

XFEL structures of the human MT₂ melatonin receptor reveal the basis of subtype selectivity

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The human MT₁ and MT₂ melatonin receptors^{1,2} are G-protein-coupled receptors (GPCRs) that help to regulate circadian rhythm and sleep patterns³. Drug development efforts have targeted both receptors for the treatment of insomnia, circadian rhythm and mood disorders, and cancer³, and MT₂ has also been implicated in type 2 diabetes^{4,5}. Here we report X-ray free electron laser (XFEL) structures of the human MT₂ receptor in complex with the agonists 2-phenylmelatonin (2-PMT) and ramelteon⁶ at resolutions of 2.8 Å and 3.3 Å, respectively, along with two structures of function-related mutants: H208^{5,46}A (superscripts represent the Ballesteros–Weinstein residue numbering nomenclature⁷) and N86^{2,50}D, obtained in complex with 2-PMT. Comparison of the structures of MT₂ with a published structure⁸ of MT₁ reveals that, despite conservation of the orthosteric ligand-binding site residues, there are notable conformational variations as well as differences in [³H]melatonin dissociation kinetics that provide insights into the selectivity between melatonin receptor subtypes. A membrane-buried lateral ligand entry channel is observed in both MT₁ and MT₂, but in addition the MT₂ structures reveal a narrow opening towards the solvent in the extracellular part of the receptor. We provide functional and kinetic data that support a prominent role for intramembrane ligand entry in both receptors, and suggest that there might also be an extracellular entry path in MT₂. Our findings contribute to a molecular understanding of melatonin receptor subtype selectivity and ligand access modes, which are essential for the design of highly selective melatonin tool compounds and therapeutic agents.

To improve the low surface expression and stability of wild-type MT₂, we introduced eight point mutations on the basis of homology to other class A receptors: D86^{2,50}N (ref. ⁹), L108^{ECL1}F, F129^{3,41}W (ref. ¹⁰), N137^{3,49}D, C140^{3,52}L, W264^{6,48}F, A305^{7,50}P, and N312^{8,47}D; these were essential for determination of the high-resolution structure of both MT₂ and MT₁⁸. To promote crystal contacts, we used a double-fusion approach, with rubredoxin¹¹ in the intracellular loop 3 (ICL3) and thermostabilized apocytochrome *b*₅₆₂RIL (BRIL)¹¹ attached to the receptor N terminus. Radioligand binding assays revealed a 120-fold reduction in melatonin binding affinity (approximately 30-fold reduction at physiological concentration of NaCl), probably owing to stabilization of the crystallized construct in an inactive ‘low agonist affinity’ state that is deficient in G-protein coupling and signalling^{9,12} (Extended Data Table 1). All four MT₂ structures were obtained using lipidic cubic phase (LCP)¹³ crystallization (Extended Data Fig. 1, Extended Data Table 2). The overall receptor conformation was similar in all four

structures (C α root mean squared deviation (r.m.s.d.) < 0.3 Å), so we used the highest resolution MT₂–2-PMT structure in the analysis below unless otherwise noted.

MT₂ adopts the canonical seven-transmembrane-domain (7TM) fold of class A receptors, with the short amphipathic helix VIII parallel to the membrane on the intracellular side (Fig. 1a). As in MT₁⁸, the 7TM bundle of MT₂ is found in an inactive conformation. Restoration of the function-impairing D86^{2,50}N mutation (Extended Data Table 3) allowed us to solve the MT₂(N86D)–2-PMT structure at a lower resolution and showed that this mutation had no major effect on the overall

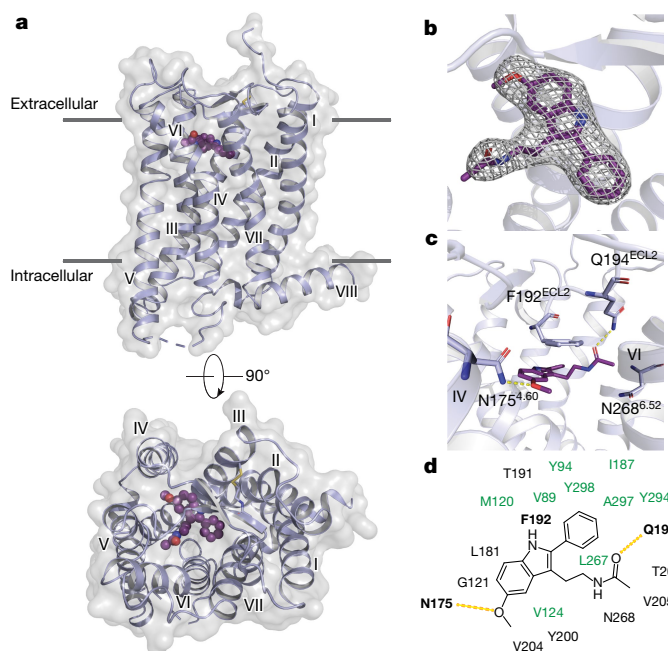


Fig. 1 | Overview of the MT₂ structure. **a**, Overview of MT₂ (violet) shows the canonical 7TM topology, with the ligand 2-PMT (purple) in the binding pocket. A 90° view shows the receptor from the extracellular side. Approximate membrane boundaries are shown as grey lines. **b**, 2mF_o – DF_c density (grey mesh) of 2-PMT contoured at 1σ. **c**, Binding pocket with key ligand interaction residues. **d**, Schematic diagram of ligand-interacting residues. Residues in the hydrophobic subpocket are coloured green. Hydrogen bonds are shown as dashed yellow lines in **c**, **d**.

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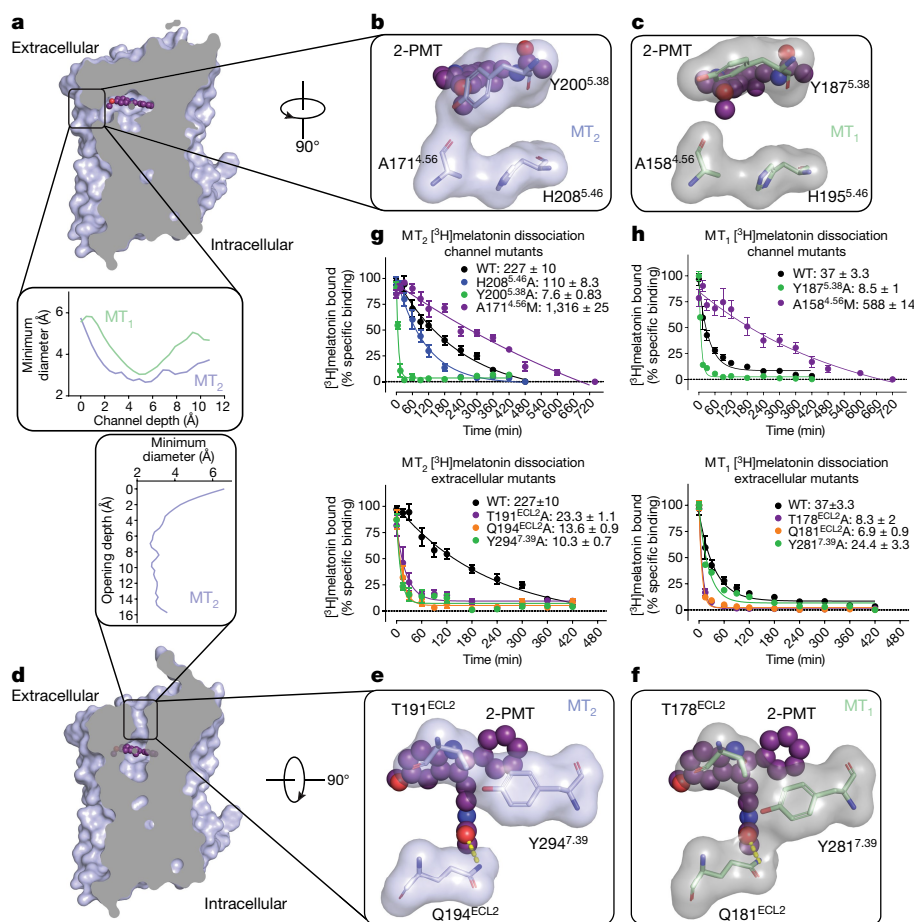


Fig. 2 | Two possible ligand entries in MT₂. **a**, View of the membrane-embedded channel in MT₂. Inset, channel diameter profile across its length for MT₁ and MT₂. **b**, A 90° view of the channel in MT₂, highlighting three residues discussed in the text. **c**, The same view as in **b** of MT₁ (green) showing a different conformation of Y187^{5.38} that widens the channel compared to MT₂. **d**, View of the ECL opening found in MT₂ (violet) with 2-PMT (purple). Inset, ECL opening profile across the length.

