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Convergence of Melatonin and Serotonin (5-HT) Signaling at MT_2/5-HT_{2C} Receptor Heteromers*

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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_2 and serotonin 5-HT_{2C} 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_2 and 5-HT_{2C} receptors into functional heteromers. This is of particular interest in light of the "synergistic" melatonin agonist/5-HT_{2C} 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_2 and 5-HT_{2C} receptor heteromers both in transfected cells and in human cortex and hippocampus. MT_2/5-HT_{2C} heteromers amplified the 5-HT-mediated G_q/phospholipase C response and triggered melatonin-induced unidirectional transactivation of the 5-HT_{2C} protomer of MT_2/5-HT_{2C} heteromers. Pharmacological studies revealed distinct functional properties for agomelatine, which shows "biased signaling." These observations demonstrate the existence of functionally unique MT_2/5-HT_{2C} 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_2/5-HT_{2C} heteromers provide a new strategy for the discovery of novel agents for the treatment of psychiatric disorders.

The monoamine serotonin (5-HT)[2] 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_1 and MT_2 receptors and with moderate affinity to the enzyme quinone reductase 2 (2). Both MT_1 and MT_2 receptors as well as all 14 classes of 5-HT receptor (except 5-HT_3) 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_1 or MT_2 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_{2A} and metabotropic glutamate-2 receptor (mGluR2) heteromers in frontal cortex implicated in schizophrenia and in the actions of antipsychotics (5) and limbic dopamine D_1 and D_2 receptor heteromers incriminated in depressed states (6).

To date, the possible existence of heteromeric associations of MT_1 or MT_2 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-

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[2] The abbreviations used are: 5-HT, serotonin; BRET, bioluminescence resonance energy transfer; PLC, phospholipase C; GPCR, G protein-coupled receptor; mGluR2, metabotropic glutamate-2 receptor; 4-PPDOT, 4-*phenyl-2-propionamidotetralin; Rluc, Renilla* luciferase; TRITC, tetramethylrhodamine isothiocyanate; IP, inositol 1-phosphate; βARK, *β-adrenergic receptor kinase*.

melatine, the first to possess a *non*-monoaminergic component of action, behaves as an agonist at G_i-coupled MT_1 and MT_2 receptors but as a neutral antagonist at $G_{q/11}$-coupled 5-HT_{2C} receptors (7, 8). Intriguingly, although the affinity of agomelatine is substantially lower at 5-HT_{2C} *versus* MT_1 and MT_2 *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-HT_{2C} and MT_1 and MT_2 receptors are necessary for expression of the antidepressant actions of agomelatine, which cannot be reproduced either by melatonin or by selective 5-HT_{2C} antagonists alone (9). For example, "synergistical" MT_1, MT_2, and 5-HT_{2C} 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-HT_{2C} 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. S20928, S21767, and agomelatine were a gift from the Institut de Recherches Servier (France).

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 culture HEK293 cells stably expressing the MT_2 receptor from 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 MT_2 receptor, the double brilliance Rluc8-βARR2-YPet sensor, and the MT_1-YFP and the 5-HT_{4d}-YFP fusion proteins were described previously (10–12). The INI isoform of the human 5-HT_{2C} coding region was fused at its C terminus with the coding region of *Renilla* luciferase (5-HT_{2C}-Rluc) or the yellow fluorescent protein (5-HT_{2C}-YFP) or at its N terminus with three HA tags (3xHA-5-HT_{2C}). The 5-HT_{2C}-S138N-Rluc mutant was obtained by mutagenesis from the 5-HT_{2C}-Rluc plasmid. All constructs were verified by sequencing.

Immunoprecipitation—For co-immunoprecipitation assays, HEK293 cells were seeded in 10-cm dishes and co-transfected with 4 μg of each indicated plasmid. 48 h after transfection, crude membranes were prepared as described previously (13). Membrane proteins were solubilized with 1% digitonin, and receptors were precipitated with the indicated antibodies (14). Immunoprecipitated proteins were eluted with 4× Laemmli buffer and immunoblotted using the indicated primary antibodies. Immunoreactivity was revealed using secondary antibodies coupled to 680 or 770 nm fluorophores using the LI-COR Odyssey infrared fluorescence scanner (ScienceTec, France). For experiments with the pork plexus choroid, membranes were prepared, and melatonin receptors were labeled with 400 pM 2-[^{125}I]iodomelatonin as described previously (15). Receptors were solubilized with 1% digitonin and precipitated with a mixture of three anti-5-HT_{2C} rabbit 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-MT_2 and 3xHA-5-HT_{2C} 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-HT_{2C} receptors was evaluated by in-cell Western experiments in intact and Triton X-100-permeabilized HEK293 cells transiently expressing the 3xHA-5-HT_{2C} construct and stably expressing the MT_2 receptor (5-HT_{2C} expression) or transiently expressing the 6xMyc-MT_2 receptor (MT_2 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-HT_{2C}-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^{TM} (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 MT_2/mg of protein and transiently expressing or not 20–30 fmol of 5-HT_{2C} or HEK293 cells transiently expressing only 5-HT_{2C} receptors were used. Inositol 1-phosphate (IP) levels were determined in cells stimulated with the indicated ligands for 1 h at 37 °C by *homogeneous time-resolved fluorescence* using the Cisbio IP-One Tb kit according to the manufacturer's instructions. Cyclic AMP levels were determined in cells treated with the indicated ligands for 30 min at room temperature in the absence or presence of 2 μM forskolin by *homogeneous time-resolved fluorescence* using the Cisbio cAMP femto Tb kit according to the manufacturer's instructions. IP and cAMP measurements were performed in triplicates, and experiments were repeated three to eight times. To determine ERK1/2 phosphorylation levels, cells were stimulated for 2, 5, 10, 15, and 30 min with 100 nM melatonin, and ERK1/2 phosphorylation was determined as described previously (16, 17).

