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The orphan GPR50 receptor specifically inhibits MT_1 melatonin receptor function through heterodimerization

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One-third of the ~400 nonodorant G protein-coupled receptors (GPCRs) are still orphans. Although a considerable number of these receptors are likely to transduce cellular signals in response to ligands that remain to be identified, they may also have ligand-independent functions. Several members of the GPCR family have been shown to modulate the function of other receptors through heterodimerization. We show that GPR50, an orphan GPCR, heterodimerizes constitutively and specifically with MT_1 and MT_2 melatonin receptors, using biochemical and biophysical approaches in intact cells. Whereas the association between GPR50 and MT_2 did not modify MT_2 function, GPR50 abolished high-affinity agonist binding and G protein coupling to the MT_1 protomer engaged in the heterodimer. Deletion of the large C-terminal tail of GPR50 suppressed the inhibitory effect of GPR50 on MT_1 without affecting heterodimerization, indicating that this domain regulates the interaction of regulatory proteins to MT_1. Pairing orphan GPCRs to potential heterodimerization partners might be of clinical importance and may become a general strategy to better understand the function of orphan GPCRs.

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Introduction

Although the formation of functional protein complexes via dimerization or oligomerization is a common theme in biology, G protein-coupled receptors (GPCRs) were assumed to exist as monomers for many years since the heterologous expression of a single GPCR was usually sufficient to produce the expected pharmacology and function. However, biochemical and structural data accumulated over the last 15 years indicate that most, if not all, GPCRs exist as functional dimers or higher oligomeric units (Bouvier, 2001; Milligan, 2004). GPCR dimerization includes the formation of homodimers (between two identical receptor protomers) and heterodimers (between two protomers of different receptors). GPCR heterodimerization has been reported for more than 40 receptor combinations (Prinster *et al*, 2005) and in most of these cases heterodimerization had a marked effect on GPCR function. Some GPCRs function as obligatory heterodimers as shown for the $GABA_B$ and taste ($T1R_{1-3}$) receptors. Other GPCRs may form heterodimers allowing for mutual regulation (trafficking, desensitization) between the two specific promoters within the heterodimer as observed in most of the known cases. Finally, heterodimerization may profoundly modify the pharmacological properties of the receptor.

Recent genome sequencing projects indicated that approximately 370 sequences belong to the nonodorant GPCR family in the human genome (Joost and Methner, 2002; Fredriksson *et al*, 2003; Vassilatis *et al*, 2003). More than 220 of these receptors have been matched with known ligands. However, over 140 receptors (≈40%) still remain as orphans despite the vast and long-standing effort of academic and industrial research to pair these receptors to potential ligands (Vassilatis *et al*, 2003).

Whereas a considerable amount of data has been accumulated on the homo- and heterodimerization of GPCRs with known ligands, nothing is known about the dimerization of orphan receptors. Heterodimerization between orphan and nonorphan GPCRs opens the interesting possibility that orphan receptors regulate the ligand binding, signaling and/or trafficking of GPCRs with known ligands.

We specifically addressed this issue by studying the dimerization of the orphan X-linked GPR50 receptor whose function is unknown (Reppert *et al*, 1996; Drew *et al*, 1998; Drew *et al*, 2001). A deletion mutant of GPR50 has been recently shown to be genetically associated with mental diseases such as bipolar affective disorder and major depressive disorder (Thomson *et al*, 2005). Moreover, some GPR50 variants are associated with higher triglyceride levels and lower HDL-cholesterol levels (Bhattacharyya *et al*, 2006). We show that GPR50 homodimerizes and forms heterodimers with MT_1 and MT_2 melatonin receptors that share the highest sequence homology with GPR50 among all GPCRs. Importantly, MT_1 loses its ability to bind to melatonin receptor-specific agonists and to couple to G proteins when engaged into GPR50/MT_1 heterodimers. Our results shed light on a previously unappreciated role of orphan receptors in the regulation of nonorphan GPCRs with known function.

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Results

Homo- and heterodimerisation of GPR50

The formation of GPR50 homodimers was first explored in Western blot experiments. In HEK 293 cell extracts expressing a fusion protein between GPR50 and the yellow fluorescent protein (YFP) (GPR50-YFP), anti-GFP antibodies detected two immunoreactive species with apparent molecular weights of approximately 90 and 180 kDa, likely corresponding to the monomeric and dimeric form of GPR50-YFP (Figure 1A). To confirm GPR50 homodimerization, we performed co-immunoprecipitation experiments with differentially tagged receptors. When immunoprecipitating GPR50-YFP, co-precipitated Flag-GPR50 was revealed in SDS–PAGE as a monomer at 70 kDa and as a SDS-resistant dimer at 140 kDa (Figure 1B).

GPR50 belongs to the melatonin receptor GPCR subfamily that in humans comprises two further subtypes, MT_1 and MT_2 (Reppert *et al*, 1996). To explore GPR50 heterodimerization with MT_1 and MT_2, GPR50-YFP was coexpressed with the N-terminally Flag-tagged MT_1 and Myc-tagged MT_2. Anti-GFP antibodies were indeed able to specifically pull down MT_1 and MT_2 proteins (Figure 1C and D). Formation of MT_1/GPR50 heterodimers was further supported by direct Western blot experiments (see Supplementary Figure 1). Taken together, Western blot and co-immunoprecipitation experiments suggest that GPR50 form homo- and heterodimers.

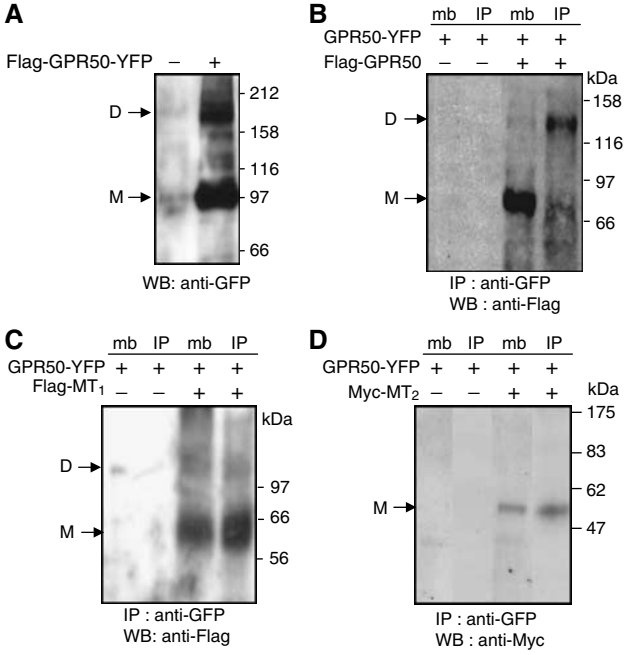


Figure 1 Detection of GPR50 homo- and heterodimers by SDS–PAGE and co-immunoprecipitation. (**A**) Lysates from HEK 293 cells stably expressing Flag-GPR50-YFP were separated by SDS–PAGE and analysis was performed by Western blot using an anti-GFP antibody. (B–D) Crude membranes were prepared from HEK 293 cells transiently expressing GPR50-YFP alone or with Flag-GPR50 (**B**), Flag-MT_1 (**C**) or Myc-MT_2 (**D**). GPR50-YFP was immunoprecipitated with a monoclonal anti-GFP antibody. Membranes and immnunoprecipitates were then separated by SDS–PAGE and analysis was performed by Western blot using polyclonal anti-Flag (B,C) or anti-Myc (D) antibodies. Similar results were obtained in three additional experiments. mb = membrane; IP = immunoprecipitation, M = monomer; D = dimer.