receptor conformation, as also supported by molecular dynamics simulations (Supplementary Fig. 1). Structural comparison of MT₂ and MT₁—which share 68% sequence identity—revealed notable overall similarity ($C\alpha$ r.m.s.d. < 0.6 Å), with all ligand-interacting residues conserved⁸ (Fig. 1d, Extended Data Fig. 2c). There is a common pharmacophore between receptor subtypes that consists of aromatic stacking of the ligand core with F192^{ECL2}, as well as hydrogen bonds between the methoxy group of 2-PMT and N175^{4.60} and between the alkylamide tail of the ligand and Q194^{ECL2} (Fig. 1c, d). The stability of these ligand-anchoring interactions was confirmed by molecular dynamics simulations (Extended Data Fig. 3). Further, mutation of F192^{ECL2} to isoleucine or alanine caused loss of ligand binding and signalling (Extended Data Tables 1, 4), as also observed for MT₁⁸. In contrast to MT₁, however, mutation of N175^{4.60} to alanine in MT₂ did not impair receptor function, which indicates that this residue has different roles in the activation of the two receptor subtypes. Mutation of either Q194^{ECL2} or N268^{6.52} to alanine had only minor effects on receptor ligand affinity, receptor activation or stability (Extended Data Tables 1, 4, 5), whereas the double mutation Q194^{ECL2}A/N268^{6.52}A results in a marked loss of receptor activity (Extended Data Tables 4, 5), which suggests that these residues show functional redundancy in MT₂. Notably, despite the binding site residues being conserved between the two receptors, we observed subtle conformational differences between them, such as in the side chains of Y200^{5.38} and Y294^{7.39} and in the backbone region surrounding P174^{4.59} (Extended Data Fig. 2d). Furthermore, the binding

e, A 90° view through the ECL opening in MT₂, highlighting three residues discussed in the text. **f**, The same view as in **e** of MT₁ (green), showing a different conformation of Y281^{7.39} that seals the ECL opening. **g**, [³H] Melatonin dissociation kinetics for MT₂ membrane channel mutants (top) and ECL opening mutants (bottom). **h**, As in **g** for MT₁. Residence time (k_{off}^{-1}) in **g** and **h** is given in minutes. Data are shown as mean ± s.e.m. for $n = 3$ independent experiments.

pocket of MT₂ is about 50 Å³ (7%) larger than that of MT₁, with most of the volume difference attributed to the region around the alkylamide tail and the hydrophobic subpocket that accommodates substituents of melatonin analogues in our structures (Fig. 1d, Extended Data Fig. 2b). These regions have a key role in MT₂ selectivity, as discussed below.

Structural analysis of MT₂ revealed an opening between helices IV and V from the orthosteric ligand binding site to the membrane (Fig. 2a). This channel is similar to that seen in MT₁, but is more constricted (around 2.6 Å in diameter at the narrowest part). Comparison of the structures of MT₂ and MT₁ revealed that Y200^{5.38} in MT₂ makes a hydrogen bond to N175^{4.60}, constricting the channel, whereas in MT₁ it adopts a different conformation, pointing towards the lipid interface (Fig. 2a–c, Extended Data Fig. 2c, d). In our MT₂(H208A)–2-PMT structure, mutating H208^{5.46} near the channel entrance to alanine further closes off the opening by an approximately 0.9 Å inward shift of helix V (Extended Data Fig. 4). This finding suggests that H208^{5.46} helps to control the channel entrance, albeit only moderately influencing ligand affinity and receptor function in MT₂ (Extended Data Tables 1, 4). Further analysis of the MT₂ structures revealed a potential secondary access route to the orthosteric binding site from the solvent-exposed extracellular (ECL) region (Fig. 2d). This second opening has a slightly larger diameter (about 2.5–3 Å) and is lined by aromatic Y294^{7.39} and hydrophilic T191^{ECL2} and Q194^{ECL2} residues (Fig. 2e). In MT₁, the corresponding residues Q181^{ECL2} and Y281^{7.39} adopt different conformations, completely sealing off this entrance (Fig. 2f).

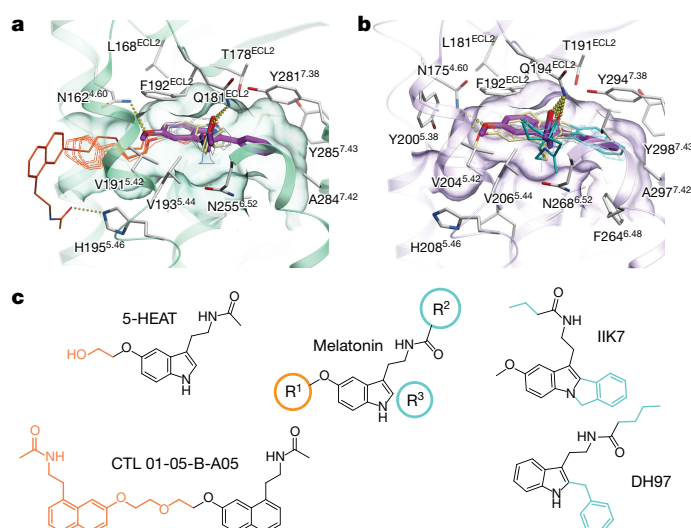


Fig. 3 | Selectivity determinants of ligands at MT₁ and MT₂. **a**, Docking of selective ligands into MT₁ (green), with 2-PMT (purple) from the crystal structure shown as reference. Ligands selective for MT₁ (compounds 63, 64, 65a, and 65b)¹⁷ are shown in grey. Two representative ligands, 5-HEAT¹⁶ and CTL 01-05-B-A05⁸ are coloured pale yellow, with their selectivity-conferring substituents (R¹ position) shown in orange. **b**, Docking of ligands into MT₂ (violet), with 2-PMT (purple) shown as reference. Non-selective (tasimelteon, TIK301¹⁷) and selective (UCM1014²⁰, K185¹⁷, and 4P-PDOT¹⁷) ligands are shown in grey. Two representative ligands, DH97¹⁸ and IIK7¹⁷ are coloured pale yellow, with selectivity-conferring substituents (R² and R³ positions) shown in cyan. Predicted hydrogen bonds are shown as dotted lines in **a**, **b**, **c**. Melatonin SAR, in which R¹ substituents confer MT₁ selectivity (orange), and substituents in R² and R³ positions confer MT₂ selectivity (cyan). See Supplementary Table 1 for a list of all docked ligands.

To test the relative importance of these two putative binding site access routes, we performed kinetic ligand dissociation studies on both receptors using [³H]melatonin as a tracer. The ligand residence time (k_{off}^{-1}) is substantially longer in wild-type MT₂ than in MT₁, which suggests that the narrower membrane entry channel restricts ligand access (Fig. 2g, h). Mutation of the membrane channel-lining residue Y^{5.38}A, designed to widen the access channel, caused a 30-fold decrease in residence time for MT₂ (with similar ligand affinity), while the corresponding mutation in MT₁ caused a more-modest decrease in residence time, consistent with the wider channel and a different conformation of Y^{5.38} in MT₁. To constrict the channel, we mutated A^{4.56}, a key residue at the interface of helices IV and V in both receptors, into a bulkier methionine. This mutation markedly increased residence time for both receptors (Fig. 2g, h), reaching up to 20 h in MT₂, which suggests that this channel is important for ligand access in both receptors.

When we tested mutations designed to widen the ECL opening in both receptors, ligand residence time was reduced more than tenfold in MT₂ mutants T191^{ECL2}A and Q194^{ECL2}A, and roughly fivefold with the equivalent ECL2 mutations in MT₁ (Fig. 2g, h). The MT₂(Y294^{7.39}A) mutant showed an even greater decrease in ligand residence time (22-fold) relative to the wild type, whereas the equivalent MT₁ mutant showed a similar residence time to the wild type. These differences can be reconciled if ligand site access through the ECL entrance is more important in MT₂ than in MT₁, consistent with the crystal structures, in which residue Y294^{7.39} adopts a different conformation in MT₂, allowing easier ligand egress through the ECL opening.

The elucidation of high-resolution structures of both melatonin receptor subtypes and published ligand structure–activity relationship (SAR) data^{14,15} allowed us to establish a model of receptor subtype ligand selectivity. To this end, we used molecular docking of several available selective ligands to both receptors. Docking of the moderately MT₁-selective compound 5-hydroxyethoxy-*N*-acetyltryptamine

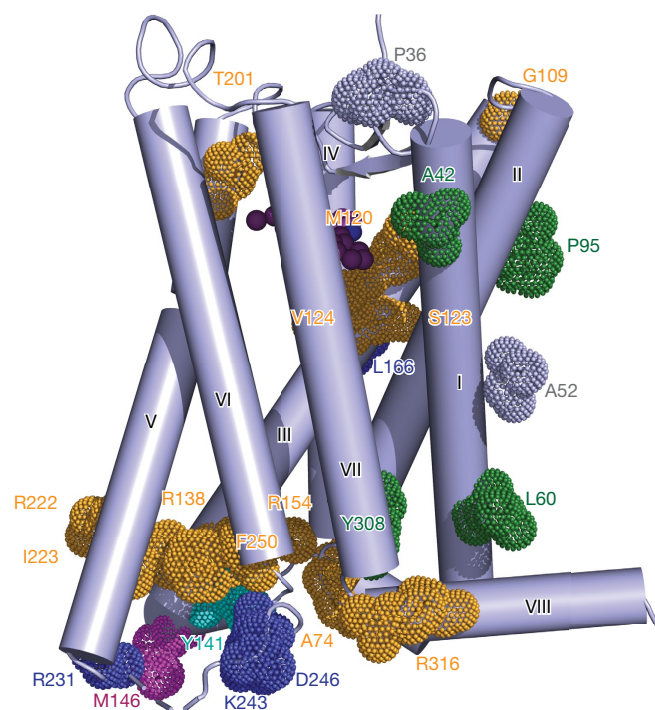


Fig. 4 | MT₂ mutations implicated in type 2 diabetes. Mapping of residues implicated in type 2 diabetes^{4,5} onto the MT₂ crystal structure. Gold, residues in which mutations lead to defects in two or more pathways; cyan, G-protein-specific defects; blue, β -arrestin-2-specific defects; magenta, ERK-specific defects; green, mutations that abolish melatonin binding; grey, mutations similar to the wild type. Type-2-diabetes-related mutations in residues not observed in the crystal structure are not shown.

