Convergence of Melatonin and Serotonin (5-HT) Signaling at MT<sub>2</sub>/5-HT<sub>2C</sub> Receptor Heteromers*

Received for publication, February 24, 2014, and in revised form, February 17, 2015. Published, JBC Papers in Press, March 13, 2015, DOI 10.1074/jbc.M114.559542

Maud Kamal, Florence Gbahou, Jean-Luc Guillaume, Avais M. Daulat, Abla Benleulmi-Chaachoua, Marine Luka, Patty Chen, Dina Kalbasi Anaraki, Marc Baroncinij, Clotilde Mannoury la Cour, Mark J. Millan, Vincent Prevojt, Philippe Delagrange, and Ralf Jockers*

From the INSERM, U1016, Institut Cochin, 75014 Paris, France, CNRS UMR 8104, 75014 Paris, France, Université Paris Descartes, Sorbonne Paris Cité, 75006 Paris, France, INSERM, Jean-Pierre Aubert Research Center, U837, 59045 Lille, France, and **Institut de Recherches Servier, 78290 Croissy/Seine, France

Background: There is cross-talk between serotonin and melatonin hormones.
Results: There is evidence for unidirectional transactivation and a heteromer-specific signaling profile for formation of functional melatonin MT<sub>2</sub> and serotonin 5-HT<sub>2C</sub> receptor heteromers.
Conclusion: A new potential target of the antidepressant agomelatine is identified.
Significance: The importance of binding of multitarget drugs to GPCR heteromers in psychiatric disorders is demonstrated.

Inasmuch as the neurohormone melatonin is synthetically derived from serotonin (5-HT), a close interrelationship between both has long been suspected. The present study reveals a hitherto unrecognized cross-talk mediated via physical association of melatonin MT<sub>2</sub> and 5-HT<sub>2C</sub> receptors into functional heteromers. This is of particular interest in light of the “synergistic” melatonin agonist/5-HT<sub>2C</sub> antagonist profile of the novel antidepressant agomelatine. A suite of co-immunoprecipitation, bioluminescence resonance energy transfer, and pharmacological techniques was exploited to demonstrate formation of functional MT<sub>2</sub> and 5-HT<sub>2C</sub> receptor heteromers both in transfected cells and in human cortex and hippocampus. MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers amplified the 5-HT-mediated G<sub>q</sub>/phospholipase C response and triggered melatonin-induced unidirectional transactivation of the 5-HT<sub>2C</sub> protomer of MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers. Pharmacological studies revealed distinct functional properties for agomelatine, which shows “biased signaling.” These observations demonstrate the existence of functionally unique MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers and suggest that the antidepressant agomelatine has a distinctive profile at these sites potentially involved in its therapeutic effects on major depression and generalized anxiety disorder. Finally, MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers provide a new strategy for the discovery of novel agents for the treatment of psychiatric disorders.

The monoamine serotonin (5-HT)<sup>2</sup> is derived from dietary tryptophan, which is transformed into 5-HT in diverse clusters of neurons in the gut and brain. 5-HT exerts its actions via 14 classes of receptors, which are broadly expressed in peripheral tissues and the central nervous system (1). Conversely, although the neurohormone melatonin is derived from 5-HT, it is mainly produced by the pineal gland in a circadian pattern under the control of hypothalamic nuclei, attaining peak levels during the night. Melatonin binds with high affinity to MT<sub>1</sub> and MT<sub>2</sub> receptors and with moderate affinity to the enzyme quinone reductase 2 (2). Both MT<sub>1</sub> and MT<sub>2</sub> receptors as well as all 14 classes of 5-HT receptor (except 5-HT<sub>1A</sub>) belong to the G protein-coupled receptor (GPCR) superfamily. Despite structural similarities between melatonin and 5-HT, melatonin does not recognize 5-HT receptors, and 5-HT fails to bind MT<sub>1</sub> or MT<sub>2</sub> receptors. Furthermore, to date, there have been only a few reports of functional cross-talk between melatonergic and serotonergic transmission: for example, melatonin inhibits the ability of 5-HT to phase shift the suprachiasmatic circadian clock (3).

Recent studies have demonstrated other more direct modes of potential functional interaction expressed not only among signaling pathways but also operating directly at the level of GPCRs, which can assemble into heteromeric complexes (4). Such complexes frequently display functional properties distinct from those of the corresponding homomers and may even transduce novel and unique cellular responses. Moreover, several classes of GPCR heteromers have been associated with the pathogenesis and control of CNS disorders like 5-HT<sub>2A</sub> and metabotropic glutamate-2 receptor (mGluR2) heteromers in frontal cortex implicated in schizophrenia and in the actions of antipsychotics (5) and limbic dopamine D<sub>1</sub> and D<sub>2</sub> receptor heteromers incriminated in depressed states (6).