Statistical Analysis—Results were analyzed by PRISM (GraphPad Software). Data are expressed as mean ± S.E. of at

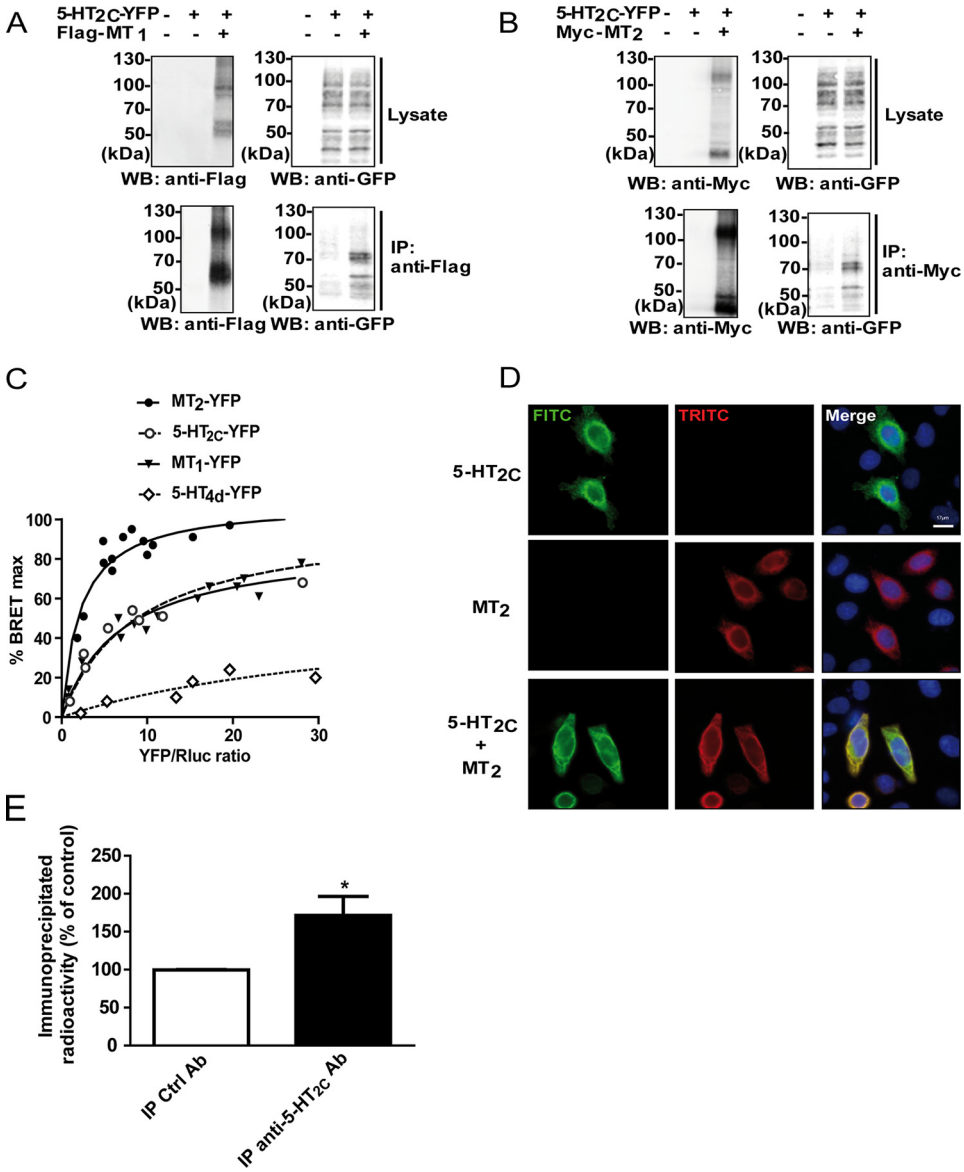

FIGURE 1. **Formation of MT_1/5-HT_{2C} and MT_2/5-HT_{2C} heteromers.** *A* and *B*, co-immunoprecipitation of MT_1/5-HT_{2C} and MT_2/5-HT_{2C} heteromers. Lysates from HEK293T cells expressing the indicated receptors were immunoprecipitated (*IP*) with antibodies recognizing Myc and FLAG epitopes, and the presence of co-precipitated 5-HT_{2C}-YFP was detected with antibodies against GFP (*lower* Western blot (*WB*)). Expression of fusion proteins in lysates was assessed by immunoblotting with the indicated antibodies (*upper* Western blots). Data are representative of three experiments. *C*, BRET donor saturation curves were determined by co-transfecting a fixed amount of 5-HT_{2C}-Rluc in the presence of increasing amounts of the indicated YFP fusion proteins in HEK293 cells. Curves were normalized to $BRET_{max}$ values. The saturation curves were obtained from three independent experiments performed in triplicates. *D*, immunofluorescence staining of permeabilized HeLa cells showing localization of 3xHA-5-HT_{2C} (*FITC*) and Myc-MT_2 (*TRITC*) expressed alone (*upper* and *middle panels*, respectively) or together (*lower panels*) (*scale bar*, 17 μm). Data are representative of three experiments. *E*, immunoprecipitation of melatonin receptors with 5-HT_{2C} receptors in pork choroid plexus; ^{125}I-labeled melatonin receptors were immunoprecipitated from solubilized membranes by anti-5-HT_{2C} antibodies (*Ab*) or control (*Ctrl*) rabbit sera (pool of five preimmune rabbit sera). Data represent the mean ± S.E. (*error bars*) of three independent experiments (*, $p < 0.05$). Representative results are shown for *A*, *C*, and *D*; similar results were obtained in two additional experiments.

least three experiments. Student's *t* test was applied for statistical analysis.

RESULTS

Physical Interaction between 5-HT_{2C} and MT_1/MT_2 Receptors—We first carried out co-immunoprecipitation experiments to assess a possible interaction between the human 5-HT_{2C} and MT_1 and MT_2 receptors. These experiments revealed that 5-HT_{2C} interacts with MT_1 and MT_2 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-HT_{2C} was fused at its C terminus to the energy donor Rluc. C-terminal YFP fusion proteins (5-HT_{2C}-YFP, MT_1-YFP, MT_2-YFP, and 5-HT_{4d}-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-HT_{4d}-YFP fusion protein for which a quasilinear increase in BRET reflecting a non-specific interaction due to random collision was observed (18, 19). Determination of $BRET_{50}$ values, corresponding to the sat-

uration of 50% of BRET donors by BRET acceptors, revealed that the relative propensity of 5-HT$_{2C}$ to form heteromers with MT$_2$ (BRET$_{50}$ = 2.2 ± 0.4) is higher than with MT$_1$ (BRET$_{50}$ = 6.9 ± 1.5) or with itself (BRET$_{50}$ = 8.8 ± 1.5), indicating preferential formation of MT$_2$/5-HT$_{2C}$ heteromers. As shown by immunofluorescence staining, 5-HT$_{2C}$ expressed alone was mainly intracellular, and a significant amount of MT$_2$ and 5-HT$_{2C}$ was present at the plasma membrane where both receptors colocalized (Fig. 1*D*). Formation of MT$_2$/5-HT$_{2C}$ heteromers was further suggested by co-immunoprecipitation studies in human cortex and hippocampus, two regions shown previously to express melatonin and 5-HT$_{2C}$ receptors (20, 21) (data not shown). Heat-inactivated anti-MT$_2$ antibodies were used as a negative control. Further evidence for the formation of MT$_2$/5-HT$_{2C}$ heteromers was obtained from choroid plexus membranes, which are known to express significant amounts of 5-HT$_{2C}$ receptors. Melatonin receptors were labeled with 2-[^{125}I]iodomelatonin, and protein complexes were solubilized and immunoprecipitated with anti-5-HT$_{2C}$ antibodies. As shown in Fig. 1*E*, significant amounts of radiolabeled melatonin receptors were precipitated with anti-5-HT$_{2C}$ antibodies as compared with a mixture of irrelevant control antibodies. Overall, our results indicate that 5-HT$_{2C}$ specifically interacts with MT$_2$ in transfected HEK293 cells and in the cortex, hippocampus, and choroid plexus.