(5-HEAT)¹⁶ and the bitopic ligand CTL 01-05-B-A05⁸ suggests that—although an extension or substitution of the R¹ position by a linear alkyl chain can be accommodated by the membrane access channel in both MT₁ and MT₂ (Fig. 3a, c)—the narrower MT₂ channel renders binding of the extended portion of the bitopic ligands suboptimal owing to potential steric clashes. Accordingly, the H208^{5.46}A mutation in MT₂ abolished the efficacy of CTL 01-05-B-A05 as a G_i agonist (Extended Data Fig. 4d), probably by further restricting the channel and/or eliminating the hydrogen bond between H208^{5.46} and CTL 01-05-B-A05 that was observed in docking to MT₁⁸. This mutation had a negligible effect on monotopic ligand binding and function (Extended Data Tables 1, 4, 5), which suggests that a sufficiently wide membrane channel (as in MT₁) is essential for accommodation of bitopic ligands.

The MT₂-selective ligands IIK7 and DH97 (both of which are about 90-fold selective)^{17,18} adopt ‘tail up’ binding modes similar to that of 2-PMT, with their alkylamide tails (R² position in Fig. 3b, c) interacting with Q194^{ECL2}. By contrast, in MT₁ the longer alkylamide tails of these ligands avoid this upward tail position owing to steric clashes, and can adopt only suboptimal ‘tail down’ conformations. Bulky substituents in the R³ position confer MT₂ selectivity by using the larger hydrophobic subpocket of the receptor (Fig. 3b, c). In summary, our analysis suggests that R¹ substituents are important for MT₁ selectivity, while R² and R³ mostly convey selectivity towards MT₂ (Fig. 3c). The slightly larger binding site in MT₂ also helps to achieve selectivity, as reflected by the larger number of compounds that are moderately selective for MT₂ (Extended Data Fig. 5).

Subtype-selective compounds are desirable owing to the involvement of MT₂ in type 2 diabetes, for which a number of single nucleotide polymorphisms (SNPs) have been reported^{4,5}. When we mapped these sites onto our MT₂ structure, we observed clustering of residues in the vicinity of the ligand binding pocket and on the receptor surface, along the membrane interface of helices I and II and the intracellular G-protein and β -arrestin binding regions (Fig. 4). The exposed positions

of these residues could point to their involvement in interactions with intracellular and membrane partners. Other instances of SNPs associated with type 2 diabetes include P95^{2,59}L of the YPYP motif, which is involved in receptor stability and function in MT₁⁸, and mutations in known microswitches such as R138^{3,50}H/L/C of the E/DRY motif¹⁹ and Y308^{7,53}S of the NPXXY motif¹². Although none of the analysed SNPs is involved in direct interactions with melatonin, the M120^{3,32}I/V and V124^{3,36}I variants are located in the hydrophobic subpocket of the receptor, which could influence ligand binding and affect subsequent signalling pathways^{4,5}.

The structural basis of melatonin receptor subtype selectivity revealed here has the potential to inspire a new generation of highly selective pharmacological tools that will help to further dissect the melatonin system. We also provide insights into differences in ligand entry between the two receptors by demonstrating the potential of MT₂ to support extracellular ligand access to the binding pocket. This difference in ligand entry can be exploited to facilitate melatonin receptor subtype selectivity, as the ECL route in MT₂ could accommodate more polar compounds compared to the membrane-buried channel. We therefore expect that our results will lead to new therapies involving these pleiotropic receptors, aimed at—but not limited to—the treatment of type 2 diabetes, cancer and sleep disorders.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41586-019-1144-0>.

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Author contributions L.C.J., B.S., V.K., and V.C. conceived the project, analysed data, and wrote the paper with contributions from all authors. L.C.J. and B.S. designed, optimized, purified and characterized receptor constructs for structural studies and crystallized the receptor, prepared crystal samples and figures, solved and refined the structures, and assisted in generating mutant constructs for binding and functional analyses. J.D.M., X.-P.H., and S.T.S. performed radioligand binding and functional experiments, assisted in making mutant and wild-type constructs, and analysed binding and functional data. L.C.J., B.S., A.B., L.Z., W.L., and V.C. collected XFEL data. S.Z. and U.W. operated the LCP injector during XFEL data collection. S.H., L.Z., and W.L. assisted in XFEL sample preparation. A.B., C.G., C.L., and N.A.Z. processed XFEL data. L.C.J., G.W.H. and A.B. determined the structures. L.C.J. and G.W.H. performed the structure refinement and quality control. N.P., J.M.G., and V.K. designed mutants for radioligand and functional studies, and performed molecular docking and molecular dynamics simulations. R.H.J.O. and A.R.T. assisted with molecular biology and functional experiments. S.Y. synthesized the bitopic compound, analysed data, and edited the paper. R.C.S. contributed to study design and selection of chemical compounds for receptor stabilization and functional characterization, supervised protein expression, and edited the paper. B.L.R. supervised pharmacological experiments and edited the paper. V.K. supervised molecular docking and molecular dynamics calculations. V.C. coordinated and supervised the whole project.

Competing interests The authors declare no competing interests.

Additional information

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Design and expression of MT₂ crystallized construct. The DNA sequence of human MT₂ receptor (UniProt²¹ identifier P49286) was synthesized by GenScript with optimization for expression in insect cells. The crystallized construct (MT₂-CC) has truncations of N-terminal residues 1–30 and C-terminal residues 341–362. The thermostabilized apocytochrome BRIL (UniProt P0ABE7) from *Escherichia coli* with mutations M7W, H102I and R106L was fused to the truncated N terminus of MT₂ with a six-residue linker (GDGARP). Another fusion protein, rubredoxin (Rub, UniProt P00268), was fused in ICL3, replacing receptor residues 232–240. For construct optimization (to increase monodispersity, thermostability and crystallizability), the following point mutations were added: D86^{2,50}N⁹, L108^{ECL1}F, F129^{3,41}W¹⁰, N137^{3,49}D, C140^{3,52}L, W264^{6,48}F, A305^{7,50}P and N312^{8,47}D (see the accompanying paper⁸ for details). The MT₂-CC coding sequence was subcloned into a modified pFastBac1 (Invitrogen) vector, with a haemagglutinin (HA) signal sequence and a Flag tag on the N terminus and a PreScission protease cleavage site followed by a 10× His tag on the C terminus. The receptor was expressed in *Spodoptera frugiperda* cells (Sf9, purchased from ATCC, CRL-1711, authenticated by supplier using morphology and growth characteristics, certified mycoplasma-free), which were collected and stored as described in the accompanying paper⁸.

Purification of MT₂-CC. Insect cell membranes were prepared by thawing frozen cell pellets in a hypotonic buffer containing 10 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl, and homemade protease inhibitor cocktail. Extensive washing of the raw membranes was performed by repeated Dounce homogenization and centrifugation in hypotonic buffer (once), followed by high osmotic buffer containing 1.0 M NaCl, 10 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl, and homemade protease inhibitor cocktail (two or three times), thereby separating soluble and membrane-associated proteins from integral membrane proteins. Stocks (100 mM) of 2-PMT (Tocris) and ramelteon (Apex Biosciences) were dissolved in DMSO. Washed membranes were resuspended into a buffer containing 50 μM 2-PMT or ramelteon, 2 mg ml⁻¹ iodoacetamide, and homemade protease inhibitor cocktail, and incubated at 4 °C for 30 min before solubilization. The membranes were then solubilized in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% (wt/vol) *n*-dodecyl-β-D-maltopyranoside (DDM, Anatrace), 0.2% (wt/vol) cholesteryl hemisuccinate (CHS, Sigma-Aldrich) at 4 °C for 3 h. The supernatant was isolated by centrifugation at 60,000g for 50 min, and incubated in 20 mM HEPES (pH 7.5), 800 mM NaCl with Talon (immobilized metal affinity chromatography IMAC) resin (Clontech) overnight at 4 °C. After binding, the resin was washed with twenty column volumes of wash buffer 1 (50 mM HEPES (pH 7.5), 50 μM 2-PMT or ramelteon, 800 mM NaCl, 10% (vol/vol) glycerol, 0.1% (wt/vol) DDM, 0.02% (wt/vol) CHS, 10 mM imidazole), followed by ten column volumes of wash buffer 2 (50 mM HEPES (pH 7.5), 50 μM 2-PMT or ramelteon, 150 mM NaCl, 10% (vol/vol) glycerol, 0.05% (wt/vol) DDM, 0.01% (wt/vol) CHS, 50 mM imidazole). The protein was then eluted in minimal volumes of elution buffer (50 mM HEPES (pH 7.5), 50 μM 2-PMT or ramelteon, 150 mM NaCl, 10% (vol/vol) glycerol, 0.02% (wt/vol) DDM, 0.01% (wt/vol) CHS, 220 mM imidazole). PD MiniTrap G-25 columns (GE Healthcare) were used to remove imidazole. The protein was then treated overnight with His-tagged PreScission protease (GenScript) to cleave the C-terminal His-tag. PreScission protease and the cleaved C-terminal fragment were removed by binding to Talon IMAC resin for 1.5 h at 4 °C. The protein was collected as the TALON IMAC column flow-through. The ligand concentration was increased to 100 μM, and the protein was concentrated to 30–40 mg ml⁻¹ with a 100-kDa molecular mass cut-off Vivaspin centrifuge concentrator (Sartorius).

