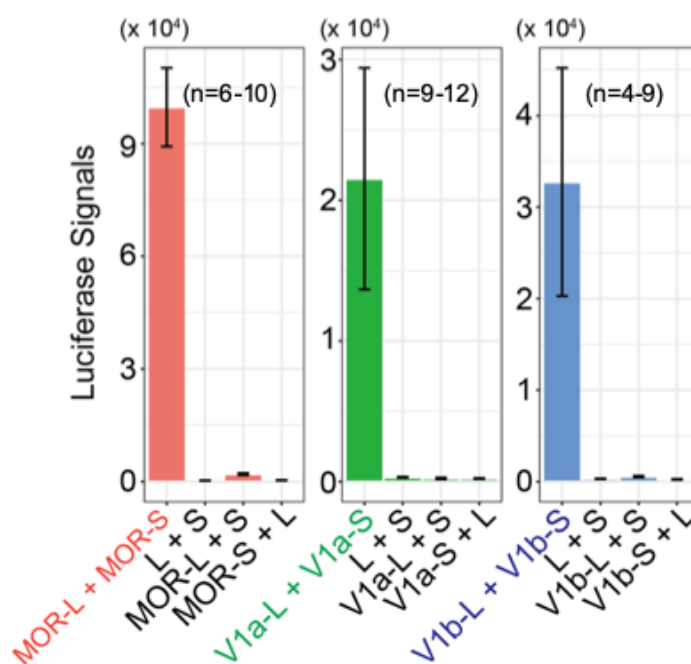


Figure 8. Assessment of receptor dimerization using split luciferase method.

HEK cells were transiently co-transfected with the plasmids for receptors tagged with LgBit and SmBit or LgBit and SmBit alone. Receptor-receptor interactions were analyzed by measuring luciferase signals after the addition of substrate. L and S represent LgBit and SmBit, respectively.

3. Time course of luciferase signals

Most GPCRs are located in the plasma membrane with the C-terminus in the cytoplasm. We found that in cells expressing receptor-connected split luciferase,

injection of luminescence substrate into cellular suspension resulted in an increase in luminescence signal over time (Figure 9a). This gradual increase is not due to limited access of the substrate into the intracellular space. After preparation of the membrane fraction from the cells expressing V1aR-LgBit and V1aR-SmBit, the addition of the substrate directly to the membrane samples generated a luminescence signal with a gradual increase (Figure 9b).

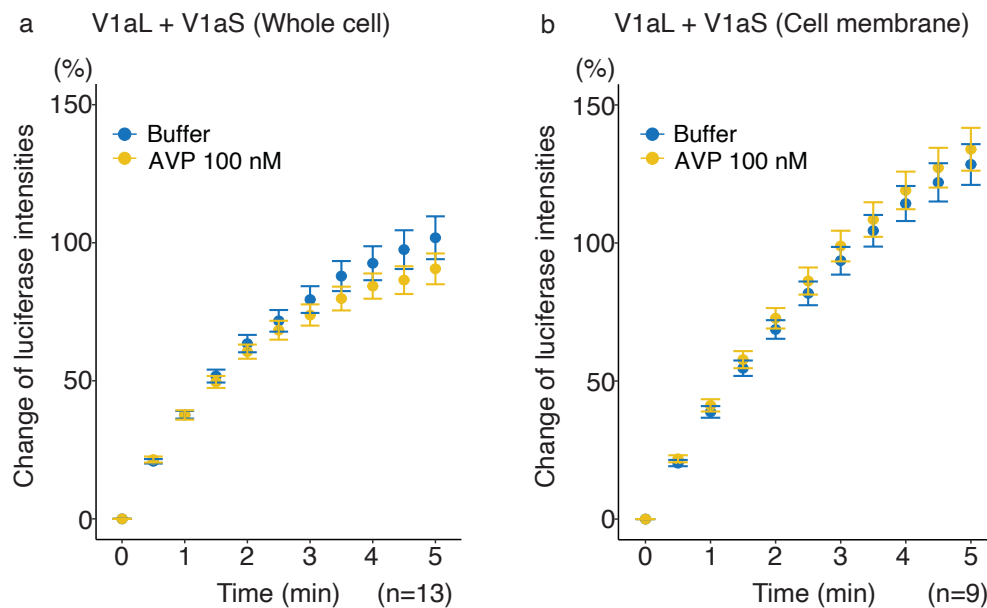


Figure 9. Luciferase signals from whole cells and cell membranes. HEK cells, which were transiently transfected with V1aR-LgBit and V1aR-SmBit, were used for measurement of luminescence signal with or without 100 nM AVP stimulation. Whole cells (a) and samples of cell membrane (b) were used. Increase in intensities from the basal signal was plotted.

4. Luciferase intensities increased during measurement of constitutively dimerized SmBit-PRKACA and LgBit-PRKAR2A

A gradual increase in luminescence after the addition of the luminescence substrate was seen in constitutively associated cAMP-dependent protein kinase type II- α

regulatory subunit and catalytic subunit pairs (Figure 10). These results suggest that the substrate might increase the activity of Nanoluciferase, which was formed by LgBit and SmBit. In this study, we averaged the luminescence intensities obtained during a measurement period of 5 min.