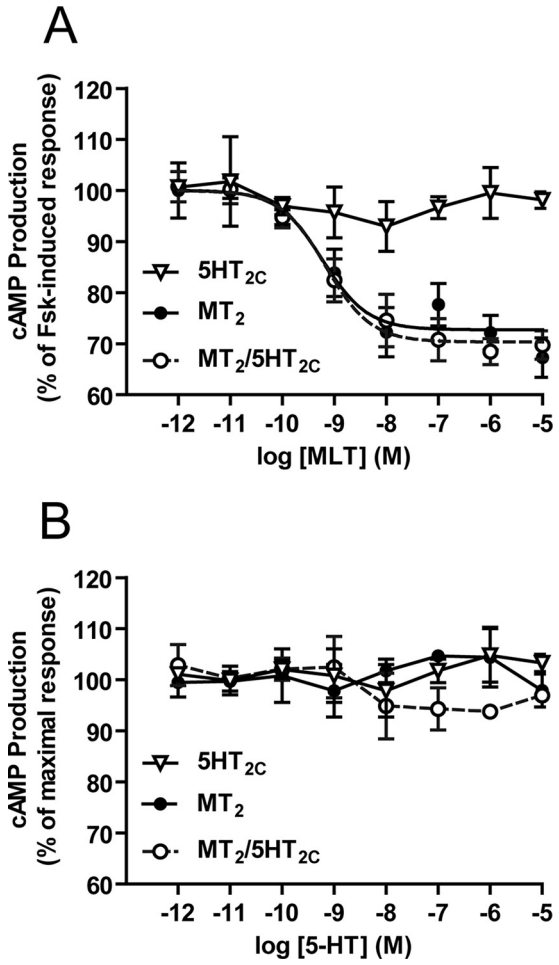

FIGURE 2. **Effect of melatonin- and 5-HT-promoted activation of MT$_2$/5-HT$_{2C}$ heteromers on the G$_i$/cAMP pathway.** HEK293 cells expressing 5-HT$_{2C}$ (▽), MT$_2$ (●), or 5-HT$_{2C}$ and MT$_2$ (○) receptors were either treated with 2 μM forskolin (*Fsk*) and increasing concentrations of melatonin (*MLT*) (*A*) or increasing concentrations of 5-HT (*B*). Data represent the mean ± S.E. (*error bars*) of at least three independent experiments performed in triplicates.

Melatonin but Not 5-HT Activates the G$_i$/cAMP Pathway through MT$_2$/5-HT$_{2c}$ Heteromers—HEK293 cells expressing equivalent and physiologically relevant levels of MT$_2$ and 5-HT$_{2C}$ (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 MT$_2$ alone as expected and in cells co-expressing MT$_2$ and 5-HT$_{2C}$ with the same efficiency (EC$_{50}$ = 0.76 ± 0.46 *versus* 0.76 ± 0.39 nM, respectively) and potency (30% reduction) (Fig. 2*A* and Table 1). Melatonin was without effect in cells expressing 5-HT$_{2C}$ alone. Stimulation with up to 10 μM 5-HT did not modify cAMP levels in any of the three cell types (Fig. 2*B*). These results indicate that activation of the MT$_2$ protomer of the MT$_2$/5-HT$_{2C}$ heteromer activates the G$_i$/cAMP pathway but that activation of the 5-HT$_{2C}$ protomer is unable to transactivate this pathway.

MT$_2$ Potentiates 5-HT-induced Signaling by Increasing Cell Surface Expression of 5-HT$_{2C}$ in the MT$_2$/5-HT$_{2C}$ Heteromer—We next investigated the capacity of MT$_2$/5-HT$_{2C}$ heteromers to activate the G$_q$/PLC pathway by monitoring 5-HT-induced IP production. Stimulation of HEK293 cells expressing 5-HT$_{2C}$ alone resulted in the expected dose-dependent increase in IP production (Fig. 3*A*). This response was potentiated (~3-fold) in cells expressing similar quantities of 5-HT$_{2C}$ receptors, but in

TABLE 1

Compared potencies and properties of melatonin receptor ligands on two signaling pathways in cells expressing MT$_2$ or MT$_2$ and 5-HT$_{2C}$ receptors

The K_i values of MTR antagonists are derived from data shown in Fig. 6 and are defined as $K_i = IC_{50}/1 + (S/X)$ where S and X represent the agonist concentration and EC$_{50}$, respectively. ND, not determined.

	EC$_{50}$ ± S.E.			
	Inhibition of cAMP production		Stimulation of inositol phosphate production	
	MT$_2$	MT$_2$/5-HT$_{2C}$	MT$_2$	MT$_2$/5-HT$_{2C}$
	nM			
MTR agonists				
MLT	0.76 ± 0.46	0.76 ± 0.39	No effect	69 ± 20
Agomelatine	0.29 ± 0.16	0.89 ± 0.35	No effect	Antagonist
MTR antagonists				
Luzindole	Antagonist (K_i = 1.12 ± 0.48 nM)	Antagonist (K_i = 7.73 ± 4.98 nM)	No effect	Full agonist (EC$_{50}$ = 223 ± 97 nM)
4-PPDOT	Antagonist (K_i = 0.14 ± 0.12 nM)	Antagonist (K_i = 0.04 ± 0.01 nM)	No effect	Partial agonist (EC$_{50}$ = 2.5 ± 1.7 nM)
S20928	Antagonist (K_i = 4.88 ± 1.5 nM)	Antagonist (K_i = 0.66 ± 0.45 nM)	No effect	Antagonist (K_i = ND)

