Monitoring of Ligand-independent Dimerization and Ligand-induced Conformational Changes of Melatonin Receptors in Living Cells by Bioluminescence Resonance Energy Transfer*\[S\]

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Several G protein-coupled receptors have been shown to exist as homo- and hetero-oligomeric complexes in living cells. However, the link between ligand-induced receptor activation and its oligomerization state as well as the proportion of the total receptor population that can engage in oligomeric complexes remain open questions. Here, the closely related human MT1 and MT2 melatonin receptors (MT1R, MT2R) were used to address these issues. Bioluminescence resonance energy transfer (BRET) experiments in living HEK 293 cells revealed that these receptors form homo- and hetero-oligomers. Constitutive energy transfer was observed for all receptor combinations at physiological expression levels and could be detected in single cell BRET experiments. Inhibition of the energy transfer by dilution of the BRET partners identified MT1R and MT2R dimers as the predominant receptor species, and this oligomerization state did not change upon agonist and antagonist binding. Agonists, neutral antagonists, and inverse agonists all promoted increases in BRET values for MT2R but not for MT1R homodimers in living cells and isolated plasma membranes. This indicates that no correlation could be inferred between the receptor activation state and the dimerization state of the receptor. This also suggests that ligand-promoted BRET increases represent specific ligand-induced conformational changes of pre-existing dimers rather than increased dimerization. The observation that ligands favored the energy transfer within the hetero-oligomer from MT1R to MT2R but not in the reverse orientation, from MT2R to MT1R, supports this view.

Membrane proteins such as tyrosine kinase, cytokine, or transforming growth factor receptors have been known for many years to form oligomers, and the link between their oligomerization and activity states has been well established (1). In contrast, G protein-coupled receptor (GPCR)\[1\] oligomerization has been documented only recently (2), and the relation between receptor activation and oligomerization is still poorly understood. Even the proportion between monomeric and oligomeric receptor species and the exact oligomerization state (dimer, trimer, tetramer, etc.) remains a matter of controversy. Currently, two models are proposed. In the first model GPCR are monomeric in their inactive state, and agonist activation induces the formation of receptor oligomers. This model is based on low basal and strong agonist-induced energy transfer signals observed in fluorescence and bioluminescence resonance energy transfer (FRET, BRET) experiments for the gonadotropin-releasing hormone (9), the somatostatin SSTR5 (4), and the SSTR5/dopamin D2R receptor oligomers (5). The second model proposes that GPCR are constitutively oligomerized and is supported by studies reporting high basal BRET or FRET signals for alpha-mating factor (6), beta2-adrenergic (beta2AR) (7), tyrothrophin-releasing hormone (8), delta-opioid (9), type A cholecystokinin (10), and dopamine D2 receptors (11). Agonist-promoted increases in signals were observed in some of these cases (7, 8, 11) but not in others (6, 9), leading some investigators to suggest that ligand-induced changes in constitutive BRET or FRET could result from conformational changes in pre-existing receptor oligomers rather than from ligand efficacy-related regulation of the oligomerization state (2, 7). If ligand-promoted increases in BRET or FRET reflect changes in oligomerization that are linked to the receptor activation state, one would predict that ligands with different efficacies would lead to distinct changes in energy transfer. In other words, agonists should increase energy transfer, while inverse agonists and neutral antagonists should promote a decrease and no change, respectively.

In the present study, the occurrence of constitutive homo and hetero-oligomerization among MT1R and MT2R was investigated in IHEK 293 cells expressing physiological levels of these receptors using BRET approaches including a novel single cell BRET assay. The oligomerization state and the effect of ligands with distinct signaling efficacy on BRET signals were also systematically assessed to directly test the hypothesis linking receptor activation and oligomerization.

