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Preferential Formation of MT_1/MT_2 Melatonin Receptor Heterodimers with Distinct Ligand Interaction Properties Compared with MT_2 Homodimers[S]

Mohammed A. Ayoub, Angélique Levoye, Philippe Delagrange, and Ralf Jockers

Department of Cell Biology, Institut Cochin, Paris, France (M.A.A., A.L., R.J.); and Institut de Recherche Servier, Suresnes, France (P.D.)

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ABSTRACT

Heterodimerization has been documented for several members of the G protein-coupled receptor (GPCR) superfamily, including the closely related MT_1 and MT_2 melatonin receptors. However, the relative abundance of hetero- versus homodimers and the specific properties, which can be attributed to each form, are difficult to determine. Using a bioluminescence resonance energy transfer (BRET) donor saturation assay, we show that half-maximal MT_1/MT_2 heterodimer formation is reached for expression levels as low as ~4000 receptors per cell. The relative propensity of MT_1 homodimer and MT_1/MT_2 heterodimer formation are similar, whereas that for the MT_2 homodimer formation is 3- to 4-fold lower. These data indicate that both the relative expression level of each receptor isoform and the affinities between monomers may determine the actual proportion of homo- and heterodimers. The specific interaction of ligands with the MT_1/MT_2 heterodimer was studied using a BRET-based assay as a readout for the conformational changes of the heterodimer. An MT_1/MT_2 heterodimer-specific profile and ligands selective for the MT_1/MT_2 heterodimer compared with the MT_2 homodimer could be identified. Classic radioligand binding and BRET studies suggest that heterodimers contain two functional ligand binding sites that maintain their respective selectivity for MT_1 and MT_2 ligands. Occupation of either binding site is sufficient to induce a conformational change within the heterodimer. Taken together, these results show that the probability of GPCR heterodimer formation may be equal to or even higher than that of the corresponding homodimers and that specific properties of heterodimers can be revealed using a BRET-based ligand/receptor interaction assay.

A growing number of observations suggest that G protein-coupled receptors (GPCR) form homodimers and heterodimerize with other members of the same receptor superfamily. Heterodimerization may have important consequences in terms of receptor function, because significant changes in ligand binding, signaling, or trafficking were observed for several heterodimers (Gazi et al., 2002). Considering that multiple receptors are expressed simultaneously in tissues and cells, it is reasonable to assume that most cells coexpress several different GPCRs that may be engaged in heterodimeric complexes. So far, little is known about the rules that govern homo- and heterodimer formation. The homo-/heterodimer ratio is expected to depend on the relative affinity of receptor subtypes for each other and on the expression level of the interacting partners. Most studies on GPCR heterodimerization did not examine the proportion of heterodimers versus homodimers. In addition, these studies were principally performed in cells expressing supraphysiological levels of recombinant receptors, in which the formation of GPCR heterodimers might be overestimated, compared with normal tissues that express endogenous levels of receptors. Recent quantitative BRET-based studies in transfected cells expressing high concentrations (1–10 pmol/mg of protein) of β_1- and β_2-adrenergic receptors (Mercier et al., 2002) or oxytocin and vasopressin receptors (Terrillon et

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ABBREVIATIONS: GPCR, G protein-coupled receptor; BRET, bioluminescence resonance energy transfer; Rluc, *Renilla reniformis* luciferase; YFP, yellow fluorescent protein, ^{125}I-MLT, 2[^{125}I]iodomelatonin; S20098, *N*-[2-(7-methoxynapht-1-yl)ethyl]acetamide; S20928, *N*-[2-(1-naphtyl) ethyl]cyclobutanecarboxamide; S22153, *N*-[2-(5-ethylbenzo[*b*]thiophen-3-yl)ethyl]acetamide; S24773, *N*-{2-[3-(3-aminophenyl)-7-methoxy-1-naphtyl]ethyl}acetamide; S26284, *N*-(2-{7-[4-({8-[2-acetylamino)ethyl]-2-naphtyl}oxy)butoxy]-1-naphtyl}ethyl)acetamide; 4P-PDOT, 4-phenyl-2-proprionamidotetraline; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; $BRET_{50}$, half-maximal bioluminescence resonance energy transfer; α_{1D}-AR, α_{1D}-adrenergic receptor.

al., 2003) suggested that the probability of forming homo- and heterodimers is similar.

Ligand binding properties of receptors may change when they are engaged in heterodimeric complexes (Jordan and Devi, 1999). Because GPCRs are major pharmacological targets, the discovery of specific ligand binding profiles for heterodimers may have important implications for the development and screening of new drugs. However, the determination of a specific binding profile for heterodimers is difficult to establish using classic radioligand competition binding assays. This limitation is particularly true for heterodimers composed of two receptors, which display similar affinities for the same radioligands.

The interaction between ligand and receptor can be studied with alternative approaches that measure the conformational changes of ligand-bound receptors such as fluorescence and electron paramagnetic resonance spectroscopy (Farrens et al., 1996; Ghanouni et al., 2001; Lee et al., 1997). Indeed, the efficiency to promote specific ligand-induced conformations (EC_{50}) is correlated, in theory, with the binding affinities of the ligands (Kenakin and Onaran, 2002). Recently, resonance energy transfer techniques, such as fluorescence resonance energy transfer and bioluminescence resonance energy transfer (BRET), have also emerged as sensitive approaches to monitor conformational changes of a wide range of proteins in living cells, including membrane receptors (Truong and Ikura, 2001; Heyduk, 2002). Energy transfer occurs if the energy donor is in close proximity (10–100 Å) to the energy acceptor and if the respective orientation of donor and acceptor is appropriate (Truong and Ikura, 2001; Heyduk, 2002). The extreme sensitivity to relatively small perturbations makes this technique an attractive approach for detecting receptor conformational changes. Two strategies have been used to study a protein of interest with this approach. In the first, both energy donor and acceptor may be fused to the same protein to be studied (intramolecular energy transfer), as reported previously to monitor calcium- and cAMP-dependent signaling, phosphorylation (Truong and Ikura, 2001; Heyduk, 2002), or the activation of parathyroid hormone and α_{2A}-adrenergic receptors (Vilardaga et al., 2003). The second strategy takes advantage of the fact that most receptors exist as dimers. The coexpression of two receptors, one fused to the energy donor and the second to the acceptor, allows for the monitoring of ligand-induced conformational changes within constitutive receptor dimers (intermolecular energy transfer) (Truong and Ikura, 2001; Heyduk, 2002). Such an approach was used to study the insulin receptor (Boute et al., 2001), the leptin receptor (Couturier and Jockers, 2003), and several GPCRs (Angers et al., 2000; Rocheville et al., 2000a; Kroeger et al., 2001). Stimulation of these receptors with the appropriate hormones modified the constitutive energy transfer in a dose-dependent manner, supporting the idea that the conformational changes modify the distance and/or the orientation between the two BRET partners.

Using a BRET-based approach, we have shown recently that MT_1 and MT_2 melatonin receptors, which share 70% sequence homology, form both homo- and heterodimers (Ayoub et al., 2002). Here, we report that MT_1/MT_2 heterodimers constitute a significant proportion of overall dimers, which can be distinguished from homodimers in living cells by their ligand-receptor interaction profile determined by a proximity-based BRET-assay.

Materials and Methods

Materials. Compounds were obtained from the following sources: melatonin was from Sigma (St Louis, MO) S20098, S20928, S22153, S24773, and S26284 were from the Institut de Recherche Servier (Suresnes, France) (Audinot et al., 2003); 2-iodomelatonin was from Sigma/RBI (Natick, MA); and luzindole (2-benzyl *N*-acetyltryptamine) and 4-phenyl-2-proprionamidotetraline (4P-PDOT) were from Tocris Cookson Inc. (Ellisville, MO).