To exclude possible artefacts associated with co-immunoprecipitation of membrane-bound receptors solubilized in detergent, we studied GPR50 dimerization in intact cells using the Bioluminescence Resonance Energy Transfer (BRET) approach. The GPR50-Rluc construct was used as BRET donor and was coexpressed with the BRET acceptor YFP fused to the C-terminus of different receptors (Figure 2A). Significant energy transfer was observed in cells coexpressing GPR50-Rluc with similar amounts of GPR50-YFP, MT_1-YFP or MT_2-YFP. These BRET signals were comparable to those obtained for the MT_1 homodimer expressed at similar levels. Stimulation of cells with melatonin did not modify the basal BRET signals (not shown). The specificity of the assay was illustrated by the absence of significant transfer between GPR50-Rluc and YFP fusion proteins of control GPCRs (β_2-adrenergic receptor (β_2-AR) and CCR5) expressed in similar amounts to those of melatonin receptor YFP fusions. Taken together, BRET experiments show that GPR50 is engaged into constitutive homo- and heterodimeric complexes with MT_1 and MT_2 in intact cells.

The relative propensity of GPR50 to form homodimers or to engage into heterodimers with MT_1 or MT_2 was then studied with the recently developed BRET donor saturation assay (Mercier *et al*, 2002; Couturier and Jockers, 2003; Ramsay *et al*, 2004). Cells were cotransfected with constant amounts of the BRET donor (receptor fused to Rluc) and increasing quantities of the BRET acceptor (receptor fused to YFP). In this assay, 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). Specific BRET signals increased as a hyperbolic function and reached an asymptote with increasing acceptor/donor ratios. Comparable $BRET_{50}$ values of 0.36 ± 0.08, 0.35 ± 0.08 and 0.41 ± 0.06 ($n = 3$–5) were obtained for GPR50 homodimers, GPR50/MT_1 heterodimers and GPR50/MT_2 heterodimers, respectively (Figure 2B–D), indicating that the propensity of GPR50 heterodimerization with MT_1 and MT_2 is similar to that of GPR50 homodimerization. For control receptors (β_2-AR and CCR5), BRET signals increased linearly and were not saturable as expected for nonspecific interactions.

Consequences of GPR50 heterodimerization on ligand binding to MT_1 and MT_2

To assess the functional consequences of GPR50 heterodimerization with MT_1 and MT_2, we generated a HEK 293 cell line stably expressing the GPR50-YFP fusion protein (HEK-GPR50). Incubation of these cells with a saturating concentration of 2(^{125}I)-iodomelatonin (^{125}I-MLT) for MT_1 and MT_2 confirmed that GPR50 has no apparent affinity for this radioligand as previously reported (Reppert *et al*, 1996) (Figure 3A). The effect of GPR50 on ^{125}I-MLT binding to MT_1 and MT_2 was studied by expressing MT_1-Rluc and MT_2-Rluc fusion proteins in HEK-GPR50 cells and wild-type HEK 293 cells (HEK-wt). Despite the presence of similar quantities of MT_1-Rluc in both cell lines, as determined by luminescence measurements, the number of ^{125}I-MLT-binding sites was decreased by more than 50% in HEK-GPR50 cells (Figure 3A). No such effect was observed when comparable quantities of MT_2-Rluc were expressed (Figure 3B). Hence, GPR50 specifically decreases ^{125}I-MLT binding to MT_1 but not to MT_2. In addition, decreased ^{125}I-MLT binding is unlikely to

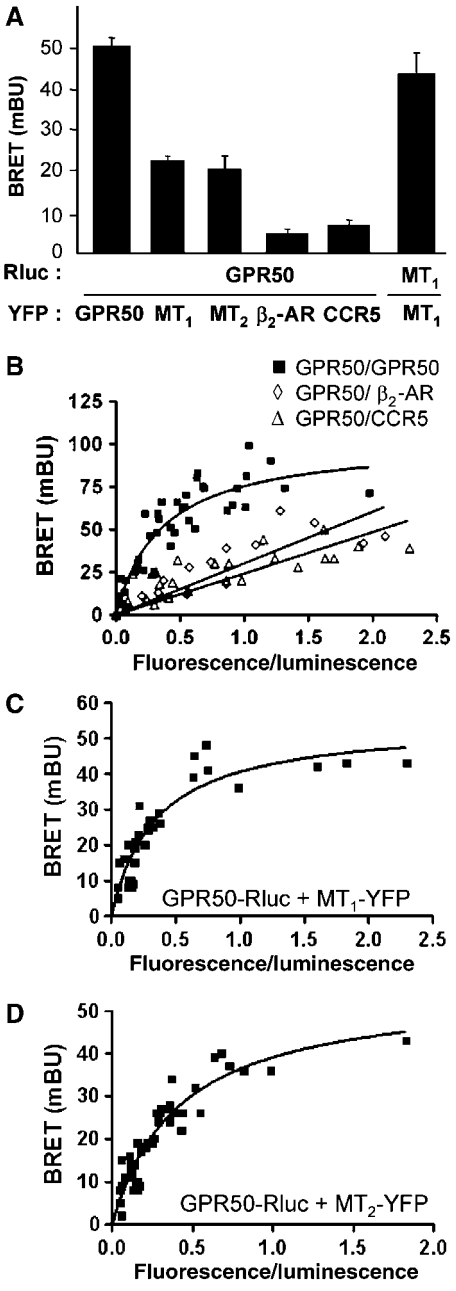


Figure 2 Constitutive dimerization of GPR50 in living HEK 293 cells. (**A**) GPR50-Rluc was transiently coexpressed in HEK 293 cells with the indicated C-terminal YFP fusion proteins expressed at comparable amounts as determined by direct fluorescence measurements (20–30 fmol of YFP fusion receptor per mg of protein as estimated from curves correlating YFP fluorescence with the number of ligand-binding sites (Ayoub *et al*, 2002)). BRET signals generated for the MT_1 homodimer (MT_1-Rluc/MT_1-YFP), when expressed at comparable amounts, were used as internal control. BRET measurements were performed in living cells by adding 5 μM coelenterazine. Data are means ± s.e.m. of at least three independent experiments each performed in duplicate. (**B**–**D**) BRET donor saturation curves were generated by transfecting transiently HEK 293 cells with a constant DNA amount of GPR50-Rluc and increasing quantities of the indicated YFP-tagged receptors. The BRET, total luminescence and total fluorescence were measured. The curves represent 3–5 individual saturation curves. Curves obtained for the BRET acceptors GPR50-YFP, MT_1-YFP and MT_2-YFP were best fitted with a nonlinear regression equation assuming a single binding site, those obtained for β_2-AR-YFP and CCR5-YFP were best fitted with a linear regression equation.

be due to receptor relocalization into intracellular compartments as the lipophilic ^{125}I-MLT easily penetrates cell membranes. To further investigate this point, GPR50-YFP was transfected in a HEK 293 cell clone stably expressing approximately 100 fmol of Flag-MT_1 per mg of protein (Figure 3C). Once again, ^{125}I-MLT binding was significantly decreased in cells expressing GPR50-YFP. A similar decrease in ^{125}I-MLT binding was observed for the untagged GPR50 showing that the YFP moiety is not responsible for this effect (Figure 3C). The decreased binding effect was dependent on the GPR50 concentration as expression of increasing amounts of GPR50-YFP progressively decreased the amount of ^{125}I-MLT-binding sites (Figure 3D). The amount of surface-expressed Flag-MT_1 in this stable cell clone remained unchanged by GPR50-YFP coexpression when monitored by flow cytometry using an antibody directed against the Flag epitope.

Dimerization between MT_1 and its known heterodimerization partner MT_2 (Ayoub *et al*, 2002) was used to assess the specificity of the inhibitory effect of GPR50 on ^{125}I-MLT binding to MT_1. In cells expressing Flag-MT_1 and MT_2-YFP, the number of ^{125}I-MLT-binding sites was higher than in cells expressing the same amount of Flag-MT_1 alone, consistent with the fact that the radioligand may bind both MT_1 and MT_2 (Figure 3C). Moreover, an MT_2-C113A-YFP mutant, which has lost the capacity of ^{125}I-MLT binding (Mseeh *et al*, 2002), still heterodimerized with MT_1 (Supplementary Figure 2) without blocking ^{125}I-MLT binding of the MT_1 protomer (Figure 3C). This suggests that ^{125}I-MLT binding to MT_1 is not modified in the MT_1/MT_2 heterodimer. Accordingly, the inhibitory effect of GPR50 on ^{125}I-MLT binding to MT_1 appears to be specific for the GPR50/MT_1 heterodimer.