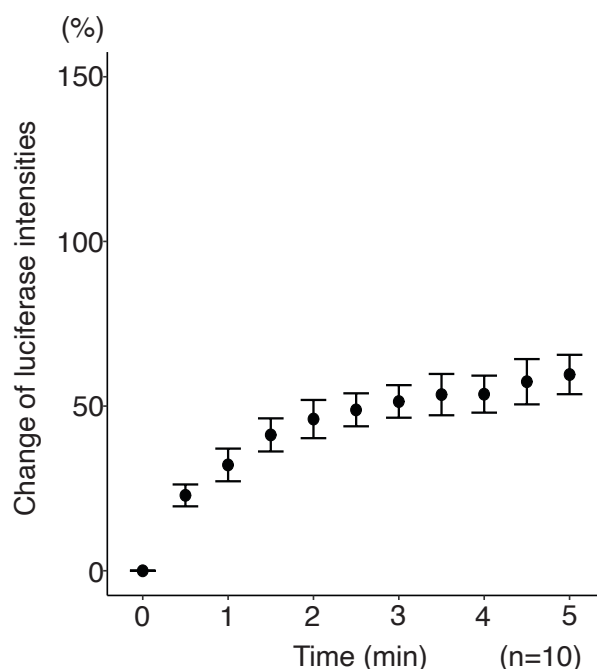


Figure 10. Luciferase signal from constitutive interaction between SmBit -PRKACA and LgBit-PRKAR2A increased during the measurement period of 5 min. The HEK cells were transfected with plasmids for LgBit-PRKAR2A and SmBit-PRKACA. The luciferase signal was measured using the plate reader.

5. Agonists did not alter the receptor dimerization

Before examining the agonist dependency in the interaction between the receptor dimer and β -arrestin 2, we determined whether agonists might change the luminescence signal from the receptor dimer. It was observed that MOR, V1aR, and V1bR tagged with whole luciferase (Nluc) or split luciferase (LgBit and SmBit) were expressed in HEK cells. The agonists, DAMGO and AVP, were used to stimulate MOR and vasopressin receptors,

respectively. Our results showed that the intensities of luciferase signals were not changed by agonist stimulation (Figure. 11).