Plasmid Constructions, Transfections, and Cell Culture. Construction of Rluc and YFP fusion proteins and Flag-MT1 and Myc- MT_2 constructs have been described elsewhere (Ayoub et al., 2002). HEK 293 cells were grown in complete medium [Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 4.5 g/l glucose, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1 mM glutamine] (all from Invitrogen, Carlsbad, CA). Transient and stable transfections were performed using the transfection reagent FuGene 6 (Roche Diagnostics, Basel, Switzerland) according to the supplier's instructions.

Radioligand Binding Experiments. Whole-cell radioligand binding assays were performed as described previously (Brydon et al., 1999b). Radioligand binding assays were performed in PBS, pH 7.4, using the lipophilic 2[^{125}I]iodomelatonin (^{125}I-MLT) (PerkinElmer Life and Analytical Sciences, Boston, MA) at 25 to 1000 pM as radioligand in saturation experiments. Specific binding was defined as binding displaced by 10 μM melatonin. Competition binding assays were carried out at 100 to 200 pM ^{125}I-MLT and with increasing concentrations of different compounds. Assays were carried out for 60 min at 37°C and then terminated by rapid filtration through Whatman GF/F glass-fiber filters (Whatman, Clifton, NJ) previously soaked in PBS, and filters were counted in a γ-counter. Competition curves were fitted using a one- or two-site nonlinear regression (GraphPad Prism; GraphPad Software Inc., San Diego, CA). IC_{50} values were transformed into K_i values using the Cheng-Prussoff formula: $K_i = IC_{50}/[1 + (L/K_d)]$, where L corresponds to the ^{125}I-MLT concentration, and K_d corresponds to the respective values obtained in^{125}I-MLT saturation binding assays.

Crude Membrane Preparation, Solubilization, and Immunoprecipitation. Crude membranes were prepared, solubilized with 1% digitonin, a detergent known to maintain melatonin receptors in a native conformation, and immunoprecipitated as described previously (Brydon et al., 1999a; Roka et al., 1999) with 2 μg/ml of the monoclonal anti c-Myc 9E10 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

SDS-PAGE/Immunoblotting. Immunoprecipitates were denatured over night in 62.5 mM Tris/HCl, pH 6.8, 5% SDS, and 10% glycerol and 0.05% bromphenol blue at room temperature. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis was carried out with the polyclonal anti-Flag antibody (2 μg/ml) (Sigma). Immunoreactivity was revealed using appropriate secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence reagent (Amersham Biosciences Inc., Aylesbury, UK).

Microplate BRET Assay. Forty-eight hours after transfection, HEK 293 cells were detached and washed with PBS. Intact cells ($1–2 \times 10^5$) were distributed in a 96-well microplate and incubated for 10 min at 25°C in the absence or presence of the indicated ligands. Coelenterazine H substrate (Molecular Probes, Eugene, OR) was added at a final concentration of 5 μM, and readings were performed with a lumino/fluorometer (Fusion; PerkinElmer) that allows the sequential integration of luminescence signals detected with two filter settings (Rluc filter, 485 ± 10 nm; YFP filter, 530 ± 12.5 nm) as described previously (Ayoub et al., 2002). The EC_{50} was defined as the ligand concentration necessary to promote 50% of the maximal ligand-induced BRET signal.

Correlation of Fluorescence and Luminescence Levels of Receptor Fusion Proteins to ^{125}I-MLT Binding Sites. Luminescence and fluorescence levels of several luciferase and green fluorescent protein receptor fusion proteins have been shown to be linearly correlated to receptor numbers (McVey et al., 2001; Ayoub et al., 2002; Mercier et al., 2002; Couturier and Jockers, 2003). Because this correlation is an intrinsic characteristic of each fusion protein, correlation curves have to be established for each construct. HEK 293 cells were transfected with increasing DNA concentrations of the melatonin receptor Rluc or YFP fusion protein constructs. Maximal luminescence was determined at 485 ± 10 nm (gain, 4; photomultiplier, 1100 V; 1.0 s) in 96-well optiplates using coelenterazine H (5 μM) as substrate in Rluc-expressing cells, and fluorescence obtained upon exogenous YFP excitation (gain, 4; photomultiplier, 1100 V; 1.0 s) was measured in 96-well HTRF plates (PerkinElmer) in YFP-expressing cells with the lumino/fluorometer Fusion. Background luminescence and fluorescence determined in wells containing untransfected cells was subtracted. To correlate the luminescence and the fluorescence values with relative receptor numbers, the total number of ^{125}I-MLT binding sites was determined in the same cells as described under "Radioligand Binding Experiments". Luminescence and fluorescence were plotted against binding sites, and linear regression curves were generated (Fig. 1 of supplemental material). To determine the expression level of YFP versus Rluc fusion proteins in cells coexpressing both proteins, the maximal luciferase activity and fluorescence were determined using the same parameters as described above, and the YFP/Rluc ratio was calculated using the corresponding standard curves. Reliable quantification of luciferase activity was possible under conditions of energy transfer between YFP and Rluc fusion proteins because the amount of energy transfer observed in the presence of YFP fusion receptors was negligible compared with the luciferase signal. Luciferase activity remained constant under conditions in which the basal energy transfer increased 1.5- to 3-fold in the presence of melatonin (see Fig. 2 of supplemental material).

Results

Evidence for MT_1/MT_2 Heterodimerization from Coimmunoprecipitation and BRET Experiments. In a previous study, we reported, using a BRET-based approach, that both MT_1 and MT_2 melatonin receptors form homodimers in living HEK 293 cells (Ayoub et al., 2002). In addition, our data suggested that these receptors may also form heterodimers. To extend these observations, coimmunoprecipitation experiments were carried out with epitope-tagged receptors. The human MT_1 receptor tagged with a Flag at its N terminus was transiently expressed in HEK 293 cells, which stably express 25 fmol/mg of total protein of MT_2 receptors tagged with an Myc epitope at their N terminus. In accordance with previous observations (Ayoub et al., 2002), Western blot analysis of membranes prepared from these cells with an anti-Flag antibody revealed two major immunoreactive forms with apparent molecular masses of 55 and 110 kDa, probably corresponding to the monomeric and the dimeric forms of the Flag-MT_1 (Fig. 1A, mb). The Flag-MT_1 was also pulled down by the immunoprecipitation of the Myc-tagged MT_2 receptor, confirming the existence of MT_1/MT_2 heterodimers in cell lysates (Fig. 1A, IP). MT_1/MT_2 heterodimerization was further studied in intact cells by BRET. Wild-type human MT_1 and MT_2 receptors were tagged at their C terminus with either Rluc (BRET donor) or YFP (BRET acceptor). Fusion proteins retained both their ligand binding (Table 1) and signaling properties (Ayoub et al., 2002). Two different combinations of MT_1 and MT_2 fused to either BRET donor or acceptor were studied. A significant BRET signal was observed for both combinations (Fig. 1B). The specificity of these signals is illustrated by the absence of significant transfer between MT_1-Rluc or MT_2-Rluc and a control β_2-adrenergic receptor YFP fusion protein expressed at comparable levels. Taken together, these data confirm our previous observation that MT_1/MT_2 heterodimers are formed in intact HEK 293 cells.