We next performed ^{125}I-MLT saturation experiments to determine the precise impact of GPR50 on the B_{max} and K_d of ^{125}I-MLT binding to MT_1 (Figure 3E). The ^{125}I-MLT saturation curves were best fitted using a nonlinear regression equation with a single binding site indicating the presence of a single pharmacological species. Comparable K_d values of 290 ± 64 pM and 335 ± 56 ($n = 3$) were obtained in cells expressing MT_1-Rluc in the presence and absence of GPR50-YFP, respectively. B_{max} values decreased to 51 ± 9.4% in the presence of GPR50-YFP. The pharmacological profile of MT_1 was then determined in the presence and absence of GPR50 in ^{125}I-MLT competition binding experiments. In cells expressing MT_1 alone, the K_i values obtained for six melatonin receptor-specific ligands were in accordance with previously reported values (Dubocovich *et al*, 1997; Audinot *et al*, 2003; Ayoub *et al*, 2004) (Table I). Very similar K_i values were obtained for the remaining ^{125}I-MLT-binding sites in the presence of GPR50. Our data suggest that the ^{125}I-MLT-binding sites observed in the presence of GPR50 most likely correspond to MT_1 homodimers, whereas MT_1/GPR50 heterodimers are unable to bind ^{125}I-MLT.

To further confirm this hypothesis, MT_1 homodimers and MT_1/GPR50 heterodimers were separated by selective co-immunoprecipitation and ^{125}I-MLT binding of the different receptor pairs was determined. MT_1-YFP or GPR50-YFP were coexpressed with Flag-MT_1, labeled with a saturating ^{125}I-MLT concentration, solubilized and adjusted to similar YFP fluorescence values. Previous studies have shown that ^{125}I-MLT remains stably bound to MT_1 under these experimental conditions (Brydon *et al*, 1999). Whereas anti-GFP-specific antibodies readily precipitated 12% of the

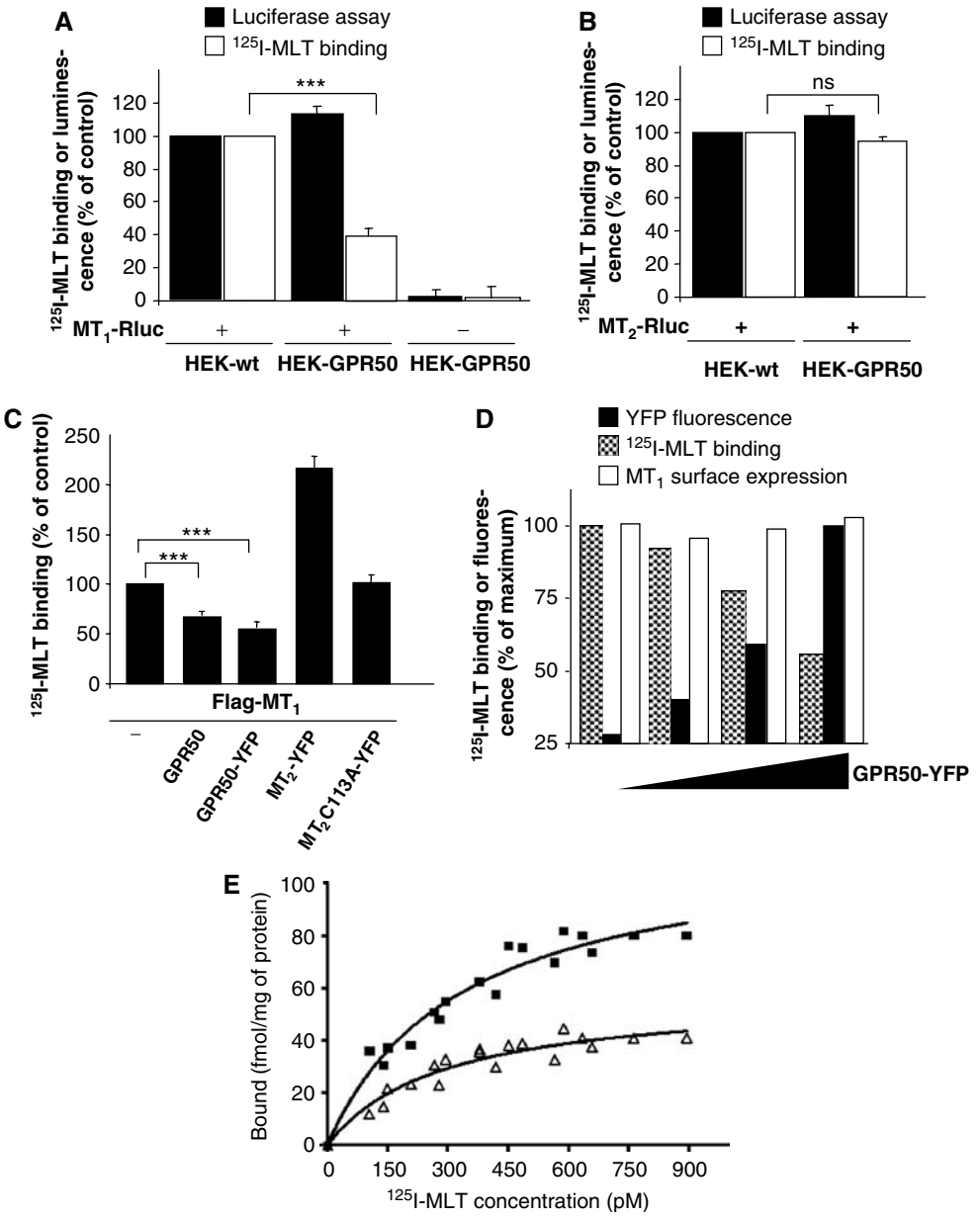


Figure 3 ^{125}I-MLT binding to MT_1 and MT_2 in the presence of GPR50. (A, B) HEK 293 cells stably expressing GPR50-YFP (HEK-GPR50) transiently expressed 5–10 fmol of MT_1-Rluc per mg of protein (**A**) or 10–15 fmol of MT_2-Rluc per mg of protein (**B**). Expression was monitored with the luciferase assay and by ^{125}I-MLT binding (500 pM). (**C**) HEK 293 cells stably expressing 70–80 fmol of Flag-MT_1 per mg of protein were transfected with untagged GPR50 or C-terminal YFP fusion constructs of GPR50, MT_2 or MT_2C113A. Expression of YFP fusion proteins was monitored by measuring YFP fluorescence, relative expression of GPR50 and GPR50-YFP was detected by Western blot using anti-GRP50-specific antibodies (not shown). Expression of Flag-MT_1 was monitored by flow cytometry using anti-Flag antibodies (not shown) and by ^{125}I-MLT binding. (**D**) Increasing concentrations of GPR50-YFP were expressed in HEK 293 cells stably expressing Flag-MT_1, and YFP fluorescence, ^{125}I-MLT binding and MT_1 surface expression were determined. (**E**) HEK 293 cells transiently expressing MT_1-Rluc in the absence (■) or presence of GPR50-YFP (△) were incubated with increasing concentrations of ^{125}I-MLT. Data are means ± s.e.m. of at least three independent experiments each performed in duplicate (A–C) or are representative of three further experiments (D, E) (***$P<0.001$; NS, $P>0.05$).

radiolabeled MT_1 homodimer (Figure 4, Flag-MT_1/MT_1-YFP), no significant amounts of ^{125}I-MLT were precipitated from the MT_1/GPR50 heterodimer sample (Figure 4, Flag-MT_1/GPR50-YFP). Western blot experiments performed with the same samples confirmed that equivalent quantities of Flag-MT_1 were co-immunoprecipitated from both samples. As expected, no radioactivity was precipitated in control experiments from cells expressing the Flag-MT_1 (−) or GPR50-YFP constructs individually, thus illustrating the specificity of the assay. Our results confirm the hypothesis that MT_1 loses its ability to bind to ^{125}I-MLT when engaged into the GPR50/MT_1 heterodimer.