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\[S\] The abbreviations used are: GPCR, G protein-coupled receptor; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein; CYP, cytochrome P450; MTR, melatonin receptor; RLuc, Renilla luciferase.
Plasmid Constructions, Transfections, and Cell Culture—Construction of β2AR-yellow fluorescent protein (YFP) and β2AR-Renilla luciferase (Rluc) fusion proteins and FLAG-tagged β2AR have been described elsewhere (7, 12). The Rluc-YFP fusion protein was generously provided by Dr. B. Sauermann (Institut Cochin, Paris). MTR-β2R MTR-YFP and MTR-β2R MTR-Rluc fusion proteins were constructed by ligating the TdR-N-terminus of the Rluc insert into the C-terminal end of the receptors. For this, the coding regions of MT1R and MT2R were inserted into the cloning sites of the pRL-CMV vector (Promega, Madison, WI) in phase with the Renilla luciferase gene or cloned in phase with the YFP coding region of the Cytoxegen®-Topaze (pGFPfpz-N1) vector (Packard, Meriden, CT). Stop codons were then deleted by site-directed mutagenesis. The DNA sequence encoding the FLAG epitope tag was inserted into the cloning sites of the pRL-CMV vector (Promega, Madison, WI) according to supplier instructions.

Radioligand Binding Experiments—Whole cell radioligand binding assays were performed as described (13). [3H]CYP was used at 200 pmol was used as the radioligand for β2AR and 2-[3H]iodomelatonin ([125I]-Mel) at 400 pmol for MTR (PerkinElmer Life Sciences). Specific binding was defined as that displaced by 10 μM propranolol (ßAR) or 1 μM melatonin. Melatonin-promoted G protein activation was determined in GTPγS binding assays with membranes prepared from HEK 293 cells stably expressing the MT2R as described recently (14).

SDS-PAGE/Immunoblotting—Membranes or immunoprecipitates were denatured in 62.5 mM Tris/HCl (pH 6.8), 5% SDS, 10% glycerol, 0.05% bromophenol blue at room temperature. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis. Immunoblotting was carried out with the monoclonal anti-FLAG M2 antibody (250 ng/ml) (Sigma) or monoclonal anti-Myc-specific 9E10 (1 μg/ml). Immunoreactivity was revealed using appropriate secondary antibodies coupled to horseradish peroxidase and the ECL chemiluminescent reagent (Amersham Biosciences).

Crude Membrane Preparation, Solubilization, and Immunoprecipitation—Crude membranes were prepared, solubilized with 1% digitonin, a detergent known to maintain MTR in a native conformation, and immunoprecipitated as described recently (13, 14) with 10 μg/ml of the FLAG-specific M2 antibody or 1 μg/ml of the polyclonal anti-green fluorescence protein antibody (CLONTECH, Palo Alto, CA). Precipitates were analyzed by assessing luciferase activity in a luminometer using coelenterazine as substrate (Molecular Probes, Eugene, OR). Western analysis was carried out with the monoclonal anti-FLAG M2 antibody or monoclonal anti-Myc-specific 9E10 (1 μg/ml). Immunoreactivity was revealed using appropriate secondary antibodies coupled to horseradish peroxidase and the ECL chemiluminescent reagent (Amersham Biosciences).

RESULTS

Detection of MT1R and MT2R in HEK 293 Cells—Western blot analysis of membranes derived from HEK 293 cells expressing N-terminally FLAG-tagged MT1R or N-terminally Myc-tagged MT2R revealed three groups of immunoreactive bands with apparent molecular masses of 45–60 kDa, 90–120 kDa, and >200 kDa, respectively (Fig. 1A). Given that similar pattern of migration on SDS-PAGE have been suggested to reflect the presence of monomeric, dimeric, and higher oligomeric states for other GPCR (2), the putative oligomerization of the MT2R was assessed using a co-immunoprecipitation approach.