Evaluation of the Proportion of MT_1 and MT_2 Homo- and Heterodimers in Living Cells. Although it is clear that MT_1 and MT_2 homo- and heterodimers are formed in HEK 293 cells, the actual proportion of homo- and heterodimers present in these cells remains unknown. To address this question, we measured the relative tendency of forming homo- and heterodimers in living cells with a BRET donor saturation assay (Mercier et al., 2002; Couturier and Jockers, 2003). Cells were cotransfected with constant amounts of cDNA coding for the BRET donor (receptor fused to Rluc) and increasing quantities of cDNA for the BRET acceptor (receptor fused to YFP). The amount of each receptor species effectively expressed in transfected cells was de-

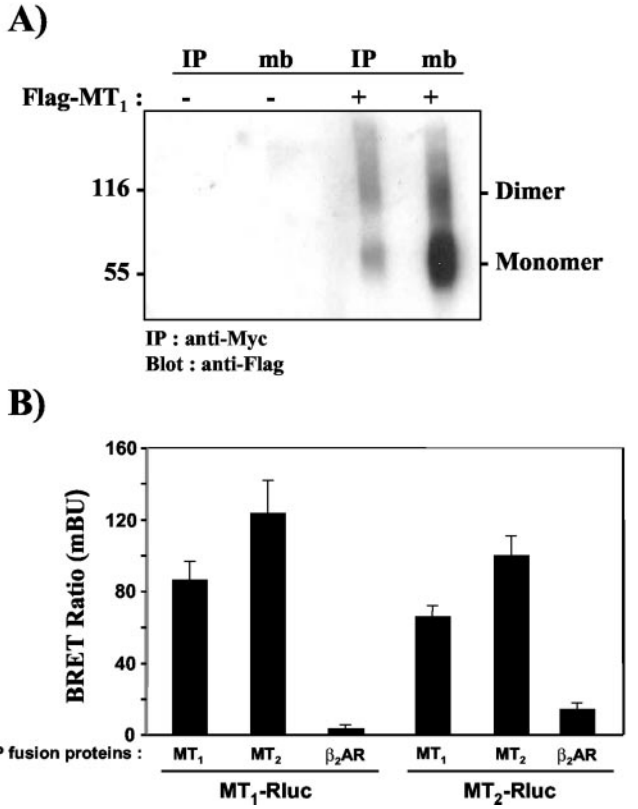

Fig. 1. Detection of MT_1/MT_2 heterodimers. A, HEK 293 cells stably expressing Myc-MT_2 were transiently transfected or not with the Flag-MT_1 construct, and crude membranes were prepared. Receptors were immunoprecipitated with a monoclonal anti-Myc antibody as described under *Materials and Methods*. Membranes and immunoprecipitates were then submitted to SDS-PAGE and revealed by Western blot analysis using a polyclonal anti-Flag antibody. B, the indicated Rluc and YFP fusion proteins were expressed at a 1:10 protein ratio in HEK 293 cells as determined using standard curves correlating ^{125}I-MLT binding sites with luminescence or YFP fluorescence (Fig. 1 of supplemental material). Energy-transfer measurements were performed in living cells by adding coelenterazine and measuring light emission in a luminometer with Rluc and YFP filter settings as described under *Materials and Methods*. Data are means ± S.E.M. of at least three independent experiments each performed in duplicate.

termined, for each individual experiment, by correlating both luminescence and fluorescence signals with ^{125}I-MLT binding sites (Fig. 1 of supplemental material). As shown in Fig. 2, BRET signals increased as a hyperbolic function of the ratio between the BRET acceptor and the BRET donor, reaching an asymptote that corresponds to the saturation of all BRET donor molecules by acceptor molecules. Assuming that the association of interacting proteins, fused to the BRET donor and the BRET acceptor, respectively, is random, the amount of acceptor required to obtain the half-maximal BRET ($BRET_{50}$) for a given amount of donor reflects the relative affinity of the two partners (Mercier et al., 2002; Terrillon et al., 2003). Comparable $BRET_{50}$ values were observed for the MT_1 homodimer and MT_1/MT_2 heterodimer formation, whereas the $BRET_{50}$ value for MT_2 homodimers was 3 to 4 times higher (Table 2). In all cases, total receptor densities at the $BRET_{50}$ were in the range of 3000 to 10,000 receptors per cell or 30 to 100 fmol/mg protein (Table 2), which corresponds to physiological values for melatonin receptors in tissues (Dubocovich and Takahashi, 1987; Morgan et al., 1994; Paul et al., 1999). Estimating an average cell surface of 240 μm^2 for HEK 293 cells, the density of dimers at the cell surface would be comprised between 10 and 50 receptors/μm^2, a value that is at least 100 times lower than that promoting nonspecific BRET in HEK 293 cells (Mercier et al., 2002). Taken together, these data support the hypothesis that MT_1/MT_2 heterodimers may form at low expression levels and indicate, in addition, that in cells which coexpress both receptor isoforms, the formation of MT_1/MT_2 heterodimers is even more probable than that of MT_2 homodimers.

Pharmacological Properties of Coexpressed MT_1 and MT_2 Receptors. To identify unambiguously MT_1/MT_2 heterodimers in tissues, it is necessary to characterize the specific binding properties of heterodimers versus those of homodimers. Such specific pharmacological properties have been documented for some GPCR heterodimers but not for others (Jordan and Devi, 1999; Rocheville et al., 2000a; Pfeiffer et al., 2001). Binding experiments with ^{125}I-MLT as radioligand were performed on cells expressing MT_1-Rluc and MT_2-YFP receptors either separately or in combination at a 1:1 protein ratio. The expression level of these receptors was monitored by measuring either luciferase activity or YFP fluorescence using calibration curves, which correlate luminescence and fluorescence signals to the number of ligand binding sites (Fig. 1 of supplemental material). Both MT_1 and MT_2 receptors bound the specific agonist ^{125}I-MLT with high affinity (K_d = 115 ± 22 and 250 ± 60 pM for MT_1-Rluc and MT_2-YFP, respectively). When receptors were coexpressed, the apparent K_d was similar (200 ± 21 pM). The pharmacological profile of melatonin receptors was then determined in ^{125}I-MLT competition binding experiments. When expressed individually, MT_1-Rluc and MT_2-YFP displayed K_i values and pharmacological profiles very similar to those reported for the corresponding wild-type receptors (Dubocovich et al., 1997; Petit et al., 1999; Audinot et al., 2003), indicating that Rluc and YFP moieties did not significantly affect receptor binding properties (Table 1 and Fig. 3 of supplemental material). In cells coexpressing MT_1-Rluc and MT_2-YFP at a 1:1 protein ratio, competition curves for melatonin, S20098, S22153, S20928, and luzindole were monophasic, with K_i values comparable with those observed for cells expressing each receptor separately. The competition profiles of the MT_2-selective ligands 4P-PDOT and S24773 were biphasic, with K_i values consistent with the binding to MT_1 and MT_2 binding sites. These data may be interpreted in different ways. According to the results obtained with the BRET donor saturation assay (Table 2), the absence of MT_1/MT_2 heterodimers can be excluded, because this receptor species represents a major receptor fraction in cells coexpressing MT_1 and MT_2 receptors at a 1:1 protein ratio. We can also exclude that MT_1/MT_2 heterodimers are unable to bind ^{125}I-MLT and that the ligand binding profile observed in cells coexpressing both receptors would correspond to the sum of competition profiles of coexisting MT_1 and MT_2 homodimers. Indeed, no decrease in ^{125}I-MLT binding has been observed in cells coexpressing both receptors compared with cells expressing equivalent amounts of both receptors individually (quantified by fluorescence/luminescence measurements), as would be expected if the heterodimer is unable to bind ^{125}I-MLT (data not shown). In addition, the effect of ligands on the BRET signal presented below shows that MT_1/MT_2 heterodimers are ligand binding-competent. Having excluded these possibilities, the competition profiles in cells coexpressing MT_1 and MT_2 receptors may be explained either by the fact that the affinity of MT_1 and MT_2 binding sites for the ligand are identical whether they are part of a homodimer or a heterodimer, or by the fact that existing differences in ligand binding properties are not revealed in this assay because of the superposition of multi-

TABLE 1
Binding affinities measured in HEK 293 cells expressing MT_1 and MT_2 receptors
HEK 293 cells expressing MT_1-Rluc or MT_2-YFP or both together at a 1:1 ratio were incubated with ^{125}I-MLT and various concentrations of the indicated compounds. K_i values were calculated as described under *Materials and Methods*. Data are means ± S.E. of three independent experiments each performed in duplicate.