GPR50 antagonizes MT_1 signaling

To verify whether GPR50 interferes also with melatonin-promoted MT_1 signaling, coupling of MT_1 to the G_i protein was assessed in the absence and presence of GPR50-YFP. A previously described $G_{\alpha i/q}$ chimera that couples G_i-coupled receptors to phospholipase C activation was used as a functional read-out for MT_1 activation (Goudet *et al*, 2004).

Table I Binding affinities measured in HEK 293 cells expressing MT_1 alone or with GPR50

Ligands	MT_1	MT_1/GPR50
	K_i (nM)	
Melatonin	0.73±0.26	0.37±0.28
S20098	0.43±0.23	0.42±0.22
S22153	131±6.24	197±66.9
S20928	254±54.3	358±5.20
S24773	1006±238	1615±612
4P-PDOT	204±6.05	217±10.0

HEK 293 cells expressing MT_1 alone or in the presence of Flag-GPR50-Rluc 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. K_i values obtained in the absence and presence of GPR50 were not statistically different according to a Student's *t* test.

The natural hormone melatonin and the synthetic melatonin receptor-specific agonist S20098 dose-dependently increased the functional response with EC_{50} values of 57.7±3.5 and 37.0±3.8 pM respectively, in agreement with published values (Godson and Reppert, 1997; Petit *et al*, 1999) (Figure 5A and B). Coexpression of GPR50-YFP had no major effect on the basal value and the EC_{50} values (39.6±2.1 and 9.3±3 pM for melatonin and S20098, respectively), but decreased the maximal response by 50 and 45% for melatonin and S20098, respectively. This decrease of the maximal response was not due to decreased expression levels of MT_1-Rluc when coexpressed with GPR50-YFP as monitored by measuring the luminescence of the Rluc fusion protein (Figure 5A inset). These results show that GPR50 antagonizes the functional response of the MT_1 receptor when stimulated by the synthetic S20098 compound and importantly the natural hormone, melatonin.

Effect of GPR50 downregulation on MT_1 function in hCMEC/D3 cells expressing endogenous receptors

The fact that melatonin is known to regulate cerebral blood flow (Regrigny *et al*, 1998; Yang *et al*, 2001) and that melatonin receptors are expressed in the vascular system (Savaskan *et al*, 2001) prompted us to search for the expression of endogenous melatonin receptors in recently immortalized human endothelial cerebral hCMEC/D3 cells (Weksler *et al*, 2005). RT–PCR experiments revealed the coexpression of MT_1 and GPR50 transcripts (Figure 6A). GPR50-selective siRNA duplexes were synthesized to investigate the effect of GPR50 downregulation on MT_1 function. The most efficient siRNA decreased the expression of GPR50-YFP in the HEK-GPR50 cells by 80% as monitored by flow cytometry (Figure 6B). Transfection of the GPR50-siRNA in hCMEC/D3 cells decreased the expression of the GPR50 transcript by 60%, whereas an Alexa Fluor 488-labeled control siRNA was without significant effect (Figure 6C). The effect of GPR50 downregulation was tested on ^{125}I-MLT binding. Whereas specific ^{125}I-MLT binding was hardly detectable in nontransfected and in control-siRNA-transfected cells, significant binding was consistently observed in GPR50-siRNA-treated cells (Figure 6D). In agreement with these observations, melatonin and S20098 inhibited forskolin-promoted cAMP accumulation only in GPR50-siRNA-treated cells (Figure 6E). The results obtained in hCMEC/D3 cells expressing both receptors endogenously further support the physiological significance of the inhibitory effect of GPR50 on MT_1 function.

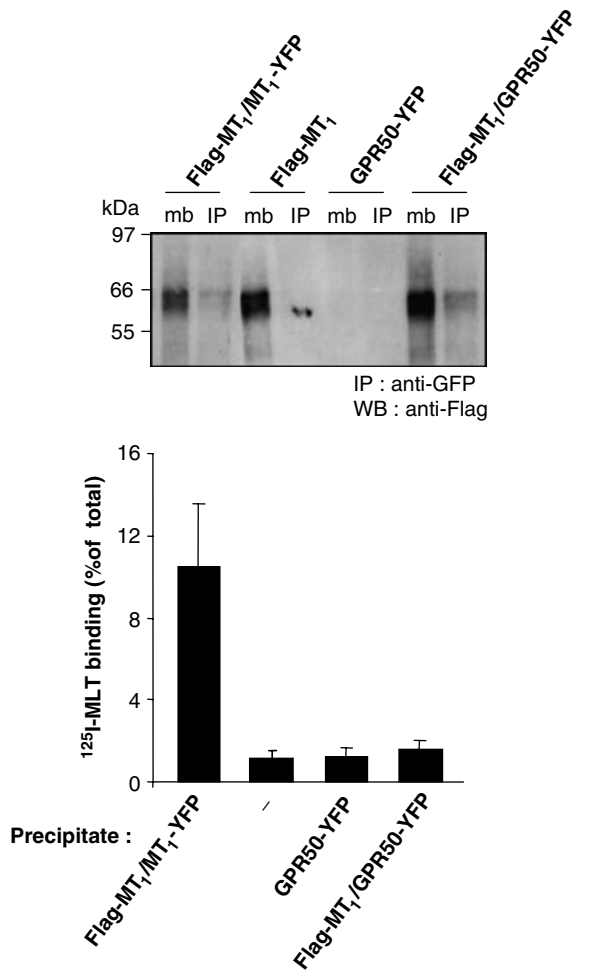


Figure 4 Absence of ^{125}I-MLT binding to GPR50/MT_1 heterodimers. Membranes from HEK 293 cells transiently expressing 30–40 fmol of Flag-MT_1 alone or in the presence of the indicated receptors were labeled with ^{125}I-MLT (500 pM), solubilized and immunoprecipitated with a monoclonal anti-GFP antibody. The amount of precipitated ^{125}I-MLT was determined in a γ-counter and precipitates were subsequently separated by SDS–PAGE. The presence of Flag-MT_1 was verified by Western blotting using polyclonal anti-Flag antibodies. Data are means of triplicates that are representative of two further experiments. mb = membranes; IP = immunoprecitate.

Mechanism of the antagonistic effect of GPR50 on MT_1 function

The decreased responsiveness of MT_1 in the presence of GPR50 could be explained by an alteration in the steady-state cell-surface expression of MT_1 owing to a change in receptor trafficking as demonstrated for other GPCR heterodimers (Jordan *et al*, 2001). However, it is not observed for MT_1/GPR50 heterodimers, as Flag-MT_1 was expressed at the cell surface irrespective of the coexpression or absence of GPR50-YFP, as quantified in a cell surface ELISA (Figure 7F) and visualized by fluorescence microscopy (Figure 7A and C). GPR50-YFP itself localized to the cell membrane

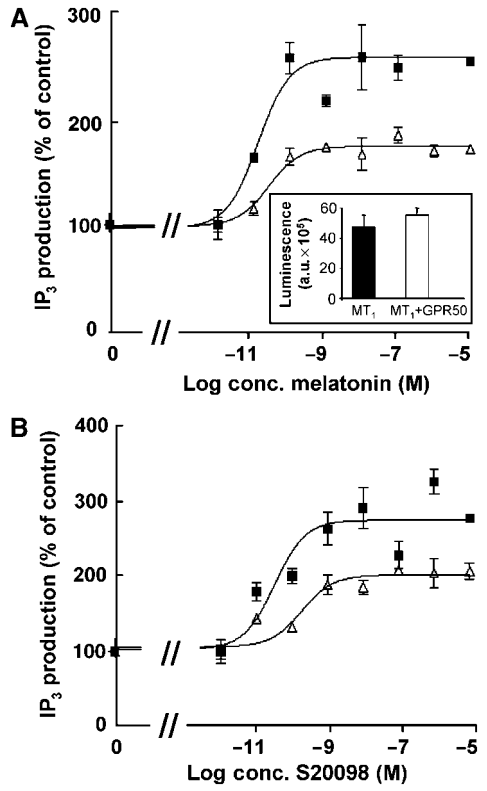


Figure 5 GPR50 antagonizes MT_1 signaling. HEK 293 cells transiently expressing MT_1-Rluc alone (■) or with GPR50-YFP (Δ) were stimulated with increasing concentrations of melatonin (**A**) or S20098 (**B**) and inositol phosphate levels were determined. MT_1-Rluc expression levels were determined by luminescence measurements (inset). Data are means ± s.e.m. of three independent experiments each performed in duplicate. A nonlinear regression equation assuming a single binding site was used to fit the data (GraphPad Prism software).

when expressed alone or with Flag-MT_1 (Figure 7B, D and G). Furthermore, stimulation with melatonin led to the internalization of the MT_1 homodimer (Figure 7F), whereas GPR50-YFP remained at the cell surface (Figure 7G). Taken together, these data suggest that MT_1 homodimers are internalized upon melatonin stimulation conversely to GPR50 homodimers and MT_1/GPR50 heterodimers.