Assessing MT1R and MT2R Oligomerization by Co-immunoprecipitation—We studied MT1R and MT2R oligomerization by co-immunoprecipitation using MT1R and MT2R fused at their C-terminus to either Rluc or YFP. The number of YFP and Rluc receptor expressed was determined for each condition as described for the “Microplate BRET Assay” (see also supplemental material). The total amount of receptor was determined using coelenterazine as substrate (Molecular Probes, Eugene, OR). The energy transfer was determined as described for the microplate BRET assay in the absence and presence of various ligands. Data were fitted by adapting the dimer, trimer, and tetramer model of energy transfer quenching proposed by Veatch and Stryer (15). Assuming that oligomer formation is random between the different receptor species, the following equations can be used: dimer (y = αx), trimer (y = (α – α2)x/α), and tetramer (y = (α – x3)/α) model, y corresponds to the BRET/BRET, x, where BRET energy transfer measured in the absence of competitor. x corresponds to the mole fraction of competitor versus the BRET partners, α corresponds to the value of x when y = 1.1 and x = 1, and y = 1 when x = 0. This value for α was than used in the equations described above to fit experimental data with the different models.

Single Cell BRET Assay—Forty-eight hours after transfection, cells were washed in phosphate-buffered saline. Coelenterazine h was added at a final concentration of 5 μM, and bioluminescence was detected with a modified Olympus upright microscope fitted with a RHE-2PCa epi-fluorescence unit recorded through a plan 40× 0.80 numerical aperture immersion lens. Images were acquired with a cooled Photonic Science (Robertsbridge, UK) extended ISIS video camera either with the Rluc or the YFP filter setting (Rluc filter: 485 ± 10 nm; YFP filter: 530 ± 12.5 nm). Areas corresponding to individual cells were randomly selected with Axon Imaging Workbench software, and the amount of light acquired for each area was integrated over 16 s and quantified for each filter setting. The BRET ratio for each cell was calculated on the basis of the difference of the emission at 530 nm/485 nm of co-transfected Rluc and YFP fusion proteins and the emission at 530 nm/485 nm of the Rluc fusion protein alone as described for the microplate assay.

Microplate BRET Assay—Forty-eight hours posttransfection, HEK 293 cells were detached and washed with phosphate-buffered saline. 1–2 × 105 intact cells or isolated plasma membranes, prepared as described above, were distributed in a 96-well microplate in the absence or presence of ligands at 25 °C. Coelenterazine h substrate (Molecular Probes) was added at a final concentration of 5 μM, and readings were performed with a lumino/fluorometer (Fusion™, Packard Instrument Company) that allows the sequential integration of luminescence signals detected with two filter settings (Rluc filter, 485 ± 10 nm; YFP filter, 530 ± 12.5 nm). The BRET ratio was defined as the difference of the emission at 530 nm/485 nm of co-transfected Rluc and YFP fusion proteins and the emission at 530 nm/485 nm of the Rluc fusion protein alone. Results were expressed in milliBRET units, 1 milliBRET corresponding to the BRET ratio values multiplied by 1000. The amount of Rluc and YFP fusion receptor expressed was determined for each condition. Maximal luciferase activity was used to determine the amount of YFP fluorescence excited by exogenous YFP excitation to determine the amount of YFP fusion receptors. Luciferase activity and YFP fluorescence were converted into receptor numbers in standard curves performed in parallel experiments relating fluorescence and luminescence signals to radioligand binding (saturating concentration) (see supplemental material at http://www.jbc.org).
Monitoring of Ligand-independent Dimerization

**Fig. 1. Detection of MT1R and MT2R oligomers.** Panel a, crude membranes of FLAG-MT1-, Myc-MT2-expressing, or mock-transfected HEK 293 cells were submitted to SDS-PAGE, and immunoreactivity was revealed using anti-FLAG M2 or anti-Myc 9E10 antibodies. HEK 293 cells were submitted to SDS-PAGE, and immunoreactivity for each antibody. Data are representative of three experiments.