Ligands	K_i			K_i Ratio		
	MT_1-Rluc	MT_2-YFP	MT_1-Rluc + MT_2-YFP (Ratio 1:1)	MT_1/MT_2	$MT_1/(MT_1 + MT_2)$	$MT_2/(MT_1 + MT_2)$
		nM				
Melatonin	0.36 ± 0.1	0.48 ± 0.1	1.13 ± 0.5	0.7	0.3	0.4
S20098	0.78 ± 0.2	0.08 ± 0.05	1.64 ± 1.5	9.7	0.5	0.05
S22153	39.4 ± 9.0	13 ± 3	31.7 ± 8.9	3	1.2	0.4
S20928	244 ± 105	210 ± 81	281 ± 114	1.2	0.9	0.7
4P-PDOT	53.5 ± 13	2 ± 0.3	1.40 ± 0.4	26	75	1.4
			70.3 ± 15 (2nd site)		0.7	0.02
Luzindole	31.6 ± 7.0	12.3 ± 4.3	10 ± 1.7	2.6	3.2	1.2
S24773	295 ± 26	3.70 ± 1.7	0.60 ± 0.1	80	491	6.2
			192 ± 23 (2nd site)	1.5		0.01

ple competition profiles caused by the different coexisting receptor species (monomers and homo- and heterodimers). To discriminate between these possibilities and to identify unambiguously the ligand binding properties of MT_1/MT_2 heterodimers, we developed an alternative approach.

Correlation between Ligand Affinity and Ligand-Induced Changes of BRET. A direct consequence of ligand binding to receptors is the induction of conformational changes within the core of the helical transmembrane domain that may be monitored with the BRET assay. For the MT_2 homodimer and the MT_1/MT_2 heterodimer, ligand-promoted modifications of BRET signals can indeed be observed in the presence of agonists and inverse agonists. Changes of the BRET signal are most likely induced by the conformational change of the receptor and does not result from dimer recruitment, receptor redistribution, or alterations in local pH (a parameter that could influence energy-transfer efficacy) (Ayoub et al., 2002). Importantly, the change of BRET signals upon ligand binding can be attributed to a specific receptor dimer because the energy transfer occurs only between BRET-competent receptors. We first verified whether the efficiency to promote ligand-induced BRET signals (EC_{50}) correlates with binding affinities of the ligands. A good correlation would be expected for receptor homodimers such as the MT_2 homodimer. To test this prediction, MT_2-Rluc and MT_2-YFP fusion proteins were coexpressed at a 1:3 ratio that corresponds to the optimized condition for BRET measurements (Ayoub et al., 2002). K_i values were determined in ^{125}I-MLT competition binding experiments for selected ligands and were shown to be similar to those observed in cells expressing MT_2-YFP alone (compare Tables 1 and 3). The same compounds increased the BRET signal in cells expressing MT_2 homodimers in a dose-dependent manner, with maximal BRET values ranging between 115 and 175% of the basal BRET (Fig. 3A). The rank order of potency of the ligands was similar in the BRET assay and the ^{125}I-MLT competition binding assay (K_i: 2-iodomelatonin = S20098 > melatonin = 4P-PDOT > S22153 = S24773 = luzindole > S20928; EC_{50}: 2-iodomelatonin ≥ S20098 = melatonin = S24773 = 4P-PDOT = S22153 > luzindole > S20928). A good correlation was obtained when EC_{50} values were plotted against the corresponding K_i values (linear regression, R^2 = 0.74) (Fig. 3B), indicating that the efficiency of a ligand to promote BRET changes within dimers is correlated with its affinity for the receptor.

Assessment of Ligand-Promoted BRET Changes of the MT_1/MT_2 Heterodimer. Similar experiments were conducted in cells expressing MT_1-Rluc/MT_2-YFP heterodimers (at a 1:3 protein ratio) for a panel of ligands (Table 4). Competition binding curves were monophasic, and K_i values were close to those measured in cells expressing MT_2-Rluc and MT_2-YFP. Again a dose-dependent ligand-induced BRET was observed for all compounds tested with maximal values ranging from 130 to 140% of the basal BRET (Fig. 4). However, no correlation could be established between EC_{50} and K_i values (R^2 = 0.02) (Fig. 5A), indicating that the efficiency of a ligand to promote BRET changes specifically within the MT_1/MT_2 heterodimer does not correlate with the apparent affinity constant measured in cells coexpressing MT_1 and MT_2 receptors. Similarly, no correlation was observed when EC_{50} values of nonselective and MT_1-selective ligands were plotted against K_i values of the MT_1 receptor (shown here

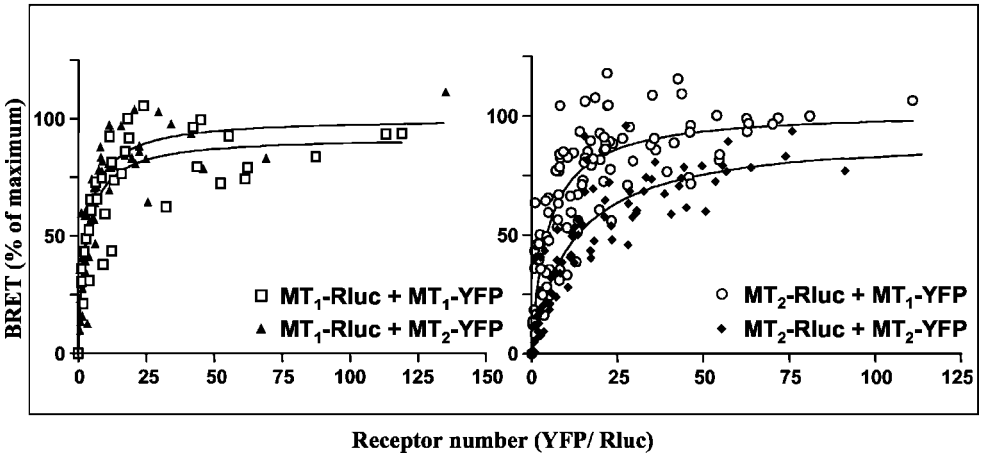

Fig. 2. BRET donor saturation curves of MT_1 and MT_2 homo- and heterodimers. BRET measurements were performed with HEK 293 cells coexpressing 12 fmol/mg of protein of the indicated Rluc fusion proteins and increasing amounts of the indicated YFP fusion proteins. BRET values were plotted as a function of the ratio of YFP/Rluc fusion proteins (as determined by transforming luminescence and fluorescence values measured for each data point into receptor numbers by using the correlation curves shown in Fig. 1 of supplemental material). The curves represent 7 to 10 individual normalized saturation curves that were fitted using a nonlinear regression equation assuming a single binding site (GraphPad Prism software).

TABLE 2

Relative affinities between two BRET partners of melatonin receptors

The $BRET_{50}$ represents the acceptor/donor ratio required to reach half-maximal BRET in BRET-donor saturation experiments. Results are the mean + S.E. of 7 to 10 independent saturation curves (Fig. 2). Receptor densities at $BRET_{50}$ are determined by averaging BRET values recorded close to the calculated $BRET_{50}$ of 7 to 10 individual saturation experiments. The luciferase activity and YFP fluorescence were used to calculate the number of ^{125}I-MLT binding sites according to standard curves shown in Fig. 1 of supplemental material; 7500 HEK 293 cells correspond to 1 μg of total protein.