Intriguingly, GPR50 displays a noncanonical C-terminal tail of 311 residues (Reppert *et al*, 1996). To study the potential role of the C-terminus on ^{125}I-MLT binding to the MT_1/GPR50 heterodimer, 264 amino acids of the intracellular domain were deleted. The first 55 amino acids of the C-terminus were conserved in the GPR50ΔCter mutant to maintain potentially important structural elements such as the eighth alpha-helical domain located at the membrane-proximal part of the C-terminus. In contrast to the full-length GPR50-YFP, expression of GPR50ΔCter-YFP or the C-terminus of GPR50 alone in cells stably expressing Flag-MT_1 failed to decrease ^{125}I-MLT binding in a context where MT_1 surface expression was not affected (Figure 8A). Immunofluorescence experiments showed that the GPR50ΔCter-YFP mutant localized exclusively at the cell surface similarly to the full-length receptor, thus excluding a defect in receptor trafficking (not shown). In agreement with the results obtained in ^{125}I-MLT-binding experiments (Figure 8B), signaling of MT_1 was affected in the presence of GPR50-YFP but was not impaired by equivalent expression levels of GPR50ΔCter-YFP (Figure 8B). BRET experiments using the GPR50ΔCter-Rluc fusion protein as BRET donor showed that the dimerization pattern of the mutant was identical to that of the full-length receptor (Figure 8C). Specific BRET signals were observed for GPR50ΔCter/GPR50, GPR50ΔCter/MT_1 and GPR50ΔCter/MT_2 dimers but not with the β_2-AR. Consequently, the effect of GPR50 on MT_1 function clearly depends on the presence of the large C-terminal tail in the full-length GPR50.

A possible mechanism of the inhibitory effect of GPR50 on MT_1 function is to hinder recruitment of intracellular interacting partners to MT_1 such as G_i proteins. Impaired G_i recruitment to MT_1 may also explain the loss of binding of the radiolabeled melatonin receptor agonist ^{125}I-MLT to MT_1 in the MT_1/GPR50 heterodimer, as high-affinity agonist binding to MT_1 depends on the presence of G_i (Barrett *et al*, 1994; Drew *et al*, 1997). To verify this hypothesis, MT_1-YFP or GPR50-YFP was expressed alone or with Rluc fusion proteins of GPR50, GPR50ΔCter or MT_1 as indicated in Figure 9A. Cell lysates containing equivalent quantities of YFP fusion proteins were immunoprecipitated and the presence of $G_{i\alpha}$ was revealed by Western blotting. $G_{i\alpha}$ was readily co-precipitated from melatonin-stimulated MT_1-YFP-containing cell lysates (Figure 9A, lane 1) confirming previous observations (Brydon *et al*, 1999). The specificity of the assay was illustrated by the absence of $G_{i\alpha}$ immunoreactivity in precipitates prepared from nontransfected cells (Figure 9A, lane 7). $G_{i\alpha}$ was also observed in co-immunoprecipitates from GPR50-YFP- and GPR50ΔCter-YFP-containing cell lysates (Figure 9A, lanes 2 and 3). At comparable receptor levels, the amount of $G_{i\alpha}$ associated with GPR50-YFP was lower compared to MT_1-YFP lysates and even lower for GPR50ΔCter-YFP. Identical amounts of $G_{i\alpha}$ were precipitated in the absence of melatonin stimulation (not shown). This indicates a certain degree of G_i preassociation to GPR50 (lane 2), which could depend on the C-terminus of GPR50 (lane 3). Preassociation of G proteins has also been observed recently for other GPCRs in intact cells (Bunemann *et al*, 2003; Gales *et al*, 2005). When MT_1 was coexpressed with GPR50 (lanes 4 and 6), less $G_{i\alpha}$ was co-precipitated compared to cell lysates prepared from cells expressing MT_1-YFP alone at comparable receptor levels (compare lanes 1 and 4). This observation suggests that the Cter of GPR50 impedes $G_{i\alpha}$ recruitment to MT_1 in the MT_1/GPR50 heterodimer. Indeed, the amount of co-precipitated $G_{i\alpha}$ clearly increased when MT_1-YFP was coexpressed with the GPR50ΔCter-Rluc construct (similar quantity as for the full-length GPR50-Rluc) (lanes 4 and 5), confirming that recruitment of $G_{i\alpha}$ to MT_1 is maintained only in the absence of the GPR50 Cter. Neither $G_{q\alpha}$, $G_{s\alpha}$ nor $G_{12\alpha}$ was found in GPR50/MT_1 precipitates (Supplementary Figure 3, lane 4), indicating that the GPR50/MT_1 heterodimer is completely devoid of G protein coupling.

Whether such a mechanism extends to other GPCR interacting partners such as β-arrestin was studied in a BRET-based β-arrestin recruitment assay. Dynamic recruitment of β-arrestin 1 to MT_1 was observed upon melatonin stimulation of cells coexpressing MT_1-Rluc and an N-terminally YFP-tagged β-arrestin 1 fusion protein (Figure 9B). A plateau was reached after approximately 15 min, which is consistent with reported β-arrestin recruitment kinetics. Coexpression of Flag-GPR50 decreased the melatonin-induced BRET signal