To date, the possible existence of heteromeric associations of MT<sub>1</sub> or MT<sub>2</sub> receptor with specific classes of 5-HT receptors has not been evaluated. Their putative existence is of particular interest inasmuch as the clinically proven antidepressant ago-receptor; mGluR2, metabotropic glutamate-2 receptor; 4-PPDOT, 4-phe-nyl-2-propionamidotetralin; Rluc, Renilla luciferase; TRITC, tetramethylrhodamine isothiocyanate; IP, inositol 1-phosphate; βARK, β-adrenergic receptor kinase.
MT₂/5-HT₂C Receptor Heteromers

melatine, the first to possess a non-monoaminergic component of action, behaves as an agonist at G₁-coupled MT₁ and MT₂ receptors but as a neutral antagonist at G₉/₁₁-coupled 5-HT₂C receptors (7, 8). Intriguingly, although the affinity of agomelatine is substantially lower at 5-HT₂C versus MT₁ and MT₂ in vitro, this apparent difference is much less pronounced in vivo, suggesting that it may exert its actions “synergistically” via these sites. Indeed, both 5-HT₂C and MT₁ and MT₂ receptors are necessary for expression of the antidepressant actions of agomelatine, which cannot be reproduced either by melatonin or by selective 5-HT₂C antagonists alone (9). For example, “synergistical” MT₁, MT₂, and 5-HT₂C receptor- transduced actions of agomelatine may account for its induction of neurogenesis and BDNF synthesis as well as its modulation of glutamate release (for a review, see Ref. 9). In light of the above observations, the present studies explored the potential formation of heteromers between melatonin receptors and 5-HT₂C receptors and specifically examined the functional profile of agomelatine at these sites.

EXPERIMENTAL PROCEDURES

Compounds—All chemicals and ligands were purchased from Sigma-Aldrich with the exception of pertussis toxin, which was purchased from Alexis Biochemicals, and 4-PPDOT, luzindole, and SB242084, which were purchased from Tocris. SU20928, SU21767, and agomelatine were a gift from the Institut de Recherches Servier (France). S20928, S21767, and agomelatine is substantially lower at 5-HT₂C versus MT₁ and MT₂.

Cell Culture and Transfection—HEK293 cells were grown in complete medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 4.5 g/liter glucose, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 1 mM glutamine) (Invitrogen). Geneticin (G418) was added at 0.4 mg/ml to the pcDNA3-CMV plasmid containing the neomycin resistance gene. Transient transfections were performed using JetPEI (Polyplus Transfection, France) according to the manufacturer’s instructions.

DNA Constructs—The pcDNA3-CMV vectors expressing the human MT₂ receptor, the double brilliance Rluc8-YBARR2-YPet sensor, and the MT₁-YFP and the 5-HT₄-YFP fusion proteins were described previously (10 – 12). The INI isoform of the human 5-HT₂C coding region was fused at its C terminus with the coding region of Renilla luciferase (5-HT₂C-Rluc) or the yellow fluorescent protein (5-HT₂C-YFP) or at its N terminus with three HA tags (3xHA-5-HT₂C). The 5-HT₂C-S138N-Rluc mutant was obtained by mutagenesis from the 5-HT₂C-Rluc plasmid. All constructs were verified by sequencing.

Immunoprecipitation—For co-immunoprecipitation assays, HEK293 cells were seeded in 12-well plates and transiently transfected with 50 ng of 5-HT₂C-Rluc and 50 – 1950 ng of the corresponding YFP plasmids. 24 h after transfection, cell lysates were transferred into a 96-well white Optiplate (Perkin Elmer Life Sciences) precoated with 10 µg/ml poly-L-lysine (Sigma) and incubated for another 24 h before BRET measurements. Luminescence and fluorescence were measured simultaneously using the lumino/fluorometer Mithras™ (Berthold) as described previously (11) using optimized filter settings (Rluc filter, 480 ± 10 nm; YFP filter, 540 ± 20 nm).

Intracellular Signaling Assays—HEK293 cells stably expressing 20 – 30 fmol of MT₂/receptor were labeled with 400 pm 2-[¹²⁵I]iodomelatonin as described previously (15). Receptors were solubilized with 1% digitonin and precipitated with a mixture of three anti-5-HT₂C rabbit polyclonal antibodies (18461-10656, 18461-10657, and 18003-42961, Genway) or a pool of control preimmune serum, and immunoprecipitated radioactivity was determined.

Immunofluorescence—HeLa cells transiently expressing 6xMyc-MT₂ and 3xHA-5-HT₂C were fixed in phosphate-buffered saline containing 2% paraformaldehyde for 15 min. Cells were permeabilized with 0.1% Triton X-100. Monoclonal anti-Myc antibody 9E10 (Santa Cruz Biotechnology) and polyclonal anti-HA antibody (Cell Signaling Technology) were applied followed by TRITC-tagged anti-mouse and fluorescein isothiocyanate-tagged anti-rabbit antibodies. Cells were examined by fluorescence microscopy.

In-cell Western Experiments—Cell surface expression of 5-HT₂C receptors was evaluated by in-cell Western experiments in intact and Triton X-100-permeabilized HEK293 cells transiently expressing the 3xHA-5-HT₂C construct and stably expressing the MT₂ receptor (5-HT₂C expression) or transiently expressing the 6xMyc-MT₂ receptor (MT₂ expression) as described previously (16) using the rabbit monoclonal anti-HA antibody (1:1000; Cell Signaling Technology), anti-Myc 9E10 antibody (1:500) and the LI-COR Odyssey infrared imaging system.