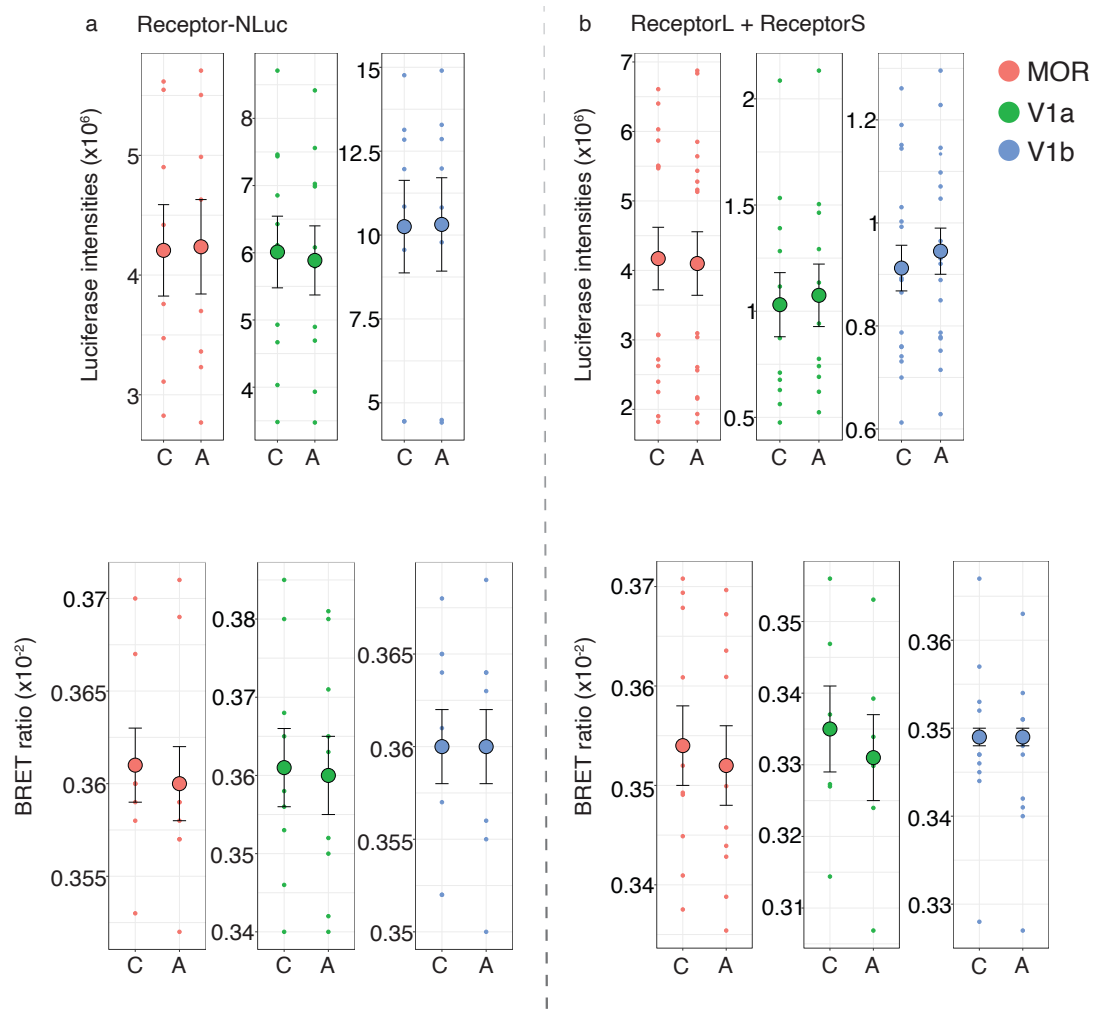


Figure 11. In cells expressing receptor-NLuc or receptor-LgBit and -SmBit, agonists did not alter luminescence signal. The HEK cells, which expressed the receptors tagged with whole luciferase (a) or split luciferase (b) had their luciferase light intensities measured with or without agonist stimulations (AVP or DAMGO 1 μ M). Signal intensity at 480 nm and BRET values of 530/480 nm were shown. L and S represent LgBit and SmBit, respectively. C, Control and A, Agonist.

6. Evaluation of non-specific interaction between the components of split luciferase

P2X2a receptor (P2X2aR) is a member of the purinergic P2X2 receptors. It is an ATP-gated ion channel, located on the plasma membrane. We co-expressed V1aR and P2X2aR in HEK cells to determine whether non-specific binding could occur between V1aR and P2X2aR. The results showed that the luciferase signals from cells expressing V1aR and P2X2aR were at background levels, compared with the interaction between P2X2aR homomers and V1aR homomers (Figure 12). Moreover, AVP did not change the luciferase signals.

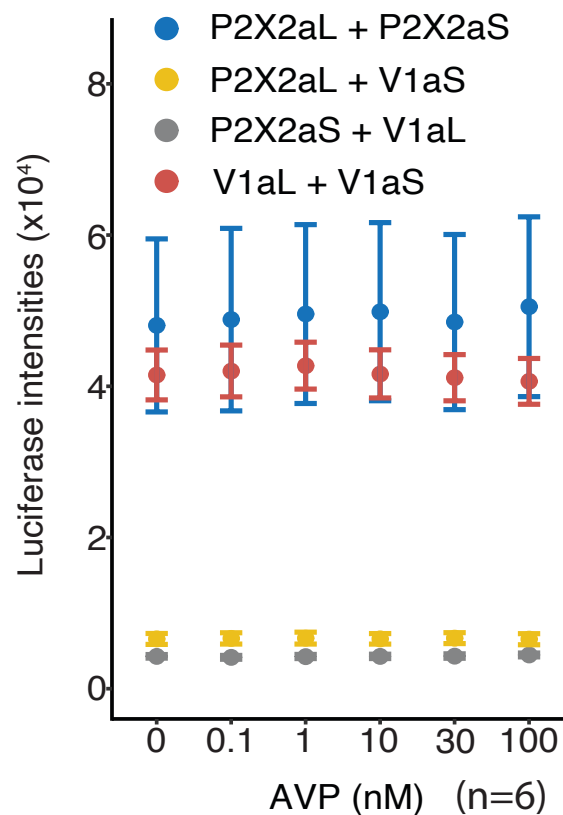


Figure 12. Evaluation of non-specific interaction in the split luciferase system. HEK cells were transfected with plasmids as indicated in the panels to evaluate non-specific interactions. Luciferase signals in the cells were measured using the plate reader.

7. Split luciferase system increased the sensitivity of determination of agonist-dependent coupling between V1aR or V1bR homomers and β -arrestin 2

When concentration-response curves of BRET measurements between receptor-Nluc and Venus- β -arrestin 2 were assessed, marked differences between V1aR and V1bR were observed (Figure 13a). The interaction between V1aR-Nluc and Venus- β -arrestin 2 was initiated by agonist and increased in an agonist-dependent manner. In contrast, basal BRET signal without agonist was high in cells expressing both V1bR-Nluc and Venus- β -arrestin 2, indicating that the interaction occurred without agonist stimulation (Figure 13a). Furthermore, an agonist-dependent increase in BRET was not observed between V1bR-Nluc and Venus- β -arrestin 2. Although β -arrestin 2 constitutively accessed V1bR, we and others previously detected agonist-dependent increases in ERK phosphorylation, which is partly mediated by β -arrestin 2 in V1bR-expressing cells, indicating that agonist stimulation of V1bR may involve further conformational changes in the V1bR- β -arrestin 2 complex. Therefore, we performed an experiment in which two parts of a split Nanoluciferase (LgBit and SmBit) were connected to the C-termini of V1bR or V1aR and the interaction between the receptor homodimer and β -arrestin 2 in a NanoBit-NanoBRET system was determined. Our data indicated that the split luciferase method increased the sensitivity of determining agonist-dependent interactions between dimerized V1aR or V1bR and β -arrestin 2 (Figure 13b).