Protein stability assays. The stability of purified MT₂-CC was analysed using a microscale thermostability assay²² using Rotorgene (QIAGEN). In brief, 1–5 μg of protein was mixed with 1.5 μM 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) dye (2.5 mM stock in DMSO) in 25 mM HEPES pH 7.5, 150 mM NaCl, 0.02% DDM (wt/vol), 0.004% CHS (wt/vol), 10% glycerol (vol/vol), and indicated concentrations of compounds to a final volume of 100 μl. Samples were incubated for 15 min at 20 °C and then heated gradually from 25 °C to 95 °C at a rate of 2 °C min⁻¹, monitoring CPM fluorescence (excitation 365 nm, emission 460 nm). The melting temperature (*T_m*) was determined using the derivative of the resulting melting temperature curve after background subtraction using Prism 7.0 (GraphPad).

Crystallization. Purified MT₂-CC in complex with 2-PMT or ramelteon was reconstituted into LCP by mixing it with molten lipid using a mechanical syringe mixer¹³. The protein-LCP mixture contained 40% (wt/wt) receptor solution, 54% (wt/wt) monoolein, and 6% (wt/wt) cholesterol. Crystallization trials were performed in 96-well glass sandwich plates (Marienfeld) using an NT8-LCP robot (Formulatrix) by dispensing 40 nl of protein-laden LCP and 800 nl of precipitant solution per well. Plates were incubated and imaged at 20 °C using an automatic

incubator/imager (RockImager 1000, Formulatrix). Initial crystal hits were identified in a condition containing 100 mM HEPES, pH 6.8, 30% (vol/vol) PEG 400, 100 mM NH₄CH₃CO₂. These crystals, approximately 30 × 30 × 70 μm³, were collected using micromounts (MiTeGen) and flash-frozen in liquid nitrogen for data collection at a microfocus synchrotron source. After extensive optimization, the best crystals diffracted to about 3.0 Å resolution, but suffered from radiation damage, resulting in a 3.5 Å complete dataset. Additives had no effect on diffraction quality. Microcrystals for SFX data collection were prepared in gas-tight syringes (Hamilton) as previously described²³. After optimization, diffraction-quality crystals were obtained from 100 mM ADA pH 5.8–6.5, 24–28% (vol/vol) PEG 400, 10–200 mM NH₄CH₃CO₂, 50 μM 2-PMT or ramelteon, by injecting 5 μl of protein-laden LCP into 50 μl precipitant in syringes. Before loading the microcrystals into the LCP injector, excess precipitant was removed and 7.9 monoacylglycerol (MAG) lipid was added to the LCP to absorb any residual precipitant solution and to prevent crystalline phase formation upon rapid cooling when injecting LCP into vacuum²⁴.

Crystallographic data collection. Data collection was performed at the Coherent X-ray Imaging (CXI)²⁵ end station of the Linac Coherent Light Source (LCLS), which operated at a wavelength of 1.3 Å (9.83 keV) delivering individual X-ray pulses of 30 and 43 fs pulse duration and approximately 10¹¹ photons per pulse focused into a spot size of approximately 1.5 μm in diameter using a pair of Kirkpatrick-Baez mirrors. Microcrystals (Extended Data Fig. 1c) of MT₂ (approximately 5 × 5 × 5 μm³) were delivered in the LCP medium using an LCP microextrusion injector²⁴ with 50-μm nozzle running at a flow rate of approximately 300 nl min⁻¹. Diffraction images were recorded at a rate of 7,200 patterns per minute (120 Hz) with the 2.3 Megapixel Cornell-SLAC Pixel Array Detector (CSPAD)²⁶. Initial diffraction frames were corrected and filtered using the software package Cheetah²⁷. A crystal 'hit' was defined as an image containing a minimum of 20 diffraction peaks with a signal-to-noise ratio above 4 and a number of pixels above 3. After further refinement of parameters (peak detection, prediction and integration), images were indexed using MOSFLM²⁸, DirAx²⁹, and XDS³⁰ and integrated and merged into a final dataset using CrystFEL v0.6.2 software suite³¹. Integration radii of 3, 5, and 6 pixels with per pattern resolution cut-offs 1.0 nm⁻¹ above the conservative resolution estimates for each crystal were applied (push-res option), otherwise default values were used. The total numbers of collected images/hits/indexed images are as follows: 2,154,963/84,928/31,677 (MT₂-CC-2-PMT), 476,863/59,071/28,130 (MT₂-CC(H208A)-2-PMT), 293,060/22,267/20,704 (MT₂-CC(N86D)-2-PMT), 727,004/60,005/28,834 (MT₂-CC-ramelteon). As a resolution cutoff, the criterion³² of CC* > 0.5 was used for all datasets (see Extended Data Table 2 for data statistics). The space group was determined to be P2₁, with two molecules per asymmetric unit.

Structure determination. To solve the 2.8 Å resolution MT₂-CC-2-PMT structure, a search model was generated as follows: the MT₂ receptor sequence was sent to the HHpred server³³, and the output models were reduced by removing all low-resolution (<3.0 Å) and NMR structures. The RCSB Protein Data Bank (PDB) files of the top ten hits were downloaded and prepared with Sculptor³⁴. The models were structurally superimposed, and the side chains were pruned, yielding the conserved receptor core model. The model that produced a successful molecular replacement solution with Phaser³⁵ (TFZ score of 14.9 and LLG of 320) was based on the C-C chemokine receptor 2 structure (PDB ID: 5T1A)³⁶. This solution containing two receptor molecules was fixed as a partial solution, and the search continued with rubredoxin (PDB ID: 1IRO), in which one molecule was placed in the asymmetric unit. The resulting three-component solution was subjected to several rounds of refining with phenix.refine³⁷ and model building with phenix.autobuild³⁸ followed by manual refinement in Coot³⁹. BRIL (PDB ID: 1M6T) was then used independently as a search model for remaining fusion partners in the asymmetric unit. The second BRIL was manually modelled into the electron density; however, no density could be found for the second rubredoxin molecule, which, therefore, was not modelled in the final structure. This rubredoxin fusion partner is likely to be disordered and does not participate in crystal contacts, however, there is space for it in the crystal lattice (Extended Data Fig. 1d). A zinc ion was modelled in rubredoxin as previously described⁴⁰. Refinement and model completion were performed by repetitive cycling between Refmac5⁴¹ or autoBUSTER v2.10.2⁴² and manual rebuilding in Coot³⁹ using both 2mF_o - DF_c and mF_o - DF_c maps. Ligand restraints for refinement of 2-PMT and ramelteon coordinates were generated by ProDRG⁴³. For the other three datasets, the MT₂-CC-2-PMT structure was used as a search model for molecular replacement, and the refinement procedure was repeated as described above. The Ramachandran plot obtained by MolProbity⁴⁴ shows that, with the exception of Y92 from the YYP motif, all residues are in the favoured/allowed regions: 95.4/4.4% of residues (MT₂-CC-2-PMT), 93/6.8% of residues (MT₂-CC(H208A)-2-PMT), 94.8/5.0% of residues (MT₂-CC(N86D)-2-PMT), 95.3/4.5% of residues (MT₂-CC-ramelteon). Data collection and refinement statistics are summarized for each structure in

Extended Data Table 2. Figures containing electron density and molecular structures were generated using PyMol⁴⁵.

Channel profile calculations. The channel diameter profile along its length was calculated with CAVER analyst v.2.0⁴⁶ using default parameters. Further details can be found in the accompanying paper⁸.

Molecular docking. MT₂ receptor ligands obtained from the ChEMBL database¹⁵ were docked into the 2-PMT-bound crystal structures using an energy based docking in ICM-Pro v3.8-6⁴⁷ as described in the accompanying paper⁸.

Molecular dynamics simulations. The experimental structure of MT₂ was prepared and subjected to molecular dynamics simulations as described in the accompanying paper⁸. The simulation periodic box had dimensions (x, y, z) of 75.5 Å, 75.5 Å, 105.4 Å, and contained lipids (129 POPC molecules), 10,281 water molecules, 26 sodium ions, and 36 chloride ions.

Radioligand binding assays. Equilibrium binding assays were performed and analysed as described in the accompanying paper⁸. HEK293T cells were obtained from ATCC (CRL-11268, authenticated by supplier using morphology, growth characteristics and short tandem repeat profiling, certified mycoplasma-free). For kinetic studies, to initiate dissociation of [³H]melatonin, 10 µl of cold excess melatonin (10 µM) was added per well at specific time points ranging from 2 min to 10 h, and immediately at time = 0 min plates were collected. Dissociation experiments were performed at 25 °C for MT₁ and 37 °C for MT₂ because of the slow kinetics in MT₂. For all assays, non-specific activity was defined by the addition of 5 µM 2-PMT. Ligand dissociation data were analysed using 'Dissociation-One phase exponential decay' to yield estimates of k_{off} using GraphPad Prism 7.0.