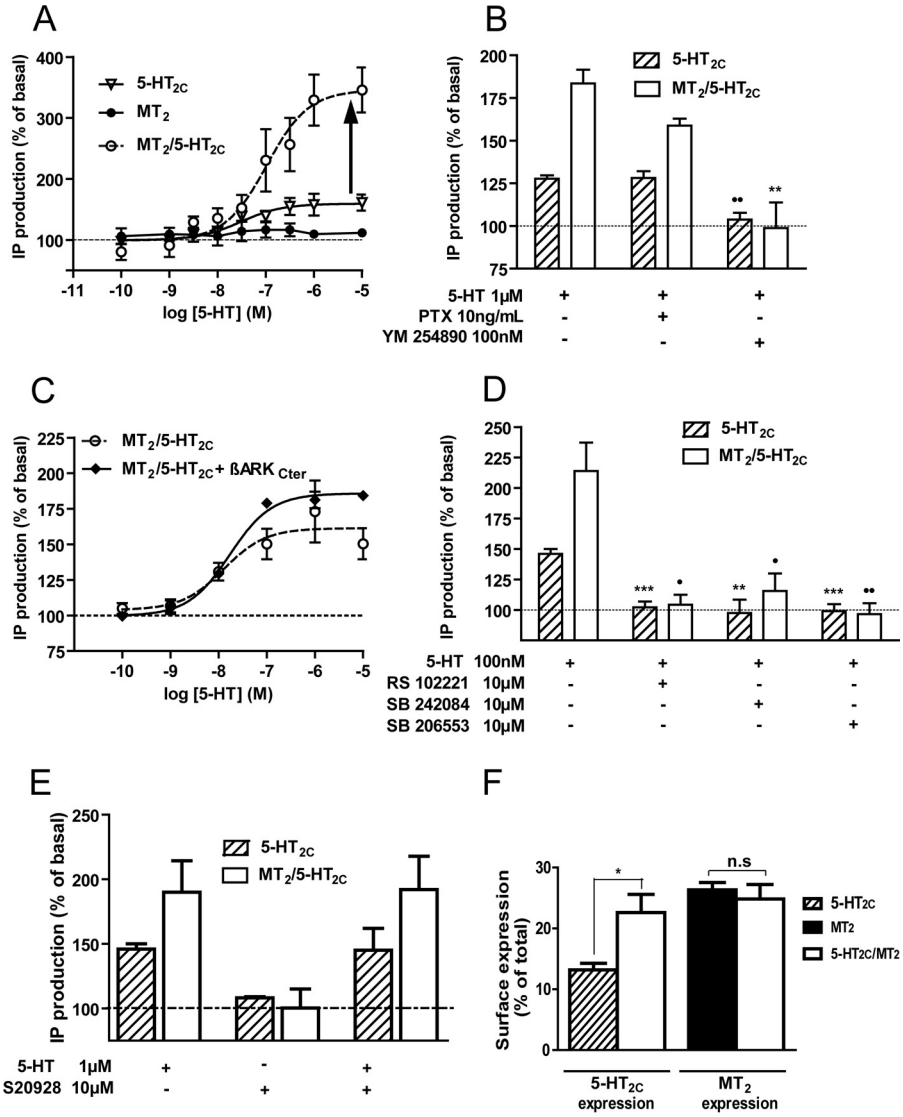

FIGURE 3. **5-HT-promoted potentiation of the G_q/PLC pathway by the $MT_2/5\text{-}HT_{2C}$ heteromer.** *A*, 5-HT-induced IP production was assessed in HEK293 cells expressing 5-HT_{2C} (▽), MT_2 (●), or 5-HT_{2C} and MT_2 (○) receptors. *B–E*, HEK293 cells expressing 5-HT_{2C} receptors alone or together with MT_2 receptors were treated with heterotrimeric G protein inhibitors (*B*), 5-HT_{2C} receptor ligands (*D*), or the melatonin receptor ligand S20928 (*E*) or co-expressed with the βARK_{Cter} $G\beta\gamma$ scavenger (*C*). *F*, cell surface expression of 5-HT_{2C} and MT_2 receptors in cells expressing 5-HT_{2C} in the absence and presence of MT_2 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-HT_{2C} cells (●, $p < 0.05$; ●●, $p < 0.01$) and $MT_2/5\text{-}HT_{2C}$ cells (**, $p < 0.01$; ***, $p < 0.001$) are indicated.

the presence of MT_2, the response was of similar potency (EC_{50} = 21 ± 11 nM (5-HT_{2C}) *versus* 68 ± 25 nM ($MT_2/5\text{-}HT_{2C}$)). 5-HT was ineffective in cells expressing MT_2 alone (Fig. 3*A*). Amplification of the 5-HT-induced response was $G_{q/11}$- but not G_i-dependent as determined by pretreating cells with YM254890 or pertussis toxin inhibitors, respectively (Fig. 3*B*). Expression of the $G\beta\gamma$ scavenger βARK_{Cter} had no significant effect (Fig. 3*C*), confirming the predominant role of $G_{q/11}\alpha$ proteins in the observed amplification. Pretreatment of cells with 5-HT_{2C} antagonists (RS102221 and SB242084) or an inverse agonist (SB206553) completely blocked the 5-HT-induced IP production in cells expressing 5-HT_{2C} alone and in the presence of MT_2 as expected (Fig. 3*D*). Pretreatment with S20928, a melatonin receptor antagonist, showed no cross-reactivity on 5-HT-induced responses (Fig. 3*E*).

Amplified 5-HT-induced responses in the context of the $MT_2/5\text{-}HT_{2C}$ heteromer might be explained by increased cell surface expression of 5-HT_{2C} receptors. In agreement with previous reports, only a minor fraction of 5-HT_{2C} receptors (13.2 ± 1.1%) was expressed at the cell surface when expressed alone as determined by in-cell Western experiments (Fig. 3*F*). In cells co-expressing 5-HT_{2C} and MT_2 receptors, the fraction of cell surface-expressed 5-HT_{2C} almost doubled (22.7 ± 3.0%), whereas the total amount of 5-HT_{2C} receptors was not modified. The total amount and the fraction of cell surface-expressed MT_2 remained constant irrespective of the presence or absence of 5-HT_{2C}. Taken together, our results indicate that MT_2 co-expression potentiates 5-HT_{2C} receptor signaling in the $MT_2/5\text{-}HT_{2C}$ heteromer by increasing the cell surface expression of 5-HT_{2C}.