Condition	Dimers	Receptor Density at $BRET_{50}$		
		$BRET_{50}$	Receptor/Cell	fmol/mg of protein
1	MT_1-Rluc/MT_1-YFP	3.7 ± 0.8	3080 ± 520	33 ± 4
2	MT_1-Rluc/MT_2-YFP	3.0 ± 1.6	3434 ± 436	43 ± 5
3	MT_2-Rluc/MT_1-YFP	4.2 ± 1.6	4554 ± 974	51 ± 12
4	MT_2-Rluc/MT_2-YFP	12.5 ± 2.2**	11318 ± 2176**	142 ± 27**

**, $P < 0.02$, compared with conditions 1, 2, and 3.

for MT_1-Rluc) (Fig. 5B) or when EC_{50} values of nonselective and MT_2-selective ligands were plotted against K_i values of the MT_2 receptor (shown here for MT_2-YFP) (Fig. 5C). This indicates that the binding properties of the MT_1 and MT_2 binding site in the heterodimer are different from those detected in the corresponding homodimers. Further evidence for this hypothesis comes from the comparison between BRET EC_{50} values of MT_2 homodimers and MT_1/MT_2 heterodimers (Fig. 5D). The absence of correlation suggests that ligand-promoted conformational changes of MT_1/MT_2 heterodimers differ from those elicited in MT_2 homodimers and indicate the existence of MT_1/MT_2 heterodimer-specific ligand binding properties. Heterodimer selectivity of ligands can be estimated by the ratio of EC_{50} values for homo- and heterodimers (Table 4). Whereas melatonin and S20098 are equally potent, EC_{50} values for S22153 and S24773 are eight times lower for the heterodimer. S20928 and luzindole are clearly more potent on MT_1/MT_2 heterodimers compared with MT_2 homodimers (26 and 126 times, respectively), and EC_{50} values for 4P-PDOT are 5 times lower for the MT_2 homodimer. Taken together, these results show that melatonin receptor-specific ligands are binding to MT_1/MT_2 heterodimers and that the potency of these ligands to induce conformational changes is similar for some ligands (melatonin and S20098) but clearly different for others (S20928 and luzindole) compared with those measured for MT_2 homodimers.

Both Ligand Binding Sites Are Functional within the MT_1/MT_2 Heterodimer. In cells coexpressing MT_1 and MT_2 receptors, ^{125}I-MLT competition binding curves for the MT_2-selective compounds S24773 and 4P-PDOT were biphasic, with an MT_2 binding site of high affinity and an MT_1 binding site with 30 to 100 times lower affinity (Table 1 and Fig. 3 of supplemental material). Accordingly, concentration-response curves of the ligand-induced BRET are expected to be biphasic for these compounds in cells expressing MT_1/MT_2 heterodimers. However, experimental BRET curves were monophasic for these compounds, and EC_{50} values corresponded to the affinity for MT_2 (Fig. 4), indicating that the ligand-promoted BRET change is caused by binding to the MT_2 binding site at this ligand concentration. The absence of the second (low-affinity) component of the BRET curves

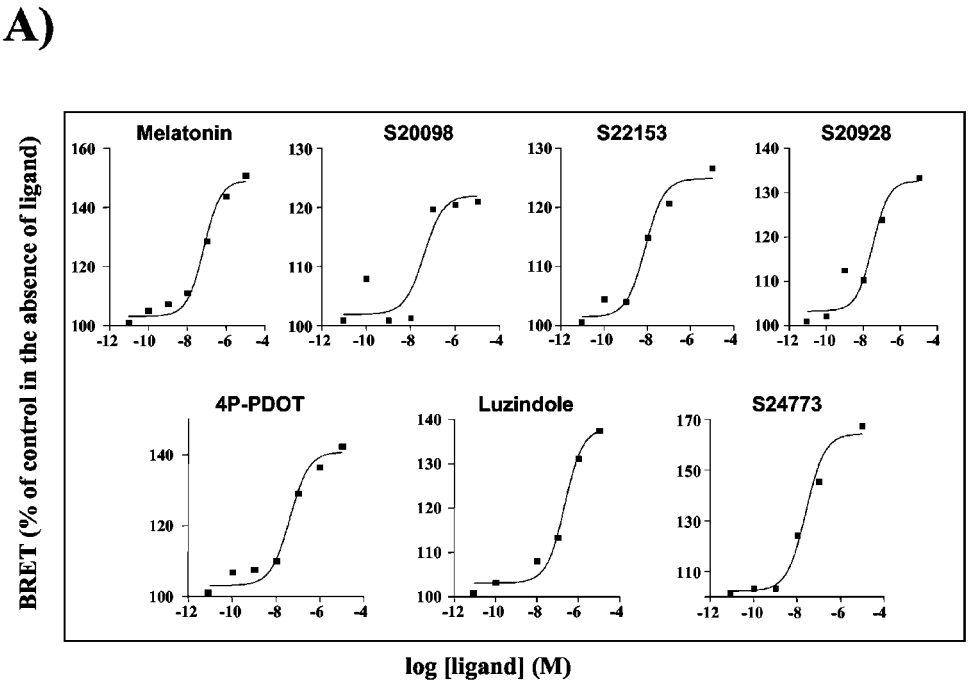

Fig. 3. Dose-response curves of the ligand-induced BRET in MT_2 homodimers. A, HEK 293 cells coexpressing MT_2-Rluc and MT_2-YFP receptors at a 1:3 protein ratio (as determined using the correlation curves shown in Fig. 1 of supplemental material) were incubated in the presence of increasing concentrations of the indicated ligands and BRET measurements performed according to the "microplate BRET assay" protocol. Data are represented as the percentage of energy transfer in the absence of ligand and are representative of at least three independent experiments each performed in triplicate. Curves were analyzed by nonlinear regression (GraphPad Prism software). B, correlation between K_i and EC_{50} values of the MT_2 homodimer. Data were fitted using a linear regression equation (GraphPad Prism software). Ligands have been numbered according to the numbers used in Table 3.

might be explained by the absence of the second (MT_1-like) functional binding site in the heterodimer. To address this point, we studied the effect of the MT_1-selective ligand S26284 (Audinot et al., 2003). The BRET dose-response curve of this compound for the MT_1/MT_2 heterodimer was monophasic, with an EC_{50} of 48 ± 3 nM (Fig. 6), which is close to the K_i value measured for MT_1-Rluc (47 ± 5 nM, n = 2) compared with the K_i value for MT_2-YFP (605 ± 420 nM, n = 2). Thus, S26284 bound to the MT_1-like binding site within the heterodimer and induced conformational changes that decrease the basal BRET signal by either increasing the distance between the BRET partners or promoting a less favorable orientation of the two partners. Binding of S26284 to the MT_1-like binding site within the heterodimer is further supported by the fact that no BRET change can be observed upon S26284 stimulation in cells co-expressing MT_2-MT_2-YFP (Fig. 6). Taken together, these data indicate that both the MT_1- and the MT_2-like binding sites within the heterodimer are functional and show that ligand binding to either of the two binding sites of the heterodimer is sufficient to induce a conformational change within the heterodimer.

Discussion

In this study, we have shown that the probability of MT_1/MT_2 heterodimer formation is similar to or even higher than those of the corresponding homodimers in cells expressing low levels of receptor and that heterodimers are competent for binding ligands. Both the MT_1 and MT_2 binding sites are functional within the heterodimer. We have also shown that the two binding sites maintain their respective selectivity for MT_1- and MT_2-selective ligands and that the ligand-interaction profile of the MT_1/MT_2 heterodimer determined by BRET is not identical with that of the MT_2 homodimer.