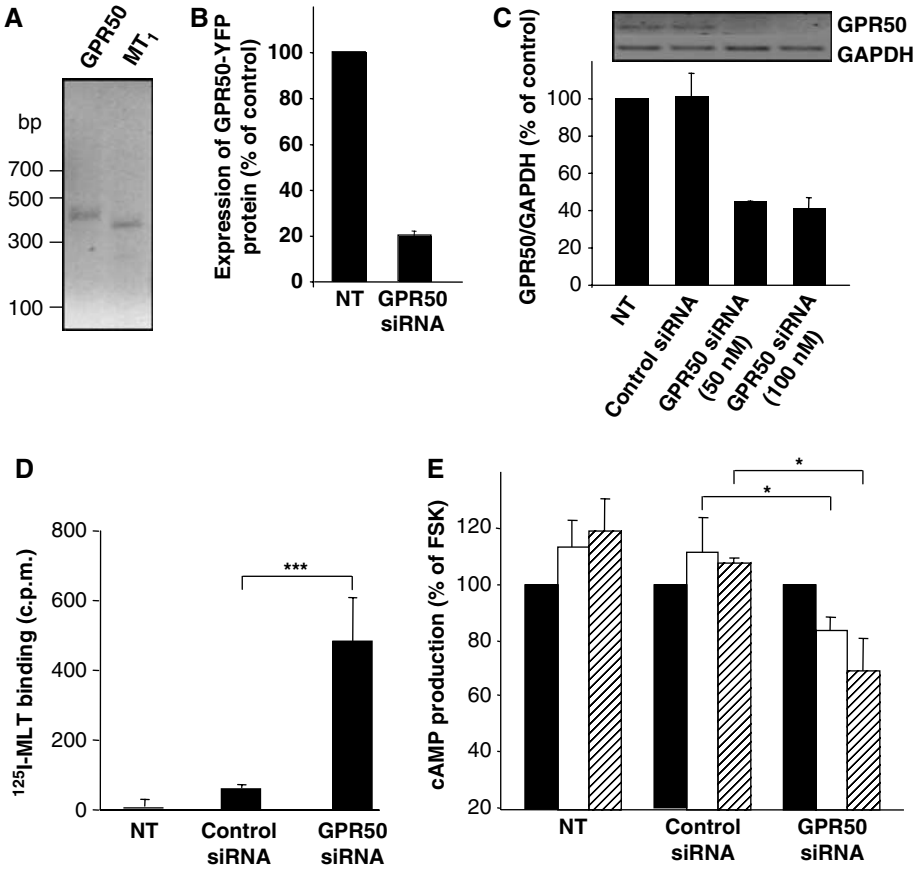


Figure 6 Downregulation of endogenously expressed GPR50 in hCMEC/D3 cells promotes MT_1 function. (**A**) GPR50 and MT_1 transcripts from hCMEC/D3 cells were reverse transcript and amplified by PCR. No amplification was observed when experiments were performed in the absence of reverse transcriptase (not shown). (**B**) HEK 293 cells stably expressing GPR50-YFP were transfected with GPR50-specific siRNA duplexes (100 nM) and GPR50-YFP fluorescence was measured by flow cytometry 48 h post-transfection. (C–E) Effect of control siRNA and GPR50-specific siRNA duplexes on GPR50 mRNA levels (**C**), ^{125}I-MLT binding (500 pM) (**D**), and forskolin-stimulated cAMP accumulation (**E**) in hCMEC/D3 cells. Stimulation with 1 μM forskolin alone (black bars) or in the presence of 1 μM melatonin (white bars) or 1 μM S20098 (hatched bars) (30 min). NT, nontransfected; bp, base pairs. Data are means ± s.e.m. of at least three independent experiments each performed in triplicate ($***P<0.001$; $*P<0.05$).

(Figure 9C, black bars) without modifying the expression levels of the two BRET partners (not shown). This result is consistent with a decrease of melatonin-sensitive MT_1 homodimers when coexpressed with GPR50 and an increase of melatonin-insensitive GPR50/MT_1 heterodimers. Interestingly, coexpression of GRP50 increased at the same time the basal BRET level (Figure 9C, white bars), suggesting a constitutive interaction between β-arrestin and the GPR50/MT_1 heterodimer.

Collectively, our data show that MT_1 is devoid of G protein coupling in the presence of GPR50, a phenomenon that might depend on the presence of the long C-terminal tail of GPR50 and on the constitutive interaction of the GPR50/MT_1 heterodimer with β-arrestins.

Discussion

It has become widely accepted that many members of the nonodorant GPCRs have the capacity to homo- and heterodimerize with important functional consequences on receptor pharmacology, signaling and regulation. So far, this has only been studied for GPCRs with known ligand and function. We are now extending the idea of GPCR dimerization towards orphan GPCRs. Using a combination of biochemical and biophysical techniques we show that the orphan GPR50 follows the general pattern of GPCR dimerization established for GPCRs with known ligands: GPR50 exists as constitutive homodimer or oligomer and heterodimerizes with other GPCRs in a specific manner (association with MT_1 and MT_2 but not with β2-AR and CCR5) with similar propensities for homo- and heterodimer formation. Importantly, engagement of the orphan GPR50 into heterodimers with MT_1, known to bind the circadian neurohormone melatonin, had profound consequences on MT_1 function. This effect appears to be specific for the GPR50/MT_1 heterodimer since it was not observed for heterodimers with the closely related MT_2.

Inhibition of GPCR function through heterodimerization has been observed for other GPCRs such as for the angiotensin II AT_1 receptor whose function is antagonized in AT_1/AT_2 and AT_1/Mas heterodimers (AbdAlla *et al*, 2001; Kostenis *et al*, 2005). In the case of the AT_1/AT_2 heterodimer, it has been suggested that the AT_2 receptor, which lacks common features of G protein coupling, stabilizes a conformation of the AT_1 receptor that can no longer undergo the conformational change necessary for G protein coupling. For the AT_1/Mas heterodimer, the molecular mechanism of

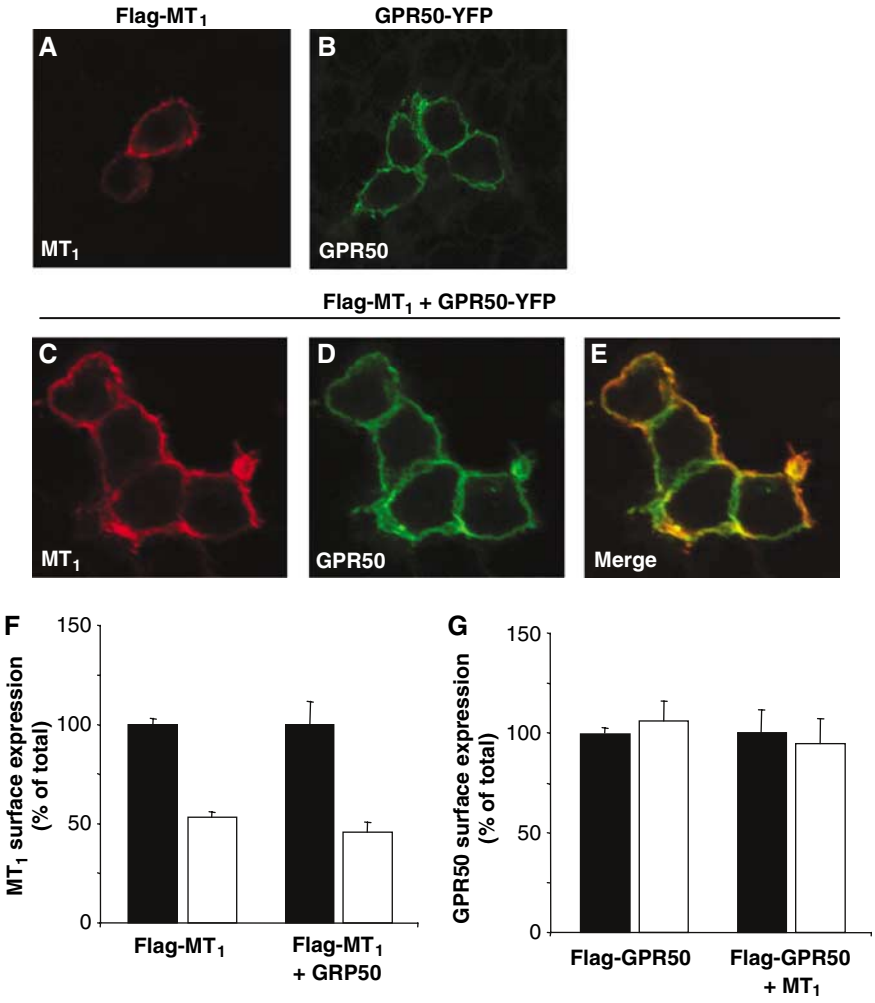


Figure 7 Subcellular localization of MT_1 and GPR50. (**A, B**) Confocal images of HEK 293 cells stably expressing Flag-MT_1 or GPR50-YFP. (**C–E**) Localization of Flag-MT_1 and GPR50-YFP in HEK 293 cells transiently coexpressing both receptors. Flag-MT_1 was detected by immunodetection after permeabilization and GPR50-YFP by measuring the YFP fluorescence. (F, G) ELISA quantification of Flag-MT_1 (**F**) and Flag-GPR50 (**G**) surface expression in the absence (black bars) and presence of melatonin (white bars). Data are means ± s.e.m. of at least three independent experiments each performed in duplicate.