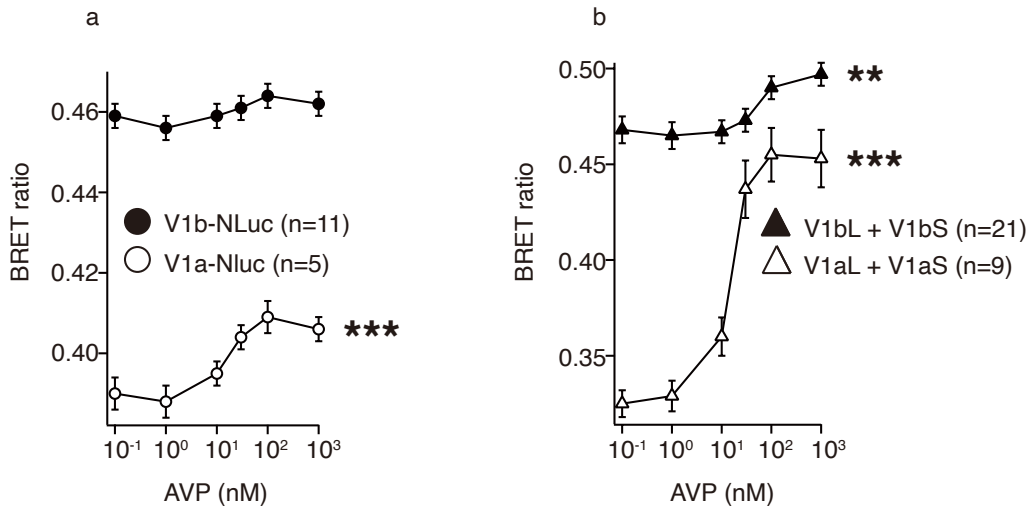


Figure 13. Determination of agonist-dependent interaction between receptor dimer and β -arrestin 2 by split luciferase system. a) HEK cells were co-transfected with V1aR-NLuc or V1bR-NLuc with Venus- β -arrestin 2. b) HEK cells were co-transfected with receptor-LgBit and -SmBit plus Venus- β -arrestin 2 as indicated. L and S indicate LgBit and SmBit, respectively. **, $P < 0.01$ and ***, $P < 0.001$ from one-way ANOVA.

8. Reduced receptor expression improved sensitivity of BRET measurements

When the quantity of plasmids for transfection of receptor-LgBit and receptor-SmBit were reduced while keeping those of Venus- β -arrestin 2 constant, the apparent BRET response generated from the receptor dimer and β -arrestin 2 improved (Figure 14a and 14b). In these experiments, an increase in the luminescence of receptor dimers resulted in lower expression of Venus- β -arrestin 2 (Figure 14c). We therefore hypothesized that the relative expression levels of receptor-connected luciferase and Venus- β -arrestin 2 might be an important factor for sensitive BRET determination.