Panel b, membranes from HEK 293 cells co-expressing the indicated receptors were solubilized and lysates adjusted to the same amount of luciferase activity. Receptors were then immunoprecipitated with the indicated antibodies and luciferase activity determined in precipitates. Values are presented as % of maximal amount of precipitated luciferase activity for each antibody. Data are representative of three experiments.

**Fig. 2. Constitutive BRET of MTR in living HEK 293 cells.** Panels a and b, the indicated fusion proteins were expressed at a 1:1 protein ratio at 20–100 fmol/mg of protein in HEK 293 cells as determined using standard curves correlating 125I-Mel binding sites with luminescence or YFP fluorescence (see supplemental material). Energy transfer measurements were performed in living cells by adding 5 μM coelenterazine and measuring light emission in a luminometer with Rluc and YFP filter settings according to the microplate BRET assay protocol described under “Materials and Methods.” Data are means ± S.E. of at least three independent experiments each performed in triplicate.

Oligomerization was assessed by the pairwise expression of receptor-Rluc and -YFP fusions, followed by precipitation using anti-green fluorescence protein antibodies and measuring co-immunoprecipitated luciferase activity. The results presented in Fig. 1b indicate that both MT1R and MT2R homo-oligomers can form.

To rule out the possibility that co-immunoprecipitation could result from spurious, receptor-independent, interaction between the YFP and the Rluc, co-immunoprecipitation was also carried out using N-terminally FLAG-tagged receptors co-expressed with C-terminal Rluc fusion receptors. Co-immunoprecipitation of melatonin receptor oligomers was confirmed as was the specificity of the interaction shown by the absence of luciferase activity in precipitates prepared from cells co-expressing FLAG-tagged β2AR and MT1R- or MT2R-Rluc fusion proteins (Fig. 1b).

**Detection of Constitutive MTR Oligomers in Living HEK 293 Cells by BRET**—To study receptor oligomerization in intact cells, we chose BRET, a technique that has recently been used to monitor protein-protein interactions (7–10). The assay relies on the observation that the degree of physical proximity between molecules can be assessed in living cells by the level of energy transfer occurring between the energy donor Rluc and a fluorescent acceptor such as the YFP (17). To monitor the transfer of energy between the MTR fusion proteins, equimolar amounts of the Rluc and YFP constructs (as assessed by generating standard curves relating fluorescence and luminescence signals to radioligand binding sites; see supplemental material) were co-expressed in HEK 293 cells. As shown in Fig 2, a and b, significant energy transfer was observed in living cells co-expressing MT1R-Rluc and MT1R-YFP as well as MT2R-Rluc and MT2R-YFP indicating that constitutive homo-oligomers exist for both receptor subtypes. Energy transfer levels detected were very similar to those observed for the β2AR-YFP/β2AR-Rluc couple (Fig. 2a) previously shown to form constitutive oligomers (7, 9, 10). Significant BRET between MT1R-Rluc and MT1R-YFP (Fig. 2a) as well as between MT2R-Rluc and MT1R-YFP (Fig. 2b) also indicated the occurrence of MT1R/MT2R hetero-oligomers when these receptor subtypes are co-expressed. The specificity of these interactions is illustrated by the absence of significant energy transfer between MT2R-Rluc and either β2AR-YFP or YFP alone.

At equimolar concentrations, the level of BRET detected was independent of the receptor expression levels (data not shown). BRET was observed at receptor levels as low as 20 fmol/mg of protein, a concentration similar to those observed in native tissues such as the retina (70 fmol/mg) (18) and the hypophysal pars tuberalis (40 fmol/mg) (19) indicating that the constitutive homo- and hetero-dimerization detected by BRET did not result from over-expression and could reflect a physiological reality.

**MTR Exist Predominantly as Constitutive Dimers**—We next wanted to determine the proportion and the nature (dimer, trimer, tetramer, etc.) of the oligomeric receptor fraction. BRET competition assays using non-tagged MTR were carried out to titrate receptor oligomers. For this, constant and equimolar amounts of MTR-YFP and MTR-Rluc (BRET receptors) were...
co-expressed with increasing concentration of the non-tagged receptor (competitor). The amount of each receptor species was determined for each data point as described under "Material and Methods" (see also supplemental material). Data were means ± S.E. of 10–20 randomly selected cells (*, p < 0.05).