Bioluminescence Resonance Energy Transfer (BRET) Measurement—For BRET donor saturation curves, HEK293 cells seeded in 12-well plates were transiently transfected with 50 ng of 5-HT₂C-Rluc and 50 – 1950 ng of the corresponding YFP plasmids. 24 h after transfection, cells were transferred into a 96-well white Optiplate (Perkin Elmer Life Sciences) precoated with 10 µg/ml poly-L-lysine (Sigma) and incubated for another 24 h before BRET measurements. Luminescence and fluorescence were measured simultaneously using the lumino/fluorometer Mithras™ (Berthold) as described previously (11) using optimized filter settings (Rluc filter, 480 ± 10 nm; YFP filter, 540 ± 20 nm).

Intracellular Signaling Assays—HEK293 cells stably expressing 20 – 30 fmol of MT₂/receptor were labeled with 400 pm 2-[¹²⁵I]iodomelatonin as described previously (15). Receptors were solubilized with 1% digitonin and precipitated with a mixture of three anti-5-HT₂C rabbit polyclonal antibodies (18461-10656, 18461-10657, and 18003-42961, Genway) or a pool of control preimmune serum, and immunoprecipitated radioactivity was determined.

Statistical Analysis—Results were analyzed by PRISM (GraphPad Software). Data are expressed as mean ± S.E. of at
least three experiments. Student’s t test was applied for statistical analysis.

RESULTS

Physical Interaction between 5-HT2C and MT1/MT2 Receptors—We first carried out co-immunoprecipitation experiments to assess a possible interaction between the human 5-HT2C and MT1 and MT2 receptors. These experiments revealed that 5-HT2C interacts with MT1 and MT2 in HEK293 cells co-expressing the respective receptors (Fig. 1, A and B). To further confirm the existence of these heteromeric complexes and to assess the propensity of heteromer formation, BRET experiments were performed. 5-HT2C was fused at its C terminus to the energy donor RLuc. C-terminal YFP fusion proteins (5-HT2C-YFP, MT1-YFP, MT2-YFP, and 5-HT4d-YFP) acted as energy acceptors (Fig. 1, C). The expected hyperbolic donor saturation curve, reflecting a specific interaction between BRET donor and acceptor pairs, was observed for all receptor combinations except for the negative control 5-HT4d-YFP fusion protein for which a quasilinear increase in BRET reflecting a nonspecific interaction due to random collision was observed (18, 19). Determination of BRET50 values, corresponding to the saturation curve, was performed in triplicates.
5-HT₂C was present at the plasma membrane where both receptors colocalized (Fig. 1D). Formation of MT₂/5-HT₂C heteromers was further suggested by co-immunoprecipitation studies in human cortex and hippocampus, two regions shown previously to express melatonin and 5-HT₂C receptors (20, 21) (data not shown). Heat-inactivated anti-MT₂ antibodies were used as a negative control. Further evidence for the formation of MT₂/5-HT₂C heteromers was obtained from choroid plexus membranes, which are known to express significant amounts of 5-HT₂C receptors. Melatonin receptors were labeled with 2-[^125]I]iodomelatonin, and protein complexes were solubilized and immunoprecipitated with anti-5-HT₂C antibodies. As shown in Fig. 1E, significant amounts of radiolabeled melatonin receptors were precipitated with anti-5-HT₂C antibodies as compared with a mixture of irrelevant control antibodies. Overall, our results indicate that 5-HT₂C specifically interacts with MT₂ in transfected HEK293 cells and in the cortex, hippocampus, and choroid plexus.

**Melatonin but Not 5-HT Activates the G₁/cAMP Pathway through MT₂/5-HT₂C Heteromers**—HEK293 cells expressing equivalent and physiologically relevant levels of MT₂ and 5-HT₂C (20–30 fmol each/mg of protein) either alone or together were treated with forskolin and increasing concentrations of melatonin followed by determination of cAMP levels. Melatonin decreased cAMP levels in cells expressing MT₂ alone as expected and in cells co-expressing MT₂ and 5-HT₂C with the same efficiency (EC₅₀ = 0.76 ± 0.46 versus 0.76 ± 0.39 nM, respectively) and potency (30% reduction) (Fig. 2A and Table 1). Melatonin was without effect in cells expressing 5-HT₂C alone. Stimulation with up to 10 μM 5-HT did not modify cAMP levels in any of the three cell types (Fig. 2B). These results indicate that activation of the MT₂ protomer of the MT₂/5-HT₂C heteromer activates the G₁/cAMP pathway but that activation of the 5-HT₂C protomer is unable to transactivate this pathway.

**MT₁ Potentiates 5-HT-induced Signaling by Increasing Cell Surface Expression of 5-HT₂C in the MT₁/5-HT₂C Heteromer**—We next investigated the capacity of MT₁/5-HT₂C heteromers to activate the G₂/PLC pathway by monitoring 5-HT-induced IP production. Stimulation of HEK293 cells expressing 5-HT₂C alone resulted in the expected dose-dependent increase in IP production (Fig. 3A). This response was potentiated (~3-fold) in cells expressing similar quantities of 5-HT₂C receptors, but in