The homo-/heterodimer ratio of MT_1 and MT_2 receptors has been determined with the BRET donor saturation assay. The engagement of MT_1 receptors into MT_1 homodimers or MT_1/MT_2 heterodimers seems to be governed exclusively by the relative expression levels of MT_1 and MT_2 receptors, because similar relative affinities were observed in BRET saturation assays. In contrast, MT_2 receptors have a higher tendency to form heterodimers than homodimers with MT_1 receptors, suggesting that MT_2 receptors may be preferentially engaged into heterodimers in cells coexpressing both receptors. The documented coexpression of MT_1 and MT_2 receptors in many melatonin-sensitive tissues, such as the hypothalamic suprachiasmatic nuclei (Reppert et al., 1988), the retina (Dubocovich, 1983), arteries (Krause et al., 1995), and adipose tissue (Brydon et al., 2001), suggests that heterodimerization could indeed occur in native mammalian tissues, assuming simultaneous expression of both receptors in the same cells. Our results indicate, for the first time, that homo- and heterodimer formation may depend not only on the relative expression levels of receptor subtypes but also on the relative affinity of the monomers for each other. For the GABA receptor B, heterodimerization between $GABA_{B1}$ and $GABA_{B2}$ subunits was shown to be obligatory for the formation of functional receptors (Jones et al., 1998). Expression of each subunit alone does not form functional receptors. However, most GPCRs clearly form functional homodimeric receptors when expressed alone. As shown for the MT_2 receptor, some receptors may form homodimers but preferentially engage into heterodimers. This may also be the case for the α_{1D}-adrenergic receptor (α_{1D}-AR) (Hague et al., 2004). This receptor forms homodimers that accumulate intracellularly when expressed alone. Coexpression of α_{1D}-AR with α_{1B}-AR caused heterodimer formation and the quantitative translocation of the α_{1D}-AR to the cell surface. A large spectrum of affinities is likely to exist for the formation of different GPCR heterodimers. Such an affinity spectrum may provide a framework for a better understanding of the formation of homo- and heterodimeric complexes in cells, which naturally coexpress several different GPCRs.

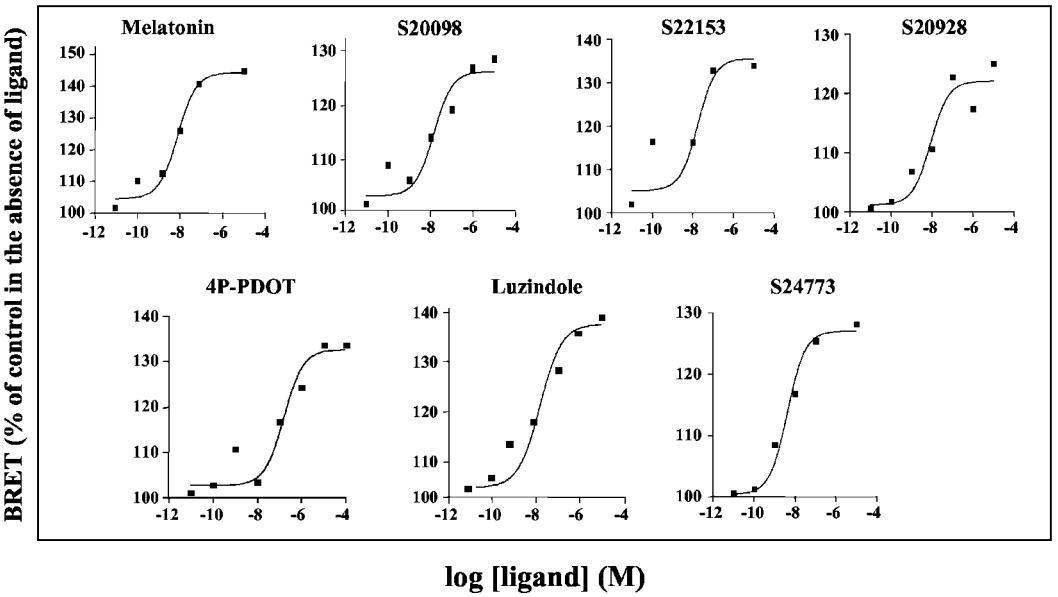

Fig. 4. Dose-response curves of the ligand-induced BRET in MT_1/MT_2 heterodimers. A, HEK 293 cells coexpressing MT_1-Rluc and MT_2-YFP receptors at a 1:3 protein ratio (as determined using the correlation curves shown in Fig. 1 of the supplemental material) were incubated in the presence of increasing concentrations of the indicated ligands and BRET measurements performed according to the "microplate BRET assay" protocol. Data are represented as the percentage of maximally induced BRET and are representative of at least three experiments each performed in triplicate. Curves were analyzed by nonlinear regression (GraphPad Prism software).

TABLE 3

Binding affinities (K_i) and EC_{50} values of ligand-induced BRET for MT_2 homodimers in HEK 293 cells

MT_2-Rluc and MT_2-YFP receptors were expressed at a 1:3 ratio (~80 fmol/mg of protein) and ^{125}I-MLT competition binding experiments and BRET measurements were performed as described under *Materials and Methods* and in Fig. 3. Data are means ± S.E. of at least three independent experiments each performed in triplicate.

Ligands	K_i	EC_{50}	E_{max}
	nM	*nM*	%
Melatonin	0.27 ± 0.01	26 ± 7.75	173 ± 20
S20098	0.06 ± 0.05	14.2 ± 13.6	143 ± 9
S22153	3 ± 1.90	47 ± 24	147 ± 18
S20928	60 ± 0.10	368 ± 234	126 ± 6
4P-PDOT	0.35 ± 0.31	40.4 ± 12	146 ± 3
Luzindole	7.23 ± 4.5	126 ± 57.2	150 ± 8
S24773	5.70 ± 1.7	32.7 ± 16.4	161 ± 6
2-iodomelatonin	0.16 ± 0.03	4.4 ± 1.95	113 ± 3

The determination of the specific pharmacological profile of heterodimers is difficult to achieve using classic radioligand competition binding assays, particularly for heterodimers composed of two receptor subtypes (MT_1 and MT_2) that display similar affinities for the same radioligand (^{125}I-MLT). The major difficulty resides in the simultaneous detection of all ligand binding-competent receptor species (monomers and homo- and heterodimers). In contrast, energy transfer assays such as the BRET assay have the unique feature to focus on one given combination of receptors (those competent for BRET). We have shown that the efficiency for a ligand to induce conformational changes in a homodimer reflects its affinity for the receptor, as observed in cells coexpressing MT_2-Rluc and MT_2-YFP fusion proteins (MT_2 homodimer). Then the efficiency of ligands to induce conformational

TABLE 4

Binding affinities (K_i) measured in HEK 293 cells coexpressing MT_1 and MT_2 receptors and EC_{50} values of ligand-induced BRET for MT_1/MT_2 heterodimers

MT_1-Rluc and MT_2-YFP receptors were expressed at a 1:3 ratio (~80 fmol/mg of protein) and ^{125}I-MLT competition binding experiments and BRET measurements were performed as described under *Materials and Methods* and in Fig. 4. Data are means ± S.E. of at least three independent experiments each performed in triplicate.