Detection of BRET in Single Cells—The degree of constitutive energy transfer detected between GPCR has been suggested to result from overexpression of the receptors (4, 8). Although the data presented above indicate that constitutive energy transfer detected between GPCR has been suggested to result from overexpression of the receptors (4, 8). Although the data presented above indicate that constitutive energy transfer detected between GPCR has been suggested to result from overexpression of the receptors (4, 8). Although the data presented above indicate that constitutive energy transfer detected between GPCR has been suggested to result from overexpression of the receptors (4, 8).

Fig. 4. Measuring BRET in single cells. Panel a, HEK 293 cells expressing the indicated proteins were incubated with 5 μM coelenterazine, and light emission was monitored in a microscope coupled to a video camera using the Rluc and YFP filter settings. Areas corresponding to individual cells were identified, and their BRET ratio was calculated by quantifying the luminescence measured with both filter settings according to the single cell BRET assay protocol. Data are means ± S.E. of 10–20 randomly selected cells (*, p < 0.05). Panel b, comparison of BRET measured in single HEK 293 cells (single cell BRET assay) and in cell populations (microplate BRET assay). Cells expressing different ratios of β2AR-Rluc and β2AR-YFP proteins were used. The amount of β2AR-Rluc and β2AR-YFP fusion proteins expressed for each condition was determined using standard curves correlating 125I-CYP binding sites with luminescence or YFP fluorescence (see supplemental material). Data are means ± S.E. of 10 randomly selected cells.
different YFP/RLuc fusion protein ratios. As can be seen in Fig. 4b, the single cell BRET measured by a microscope was identical to the BRET determined on the total population of cells using the microplate assay format, and this for all β2AR-YFP/β2AR-RLuc ratios. This indicates that the mean BRET values observed in heterogeneous population of cells do not reflect events occurring in a small subpopulation of cells expressing higher levels of receptors. Interestingly, BRET increased linearly (r = 0.926) with increasing YFP/RLuc fusion protein ratios. This is expected because the BRET will increase as a function of the proportion of RLuc fusion interacting with YFP fusion proteins until all available RLuc-receptors are bound by YFP-receptors. Such saturation of BRET occurred at β2AR-YFP/β2AR-RLuc ratios of 3 (data not shown).

**Ligand-promoted Change in BRET—**Agonist-promoted FRET or BRET observed for several GPCR have been attributed to an increased oligomer formation by many authors (3–5, 7, 8, 11). However, several studies failed to detect such changes in BRET or FRET signals upon agonist stimulation of certain receptors (6, 9). If agonist-promoted increase in energy transfer truly reflects an increased dimerization, one would need to argue that a relation between receptor dimerization and activation exists for some receptors but not for others. Alternatively, agonist-promoted changes in energy transfer, could reflect conformational changes within pre-existing dimers that could be detected for some constructs but not others. In an effort to formally test these two possibilities, the effects of ligands with various efficacies were assessed on the BRET signals for MT1R and MT2R homo-dimers in whole cells. As shown in Fig. 5a, melatonin stimulation promoted a dose-dependent increase in BRET for the MT2 homodimer with an EC50 value of 1 nM, consistent with the affinity of melatonin for this receptor (20). In contrast, no change in constitutive BRET was observed for the MT1R homo-dimer. Similarly, the synthetic non-selective MTR agonist S20928 increased BRET for MT2R (Fig. 5b) but was without effect on MT1R (not shown). These data could be interpreted as an indication that receptor activation is accompanied by increased receptor dimerization for MT2R but not MT1R. Alternatively, conformational changes imposed by agonists may be responsible for the increase in BRET by changing the position and/or orientation of the RLuc and YFP moieties within pre-existing MT2R dimers. The lack of change in BRET for the MT1R would then indicate that the conformational switch within this dimer is not accompanied by significant changes in the position and/or orientation of RLuc and YFP moieties.