---

**TABLE 1**

Compared potencies and properties of melatonin receptor ligands on two signaling pathways in cells expressing MT₂ or MT₂ and 5-HT₂C receptors

| MTR agonists | Inhibition of cAMP production | Stimulation of inositol phosphate production |
|--------------|-------------------------------|---------------------------------------------|
|               | EC₅₀ ± S.E. (nM)              |                                             |
| MT₂          | MT₂/5-HT₂C                    |                                             |
| Melatonin    | 0.76 ± 0.46                   | 0.76 ± 0.39                                 |
| Agomelatine  | 0.29 ± 0.16                   | 0.89 ± 0.35                                 |
|               |                               |                                             |
| MTR antagonists |                                |                                             |
| Luzindole    | Antagonist (Kᵢ = 1.12 ± 0.48 nM) | Antagonist (Kᵢ = 7.73 ± 4.98 nM)           |
| 4-PPDOT      | Antagonist (Kᵢ = 0.14 ± 0.12 nM) | Antagonist (Kᵢ = 0.04 ± 0.01 nM)           |
| S2D928       | Antagonist (Kᵢ = 4.88 ± 1.5 nM) | Antagonist (Kᵢ = 0.66 ± 0.45 nM)           |

**EC₅₀ values of MTR antagonists are derived from data shown in Fig. 6 and are defined as EC₅₀ = IC₅₀/1 + (S/X) where S and X represent the agonist concentration and EC₅₀, respectively. ND, not determined.**
the presence of MT₂, the response was of similar potency (EC₅₀ = 21 ± 11 nM (5-HT₂C) versus 68 ± 25 nM (MT₂/5-HT₂C)). 5-HT was ineffective in cells expressing MT₂ alone (Fig. 3A). Amplification of the 5-HT-induced response was G₉/₁₁-dependent as determined by pretreating cells with YM254890 or pertussis toxin inhibitors, respectively (Fig. 3B). Expression of the Gβγ scavenger βARK₉/₁₁ had no significant effect (Fig. 3C), confirming the predominant role of G₉/₁₁α proteins in the observed amplification. Pretreatment of cells with 5-HT₂C antagonists (RS102221 and SB242084) or an inverse agonist (SB206553) completely blocked the 5-HT-induced IP production in cells expressing 5-HT₂C alone and in the presence of MT₂ as expected (Fig. 3D). Pretreatment with S20928, a melatonin receptor antagonist, showed no cross-reactivity on 5-HT-induced responses (Fig. 3E).

Amplified 5-HT-induced responses in the context of the MT₂/5-HT₂C heteromer might be explained by increased cell surface expression of 5-HT₂C receptors. In agreement with previous reports, only a minor fraction of 5-HT₂C receptors (13.2 ± 1.1%) was expressed at the cell surface when expressed alone as determined by in-cell Western experiments (*, p < 0.05; n.s., not significantly different). Data represent the mean ± S.E. (error bars) of at least three independent experiments performed in triplicates. Results that are statistically different compared with 5-HT alone for 5-HT₂C cells (●, p < 0.05; ●●, p < 0.01) and MT₂/5-HT₂C cells (**, p < 0.01; ***, p < 0.001) are indicated.

**FIGURE 3.** 5-HT-promoted potentiation of the Gq/PLC pathway by the MT₂/5-HT₂C heteromer. A, 5-HT-induced IP production was assessed in HEK293 cells expressing 5-HT₂C (●), MT₂ (●), or 5-HT₂C and MT₂ (●) receptors. B–E, HEK293 cells expressing 5-HT₂C receptors alone or together with MT₂ receptors were treated with heterotrimeric G protein inhibitors (B), 5-HT₂C receptor ligands (D), or the melatonin receptor ligand S20928 (E) or co-expressed with the βARK₉/₁₁ Gβγ scavenger (C). F, cell surface expression of 5-HT₂C and MT₂ receptors in cells expressing 5-HT₂C in the absence and presence of MT₂ receptors measured by in-cell Western experiments (*, p < 0.05; n.s., not significantly different). Data represent the mean ± S.E. (error bars) of at least three independent experiments performed in triplicates. Results that are statistically different compared with 5-HT alone for 5-HT₂C cells (●, p < 0.05; ●●, p < 0.01) and MT₂/5-HT₂C cells (**, p < 0.01; ***, p < 0.001) are indicated.
Activation of the MT₂ Protomer Transactivates the PLC Pathway and Improves /H9252-Arrestin Recruitment in the MT₂/5-HT₂C Heteromer—To explore the possible effect of MT₂ activation in the MT₂/5-HT₂C heteromer on the Gq/PLC pathway, we stimulated HEK293 cells expressing MT₂ and 5-HT₂C either alone or together with melatonin and determined IP production. Whereas melatonin had no effect in cells expressing either receptor alone, a dose-dependent increase in IP production was observed in cells co-expressing both receptors with an EC₅₀ of 69 ± 20 nM (Fig. 4A). To verify that melatonin-induced IP production is not due to the signaling cross-talk between MT₂ and any Gq-coupled GPCR, we co-expressed MT₂ with the Gq-coupled M₁ muscarinic receptor, which does not form heteromers with MT₂ (data not show). In these cells, melatonin did not increase IP production, indicating that melatonin-induced IP production is specific for the MT₂/5-HT₂C heteromer (Fig. 4B). Functional expression of M₁ receptors was shown by the expected increase in IP production in the presence of acetylcholine (Fig. 4B).