Ligands	K_i	EC_{50}	E_{max}	EC_{50} Ratio of MT_2 Homodimer/Heterodimer
	nM	*nM*	%	
Melatonin	0.40 ± 0.16	16.8 ± 9.7	142 ± 9	1.5
S20098	0.23 ± 0.17	7.7 ± 4.6	136 ± 6	1.8
S22153	8.87 ± 2.34	6.4 ± 5.6	132 ± 10	7.3
S20928	116.6 ± 1.75	14 ± 9.5	136 ± 7	26.3
4P-PDOT	8.60 ± 7.40	211 ± 159	141 ± 4	0.2
Luzindole	32.4 ± 9.20	1.0 ± 0.1	134 ± 13	126
S24773	4.5 ± 1.0	4.1 ± 2.2	137 ± 4	8

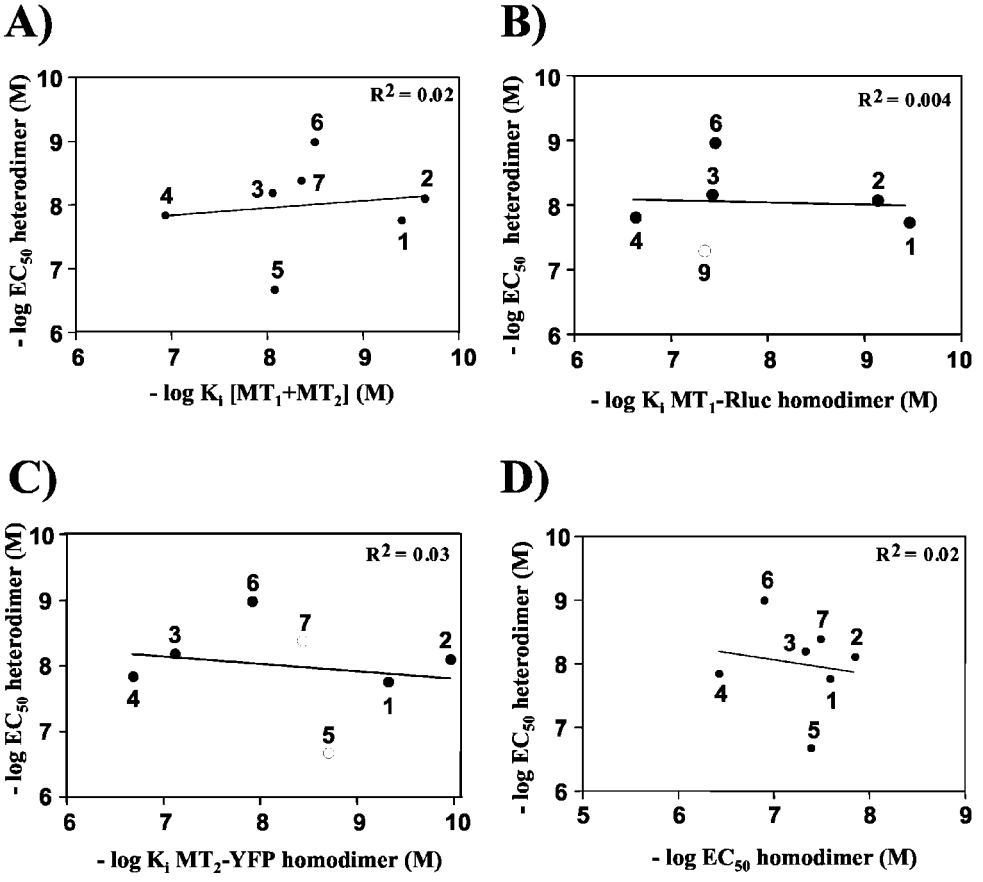

Fig. 5. Correlations between EC_{50} values of MT_1/MT_2 heterodimers and K_i values of MT_1 and MT_2 receptors. EC_{50} values of the MT_1/MT_2 heterodimer shown in Table 4 were plotted against K_i values determined in cells coexpressing MT_1 and MT_2 receptors (A) or expressing MT_1-Rluc (B) or MT_2-YFP (C) receptors individually. D, correlation between EC_{50} values of the MT_2 homodimer and the MT_1/MT_2 heterodimer. Ligands: 1, melatonin; 2, S20098; 3, S22153; 4, S20928; 5, 4P-PDOT; 6, luzindole; 7, S24773; 8, 2-iodomelatonin. B and C, nonselective (●) and selective (○) compounds. Ligand 9 shown in B corresponds to the MT_1-selective S26284 compound. Data were fitted using a linear regression equation (GraphPad Prism software).

changes was compared between MT_2 homodimers and MT_1/MT_2 heterodimers. MT_1 homodimers could not be studied directly because the ligand-induced conformational change does not translate into alterations of the BRET signal for this specific subtype (Ayoub et al., 2002). Some ligands, including the natural hormone melatonin, showed similar efficiencies to induce BRET changes in MT_2 homodimers and heterodimers, whereas several synthetic compounds (S20928, luzindole, and S26284) caused clearly different effects on homo- and heterodimers, showing that the efficiency to promote ligand-induced conformational changes of MT_1/MT_2 heterodimers differs from that of MT_2 homodimers.

Subtype-selective ligands are frequently used to define the specific melatonin receptor subtype involved in the physiological effects of melatonin (Masana and Dubocovich, 2001). We have shown that both the MT_2-selective 4P-PDOT and S24773 and the MT_1-selective S26284 bind with high affinity to MT_1/MT_2 heterodimers. This may have important implications for the interpretation of data obtained in cells coexpressing the two melatonin receptor subtypes because these compounds will bind with high affinity not only to the selected homodimer but also to the MT_1/MT_2 heterodimer.

GPCR dimers are potentially composed of two functional ligand binding sites. Whether both sites are indeed functional and whether ligand binding to both sites is necessary for receptor activation are critical questions to understand the activation mechanism of GPCRs. ^{125}I-MLT binding and BRET experiments with subtype-selective ligands in cells coexpressing MT_1 and MT_2 receptors suggested that MT_1/MT_2 heterodimers are composed of two functional ligand binding sites with distinct properties, an MT_1-like binding site and an MT_2-like binding site. The conservation of two ligand binding sites within GPCR dimers showing variable changes of the pharmacological properties have also been observed for other heterodimers [δ and κ opioid (Jordan and Devi, 1999), μ and δ opioid (George et al., 2000), somatostatin sst1 and sst5 receptors (Patel et al., 2002), adenosine A1 and dopamine D1 (Ferre et al., 1998), somatostatin sst5, and dopamine D2 (Rocheville et al., 2000a)].

Whether ligand binding to both sites is necessary for receptor activation has been a difficult issue to address. Early studies on the GABA receptor B, which forms obligatory heterodimers between the $GABA_{B1}$ and $GABA_{B2}$ subunits, showed that ligand binding to the $GABA_{B1}$ subunit is sufficient to promote G protein transactivation through the $GABA_{B2}$ subunit (Kniazeff et al., 2002). Further evidence comes from internalization studies of somatostatin receptor sst1/sst5 heterodimers. Although sst5 receptors can be internalized, sst1 receptors are unable to be internalized when expressed alone. However, after binding of an sst1 selective ligand to the sst1/sst5 heterodimer, the sst1 receptor was reported to be internalized (Rocheville et al., 2000b), indicating that ligand binding to one monomeric unit (sst1) of the heterodimer is sufficient to induce the conformational change and receptor internalization of the dimer. Similar observations were made for the internalization of the V1a/V2 vasopressin receptor heterodimer, in which the fate of the internalized heterodimer depends on the activation of the specific monomeric unit within the heterodimer (Terrillon et al., 2004). Similarly, the induction of conformational changes upon binding of subtype-selective ligands to either MT_1- or MT_2-like binding sites of the MT_1/MT_2 heterodimer is also consistent with the model that occupation of only one ligand binding site within the dimer may be sufficient for receptor activation.