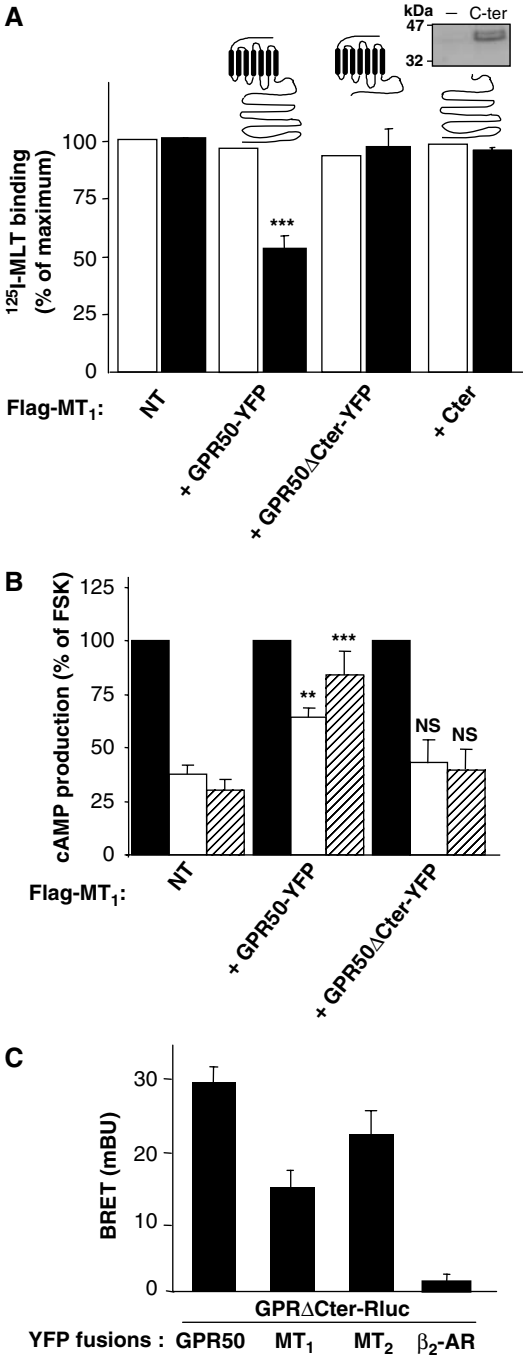
antagonism has not been clarified yet. In the present report, we establish a novel mechanism for the inhibition of GPCR function by heterodimerization, through inhibition of the recruitment of intracellular interacting partners such as G proteins owing to sterical hindrance by the large C-terminal domain of GPR50. β-Arrestin which could be constitutively recruited to the heterodimer might also contribute to the impaired G protein coupling of the heterodimer since both proteins have overlapping interaction sites with the receptor. Whether the constitutive interaction of β-arrestin with the GPR50/MT_1 heterodimer induces β-arrestin-dependent signaling events remains to be clarified in future studies.

The GPR50 mRNA is widely expressed in the brain with preferential expression in the hypothalamic–pituitary–adrenocortical system (Reppert *et al*, 1996; Drew *et al*, 1998, 2001; Vassilatis *et al*, 2003) where overlapping expression patterns with MT_1 and MT_2 have been observed. This point is further supported by our studies in human endothelial cerebral hCMEC/D3 cells.

Inhibition of MT_1 function by heterodimerization with GPR50 raises the question of the regulation of this process. Our data showed that the relative propensity of GPR50/MT_1 heterodimer formation compared to GPR50 homodimers depends on the relative expression levels of both receptors when coexpressed in the same cells. Coexpression of receptors such as the MT_2 that heterodimerize with GPR50 without any obvious functional consequences may behave as a competitor of the GPR50/MT_1 heterodimer. Little is known about the expression profile of GPR50 but differential regulation during development or during the day–night cycle can be postulated as shown for MT_1 (Poirel *et al*, 2002; Danilova *et al*, 2004). Regulation of GPR50 mRNA levels by microRNA molecules is also likely since expression of an miRNA sequence specifically targeting the 3′ untranslated region of the GPR50 transcript has been shown in the brain including the hypothalamic–pituitary–adrenocortical system (John *et al*, 2004; Sempere *et al*, 2004).

Several features of GPR50 suggest that orphan GPCRs may have important functions even in the absence of any known ligand binding properties. GPR50 was originally cloned from human pituitary cDNA library using degenerated PCR primers based on MT_1 and MT_2 (Reppert *et al*, 1996). The high degree of sequence homology with MT_1 and MT_2, the presence of shared amino-acid motifs and the conservation of

the overall gene structure clearly confirmed the classification of GPR50 in the melatonin receptor subfamily. Despite intensive efforts to deorphanize GPR50 with the latest state of the art GPCR ligand screening technology, the ligand of GPR50 remains elusive, even 9 years after its cloning and classification. Unusual features of GPR50 that may hint at important ligand-independent functions include the long C-terminus that is more than 300 amino acids long. It is likely that the C-terminus may either inhibit protein recruitment as shown in the GPR50/MT_1 heterodimer or function as a protein scaffold for other intracellular proteins. The recently discovered insertion/deletion polymorphism genetically associated with psychiatric disorders is located in the C-terminus underlining the functional importance of this domain.



Our finding raises the interesting possibility that some orphan GPCRs are not capable of binding any endogenous ligand but rather regulate the function of non-orphan GPCRs through heterodimerization or other mechanisms. Although this hypothesis is obviously difficult to prove, several recent observations are in favor of such mechanisms. The recent completion of the human genome allowed a comprehensive classification of GPCRs. In total, 12 human and 49 mice orphan GPCRs were assigned to subfamilies with known ligands (Vassilatis *et al*, 2003). Two further studies analyzing either sequences of the human rhodopsin GPCR subfamily or comparing human and drosophila GPCRs came to a similar conclusion (Fredriksson and Schioth, 2005; Metpally and Sowdhamini, 2005). Despite the fact that a good prediction for the putative ligand could be made, most of the receptors still remain as orphans (Civelli, 2005) consistent with the hypothesis that some receptors are unable to bind any ligand.

Additional support for the potential functional significance of heterodimers between orphan and nonorphan GPCRs comes from obligatory heterodimers observed in other GPCR classes. The functional $GABA_B$ receptor is composed of two homologous subunits called $GABA_{B1}$ and $GABA_{B2}$. Interestingly, each subunit of this obligatory heterodimer has a distinct function, whereas $GABA_{B1}$ provides ligand binding, $GABA_{B2}$ promotes efficient trafficking of $GABA_{B1}$ to the cell surface (White *et al*, 1998). Mechanistically, $GABA_{B2}$ can thus be considered as an 'orphan' receptor in the heterodimer. $T1R_1/T1R_3$ and $T1R_2/T1R_3$ heterodimers of taste receptors appear also to function as heterodimers between orphan and nonorphan receptors. $T1R_1$ and $T1R_2$, originally discovered as orphan receptors, have subsequently been 'deorphenized' by the discovery of their heterodimerization partner $T1R_3$. According to the current working model, taste receptor heterodimers are composed of $T1R_1$ and $T1R_2$, which bind the ligand, and the orphan $T1R_3$ (Xu *et al*, 2004). Similar observations have been made for the insect odorant receptor DOR83b that has no apparent affinity for a large panel of odorants but strongly increases the functionality of other odorant receptors such as DOR43 by heterodimerization (Neuhaus *et al*, 2005).

Figure 8 The inhibitory effect of GPR50 on MT_1 function in the GPR50/MT_1 heterodimer depends on the C-terminus of GPR50. (**A, B**) HEK 293 cells stably expressing 70–80 fmol of Flag-MT_1 per mg of protein were coexpressed with the indicated proteins. Expression of comparable amounts of YFP fusion proteins was verified by measuring YFP fluorescence (not shown). Expression of the Cter was monitored by Western blot using anti-GPR50 antibodies. (A) Surface expression of Flag-MT_1 was determined by flow cytometry using anti-Flag antibodies (white bars) and by radioligand binding using a saturating concentration of ^{125}I-MLT (black bars) (***$P<0.001$ compared to NT condition). (B) Cells were stimulated for 30 min with 1 μM forskolin alone (black bars) or in the presence of 1 μM melatonin (white bars) or 1 μM S20098 (hatched bars) and cAMP levels were determined (**$P<0.01$; NS, $P>0.05$ compared with corresponding NT condition). (**C**) GPR50ΔCter-Rluc was transiently coexpressed in HEK 293 cells with the indicated C-terminal YFP fusion proteins expressed at comparable amounts as determined by direct fluorescence measurements (20–30 fmol of YFP fusion receptor per mg of protein as estimated from curves correlating YFP fluorescence with the number of ^{125}I-MLT-binding sites (Ayoub *et al*, 2002)). BRET measurements were performed in living cells by adding 5 μM coelenterazine. All data are means ± s.e.m. of at least three independent experiments each performed in duplicate. NT, nontransfected.