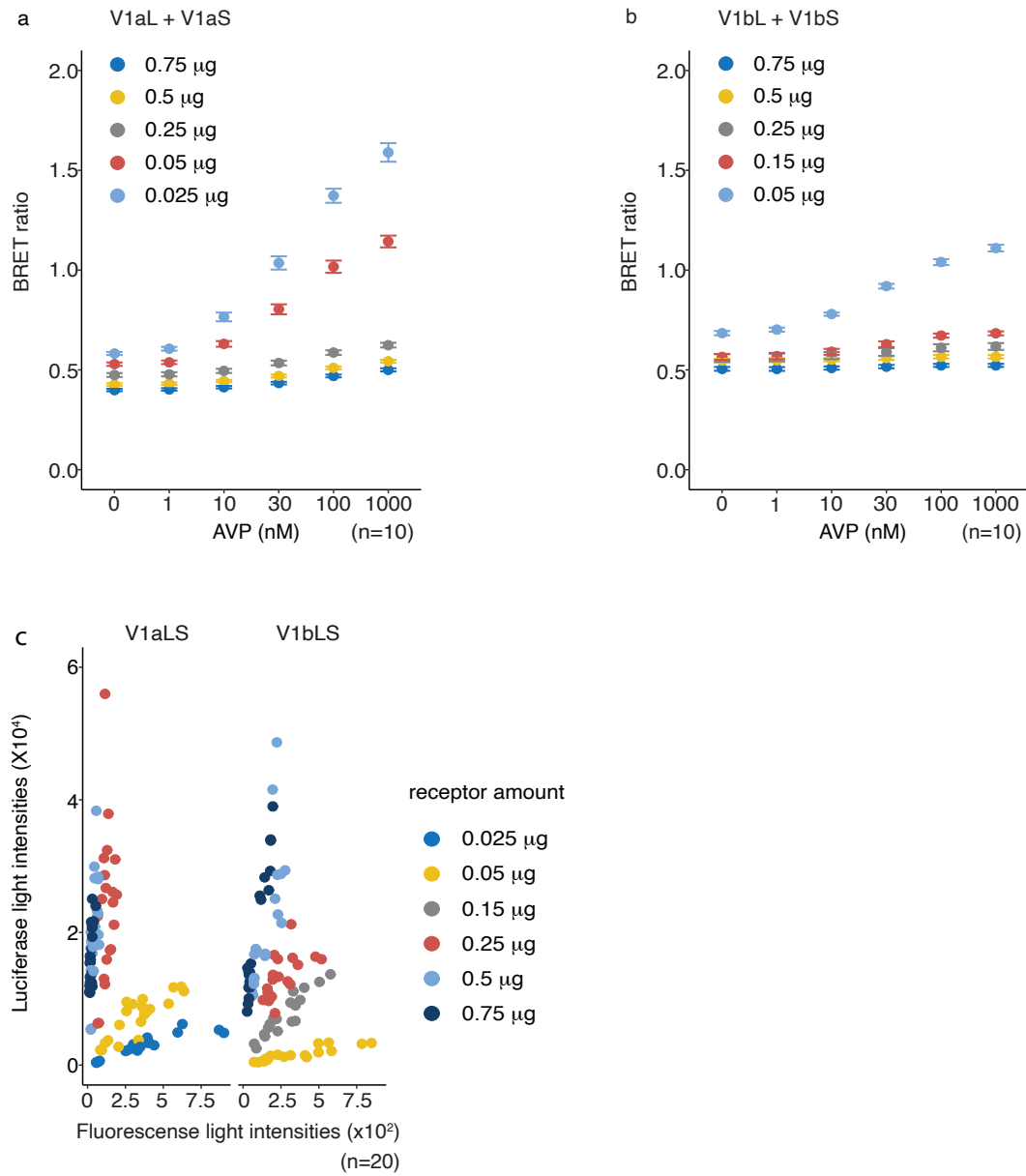


Figure 14. Reduced receptor expression improved sensitivity of BRET measurements. HEK cells were expressed with V1aR (a) and V1bR (b) tagged with split luciferase at different levels with a fixed amount of Venus-β-arrestin 2. BRET responses were evaluated at different concentrations of AVP stimulations. Figure c shows the relationship between the luciferase signals and Venus-β-arrestin 2 fluorescence in the experiments shown in (a) and (b).

9. Increase in Venus- β -arrestin 2 expression improved sensitivity of BRET measurements

Next, we changed the expression levels of Venus- β -arrestin 2 at a fixed level of the receptor-LgBit and -SmBit. The results showed that increasing the level of plasmid Venus- β -arrestin 2 increased the BRET signal in an agonist-dependent manner (Figure 15a and 15b). Basal BRET levels without AVP administration were higher in V1bR homodimers than those in V1aR, indicating that V1bR homodimers were more likely to bind with β -arrestin 2 at basal conditions. Luminescence intensities from receptor dimers tended to be low when expression of Venus- β -arrestin 2 was high, resulting in a high amplitude of the agonist-induced BRET signal (Figure 15c and 15d).