Melatonin-induced IP production in cells co-expressing MT₂ and 5-HT₂C receptors was not affected by the expression of the Gβγ scavenger βARK₇ (Fig. 4C), but it was partially and completely abrogated by pertussis toxin and YM254890 treatment, respectively (Fig. 4D). This indicates the predominant role of Gqα proteins, which can be assisted by the presence of Giα proteins. This result raises the intriguing possibility that melatonin binding to the MT₂ protomer of the MT₂/5-HT₂C heteromer transactivates the 5-HT₂C protomer, which then activates Gqα.

To verify this hypothesis, we evaluated whether the melatonin-induced response depended on the activation state of the 5-HT₂C protomer. Pretreatment of cells with 5-HT₂C neutral antagonists (RS102221 and SB242084) prior to melatonin addition had no effect on melatonin-induced IP production, whereas the inverse agonist SB206553 blocked the effect (Fig. 5A). This is consistent with the notion that the melatonin-induced effect is independent of the occupation of the 5-HT₂C receptor binding site but dependent on the constitutive activity of the 5-HT₂C protomer.

To further verify the transactivation hypothesis, we used the 5-HT₂C-S138N mutant, which does not bind 5-HT and exhibits decreased constitutive activity due to decreased coupling to Gq proteins compared with the wild-type receptor (22). The absence of 5-HT-promoted IP production was confirmed in cells expressing 5-HT₂C-S138N and MT₂ (Fig. 5B). When cells co-expressing this mutant and MT₂ receptors were stimulated with melatonin, the anticipated reduction in amplitude and efficiency (EC₅₀ = 550 ± 187 versus 69 ± 20 nM for MT₂/5-HT₂C-S138N and MT₂/5-HT₂C, respectively (p < 0.001)) was observed compared with 5-HT₂C wild-type receptor-expressing cells (Fig. 5C). Decreased activity was not due to less efficient heteromerization with MT₂ as comparable BRET5₀ values (2.2 ± 0.4 versus 3.5 ± 0.6 for MT₂/5-HT₂C and MT₂/5-HT₂C-S138N, respectively) were observed (Fig. 5D). The decreased response to melatonin in the presence of the 5-HT₂C-S138N mutant further supports the transactivation mechanism in which the melatonin-induced conformational change in the MT₂ protomer is transmitted to the 5-HT₂C protomer and then further downstream to the Gq/PLC pathway.

Apart from interacting with heterotrimeric G proteins, GPCRs are also known to recruit β-arrestins. We therefore...
studied the ability of melatonin to activate the previously described β-arrestin 2 BRET sensor (11, 23) in cells expressing either MT2 alone or together with 5-HT2C. As seen in Fig. 5E, MT2 alone only weakly activated the BRET sensor with an EC50 of 260 ± 170 nM. In contrast, melatonin potently recruited the sensor in cells co-expressing the 5-HT2C-S138N mutant and MT2 receptor. C, melatonin-induced transactivation of 5-HT2C wild-type and 5-HT2C-S138N mutant receptors in the presence of MT2. D, BRET donor saturation curves of HEK293 cells expressing MT2–Rluc and 5-HT2C–Rluc or 5-HT2C-S138N–Rluc. Melatonin-induced (E) and 5-HT-induced (F) recruitment of β-arrestin 2 measured by BRET in HEK293 cells expressing 5-HT2C (∇), MT2 (●), or 5-HT2C and MT2 (○) receptors is shown. Data represent the mean ± S.E. (error bars) of at least three independent experiments performed in triplicates. mBu, milli-BRET units.

FIGURE 5. Transactivation of the Gq/PLC pathway by melatonin in the MT2/5-HT2C heteromer. A, melatonin (MLT)-induced IP production in the presence of 5-HT2C ligands in HEK293 cells expressing MT2 receptors alone or co-expressing 5-HT2C receptors (**, p < 0.05: melatonin versus melatonin + SB206553 on heteromers). B, 5-HT-induced IP production in cells co-expressing the 5-HT2C-S138N mutant and MT2 receptor. C, melatonin-induced transactivation of 5-HT2C wild-type and 5-HT2C-S138N mutant receptors in the presence of MT2. D, BRET donor saturation curves of HEK293 cells expressing MT2–Rluc and 5-HT2C–Rluc or 5-HT2C-S138N–Rluc. Melatonin-induced (E) and 5-HT-induced (F) recruitment of β-arrestin 2 measured by BRET in HEK293 cells expressing 5-HT2C (∇), MT2 (●), or 5-HT2C and MT2 (○) receptors is shown. Data represent the mean ± S.E. (error bars) of at least three independent experiments performed in triplicates. mBu, milli-BRET units.
pathway (EC\textsubscript{50} = 2.5 ± 1.7 nM and EC\textsubscript{50} = 223 ± 97 nM, respectively) (Fig. 6, A and C, and Table 1). Taken together, these data suggest that S20928 is an antagonist at both pathways with some partial agonistic activity at very high concentrations and that 4-PPDOT and luzindole are G\textsubscript{q}/PLC pathway-biased ligands of MT\textsubscript{2}/5-HT\textsubscript{2C} receptors.