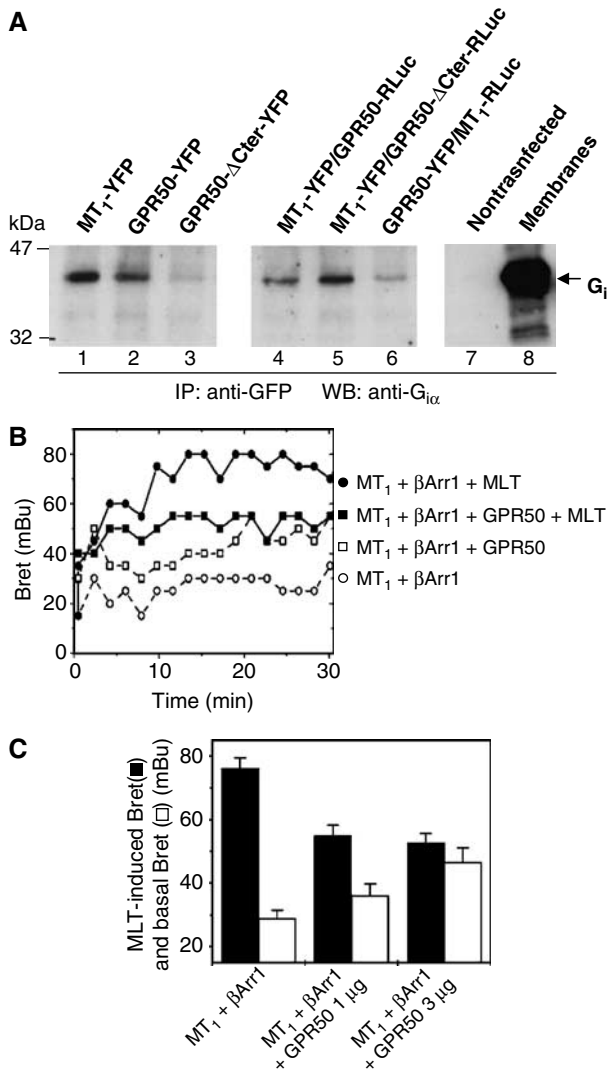


Figure 9 Binding of the GPR50/MT_1 heterodimer to G_i proteins and β-arrestin. (**A**) Membranes from HEK 293 cells transiently expressing 5–10 fmol of MT_1-YFP alone or in the presence of the indicated receptors were incubated with melatonin and solubilized. Equivalent quantities of YFP fusion proteins were immunoprecipitated, separated by SDS–PAGE and analyzed by Western blot. Data are representative of three experiments. (**B**) Dynamics of the interaction between MT_1 and β-arrestin. BRET signals were monitored during 30 min after the addition of melatonin (1 μM) in HEK 293 cells coexpressing MT_1-Rluc (MT_1) and YFP-β-arrestin (βArr1) with or without Flag-GPR50 (3 μg vector). (**C**) Graphic representation of mean melatonin-induced and basal BRET signals measured 15–30 min after melatonin addition. Values are depicted from curves presented in (B) and from additional curve generated from cells transfected with 1 μg of GPR50 expression vector. Data are means ± s.e.m. of at least three independent experiments each performed in duplicate.

In conclusion, the orphan GPR50 antagonizes the function of MT_1, which constitutes the first example of functional heterodimer formation between an orphan GPCR and a member of the large rhodopsin-like GPCR family with a known ligand. We propose that some orphan GPCRs could have ligand-independent functions such as the regulation of other GPCRs through heterodimerization. In this respect, evolutionary conservation of orphan receptors without any ligand binding may be considered as an evidence for the physiological importance of GPCR heterodimerization. Studying the heterodimerization profile of orphan GPCRs may become a new strategy to obtain better insights in the biology of GPCRs with known ligands and functions.

Materials and methods

Chemicals and reagents

S20098 was kindly provided by Institut de Recherches Servier. Anti-GPR50 antibodies were from Lifespan Biosciences (LS-A6621) (Interchim, Montluçon, France). Negative control siRNA Alexa Fluor 488 was from Qiagen (Cat No 1022563).

Receptor constructs

MT_1-Rluc, MT_2-Rluc, MT_1-YFP, MT_2-YFP, Flag-MT_1, Myc-MT_2 (Ayoub *et al*, 2002), β_2-AR-YFP (Angers *et al*, 2000), CCR5-YFP (Issafras *et al*, 2002) and YFP-β-arrestin-1 (Storez *et al*, 2005) have been described elsewhere. All GPR50 constructs were obtained by PCR using the described N-terminally Flag-tagged human GPR50 construct (Conway *et al*, 2000). GPR50ΔCter constructs are composed of GPR50 amino acids 1–349 and the Cter construct contains the amino acids 307–613.

Cell culture, transfection and RNA interference

HEK 293 cells were grown and transfected as described (Ayoub *et al*, 2002). Human endothelial cerebral hCMEC/D3 cells were cultured as described (Weksler *et al*, 2005). GPR50-specific siRNA duplexes are available upon request.

Reverse transcription–polymerase chain reaction

Total RNA was isolated using the TRIZOL Reagent (Invitrogen) as described by the manufacturer. PCR primers and conditions are available upon request.

Determination of intracellular inositol phosphates and cAMP levels

Inositol phosphate accumulation was performed in 96-well microplates after cell labeling overnight with [^{3}H]myo-inositol, 0.5 μCi/well (Amersham Biosciences) in the medium as described previously (Goudet *et al*, 2004). Cyclic AMP levels were determined by HTRF using the Cisbio 'cAMP femto2' kit according to the manufacturer's instructions.

Binding assays

Whole-cell ^{125}I-MLT radioligand binding assays were performed as described previously (Ayoub *et al*, 2002). IC_{50} values were transformed into K_i values using the Cheng–Prussof formula: $K_i = IC_{50}/[1 + (L/K_d)]$.

Immunoprecipitation and Western blotting

Co-immunoprecipitation: Crude membranes were solubilized with 1% digitonin and immunoprecipitated with the monoclonal anti-GFP antibody (4 μg/ml) (Roche, Basel, Switzerland) as described previously (Ayoub *et al*, 2004). G protein immunoprecipitation were carried out as described by Brydon *et al* (1999) using monoclonal anti-GFP (4 μg/ml) and polyclonal anti-$G_{\alpha i3}$ C-10 (0.4 μg/ml) (Santa Cruz, CA) antibodies.

BRET assay, luminescence and fluorescence measurements

BRET experiments, luminescence and fluorescence measurements were performed as described (Ayoub *et al*, 2002, 2004).

Immunofluorescence microscopy and cell surface ELISA

Fixed cells were permeabilized by 0.2% Triton X-100 first incubated with monoclonal anti-Flag antibody (Sigma, St Louis, MO) (2 μg/ml) and subsequently with a Cy3-coupled secondary antibody. For detecting GFP and Cy3 fluorescence, samples were excited using an argon laser at a wavelength of 488 nm for GFP and 514 nm for Cy3. Cell surface ELISA were performed as described (Brydon *et al*, 1999) using anti-Flag M2 (3 μg/ml) antibodies.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Note added in proof

While this manuscript was under revision, Milasta *et al*. (2006, *Mol Pharmacol* **69:** 479–491) published that the orphan MrgE receptor dimerizes with the MrgD receptor for β-alanine.

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