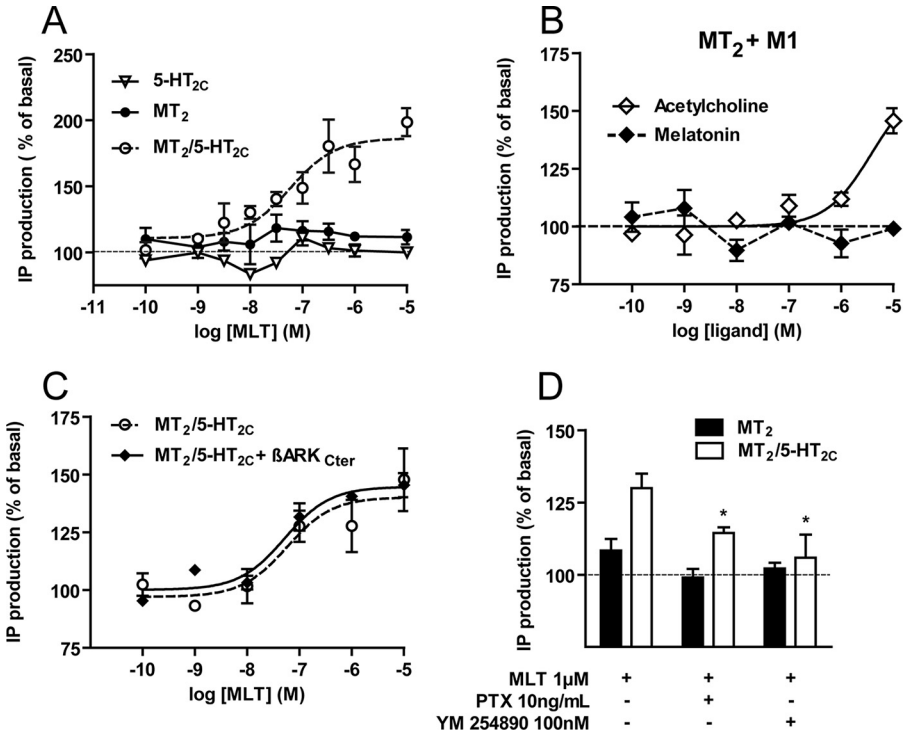

FIGURE 4. **Melatonin activates the G_q/PLC pathway in the MT$_2$/5-HT$_{2C}$ heteromer.** *A*, melatonin (*MLT*)-induced IP production was assessed in HEK293 cells expressing 5-HT$_{2C}$ (▽), MT$_2$ (●), or 5-HT$_{2C}$ and MT$_2$ (○) receptors. *B*, HEK293 cells co-expressing MT$_2$ receptors and M1 muscarinic receptors were stimulated with melatonin or acetylcholine, and IP production was measured. The influence of the βARK$_{Cter}$ G$\beta\gamma$ scavenger (*C*) or heterotrimeric G protein inhibitors (*D*) on melatonin-promoted IP production of the MT$_2$/5-HT$_{2C}$ heteromer is shown. Data represent the mean ± S.E. (*error bars*) of at least three independent experiments performed in triplicates. Results that are statistically different compared with melatonin alone for MT$_2$/5-HT$_{2C}$ cells (*, $p < 0.05$) are indicated.

Activation of the MT$_2$ Protomer Transactivates the PLC Pathway and Improves β-Arrestin Recruitment in the MT$_2$/5-HT$_{2C}$ Heteromer—To explore the possible effect of MT$_2$ activation in the MT$_2$/5-HT$_{2C}$ heteromer on the G$_q$/PLC pathway, we stimulated HEK293 cells expressing MT$_2$ and 5-HT$_{2C}$ 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 EC$_{50}$ of 69 ± 20 nM (Fig. 4*A*). To verify that melatonin-induced IP production is not due to the signaling cross-talk between MT$_2$ and any G$_q$-coupled GPCR, we co-expressed MT$_2$ with the G$_q$-coupled M1 muscarinic receptor, which does not form heteromers with MT$_2$ (data not show). In these cells, melatonin did not increase IP production, indicating that melatonin-induced IP production is specific for the MT$_2$/5-HT$_{2C}$ heteromer (Fig. 4*B*). Functional expression of M1 receptors was shown by the expected increase in IP production in the presence of acetylcholine (Fig. 4*B*).

Melatonin-induced IP production in cells co-expressing MT$_2$ and 5-HT$_{2C}$ receptors was not affected by the expression of the G$\beta\gamma$ scavenger βARK$_{Cter}$ (Fig. 4*C*), but it was partially and completely abrogated by pertussis toxin and YM254890 treatment, respectively (Fig. 4*D*). This indicates the predominant role of G$_{q/11}\alpha$ proteins, which can be assisted by the presence of G$_i\alpha$ proteins. This result raises the intriguing possibility that melatonin binding to the MT$_2$ protomer of the MT$_2$/5-HT$_{2C}$ heteromer transactivates the 5-HT$_{2C}$ protomer, which then activates G$_q$.

To verify this hypothesis, we evaluated whether the melatonin-induced response depended on the activation state of the 5-HT$_{2C}$ protomer. Pretreatment of cells with 5-HT$_{2C}$ 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. 5*A*). This is consistent with the notion that the melatonin-induced effect is independent of the occupation of the 5-HT$_{2C}$ receptor binding site but dependent on the constitutive activity of the 5-HT$_{2C}$ protomer.

To further verify the transactivation hypothesis, we used the 5-HT$_{2C}$-S138N mutant, which does not bind 5-HT and exhibits decreased constitutive activity due to decreased coupling to G$_q$ proteins compared with the wild-type receptor (22). The absence of 5-HT-promoted IP production was confirmed in cells expressing 5-HT$_{2C}$-S138N and MT$_2$ (Fig. 5*B*). When cells co-expressing this mutant and MT$_2$ receptors were stimulated with melatonin, the anticipated reduction in amplitude and efficiency (EC$_{50}$ = 550 ± 187 *versus* 69 ± 20 nM for MT$_2$/5-HT$_{2C}$-S138N and MT$_2$/5-HT$_{2C}$, respectively ($p < 0.001$)) was observed compared with 5-HT$_{2C}$ wild-type receptor-expressing cells (Fig. 5*C*). Decreased activity was not due to less efficient heteromerization with MT$_2$ as comparable BRET$_{50}$ values (2.2 ± 0.4 *versus* 3.5 ± 0.6 for MT$_2$/5-HT$_{2C}$ and MT$_2$/5-HT$_{2C}$-S138N, respectively) were observed (Fig. 5*D*). The decreased response to melatonin in the presence of the 5-HT$_{2C}$-S138N mutant further supports the transactivation mechanism in which the melatonin-induced conformational change in the MT$_2$ protomer is transmitted to the 5-HT$_{2C}$ protomer and then further downstream to the G$_q$/PLC pathway.