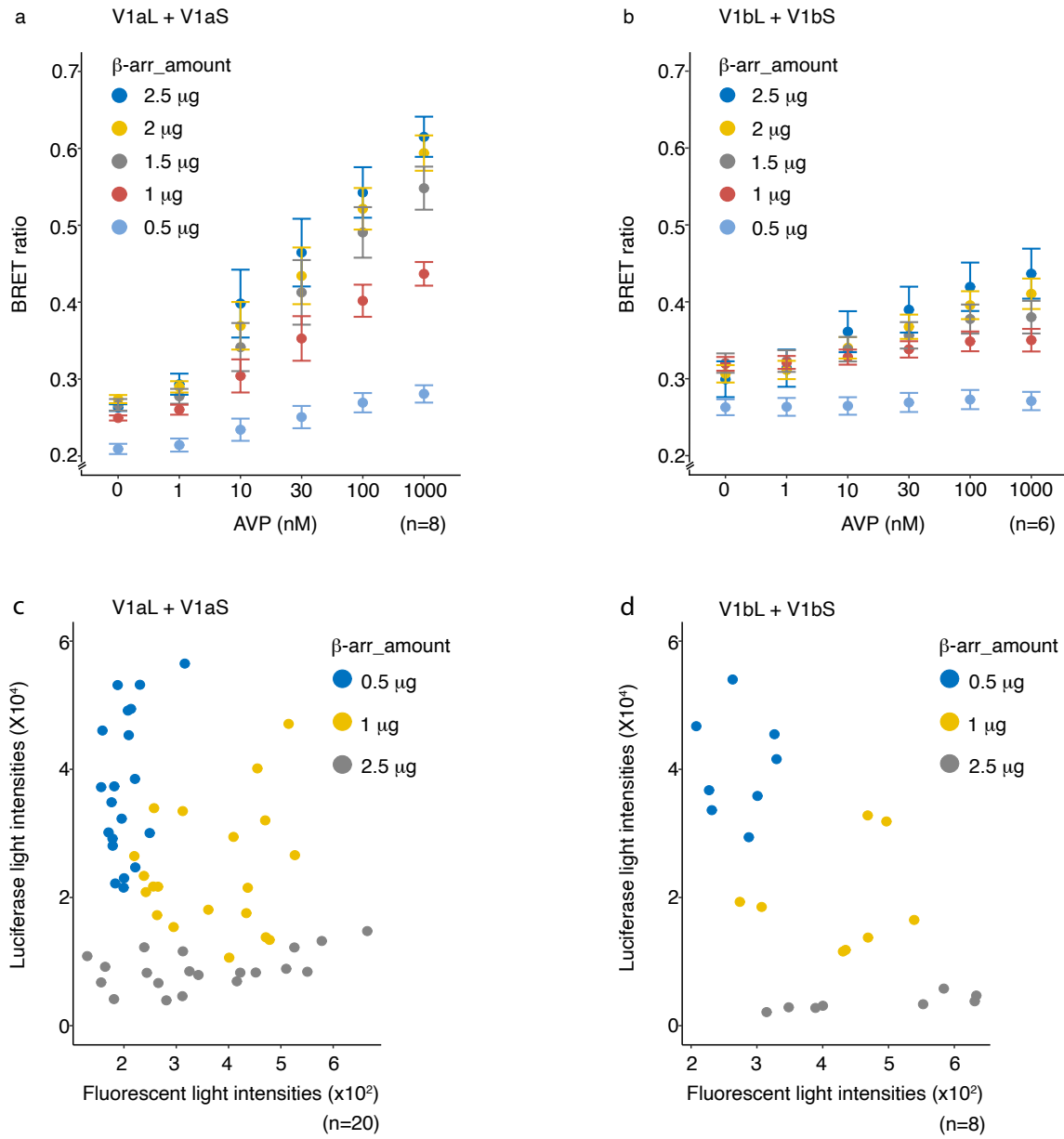


Figure 15. Increase in β-arrestin expression increased BRET responses. HEK cells were transiently co-transfected with varying levels of Venus-β-arrestin 2 plasmid and a constant level (1.5 μg/35-mm dish) of V1aR (a) or V1bR (b) tagged with split luciferase. (c) and (d) are the relationships between luciferase signals and Venus-β-arrestin 2 fluorescence.

10. High affinity binding of HiBit to LgBit did not improve the amplitude of BRET signal

HiBit shows a higher affinity for LgBit than SmBit. We examined whether increasing the affinity between the receptor C-termini increases BRET between the receptor dimer and β -arrestin 2. When expressed with V1bR-LgBit, V1bR-HiBit generated stronger luminescence than V1bR-SmBit. In contrast, the maximum BRET values of cells expressing V1bR-HiBit were lower than those expressing V1bR-SmBit (Figure 16), although reducing levels of V1bR-LgBit and V1bR-HiBit also increased the maximum levels of BRET signal.

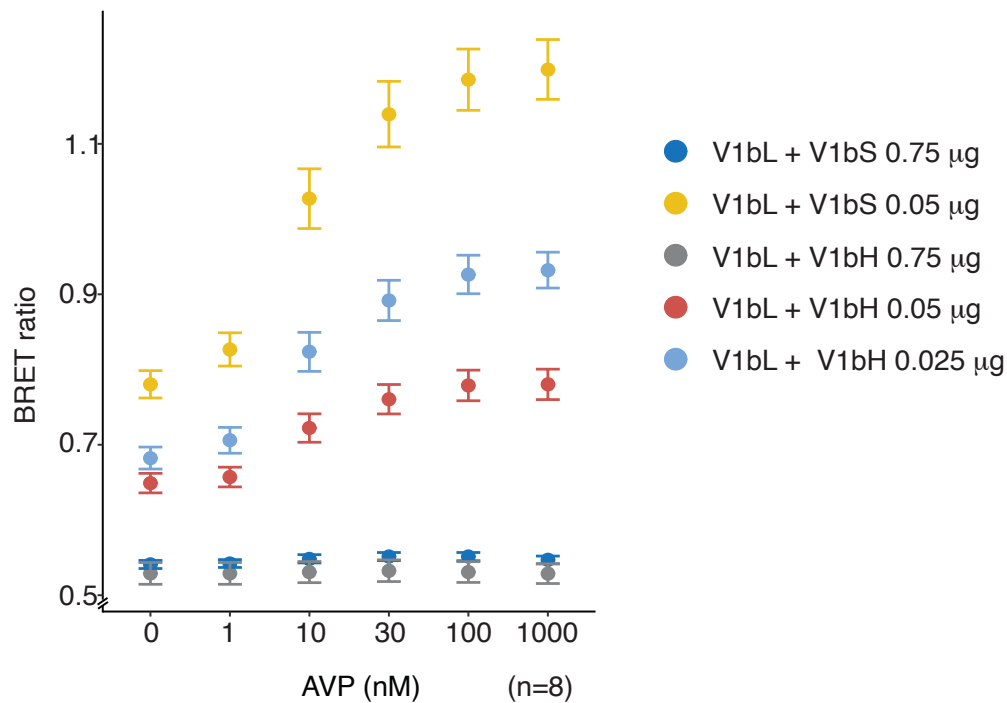


Figure 16. High affinity of HiBit did not increase the amplitude of BRET response.

HEK cells were transfected with the indicated quantities of plasmids for a pair of V1bRs tagged with LgBit and SmBit or with LgBit and HiBit and together with 1.5 μ g of Venus- β -arrestin 2. BRET levels were measured at different concentrations of AVP.

11. High β -arrestin expression increased BRET signals in cells

To determine the effect of β -arrestin expression and agonist stimulation on BRET signal, HEK cells were transiently transfected with various quantities of V1aR-Nluc plasmid with or without Venus- β -arrestin 2 plasmid. Increased agonist-induced BRET and basal BRET of β -arrestin 2 (Figure 17). BRET signals at 530/480 nm did not change the expression levels of V1aR-Nluc, when the plasmid for Venus- β -arrestin 2 was not included in transient expression (Figure 17, left panel).