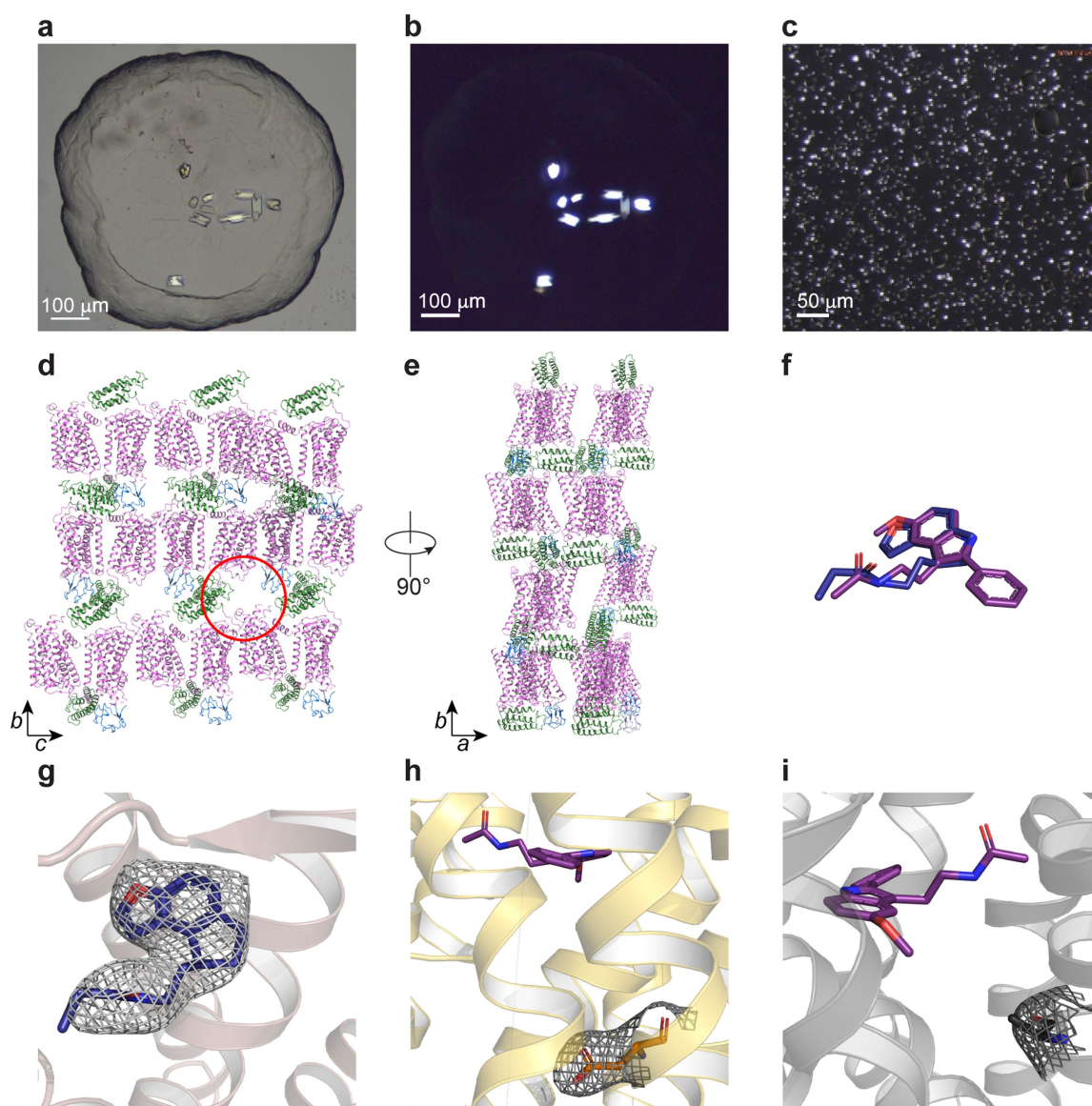
MT₂ G_{i/o}-mediated cAMP inhibition assay. MT₂ G_{i/o}-mediated cAMP inhibition assays were performed in HEK293T cells as described in the accompanying paper⁸.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

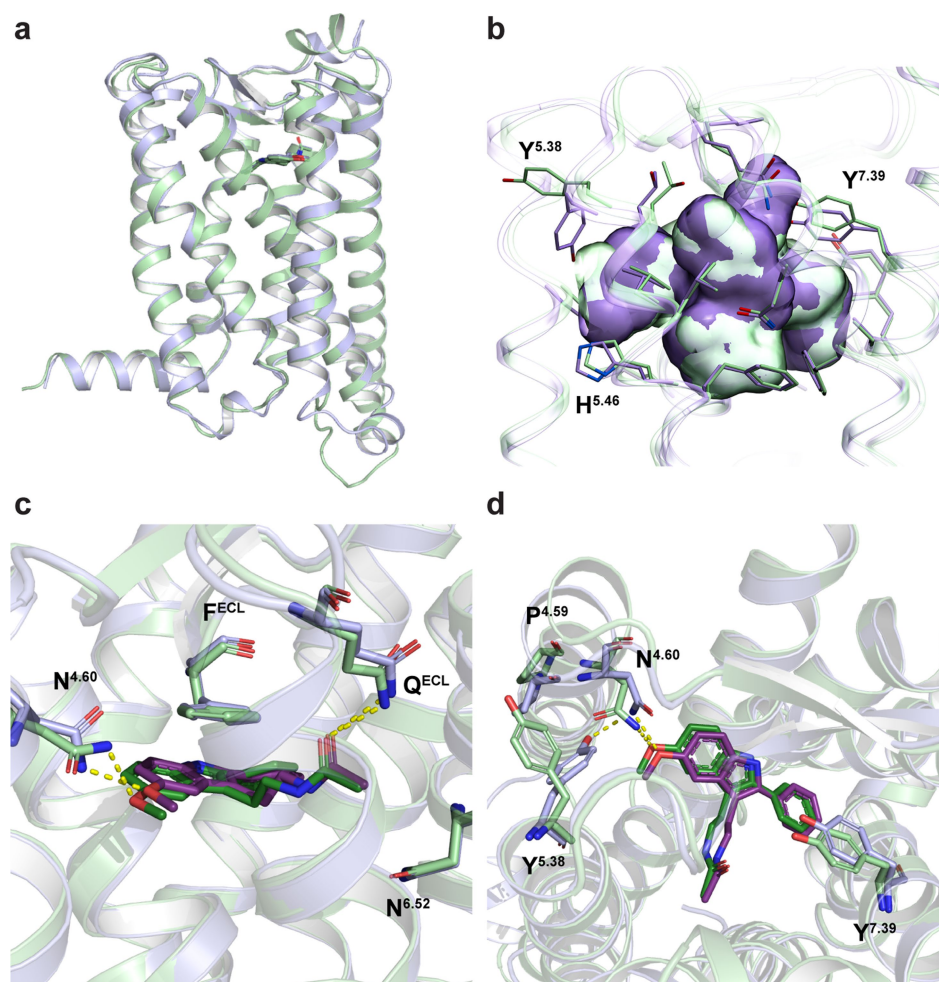
Structure factors and coordinates were deposited in the Protein Data Bank under the following accession codes: 6ME6 (MT₂-CC-2-PMT), 6ME7 (MT₂-CC(H208A)-2-PMT), 6ME8 (MT₂-CC(N86D)-2-PMT), 6ME9 (MT₂-CC-ramelteon).