To determine whether the agonist-promoted change in BRET observed for the MT2R is linked to the activation process, the signaling efficacy, as assessed in a GTPγS binding assay and the effects on BRET signals, were determined in parallel for a series of MTR ligands. In agreement with published data (20), GTPγS binding experiments classified S20928 as neutral antagonists, 4P-PDOT as a weak inverse agonist, and melatonin and S20098 as full agonists (Fig. 5b). All compounds increased BRET for the MT2R homo-dimer (Fig. 5b) but had no detectable effect on MT1R in intact cells (data not shown). To exclude the possibility that the lack of effect of the ligands on the MT1R could result from the inaccessibility of large intracellular receptor population, BRET measurements were carried out in isolated plasma membrane preparations. As shown in Fig. 5c, identical results were obtained. Basal energy transfer was obtained for both MT1R and MT2R homo-dimers, while ligands promoted BRET increase for the MT2R but not the MT1R. In addition, to exclude receptor inaccessibility as the cause for the ligand-insensitive BRET of the MT1R homo-dimer, these results demonstrate that the ligand-promoted BRET changes observed in whole cells for the MT2R did not result from alterations in local pH (a parameter that could influence energy transfer efficacy) because they could be recapitulated in buffered membrane preparations. Taken together, the observations that agonist, neutral antagonists, and inverse agonists all cause an increase in the MT2R homo-dimer BRET signal clearly dissociate BRET changes from receptor activation.

Based on the conclusion reached above that most if not all MT2R receptors exist as constitutive dimers, ligand-induced change in the receptor monomer/dimer ratio is unlikely to explain the BRET increases caused by the ligands. However, a ligand-promoted change from dimer to higher oligomeric states can not be excluded. To address this possibility, we performed
When considering the effects of the ligands on the BRET signals observed for the MT1R/MT2R hetero-dimer drastically different results were obtained for the two possible transfer orientations. Whereas energy transfer from MT2R-Rluc toward MT1R-YFP was ligand-insensitive, all compounds increased BRET for the reverse configuration (i.e. MT1R-Rluc toward MT2R-YFP) (Fig. 7). This cannot be explained by the loss of ligand binding to these receptors because all fusion proteins retained high affinity ligand binding. Once more, these data are incompatible with the notion that the increase in BRET signal reflects ligand-promoted dimerization because equivalent results would be expected for the two hetero-dimer combinations. However, this result is entirely compatible with the notion that the BRET changes may reflect conformational changes within pre-existing dimers that can be more easily detected for specific pair orientations due to structural constraints that may or may not translate conformational changes into more favorable dipole orientation between energy donors and acceptors.

**DISCUSSION**

Taken together, the data presented here demonstrate that MT1R and MT2R can exist as constitutive homo- and hetero-oligomers in living cells. Results from quantitative BRET competition experiments revealed that, when expressed at physiological levels, most if not all MTR are organized in specific receptor dimers under basal as well as agonist-stimulated conditions. The ligand-induced changes in BRET observed for the MT2R homo-dimer and for the MT1R/MT2R hetero-dimer most likely reflect conformational changes within pre-existing dimers that cannot be directly correlated with the activation state of the receptor.