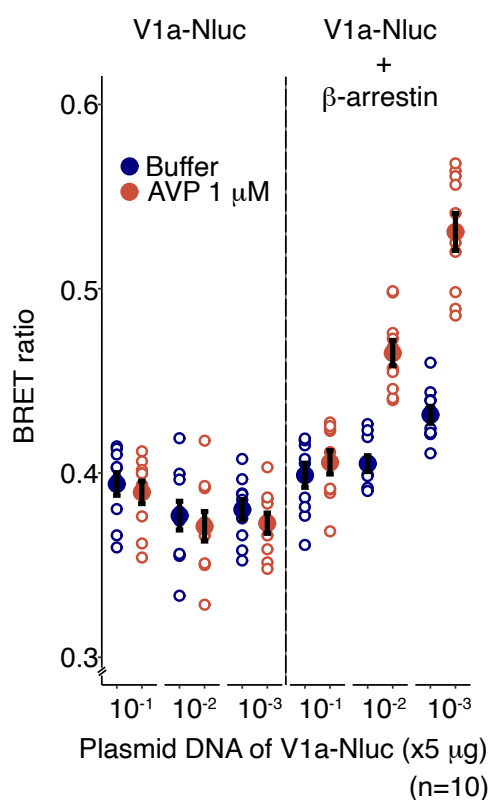


Figure 17. β -arrestin 2 was necessary for increasing BRET signal in cells, which expressed V1aR-Nluc. Cells in a 35-mm dish were transfected with different quantities of plasmid for V1aR-Nluc with or without plasmid for Venus- β -arrestin 2 (1.5 µg). BRET signals were measured after treatment with 1 µM AVP or buffer.

12. Exchanging LgBit and SmBit at receptor C-termini resulted in different BRET responses in cells expressing MOR-V1bR heterodimer and β -arrestin 2

Previous studies have shown a high basal BRET ratio in cells expressing V1bR and MOR tagged with whole luciferase and Venus- β -arrestin 2²¹. To determine the agonist effect on the interactions of the three molecules, HEK cells were transfected with plasmids of the receptors tagged with a pair of split luciferase fragments and a plasmid of Venus- β -arrestin 2. We compared receptor dimers composed of V1bR-LgBit_MOR-SmBit and those of V1bR-SmBit_MOR-LgBit in terms of efficiency to couple with β -arrestin 2. In both agonists for V1bR and MOR, V1bR-LgBit_MOR-SmBit heteromer generated larger responses, as seen in Figure 18a and 18b than the opposite combination of receptor tags.

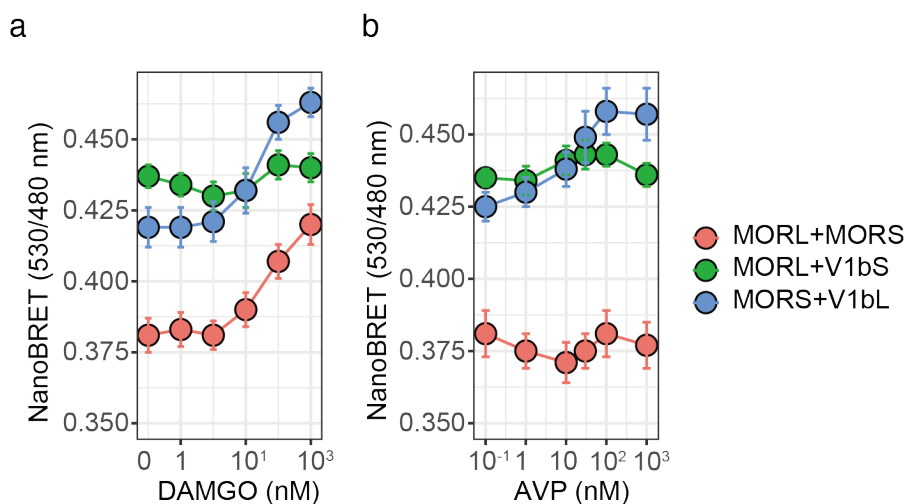


Figure 18. Agonists for MOR and V1b receptors stimulated the access of β -arrestin 2 to MOR-V1b receptor heteromers. Split luciferase components were connected to MOR and V1b receptors. Plasmid DNAs (0.75 μ g for each receptor construct) were co-expressed in HEK cells in 35 mm dish together with 1.5 μ g of Venus- β -arrestin 2. After 48 h the cells were stimulated with the indicated concentrations of (a) DAMGO or (b) AVP.

Discussion

Agonist did not change receptor dimerization

Our experiment demonstrated that the receptors constitutively dimerized independently of the agonist. Ligands may or may not regulate receptor dimerization^{15, 29-31, 32-34}. Because the intrinsic affinity between LgBit and SmBit has been reported to be 190 μ M, its effect was evaluated in our system. To avoid assessing an artificial interaction, we first evaluated interactions between two constructs connected with Nluc and Venus in a preliminary experiment because intrinsic affinity was not reported for this pair of molecular tags. V1a, V1b, and MOR receptors dimerized independently of the tag used for assessment. During agonist stimulation and receptor-receptor interaction, the peak wavelength of the luminescent light and BRET signal at 530/480 nm may change even without Venus- β -arrestin 2. However, this is not the case in our experiments. The interaction between receptor C-termini was stable in receptor homomers and heteromeric V1bRMOR receptors. Therefore, agonist-induced changes in BRET levels in cells indicated interactions between the receptor and β -arrestin 2.

Specific detection of V1aR, V1bR, and MOR dimerization

From the dimerized receptor with split luciferase, specific signal was determined. To exclude the possibility of non-specific interaction between receptors in the plasma membrane, we used the P2X2a purinergic receptor as a negative control³⁵. The P2X2a receptor is an adenosine triphosphate-gated ion channel with four transmembrane receptors. P2X2aR, connected with split luciferases, produced only the background signal, when expressed with seven transmembrane receptors, such as V1aR or MOR. Therefore, our experimental conditions revealed receptor-receptor interactions in the same structural group.