Apart from interacting with heterotrimeric G proteins, GPCRs are also known to recruit β-arrestins. We therefore

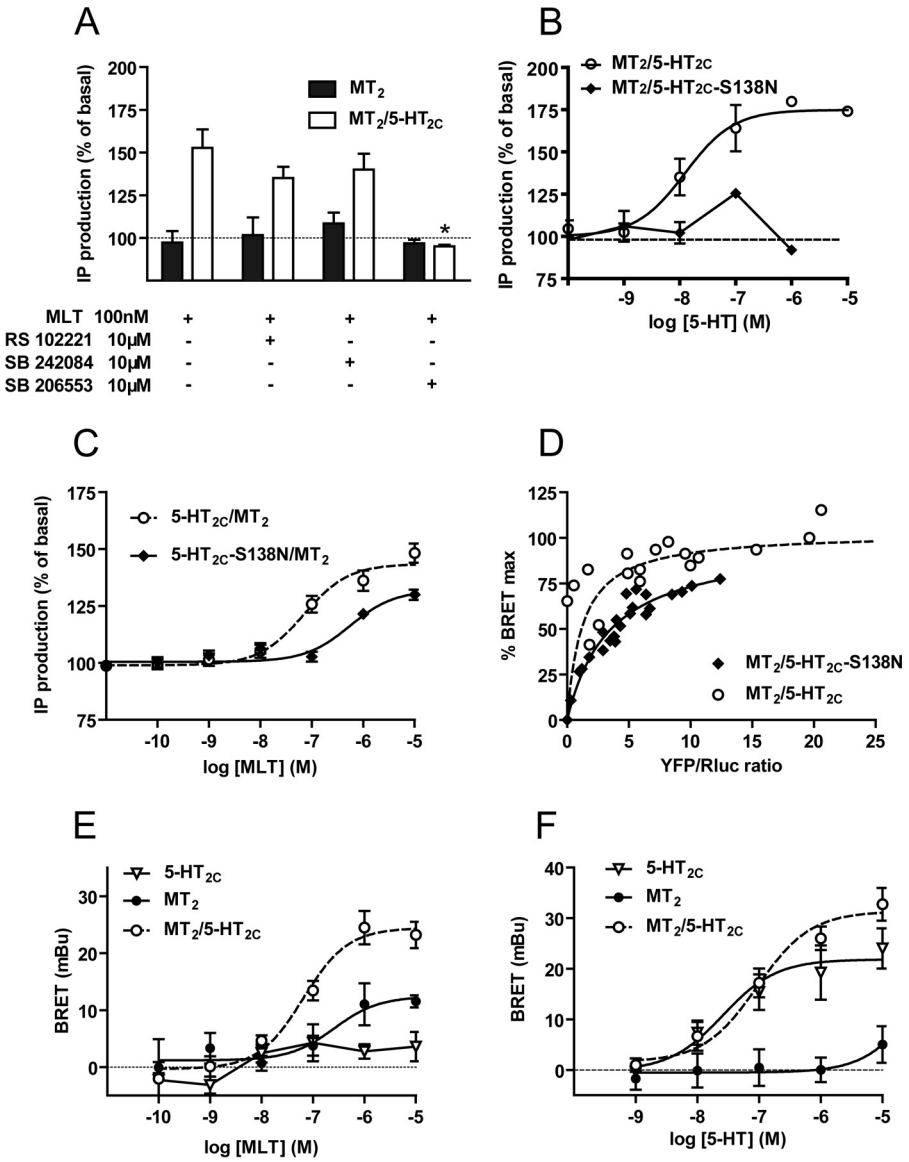

FIGURE 5. **Transactivation of the G_q/PLC pathway by melatonin in the $MT_2/5\text{-}HT_{2C}$ heteromer.** *A*, melatonin (*MLT*)-induced IP production in the presence of 5-HT_{2C} ligands in HEK293 cells expressing MT_2 receptors alone or co-expressing 5-HT_{2C} receptors (*, $p < 0.05$: melatonin *versus* melatonin + SB206553 on heteromers). *B*, 5-HT-induced IP production in cells co-expressing the 5-HT_{2C}-S138N mutant and MT_2 receptor. *C*, melatonin-induced transactivation of 5-HT_{2C} wild-type and 5-HT_{2C}-S138N mutant receptors in the presence of MT_2. *D*, BRET donor saturation curves of HEK293 cells expressing MT_2-Rluc and 5-HT_{2C}-Rluc or 5-HT_{2C}-S138N-Rluc. Melatonin-induced (*E*) and 5-HT-induced (*F*) recruitment of β-arrestin 2 measured by BRET in HEK293 cells expressing 5-HT_{2C} (▽), MT_2 (●), or 5-HT_{2C} and MT_2 (○) receptors is shown. Data represent the mean ± S.E. (*error bars*) of at least three independent experiments performed in triplicates. *mBu*, milli-BRET units.

studied the ability of melatonin to activate the previously described β-arrestin 2 BRET sensor (11, 23) in cells expressing either MT_2 alone or together with 5-HT_{2C}. As seen in Fig. 5*E*, MT_2 alone only weakly activated the BRET sensor with an EC_{50} of 260 ± 170 nM. In contrast, melatonin potently recruited the sensor in cells co-expressing MT_2 and 5-HT_{2C} (EC_{50} = 65 ± 23 nM). No effect was seen in cells expressing 5-HT_{2C} receptors alone as expected. Stimulation with 5-HT activated the sensor in cells expressing 5-HT_{2C} irrespective of the presence of MT_2 (Fig. 5*F*). These results show that melatonin-induced β-arrestin recruitment to MT_2 is potentiated in the $MT_2/5\text{-}HT_{2C}$ heteromer.

Agomelatine Exhibits a Unique Functional Profile at $MT_2/5\text{-}HT_{2C}$ Heteromers—To further explore the functional profile of $MT_2/5\text{-}HT_{2C}$ heteromers, we studied the effect of several synthetic melatonin receptors ligands. S20928, a well know melatonin receptor antagonist (24), antagonized the melatonin-induced inhibition of cAMP levels as expected in cells expressing MT_2 alone (K_i = 4.88 ± 1.50 nM) but also in cells co-expressing MT_2 and 5-HT_{2C} receptors (K_i = 0.66 ± 0.45 nM) (Table 1 and Fig. 6*A*). Similarly, IP production was also antagonized by S20928 under both conditions (Fig. 6*B*), suggesting that S20928 behaves as an antagonist of $MT_2/5\text{-}HT_{2C}$ heteromers for both pathways. Some agonistic activity is seen at micromolar concentrations. In contrast, 4-PPDOT and luzindole, two reported antagonists of MT_2 on the G_i/cAMP pathway (7, 25), showed pathway-biased properties on $MT_2/5\text{-}HT_{2C}$ heteromers as they behaved as full antagonists on the G_i/cAMP pathway (K_i = 0.04 ± 0.01 nM and K_i = 7.73 ± 4.98 nM, respectively) but as a partial (4-PPDOT) or full (luzindole) agonist on the G_q/PLC