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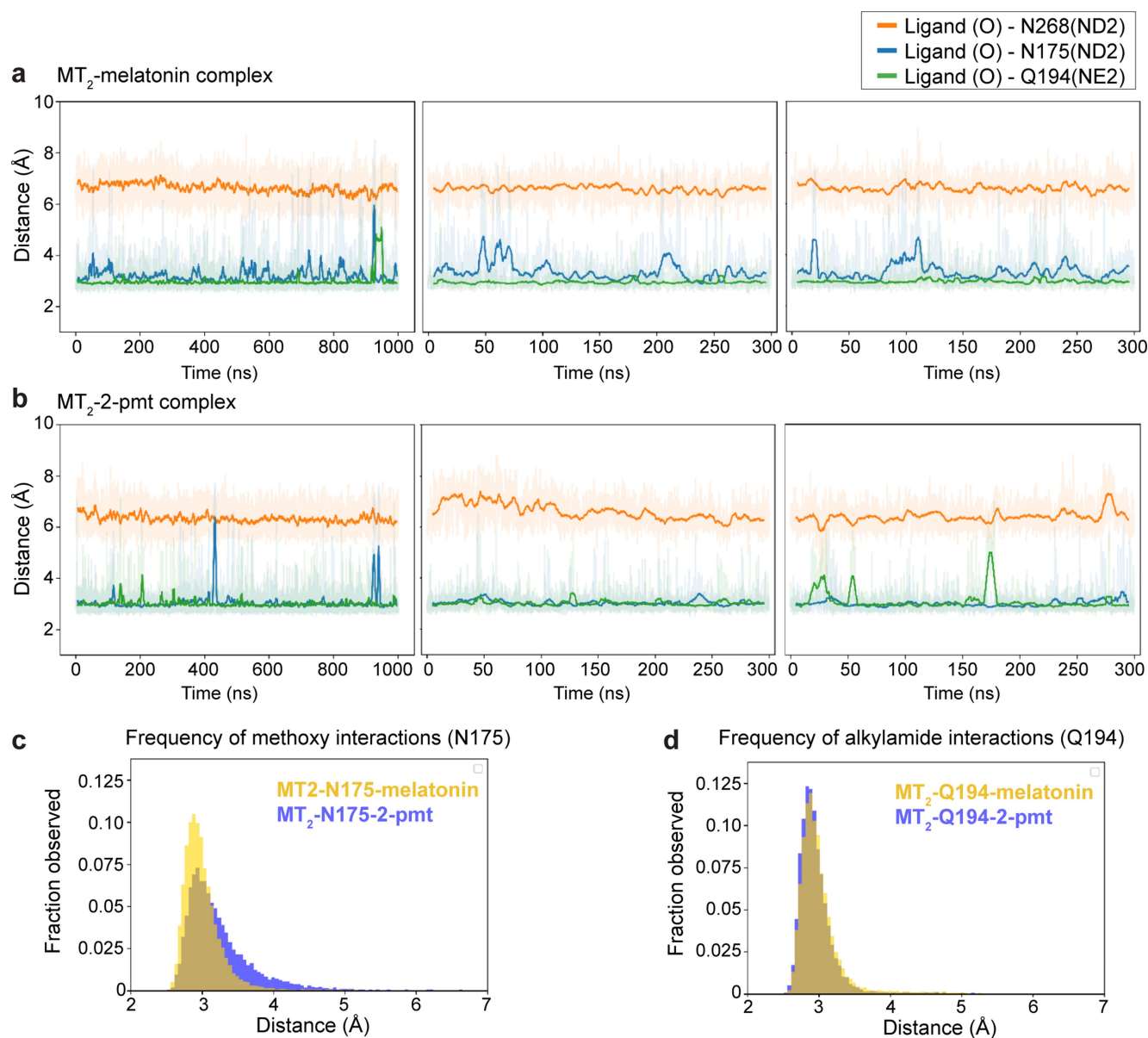
Extended Data Fig. 1 | Crystallization of MT₂: crystals, crystal packing and electron density. **a, b**, Bright field (**a**) and cross-polarized images (**b**) of representative MT₂-2-PMT crystals optimized for synchrotron data collection (representing three independent crystallization setups). **c**, Cross-polarized image of representative MT₂(N86D)-2-PMT crystals used for XFEL data collection (representing three independent crystallization setups). See Extended Data Table 2 for data collection

statistics. **d, e**, Crystal packing (receptor, purple; BRIL, green; rubredoxin, blue). Space for missing rubredoxin in molecule B of the asymmetric unit is indicated with a red circle. Lattice rotated 90° is shown in **e**. **f**, Overlay of 2-PMT (purple) and ramelteon (blue) ligands of MT₂. **g-i**, $2mF_o - DF_c$ density (grey) contoured at 1σ of ramelteon (**g**), N86^{2.50}D mutation (**h**), and H208^{5.46}A mutation (**i**). 2-PMT is shown in purple.



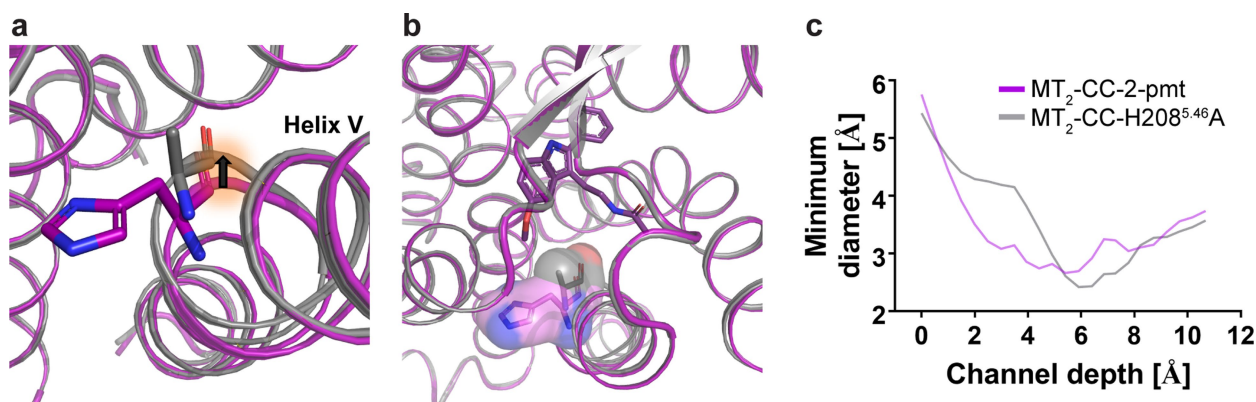
Extended Data Fig. 2 | Structural differences between MT₁ and MT₂.
a, Overlay of MT₁-2-PMT (green) and MT₂-2-PMT (violet) structures (C α r.m.s.d = 0.6 Å). **b**, Comparison of MT₁ (green) and MT₂ (violet) binding pockets. Overall, the binding pocket in MT₂ is about 50 Å³ larger than in MT₁. **c**, Comparison of 2-PMT ligand conformations in

MT₁ (green) and MT₂ (violet). Hydrogen bonds are shown as yellow dashed lines. **d**, Overlay of MT₁ and MT₂, showing residues with different conformations in the vicinity of the binding pocket. N^{4.60} makes a hydrogen bond with Y^{5.38} in MT₂ but not in MT₁.



Extended Data Fig. 3 | Molecular dynamics simulations. **a, b**, Distance plots for interactions between residues in MT₂ (N175^{4,60}, atom ND2; Q194^{ECL2}, atom NE2; N268^{6,52}, atom ND2), and closest oxygen atoms of the ligand methoxy and acetyl groups, respectively, in complexes with melatonin (**a**) and 2-PMT (**b**) from three independent simulation

runs. **c**, Distance histograms for interactions of N175^{4,60} with methoxy of melatonin (yellow) and 2-PMT (violet). **d**, Distance histograms for interactions of Q194^{ECL2} with alkylamide tail of melatonin (yellow) and 2-PMT (violet).



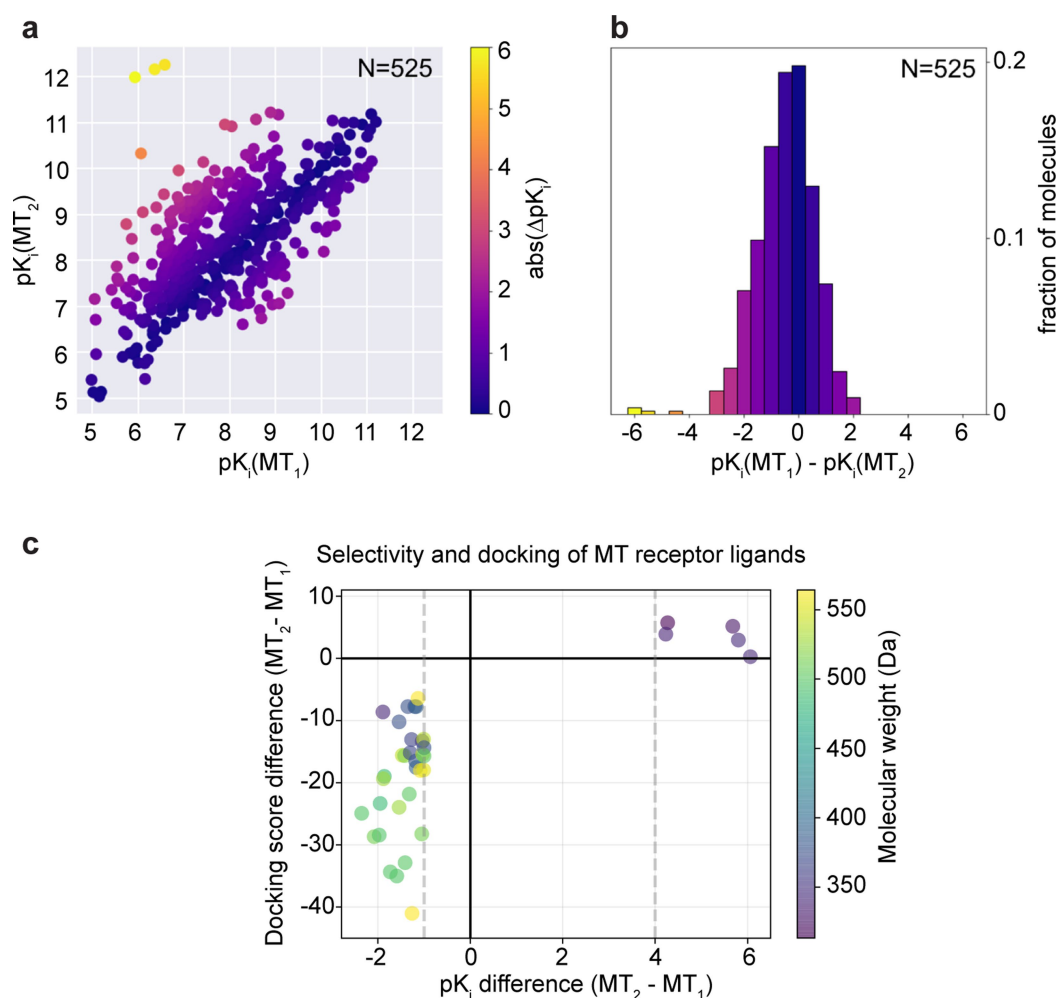
d

Mutant	melatonin			CTL 01-05-B-A05		
	EC ₅₀ , nM [n] (pEC ₅₀ ± s.e.m.)	%E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} /EC ₅₀) (mutant-wt)	EC ₅₀ , nM [n] (pEC ₅₀ ± s.e.m.)	%E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} /EC ₅₀) (mutant-wt)
WT	0.028 [16] (10.56±0.09)	100 (100)	0	16.59 [6] (7.78±0.07)	100 (63±7)	0
H208A (5.46)	0.190 [9] (9.72±0.26)	80±9	-0.93	No agonist activity		