Several lines of evidence suggest that constitutive oligomerization is a general feature of GPCR (2). Such basal oligomerization has been reported for the b2-adrenergic (7, 9, and this report), the a-mating factor (6), the gonadotropin releasing hormone (3), the somatostatin (4), the thyrotropin-releasing hormone (8), the d-opioid (9), the type A cholecystokinin (10), and the dopamine D2 (11) receptors. In particular, constitutive hetero-oligomerization between GABA_AR1 and GABA_AR2 was shown to be a prerequisite for the expression of a functional metabotropic GABA_AR (21). Dominant negative effects of several GPCR mutants have also been explained by their ability to form constitutive oligomers with wild-type receptors thus interfering with the normal trafficking and cell surface expression of the latter (22–24). Detailed pharmacological characterization of several dopamine ligands also led some authors to conclude to the existence of constitutive D2-dopamine receptor dimers or oligomers (25). Constitutive homo-oligomerization of cell surface proteins is not restricted to GPCR, and several examples including the human serotonin transporter, the rat GABA transporter 1 and the erythropoietin receptor have been reported (26–28).
However, the occurrence of constitutive GPCR oligomerization has not been universally observed. Indeed, several studies that used energy transfer approaches concluded that no or very little constitutive oligomerization occurs for some GPCR but that it could be induced by agonist treatment (4, 5, 8). This lack of evidence of constitutive oligomerization may either reflect specific characteristics of the receptors considered in these studies or be due to experimental conditions that did not allow detection of constitutive oligomerization. In that respect, it should be emphasized that the lack of energy transfer is not a proof of the absence of oligomerization because the energy donor and acceptor may be too far apart or inadequately oriented within the oligomer to allow efficient transfer. The ligand-promoted energy transfer could then result from a change in conformation that now places the donor and acceptor into permissive conditions. In most of these studies, a link between agonist-induced increase in energy transfer, the state of oligomerization, and the activation of the receptor were inferred. No such link could be established for the MT2R in the present study because no correlation existed between ligand signaling efficacy and their ability to promote BRET. Taken with the observation that, under basal conditions, dimers represented a preponderant fraction of the total receptor population, we conclude that conformational changes within pre-existing dimers most likely underlie ligand-promoted changes in BRET. The observation that ligands promoted an increase in BRET for the MT1R/MT1-YFP and MT2-Rluc/MT2-YFP pairs. This suggests that the energy transfer changes reflect the stabilizing energy transfer on both the distance between the donor and acceptor and their relative orientation (29) makes it very plausible that slight structural differences between the fusion proteins may account for the different sensitivity to conformational changes.

Given that agonists, neutral antagonists, and inverse agonists are believed to promote/stabilize distinct receptor conformations (30, 31), it may be surprising that representatives of these three classes of ligands all led to increases in BRET for MT2-Rluc/MT2-YFP and MT1Rluc/MT2-YFP pairs. This suggests that the energy transfer changes reflect the stabilizing effects of ligand binding rather than conformational changes linked to the activation or inactivation processes. One could hypothesize that ligand binding increases BRET by restraining the conformational space of receptors thus decreasing the mean distance between the energy donor and acceptor. In a recent study, Boute et al. (32) demonstrated the usefulness of BRET to monitor ligand-promoted conformational changes within the preformed insulin receptor dimer. However, the observation, in the present study, that conformational changes can be translated in BRET variations for some receptor pairs but not for others, indicates that the use of BRET as a conformational sensor will need to be evaluated on a case by case basis.

The observation that MT1R and MT2R are predominantly expressed as dimers in living cells is consistent with the notion that they represent functional signaling units. Thus, although receptor dimerization is not modulated by ligand binding, dimers may still be necessary for biological function. This is supported by the observation that peptides that interfered with β2AR dimerization also inhibited agonist-promoted signaling (33). In addition to signaling, receptor dimerization may also play a role in trafficking as has been shown for the GABA_A receptor (21).

The herein demonstration of MT1R/MT2R hetero-dimerization is the latest of a growing list that suggest that it could be a general trait of GPCR. Although the physiological significance of MT1R/MT2R hetero-dimerization remains to be investigated, co-expression of both subtypes in hypothalamic supra-chiasmatic nuclei (34) (where they regulate the circadian clock (35)) and in the retina (where they inhibit dopamine release (36)) suggests that hetero-oligomerization could occur in native mammalian tissues.

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