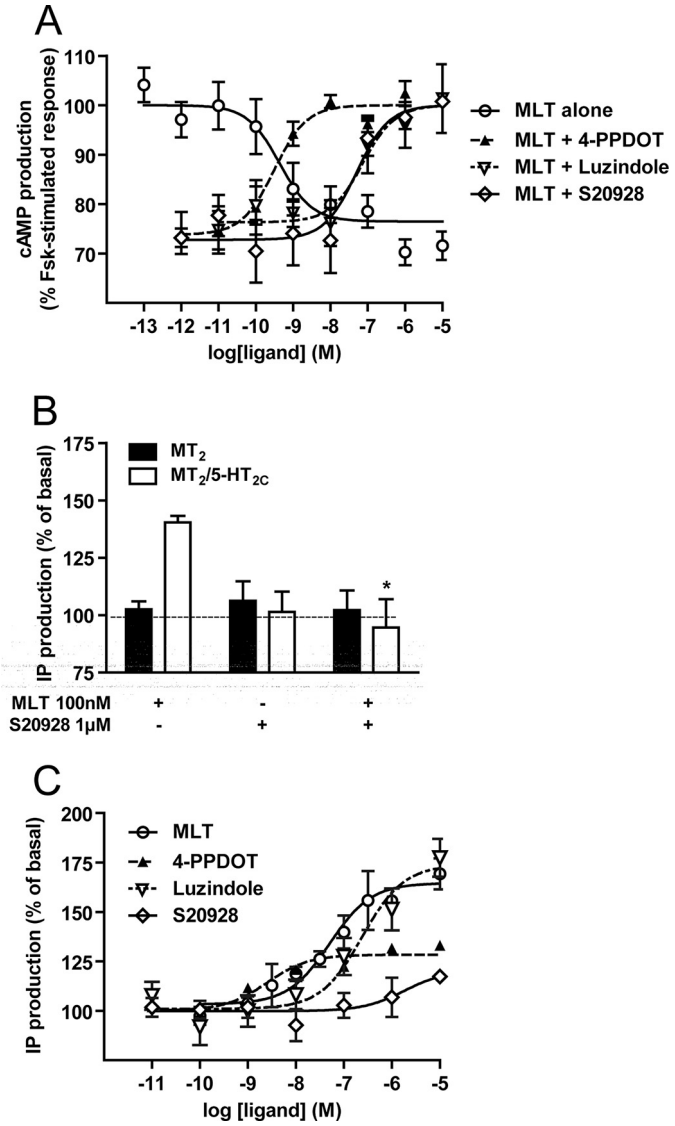

FIGURE 6. **Biased ligands reveal unique functional profile of MT_2/5-HT_{2C} heteromers.** *A*, cAMP production in HEK293 cells co-expressing MT_2 and 5-HT_{2C} receptors in the presence of 2 μM forskolin (*Fsk*) and different concentrations of melatonin (*MLT*) or 10 nM melatonin and different concentrations of 4-PPDOT, luzindole, or S20928. *B*, antagonistic effect of S20928 on melatonin-induced IP production in HEK293 cells expressing MT_2 alone or together with 5-HT_{2C} receptors. *C*, IP production in HEK293 cells co-expressing MT_2 and 5-HT_{2C} receptors in the presence of different concentrations of the indicated ligands. Data represent the mean ± S.E. (*error bars*) of at least three independent experiments performed in triplicates.

pathway (EC_{50} = 2.5 ± 1.7 nM and EC_{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_q/PLC pathway-biased ligands of MT_2/5-HT_{2C} heteromers.

We next studied the effect of agomelatine, the antidepressant with agonistic activity at MT_2 receptors and neutral antagonistic properties at 5-HT_{2C} receptors (7, 8). In cells expressing MT_2 in the absence or presence of 5-HT_{2C} receptors, agomelatine behaved as an agonist of the G_i/cAMP pathway (EC_{50} = 0.29 ± 0.16 nM and EC_{50} = 0.89 ± 0.35 nM, respectively) (Fig. 7*A* and Table 1) but as an antagonist for the melatonin-induced activation of the G_q/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. 7*D*). This clearly shows that agomelatine has a distinct action profile on MT_2/5-HT_{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_2 and 5-HT_{2C} receptors. MT_2/5-HT_{2C} heteromers have unique functional properties and are formed preferentially compared with the corresponding homomers. MT_1 and 5-HT_{2C} receptors also form heteromers in transfected cells, but we focus herein on MT_2/5-HT_{2C} heteromers. Within these heteromers, melatonin is able to activate distinct cellular cascades: not only the G_i/cAMP pathway as for MT_2 homomers but also the G_q/PLC pathway by transactivation of the 5-HT_{2C} protomer. This transactivation was unidirectional and not observed for the MT_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_2/5-HT_{2C} heteromers that are biased toward the G_i/cAMP pathway and thus distinct from those of melatonin- and 5-HT_{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_i-coupled MT_1 and MT_2 receptors and as a neutral antagonist at $G_{q/11}$-coupled 5-HT_{2C} receptors (7, 8). Our data indicate that agomelatine preserves these properties in MT_2/5-HT_{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_{2C} receptors. Importantly, these properties clearly distinguish agomelatine from melatonin or any other tested compound including 5-HT_{2C} receptor antagonists.

Interestingly, the MT_2/5-HT_{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_{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_{2C} receptor. Limited surface expression of 5-HT_{2C} receptors is in agreement with previous studies showing a high level of constitutive internalization for this receptor (26). In the MT_2 protomer, melatonin stimulation not only activates the G_i/cAMP pathway as for MT_2 homomers but also transactivates the G_q/PLC pathway through the 5-HT_{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