Determination of three-molecular interactions between dimerized receptor and β -arrestin 2

A question that remained in our previous study was why V1bR, in contrast with V1aR, interacted with β -arrestin 2 without agonist and agonist did not increase BRET values. In this study, we determined three molecular interactions between receptor homomers and

β -arrestin 2 in V1aR, V1bR, and MOR. At basal BRET levels, V1b homomeric receptors interacted with β -arrestin 2 even when the expression level of the receptors was high. In contrast, we found that the interaction between V1a homomer and β -arrestin 2 at basal levels was dependent on the ratio of V1aR to β -arrestin 2. AVP stimulation further increased the BRET values in the V1a and V1b homomer receptors. Our study added new information on agonist dependence between a dimerized receptor and β -arrestin 2. To the best of our knowledge, this is the first report, of agonist-dependent access of β -arrestin 2 to V1bR. In our study, we also improved the sensitivity of agonist-dependent BRET responses. Reducing receptor expression and increasing β -arrestin 2 expression both improved BRET levels. We also applied this sensitive method to the analysis of the interaction between MOR-V1bR heteromer and β -arrestin 2, which is involved in chronic morphine tolerance,²¹. Our data showed that these three molecules were in close contact when they were expressed together. Moreover, swapping split luciferase between MOR and V1bR had a significant influence on the BRET response. V1bR-LgBit plus MOR-SmBit had larger BRET signals than those of opposite receptor tags.

Reduced receptor expression and increase in β -arrestin 2 expression resulted in large BRET signals

We developed a new protocol for determining agonist-dependent BRET. Reduced receptor plasmid levels and increased β -arrestin 2 plasmid quantities for transfection both improved BRET responses. To obtain a better response, the quantities of receptor-LgBit and -SmBit plasmids for transfection can be decreased to about 3%. Brightness of Nanoluciferase made it possible to determine this even at low expression levels. The intensities of light increased in accordance with a wide range of expression levels in this study. High β -arrestin 2 levels relative to V1bR in our experiment might be an artificial condition compared with native tissue. However, our sensitive method has an advantage to determine partial agonists and antagonists, which are directed to the AVP binding site in the MOR-V1b receptor.

Dimerized receptor tagged with SmBit effectively showed positive signals than HiBit

HiBit has 11 amino acids and is a part of a split luciferase protein. It couples with LgBit at a higher affinity than SmBit. When V1bR-HiBit and V1bR-SmBit were compared, the V1bR-HiBit showed a lower BRET signal than V1bR-SmBit. This shows that increasing interaction between receptor C-termini did not improve BRET between V1bR homomer and β -arrestin 2. This new finding is useful for designing future BRET experiments.

Conclusion

We assessed three molecular interactions between the receptor dimer and β -arrestin 2. To the best of our knowledge, this is the first report on this subject. It is important to mention that dimerization of receptors should be carefully evaluated prior to the assessment of BRET responses. Agonist-dependence between the dimerized receptor and β -arrestin 2 was validated in our study. From the results on the swapping of a pair of split luciferase, both combinations of tagging to the receptor heteromers should be examined for its efficacy. The agonist effect on the MOR-V1b receptor heteromer is useful in the search for an antagonist, which can reduce the BRET response from the dimerized receptors and β -arrestin 2 and reduce tolerance to morphine analgesia.

Acknowledgement

I have been pursuing this PhD program at Jichi Medical University in Japan over the past four years and I have received a great deal of support.

I would first like to thank my supervisor, Professor Koshimizu Taka-aki, who guided me throughout the four years. He always encouraged me to focus on the experiments and gave me the opportunity to present our research in many conferences. His expertise is invaluable in formulating research questions and methodology. His insightful feedback stimulated me to sharpen my thinking and improved the quality of my work. I thank him for his patience, and for all the opportunities given to improve my research.

I would like to acknowledge my teachers and my friends in the Molecular Pharmacology department of Jichi Medical University. Tsuchiya sensei, Azuma sensei, Mochimaru sensei, and Kaminaga sensei, for their valuable guidance throughout my studies.

I would like to thank Miss Oyama Yuki, who took care of me as a sister throughout my stay in Japan. She helped me not only in the conduction of experiments, but also, my daily life in Japan. She always gave me wise counsel and was always ready to listen to me.

I would also like to thank my tutors, Dr. Vachira Hunprasit and Dr. Praew Kotruchin, who was my Thai guidance teacher throughout my studies, and for the immense help offered during the statistical analysis of my research.

In addition, I would like to thank my family including my parents, my two sisters, and my relatives who live in Thailand. They always sent me words of encouragement and gave me wise counsel. Moreover, I would like to thank my boyfriend (Mr. Krissda Bunaramrueng). He has been very patient in dealing with my bad emotions. He has been with me in moments when I felt disheartened and discouraged in this PhD program. He encouraged me in difficult moments that challenges in life are surmountable. He has been my strength whenever I was weak. Finally, I could not have completed this dissertation without the support of my high school friends (Toon and Ti) and friends from Veterinary University (Jib and Bo), and Japanese, Mongolia, and South-Africa friends whom I met in Japan.

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