We next studied the effect of agomelatine, the antidepressant with agonistic activity at MT\textsubscript{2} receptors and neutral antagonistic properties at 5-HT\textsubscript{2C} receptors (7, 8). In cells expressing MT\textsubscript{2} in the absence or presence of 5-HT\textsubscript{2C} receptors, agomelatine behaved as an agonist of the G\textsubscript{i}/cAMP pathway (EC\textsubscript{50} = 0.29 ± 0.16 nM and EC\textsubscript{50} = 0.89 ± 0.35 nM, respectively) (Fig. 7A and Table 1) but as an antagonist for the melatonin-induced activation of the G\textsubscript{i}/PLC pathway (Fig. 7, B and C). Agomelatine also antagonized the 5-HT response on this pathway, which is compatible with the properties of this compound (Fig. 7D). This clearly shows that agomelatine has a distinct action profile on MT\textsubscript{2}/5-HT\textsubscript{2C} heteromers as compared with melatonin and 5-HT.

### DISCUSSION

We describe here a previously unappreciated dimension of cross-talk between melatonin and 5-HT that is mediated by heteromers of MT\textsubscript{2} and 5-HT\textsubscript{2C} receptors. MT\textsubscript{2}/5-HT\textsubscript{2C} heteromers have unique functional properties and are formed preferentially compared with the corresponding homomers. MT\textsubscript{1} and 5-HT\textsubscript{2C} receptors also form heteromers in transfected cells, but we focus herein on MT\textsubscript{2}/5-HT\textsubscript{2C} heteromers. Within these heteromers, melatonin is able to activate distinct cellular cascades: not only the G\textsubscript{i}/cAMP pathway as for MT\textsubscript{2} homomers but also the G\textsubscript{q}/PLC pathway by transactivation of the 5-HT\textsubscript{2C} protomer. This transactivation was unidirectional and not observed for the MT\textsubscript{2} protomer upon 5-HT stimulation. Whereas melatonin activates both pathways, other ligands have a more restricted profile using either the direct activation or transactivation mode. Interestingly, the clinically active antidepressant agomelatine shows functional properties on MT\textsubscript{2}/5-HT\textsubscript{2C} heteromers that are biased toward the G\textsubscript{i}/cAMP pathway and thus distinct from those of melatonin- and 5-HT\textsubscript{2C}-specific antagonists.

GPCR heteromers are indeed increasingly recognized as independent pharmacological entities participating in physiological functions and drug action (4). Ligands such as agomelatine are particularly interesting in this context as they have the potential to bind to both protomers. Previous studies established that agomelatine behaves as an agonist at G\textsubscript{i}-coupled MT\textsubscript{1} and MT\textsubscript{2} receptors and as a neutral antagonist at G\textsubscript{q/11}-coupled 5-HT\textsubscript{2C} receptors (7, 8). Our data indicate that agomelatine preserves these properties in MT\textsubscript{2}/5-HT\textsubscript{2C} heteromers and behaves as a competitive antagonist on the 5-HT binding site and as an agonist on the melatonin binding site. However, not all effects of melatonin are mimicked by agomelatine because it is unable to transactivate 5-HT\textsubscript{2C} receptors. Importantly, these properties clearly distinguish agomelatine from melatonin or any other tested compound including 5-HT\textsubscript{2C} receptor antagonists.

Interestingly, the MT\textsubscript{2}/5-HT\textsubscript{2C} heteromer appears to behave in an asymmetric manner as the properties of the two protomers are differently affected by heteromerization. In the case of the 5-HT\textsubscript{2C} protomer, no modification of the signaling profile per se was observed but rather an amplification of known 5-HT-promoted responses most likely due to increased surface expression of the 5-HT\textsubscript{2C} receptor. Limited surface expression of 5-HT\textsubscript{2C} receptors is in agreement with previous studies showing a high level of constitutive internalization for this receptor (26). In the MT\textsubscript{2} protomer, melatonin stimulation not only activates the G\textsubscript{i}/cAMP pathway as for MT\textsubscript{2} homomers but also transactivates the G\textsubscript{q}/PLC pathway through the 5-HT\textsubscript{2C} protomer in a unidirectional manner (not seen upon 5-HT stimulation). Transactivation between protomers is a unique property of GPCR dimers that has been observed in a limited...
number of other cases such as the GABA<sub>B</sub> receptor, which is an obligatory heteromer composed of two subunits with one subunit binding the ligand and the other activating the G protein (27). Another example of this activation mode is the dopamine D<sub>2</sub> receptors (28). Notably, the present functional characterization allowed us to identify biased ligands. Whereas luzindole and 4-PPDOT behave as agonists in the transactivation mode of the 5-HT<sub>2C</sub> protomer by the MT<sub>2</sub> protomer, agomelatine is completely inactive in this mode but still able to fully activate the MT<sub>2</sub> protomer.