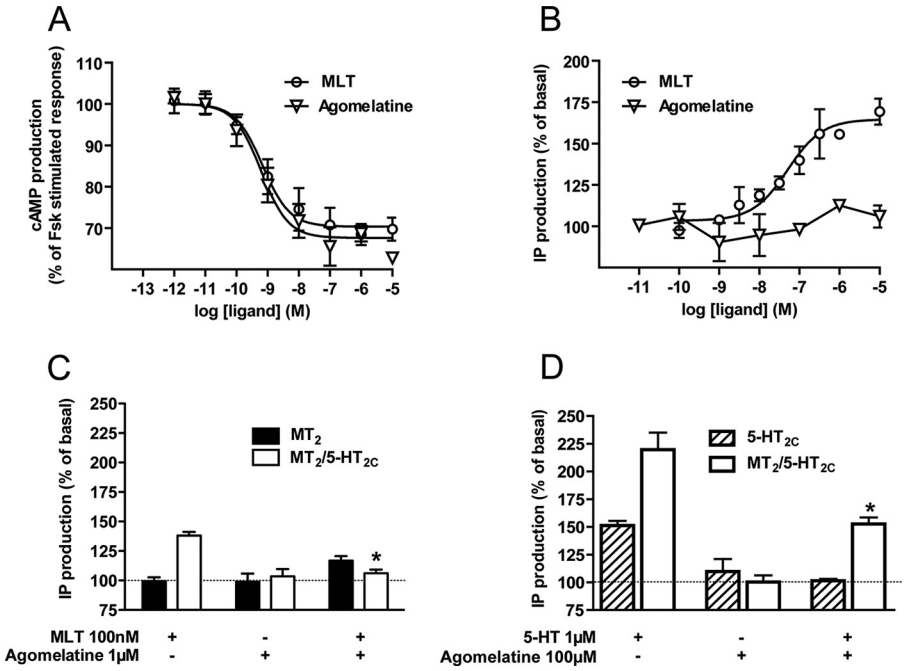

FIGURE 7. **Effect of agomelatine on the MT_2/5-HT_{2C} heteromer signaling.** Effect of melatonin (*MLT*) and agomelatine on cAMP (*A*) and IP (*B*) production in HEK293 cells co-expressing MT_2 and 5-HT_{2C} receptors. *C*, effect of agomelatine on melatonin-promoted IP production in cells expressing MT_2 receptors alone or together with 5-HT_{2C} receptors. *D*, effect of agomelatine on 5-HT-promoted IP production in cells expressing 5-HT_{2C} receptors alone or together with MT_2 receptors. Data represent the mean ± S.E. (*error bars*) of at least three independent experiments performed in triplicates. Results that are statistically different compared with melatonin (*C*) or 5-HT (*D*) alone for MT_2/5-HT_{2C} cells (*, $p < 0.05$) are indicated. *Fsk*, forskolin.

number of other cases such as the $GABA_B$ 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_2 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_{2C} protomer by the MT_2 protomer, agomelatine is completely inactive in this mode but still able to fully activate the MT_2 protomer.

Simultaneous activation of G_i- and G_q-dependent signaling by melatonin is a distinctive feature of MT_2/5-HT_{2C} heteromers compared with the corresponding homomers. Notably, the balance between G_i- and G_q-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_{2A} heteromers, which are composed of the G_q-coupled 5-HT_{2A} receptor and the G_i-coupled mGluR2 receptor (5) and for which the balance between G_i- and G_q-dependent signaling predicts the anti- or propsychotic activity of drugs targeting mGluR2 and 5-HT_{2A} receptors. Antipsychotic drugs have a high G_i/G_q activation ratio regardless of which receptor they target, whereas propsychotic drugs have a low ratio. A similar balance between G_i and G_q activation can be proposed for ligands acting on MT_2/5-HT_{2C} heteromers. According to our current functional characterization, agomelatine is unique inasmuch as it possesses the highest G_i/G_q activation ratio (activation of G_i pathway and allosteric antagonistic effect on 5-HT-induced G_q pathway activation), whereas melatonin would have a more balanced G_i/G_q activation ratio (activation of both pathways), and luzindole, 4-PPDOT, and 5-HT would have a low ratio, exclusively activating the G_q pathway.

In conclusion, this present work revealed the capacity of MT_2 and 5-HT_{2C} receptors to assemble into functional heteromers. The binding and coupling properties of MT_2/5-HT_{2C} 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_2/5-HT_{2C} 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).

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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.,

Gingrich, J. A., Filizola, 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., Bonnaud, A., Le Gall, A., Amossé, C., Dromaint, S., Rodriguez, M., Nagel, N., Galizzi, J. P., Malpaux, B., Guillaumet, G., Lesieur, D., Lefoulon, F., Renard, P., Delagrange, P., and Boutin, J. A. (2003) New selective ligands of human cloned melatonin MT1 and MT2 receptors. *Naunyn Schmiedebergs 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. (2003) 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., Froguel, P., Jockers, R., and Couturier, C. (2009) Improved donor/acceptor BRET couples for monitoring β-arrestin recruitment to G protein-coupled receptors. *Biotechnol. J.* **4,** 1337–1344
12. Berthouze, M., Ayoub, M., Russo, O., Rivail, L., Sicsic, S., Fischmeister, R., Berque-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-Spahn, 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., Levoye, A., Migaud, M., Brydon, L., Malpaux, B., Borg-Capra, C., and Jockers, R. (2008) The PDZ protein mupp1 promotes G_i 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., Guillaume, J. L., Dechaume, A., Payne, F., Roussel, R., Czernichow, S., Hercberg, S., Hadjadj, S., Balkau, B., Marre, M., Lantieri, O., Langenberg, C., Bouatia-Naji, N., Meta-Analysis of Glucose and Insulin-Related Traits Consortium (MAGIC), Charpentier, G., Vaxillaire, M., Rocheleau, G., Wareham, N. J., Sladek, R., McCarthy, M. I., Dina, C., Barroso, I., Jockers, R., and Froguel, P. (2012) Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. *Nat. Genet.* **44,** 297–301
17. Chaste, P., Clement, N., Mercati, O., Guillaume, J. L., Delorme, R., Botros, H. G., Pagan, C., Périvier, S., Scheid, I., Nygren, G., Anckarsäter, H., Rastam, M., Ståhlberg, O., Gillberg, C., Serrano, E., Lemière, N., Launay, J. M., Mouren-Simeoni, M. C., Leboyer, M., Gillberg, C., Jockers, R., and Bourgeron, T. (2010) Identification of pathway-biased and deleterious melatonin receptor mutants in autism spectrum disorders and in the general population. *PLoS One* **5,** e11495
18. Mercier, J. F., Salahpour, A., Angers, S., Breit, A., and Bouvier, M. (2002) Quantitative assessment of β_1- and β_2-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., Sözer-Topcular, N., Jockers, R., Ravid, R., Angeloni, D., Fraschini, F., Eckert, A., Müller-Spahn, F., and Savaskan, E. (2006) Pineal and cortical melatonin receptors MT1 and MT2 are decreased in Alzheimer's disease. *Eur. J. Histochem.* **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. J., 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. Charest, 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., Delagrange, P., Mocaer, E., Renard, P., and Guardiola-Lemaitre, 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. S., Melan, M. A., Passey, 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., Bettler, B., Prézeau, L., and Pin, J. P. (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal $GABA_B$ receptor function. *EMBO J.* **20,** 2152–2159
28. Han, Y., 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., MacKerell, A. D., Jr., Brezina, V., Sealfon, S. C., Filizola, 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,** 135–370
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