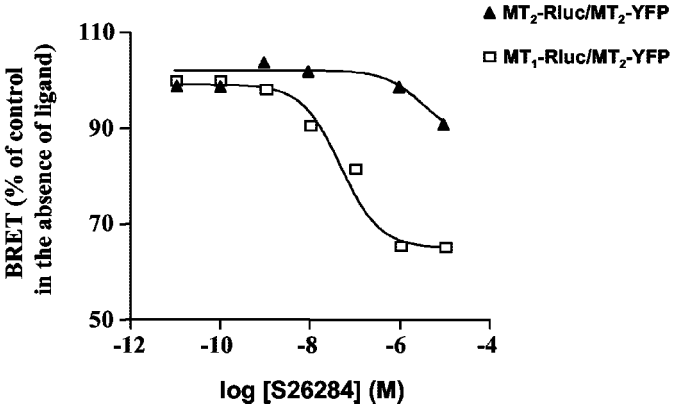

Fig. 6. Ligand-dependent BRET changes of S26284 in cells expressing MT_1 and MT_2 homo- and heterodimers. HEK 293 cells coexpressing MT_1-Rluc and MT_2-YFP (□) or MT_2-Rluc and MT_2-YFP (▲) receptors at a 1:3 protein ratio were incubated in the presence of increasing concentrations of S26284 and BRET experiments performed as described under *Materials and Methods*. Data are representative of at least three independent experiments each performed in triplicate. Curves were analyzed by nonlinear regression (GraphPad Prism software).

Although ligand-induced conformational changes are supposed to be a general phenomenon, ligand-induced energy transfer has not been observed for all receptors studied thus far (Issafras et al., 2002; Terrillon et al., 2003), indicating that conformational changes do not always result into energy-transfer variations. Indeed, the ligand-induced conformational change within the receptor moiety may have little effect on the position and the orientation of the energy-transfer partners. Data available thus far indicate that the development of energy-transfer assays as conformational sensors needs some optimization of the assay conditions and of the fusion protein design (Boute et al., 2001; Couturier and Jockers, 2003). In this respect, the introduction of energy donors and acceptors at sites other than the C terminus of GPCRs may represent an interesting alternative, as reported for the receptor "chameleon" constructs carrying cyan fluorescent protein and YFP, respectively, in the third intracellular loop and after the C terminus of the parathyroid hormone and α_{2A} adrenergic receptors. These constructs were still functional and highly sensitive to ligand-induced conformational changes, in agreement with the predicted movement of the third intracellular loop away from the C terminus (Vilardaga et al., 2003). Ligand-promoted BRET changes were also observed for tyrosine kinase receptors (Boute et al., 2001) and cytokine receptors (Couturier and Jockers, 2003), demonstrating the general interest of bioluminescence/fluorescence resonance energy transfer approaches to monitor ligand-induced conformational changes.

In conclusion, we investigated melatonin receptor heterodimerization using the BRET technology. The relative propensity for melatonin receptor homo- and heterodimer formation was determined in a BRET donor saturation assay and showed that MT_1/MT_2 heterodimers are formed at low expression levels and at probability equal to or higher than the corresponding homodimers. We have shown that the efficiency to promote ligand-induced variations of the BRET signal correlates with the binding affinities of ligands to the

receptor. From this correlation, we developed a BRET-based approach to study the specific ligand binding properties of MT_1/MT_2 heterodimers. This approach may be potentially applied to a wide range of ligand-regulated receptors.

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Address correspondence to: Dr. R. Jockers, Institut Cochin, 22 rue Méchain, 75014 Paris, France. E-mail: jockers@cochin.inserm.fr

MOL PHARM

Correction to "Preferential formation of MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction properties compared with MT2 homodimers"

In the above article [Ayoub MA, Levoye A, Delagrange P, and Jockers R (2004) *Mol Pharmacol* **66:**312–321], information regarding the ligands in Fig. 5 was removed in error during copyediting. A corrected version of Fig. 5 appears below. The online version has been corrected in departure from the print version.

We regret this error and apologize for any confusion or inconvenience it may have caused.

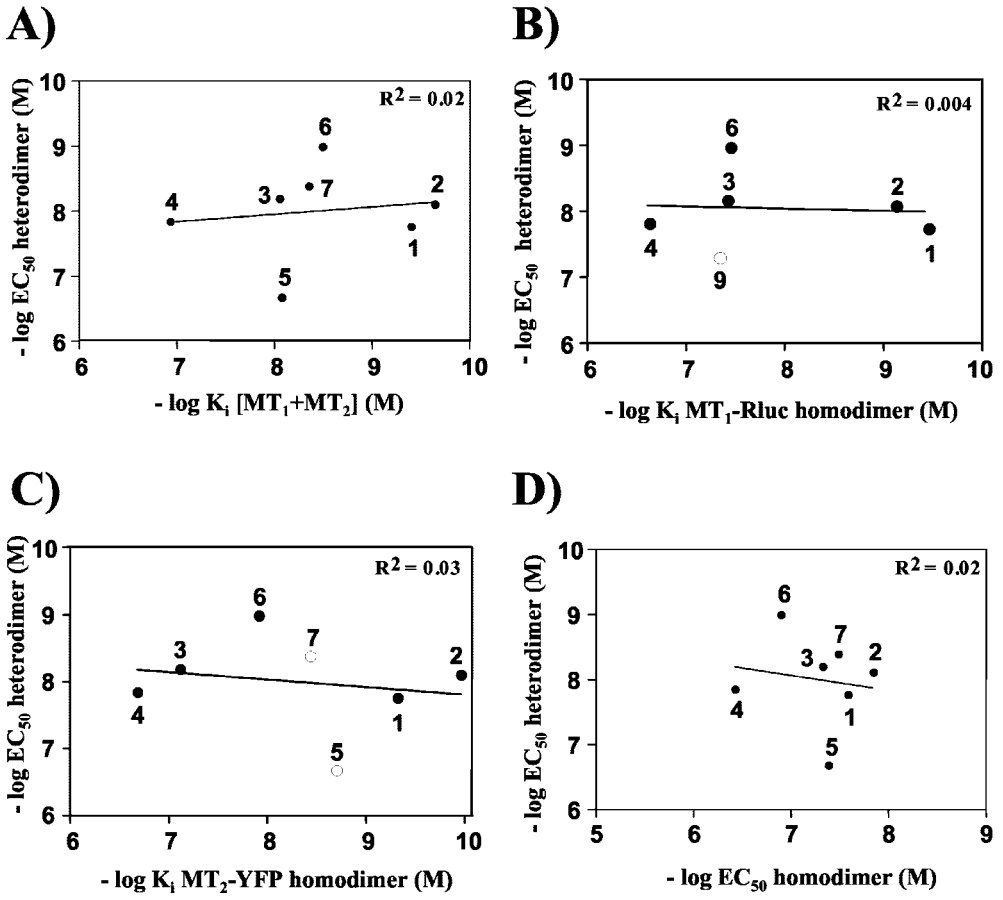

Fig. 5. Correlations between EC_{50} values of MT_1/MT_2 heterodimers and K_i values of MT_1 and MT_2 receptors. EC_{50} values of the MT_1/MT_2 heterodimer shown in Table 4 were plotted against K_i values determined in cells coexpressing MT_1 and MT_2 receptors (A) or expressing MT_1-Rluc (B) or MT2-YFP (C) receptors individually. D, correlation between EC_{50} values of the MT_2 homodimer and the MT_1/MT_2 heterodimer. Ligands: 1, melatonin; 2, S20098; 3, S22153; 4, S20928; 5, 4P-PDOT; 6, luzindole; 7, S24773; 8, 2-iodomelatonin. B and C, nonselective (●) and selective (○) compounds. Ligand 9 shown in B corresponds to the MT_1-selective S26284 compound. Data were fitted using a linear regression equation (GraphPad Prism software).