Simultaneous activation of G<sub>i</sub>- and G<sub>q</sub>-dependent signaling by melatonin is a distinctive feature of MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers compared with the corresponding homomers. Notably, the balance between G<sub>i</sub>- and G<sub>q</sub>-dependent signaling has been recently suggested to be an important parameter determining the ligand action on GPCR heteromers (29). This has been shown for mGluR2/5-HT<sub>2A</sub> heteromers, which are composed of the G<sub>q</sub>-coupled 5-HT<sub>2A</sub> receptor and the G<sub>i</sub>-coupled mGluR2 receptor (5) and for which the balance between G<sub>i</sub>- and G<sub>q</sub>-dependent signaling predicts the anti- or propychoptic activity of drugs targeting mGluR2 and 5-HT<sub>2A</sub> receptors. Antipsychotic drugs have a high G<sub>i</sub>/G<sub>q</sub> activation ratio regardless of which receptor they target, whereas propychoptic drugs have a low ratio. A similar balance between G<sub>i</sub> and G<sub>q</sub> activation can be proposed for ligands acting on MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers. According to our current functional characterization, agomelatine is unique inasmuch as it possesses the highest G<sub>i</sub>/G<sub>q</sub> activation ratio (activation of both pathways), and luzindole, 4-PPDOT, and 5-HT would have a low ratio, exclusively activating the G<sub>q</sub> pathway.

In conclusion, this present work revealed the capacity of MT<sub>2</sub> and 5-HT<sub>2C</sub> receptors to assemble into functional heteromers. The binding and coupling properties of MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers and the cellular pathway-biased ligands identified in the present study provide a solid framework for the study of the potential involvement of MT<sub>2</sub>/5-HT<sub>2C</sub> heteromers in the beneficial action of agomelatine in the treatment of major depression and generalized anxiety disorder. Targeting of GPCR heteromers might be of general importance for the increasing number of multitarget drugs developed in particular to treat psychiatric diseases (30, 31).

Acknowledgments—We thank Drs. Jean A. Boutin, Olivier Nosjean, and Francis Cogé (Servier, Croissy, France) for help during the initial phase of the project; Erika Cecon (University of Sao Paulo, Brazil) for comments on the manuscript.

REFERENCES
1. Millan, M. J., Marin, P., Bockaert, J., and Mannoury la Cour, C. (2008) Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. Trends Pharmacol. Sci. 29, 454–464
2. Jockers, R., Maurice, P., Boutin, J. A., and Delagrange, P. (2008) Melatonin receptors, heterodimerization, signal transduction and binding sites: what’s new? Br. J. Pharmacol. 154, 1182–1195
3. Prosser, R. A. (1999) Melatonin inhibits in vitro serotonergic phase shifts of the suprachiasmatic circadian clock. Brain Res. 818, 408 – 413
4. Milligan, G. (2009) G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. Br. J. Pharmacol. 158, 5–14
5. González-Maeso, J., Ang, R. L., Yuen, T., Chan, P., Weisstaub, N. V., López-Giménez, J. F., Zhou, M., Okawa, Y., Callado, L. F., Milligan, G.,
MT₂/5-HT₂C Receptor Heteromers

Gingrich, J. A., Filzola, M., Meana, J. J., and Sealfon, S. C. (2008) Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452, 93–97

6. Pei, L., Li, S., Wang, M., Diwan, M., Anisman, H., Fletcher, P. J., Nobrega, J. N., and Liu, F. (2010) Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. *Nat. Med.* 16, 1393–1395

7. Audinot, V., Mailliet, F., Lahaye-Brasseur, C., Bonnau, A., Le Gall, A., Amossé, C., Dromaint, S., Rodriguez, M., Nagel, N., Galizzi, J. P., Malpau, B., Guillaumet, G., Lesieur, D., Lefoulon, F., Renard, P., Delargrange, P., and Boutin, J. A. (2003) New selective ligands of human cloned melanotin MT1 and MT2 receptors. *Naunyn Schmiedeberg Arch. Pharmacol.* 367, 553–561

8. Millan, M. J., Gobert, A., Lejeune, F., Dekeyne, A., Newman-Tancredi, A., Pasteau, V., Rivet, J. M., and Cussac, D. (2005) The novel melatonin agonist agomelatine (S20098) is an antagonist at 5-hydroxytryptamine 2C receptors, blockade of which enhances the activity of frontocortical dopaminergic and adrenergic pathways. *J. Pharmacol. Exp. Ther.* 306, 954–964

9. Racagni, G., Riva, M. A., Molteni, R., Musazzi, L., Calabrese, F., Popoli, M., and Tardito, D. (2011) Mode of action of agomelatine: Synergy between melatonergic and 5-HT2C receptors. *World J. Biol. Psychiatry* 12, 574–587

10. Petit, L., Lacroix, I., de Coppet, P., Strosberg, A. D., and Jockers, R. (1999) Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3'-5'-monophosphate pathway. *Biochem. Pharmacol.* 58, 633–639

11. Kamal, M., Marquez, M., Vauthier, V., Leloire, A., Farguel, P., Jockers, R., and Couturier, C. (2009) Improved donor/acceptor BRET couples for monitoring β-arrestin recruitment to G protein-coupled receptors. *Bio-technol. J.* 4, 1337–1344

12. Berthouze, M., Ayoub, M., Russo, O., Rivail, L., Sicsic, S., Fischmeister, R., Bérque-Bestel, I., Jockers, R., and Lezoualc'h, F. (2005) Constitutive dimerization of human serotonin 5-HT4 receptors in living cells. *FEBS Lett.* 579, 2973–2980

13. Jockers, R., Issad, T., Zilberfarb, V., de Coppet, P., Marullo, S., and Strosberg, A. D. (1998) Desensitization of the β-adrenergic response in human brown adipocytes. *Endocrinology* 139, 2676–2684