Extended Data Fig. 4 | Structural and functional differences

between MT₂-2-PMT and MT₂(H208A^{5.46})-2-PMT. **a**, Overlay of the MT₂-2-PMT (purple) structure with that of MT₂(H208^{5.46}A)-2-PMT (grey) reveals an inward shift of helix V of about 0.9 Å, due to the H208^{5.46}A mutation (black arrow). **b**, Surface representation of the H208^{5.46} and H208^{5.46}A residues. Rotation of helix V renders the binding pocket volume about 50 Å³ smaller for the H208^{5.46}A structure (binding site volume for MT₂-2-PMT: 766 Å³ compared to 716 Å³ for the MT₂(H208^{5.46}A)-2-PMT structure). **c**, Comparison of the channel profiles (from the outside of the protein towards the ligand) for

MT₂-2-PMT (purple) and MT₂(H208^{5.46}A)-2-PMT (grey) reveals a narrowing of the MT₂(H208^{5.46}A)-2-PMT channel at a depth of around 6 Å as a consequence of the mutation and subsequent inward rotation of helix V. **d**, Functional data for wild-type and H208^{5.46}A mutant MT₂ expressed in HEK293T cells by using GloSensor to measure G_{i/o}-mediated inhibition of cAMP. Data represent mean ± s.e.m. for *n* independent experiments as indicated in square brackets. %E_{MAX} (maximum effect of a drug) is relative to wild-type receptor (in columns), and %E_{MAX}* is relative to melatonin activity (in rows). See Methods for further information and Supplementary Fig. 6 for dose-response curves.



Extended Data Fig. 5 | Selectivity analysis of melatonergic compounds.

a, Binding affinities of ligands for MT_1 (ChEMBL target identifier CHEMBL1945) and MT_2 (ChEMBL1946) were retrieved from the ChEMBL database¹⁵ (v. 24) of experimental literature values. Of these ligands, 525 have affinities reported for both receptor subtypes. For ligands with multiple reported affinity values for a given receptor, pK_i values were averaged. MT_1 -selective ligands are in the lower right quadrant; MT_2 -selective ligands are in the upper left quadrant. Data points are

coloured by absolute pK_i difference between subtypes (that is, selectivity). **b**, Histogram of observed ligand selectivities. MT_2 selective ligands are on the left of the panel, MT_1 selective ligands are on the right. **c**, Plot of the docking score difference of select ligands that were docked between MT_2 and MT_1 versus their pK_i difference ($MT_2 - MT_1$). Dashed lines indicate pK_i selectivity cutoff criteria (MT_1 : -1 and MT_2 : 4). Data points are coloured by molecular mass (Da). See Supplementary Table 1 for details of docked ligands.

Extended Data Table 1 | Ligand affinity data for MT₂ mutants

Mutant	melatonin	2-pmt	ramelteon	agomelatine
	K _d , nM [n] (pK _d ± s.e.m.)	K _i , nM [n] (pK _i ± s.e.m.)	K _i , nM [n] (pK _i ± s.e.m.)	K _i , nM [n] (pK _i ± s.e.m.)
WT	0.54 [10] (9.27±0.12)	0.17 [10] (9.78±0.11)	0.23 [4] (9.66±0.10)	0.24 [4] (9.63±0.08)
WT + NaCl	1.56 [6] (8.81 ± 0.18)	ND	ND	ND
MT ₂ -CC (Sf9)	63.10 [3] (7.20±0.06)	3.14 [4] (8.50±0.06)	2.60 [4] (8.59±0.02)	6.88 [4] (8.16±0.05)
MT ₂ -CC (sf9) + NaCl	48.23 [3] (7.32±0.03)	ND	ND	ND
MT ₂ -CC-N86 ^{2.50} D (Sf9)	29.40 [6] (7.53±0.34)	6.46 [4] (8.19±0.15)	7.37 [4] (8.13±0.09)	26.76 [4] (7.57±0.05)
MT ₂ -CC-H208 ^{5.46} A (Sf9)	10.81 [6] (7.97±0.16)	3.57 [6] (8.45±0.03)	2.03 [6] (8.69±0.13)	4.98 [6] (8.30±0.04)
D86N (2.50)	5.80 [3] (8.24±0.04)	0.33 [3] (9.48±0.12)	ND	ND
D86N (2.50) + NaCl	3.26 [3] (8.49±0.09)	ND	ND	ND
L108F (ECL1)	0.94 [3] (9.03±0.11)	0.13 [3] (9.87±0.15)	ND	ND
F129W (3.41)	2.84 [3] (8.55±0.04)	0.22 [3] (9.65±0.08)	ND	ND
N137D (3.49)	1.24 [3] (8.91±0.13)	0.12 [3] (9.92±0.00)	ND	ND
C140L (3.52)	0.21 [3] (9.68±0.05)	0.03 [3] (10.50±0.02)	ND	ND
W264F (6.48)	0.88 [3] (9.06±0.14)	0.06 [3] (10.25±0.26)	ND	ND
A305P (7.50)	3.94 [3] (8.40±0.19)	0.47 [3] (9.32±0.06)	ND	ND
N312D (7.57)	2.85 [3] (8.54±0.07)	0.36 [3] (9.44±0.04)	ND	ND
P95A (2.59)	<i>No specific binding up to 7 nM [³H]-melatonin</i>			
M120A (3.32)	0.44 [3] (9.42±0.16)	0.028 [3] (10.7±0.3)	0.055 [3] (10.28±0.09)	0.052 [3] (10.35±0.18)
N175A (4.60)	0.86 [3] (9.2±0.3)	0.09 [3] (10.06±0.06)	0.08 [3] (10.12±0.11)	0.25 [3] (9.60±0.04)
F192A (ECL2)	<i>Low expression, no specific binding up to 7 nM [³H]-melatonin</i>			
F192I (ECL2)	<i>No specific binding up to 7 nM [³H]-melatonin</i>			
Q194A (ECL2)	0.62 [3] (9.4±0.3)	0.043 [3] (10.38±0.07)	0.051 [3] (10.4±0.2)	0.12 [3] (9.94±0.09)
Y200A (5.38)	0.63 [3] (9.3±0.3)	0.14 [3] (9.86±0.01)	0.19 [3] (9.73±0.03)	0.67 [3] (9.18±0.02)
A203F (5.41)	0.82 [5] (9.09±0.01)	0.12 [5] (9.94±0.06)	0.19 [5] (9.47±0.19)	0.42 [5] (9.37±0.19)
H208A (5.46)	1.24 [3] (8.94±0.13)	0.17 [3] (9.79±0.09)	0.18 [3] (9.77±0.11)	0.22 [3] (9.68±0.11)
N268A (6.52)	0.96 [3] (9.3±0.4)	0.09 [3] (10.08±0.09)	0.12 [3] (9.92±0.05)	0.20 [3] (9.69±0.03)
Y294A (7.39)	1.07 [3] (8.99±0.09)	0.042 [3] (10.38±0.04)	0.049 [3] (10.33±0.09)	0.10 [3] (10.04±0.10)
Y308S (7.53)	<i>No specific binding up to 7 nM [³H]-melatonin</i>			

Data were acquired with wild-type (WT) MT₂ and mutants expressed in HEK293T cells by radioligand competition binding using [³H]melatonin to yield K_d or K_i affinity estimates. Data represent mean ± s.e.m. for *n* independent experiments as indicated in square brackets. Crystal constructs (CC) were expressed in Sf9 cells. ND, not determined. Binding isotherms are shown in Supplementary Fig. 2. For determining the effect of NaCl, binding assays were performed in the presence of 147 nM NaCl (binding isotherms in Supplementary Fig. 3).