14. Savaskan, E., Ayoub, M. A., Ravid, R., Angeloni, D., Fraschini, F., Meier, F., Eckert, A., Müller-Spaßn, F., and Jockers, R. (2005) Reduced hippocampal MT2 melatonin receptor expression in Alzheimer’s disease. *J. Pineal Res.* 38, 10–16

15. Guillaume, J. L., Daulat, A. M., Maurice, P., Levoeye, A., Migaud, M., Brydon, L., Malpau, B., Borg-Capra, C., and Jockers, R. (2008) The PDZ protein muppl promotes G₁ coupling and signaling of the MT1 melatonin receptor. *J. Biol. Chem.* 283, 16762–16771

16. Bonnefond, A., Clément, N., Fawcett, K., Yengo, L., Vaillant, E., Guillemet, A. D. (1998) Desensitization of the MT2/5-HT2C receptor heteromers. *J. Pineal Res.* 29, 93–97

17. Chaste, P., Clément, N., Mercati, O., Guillaume, J. L., Delorme, R., Botros, H. G., Pagan, C., Perivier, S., Scheid, I., Nygren, G., Anckarsäter, H., Ramstedt, M., Ståhlberg, O., Gillberg, C., Serrano, E., Lemièvre, N., Launay, J. M., Mouren-Simeoni, M. C., Leboyer, M., Gillberg, C., Jockers, R., and Bourgeron, T. (2010) Identification of pathwaybiased and deleterious melatonin receptor mutants in autism spectrum disorders and in the general population. *PLoS One* 5, e11495

18. Mercier, J. F., Salhbour, A., Angers, S., Breit, A., and Bouvier, M. (2002) Quantitative assessment of β₁- and β₂-adrenergic receptor homo- and hetero-dimerization by bioluminescence resonance energy transfer. *J. Biol. Chem.* 277, 44925–44931

19. Couturier, C., and Jockers, R. (2003) Activation of leptin receptor by a ligand-induced conformational change of constitutive receptor dimers. *J. Biol. Chem.* 278, 26604–26611

20. Brunner, P., Sóza–Topcular, N., Jockers, R., Ravid, R., Angeloni, D., Fraschini, F., Eckert, A., Müller-Spaßn, F., and Savaskan, E. (2006) Pineal and cortical melatonin receptors MT1 and MT2 are decreased in Alzheimer’s disease. *Eur. J. Histochern.* 50, 311–316

21. Pasqualetti, M., Ori, M., Castagna, M., Marazziti, D., Cassano, G. B., and Nardi, I. (1999) Distribution and cellular localization of the serotonin type 2C receptor messenger RNA in human brain. *Neuroscience* 92, 601–611

22. Herrick-Davis, K., Grinde, E., Harrigan, T. I., and Mazurkiewicz, J. E. (2005) Inhibition of serotonin 5-hydroxytryptamine 2C receptor function through heterodimerization: receptor dimers bind two molecules of ligand and one G-protein. *J. Biol. Chem.* 280, 40144–40151

23. Char est, P. G., Terrillon, S., and Bouvier, M. (2005) Monitoring agonist-promoted conformational changes of β-arrestin in living cells by intramolecular BRET. *EMBO Rep.* 6, 334–340

24. Ying, S. W., Rusak, B., Delargrange, P., Mocera, E., Renard, P., and Guirdla-Lemaître, B. (1996) Melatonin analogues as agonists and antagonists in the circadian system and other brain areas. *Eur. J. Pharmacol.* 296, 33–42

25. MacKenzie, R. M., Meana, M. A., Passy, D. K., and Witt–Enderby, P. A. (2002) Dual coupling of MT1 and MT2 melatonin receptors to cyclic AMP and phosphoinositide signal transduction cascades and their regulation following melatonin exposure. *Biochem. Pharmacol.* 63, 587–595

26. Marion, S., Weiner, D. M., and Caron, M. G. (2004) RNA editing induces variation in desensitization and trafficking of 5-hydroxytryptamine 2C receptor isoforms. *J. Biol. Chem.* 279, 2945–2954

27. Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettle r, B., Prézeau, L., and Pin, J. P. (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA₆ receptor function. *EMBO J.* 20, 2152–2159

28. Yu, H., Moreira, I. S., Urizar, E., Weinstein, H., and Javitch, J. A. (2009) Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. *Nat. Chem. Biol.* 5, 688–695

29. Fribourg, M., Moreno, J. L., Holloway, T., Provasi, D., Baki, L., Mahajan, R., Park, G., Adney, S. K., Hatcher, C., Eltit, J. M., Ruta, J. D., Albizu, L., Li, Z., Umali, A., Shim, J., Fabiato, A., Mackrell, A. D., Jr., Brezina, V., Sealfon, S. C., Filzola, M., González-Maeso, J., and Logothetis, D. E. (2011) Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell* 147, 1011–1023

30. Millan, M. J. (2006) Multi-target strategies for the improved treatment of depressive states: conceptual foundations and neuronal substrates, drug discovery and therapeutic application. *Pharmacol. Ther.* 110, 155–370

31. Wong, E. H., Tarazi, F. I., and Shahid, M. (2010) The effectiveness of multi-target agents in schizophrenia and mood disorders: relevance of receptor signature to clinical action. *Pharmacol. Ther.* 126, 173–185