Extended Data Table 2 | MT₂ Crystallographic data collection and refinement statistics

	MT ₂ -CC-2-pmt ^a		MT ₂ -CC-H208 ^{5,46} A-2-pmt ^b		MT ₂ -CC-N86 ^{2,50} D-2-pmt ^c		MT ₂ -CC-ramelteon ^d	
Data collection	P2 ₁		P2 ₁		P2 ₁		P2 ₁	
Space group	P2 ₁		P2 ₁		P2 ₁		P2 ₁	
Cell dimensions								
<i>a</i> , <i>b</i> , <i>c</i> (Å)	69.5, 146.2, 77.3		69.2, 146.2, 77.3		68.7, 145.8, 77.0		69.4, 145.7, 77.2	
α , β , γ (°)	90, 111.7, 90		90, 105.2, 90		90, 107.4, 90		90, 106.2, 90	
Resolution (Å)	21.99-2.80 (2.88-2.80)		21.99-3.20 (3.34-3.20)		22.0-3.10 (3.23-3.10)		22.0-3.30 (3.46-3.30)	
<i>R</i> _{split}	0.146 (4.31)		0.181 (3.26)		0.189 (4.70)		0.201 (2.90)	
<i>I</i> / σ <i>I</i>	3.07 (0.46)		4.02 (0.39)		3.87 (0.3)		3.67 (0.42)	
CC*	0.999 (0.52)		0.997 (0.54)		0.997 (0.60)		0.997 (0.54)	
Completeness (%)	100 (100)		100 (100)		100 (100)		100 (100)	
Redundancy	571.2 (141.3)		196.6 (39)		133 (38.2)		221.1 (84.6)	
Refinement								
Resolution (Å)	21.99-2.80		21.99-3.20		22.0-3.10		22.0-3.30	
No. reflections	35,193		24,439		26,179		22,122	
<i>R</i> _{work} / <i>R</i> _{free}	0.219/0.249		0.224/0.250		0.234/0.262		0.248/0.270	
No. atoms	A	B	A	B	A	B	A	B
Protein	3,333	2,852	3,343	2,786	3,293	2,752	3,227	2,738
Ligand/Zn ⁺²	23/1	23/0	23/1	23/0	23/1	23/0	19/1	19/0
Lipid and other	0	11	0	12	0	0	0	0
B-factors (Å ²)								
Receptor	116.4	121.0	95.0	99.6	114.2	117.2	114.1	118.2
BRIL	162.3	188.9	143.2	176.4	167.6	208.8	185.3	248.1
Rubredoxin	114.7	n/a	100.3	n/a	116.2	n/a	118.6	n/a
Ligand/Zn ⁺²	101.6/114.7	106.1/n/a	73.3/91.2	88.7/n/a	96.9/112.3	102.1/n/a	94.9/117.7	105.6/n/a
Lipids and other	n/a	138.8	n/a	111.0	n/a	n/a	n/a	n/a
R.m.s. deviations								
Bond lengths (Å)	0.009		0.010		0.009		0.009	
Bond angles (°)	0.97		1.06		0.96		0.95	

Number of crystals used for structure determination: ^a31,677, ^b28,130, ^c20,704 and ^d28,834. Values in parentheses are for highest-resolution shell.

Extended Data Table 3 | Functional data ($G_{i/o}$ GloSensor) for MT₂ crystal construct mutants

Mutant	melatonin			2-pmt		
	EC ₅₀ , nM [n] (pEC ₅₀ ± s.e.m.)	%E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} / EC ₅₀) (mutant-WT)	EC ₅₀ , nM [n] (pEC ₅₀ ± s.e.m.)	% E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} / EC ₅₀) (mutant-WT)
WT	0.028 [16] (10.56±0.09)	100 (100)	0	0.018 [14] (10.75±0.11)	100 (100±5)	0
MT ₂ -CC (Sf9)	<i>No activity</i>					
MT ₂ -Rub	<i>No activity</i>					
D86N (2.50)	3.951 [3] (8.40±0.34)	80±18 (100)	-2.25	1.995 [3] (8.70±0.11)	101±17 (126±21)	-2.04
L108F (ECL1)	0.029 [5] (10.54±0.10)	72±4 (100)	-0.16	0.011 [6] (10.95±0.19)	80±8 (110±11)	0.11
F129W (3.41)	0.011 [6] (10.95±0.17)	128±9 (100)	+0.50	0.007 [6] (11.16±0.20)	128±7 (99±6)	0.52
N137D (3.49)	0.019 [6] (10.72±0.12)	88±6 (100)	-0.11	0.016 [6] (10.81±0.10)	90±5 (103±5)	0.19
C140L (3.52)	0.072 [7] (10.15±0.16)	90±8 (100)	-0.46	0.035 [6] (10.45±0.16)	89±6 (101±4)	-0.34
W264F (6.48)	0.044 [7] (10.36±0.09)	117±6 (100)	-0.13	0.020 [5] (10.70±0.40)	118±8 (101±6)	-0.03
A305P (7.50)	0.141 [7] (9.85±0.16)	129±5 (100)	-0.60	0.073 [5] (10.14±0.26)	143±4 (91±6)	-0.45
N312D (7.57)	0.069 [5] (10.16±0.15)	135±6 (100)	-0.26	0.041 [3] (10.39±0.18)	138±10 (102±8)	-0.21

Data were acquired with wild-type MT₂ and mutants expressed in HEK293T cells by using GloSensor to measure $G_{i/o}$ -mediated cAMP inhibition via isoproterenol stimulation. Data represent mean ± s.e.m. for *n* independent experiments as indicated in square brackets. %E_{MAX} is relative to wild-type receptor (in columns), and %E_{MAX}* is relative to melatonin (in rows). Mutant effects were calculated by the change in relative activity or log(E_{max}/EC₅₀), subtracting wild type from mutant. Dose-response curves are shown in Supplementary Fig. 4.

Extended Data Table 4 | Functional data ($G_{i/o}$ GloSensor) for MT₂ mutants

Mutant	melatonin			2-pmt			ramelteon			agomelatine		
	EC ₅₀ , nM [n] (pEC ₅₀ ±s.e.m.)	% E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} / EC ₅₀) (mutant- wt)	EC ₅₀ , nM [n] (pEC ₅₀ ±s.e.m.)	% E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} / EC ₅₀) (mutant- wt)	EC ₅₀ , nM [n] (pEC ₅₀ ±s.e.m.)	% E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} / EC ₅₀) (mutant- wt)	EC ₅₀ , nM [n] (pEC ₅₀ ±s.e.m.)	% E _{MAX} (%E _{MAX} *)	Δlog(E _{MAX} / EC ₅₀) (mutant- wt)
WT	0.028 [16] (10.56±0.09)	100 (100)	0	0.018 [12] (10.75±0.11)	100 (100±4)	0	0.016 [12] (10.81±0.14)	100 (108±3)	0	0.018 [10] (10.75±0.12)	100 (105±4)	0
P95A (2.59)	<i>No activity</i>											
A171M (4.56)	0.075 [9] 10.13±0.14	66±9 (100)	-0.61	0.032 [8] 10.49±0.11	62±9 (94±14)	-0.46	0.031 [7] 10.51±0.19	67±9 (90±12)	-0.47	0.025 [6] 10.60±0.16	75±10 (107±15)	-0.27
N175A (4.60)	0.070 [9] (10.16±0.15)	74±10 (100)	-0.53	0.0192 [8] (10.72±0.12)	67±14 91±19	-0.20	0.010 [7] (11.00±0.21)	72±12 (87±14)	+0.05	0.015 [7] (10.82±0.13)	71±7 (91±10)	0.08
F192A (ECL2)	99.235 [6] (7.00±0.26)	122±7 (100)	-3.46	4.808 [10] 8.32±0.09	139±3 (114±6)	-2.28	4.799 [9] 8.32±0.04	150±4 (109±3)	-2.31	5.316 [9] 8.27±0.05	145±2 (111±2)	-2.31
F192I (ECL2)	3.00 [3] (8.52±0.30)	159±4 (100)	-1.83	0.211 [3] (9.68±0.07)	159±3 (100±1)	-0.87	0.571 [3] (9.24±0.16)	169±5 (94±3)	-1.34	2.754 [3] (8.56±0.05)	160±8 (94±5)	-1.98
Q194A (ECL2)	0.025 [3] (10.60±0.22)	131±4 (100)	+0.16	0.011 [3] (10.96±0.08)	130±3 (99±3)	+0.33	0.006 [3] (11.23±0.10)	130±6 (88±4)	+0.54	0.005 [3] (11.30±0.13)	110±15 (78±10)	+0.59
Y200A (5.38)	0.517 [3] (9.29±0.32)	161±10 (100)	-1.06	0.009 [3] (11.07±0.08)	158±16 (98±10)	+0.53	0.014 [3] (10.86±0.07)	164±21 (90±10)	0.26	0.314 [3] (9.50±0.31)	149±14 (87±8)	-1.07
H208A (5.46)	0.190 [9] (9.72±0.26)	80±9 (100)	-0.93	0.101 [10] (10.00±0.15)	75±9 (93±11)	-0.88	0.035 [8] (10.45±0.28)	79±11 (88±12)	-0.46	0.083 [8] (10.08±0.26)	76±2 (89±3)	-0.79
N268A (6.52)	0.046 [3] (10.33±0.27)	141±10 (100)	-0.08	0.013 [3] (10.87±0.10)	140±8 (99±6)	+0.28	0.009 [3] (11.05±0.10)	132±9 (83±6)	+0.36	0.007 [3] (11.16±0.10)	112±4 (75±3)	+0.46
Q194A (ECL2)/ N268A (6.52)	2.405 [9] 8.62±0.21	116±7 (100)	-1.88	0.033 [10] 10.49±0.18	112±6 (96±5)	-0.21	0.136 [9] 9.87±0.15	121±7 (93±5)	-0.86	0.759 [8] 9.12±0.14	116±4 (94±4)	-1.56
Y294A (7.39)	0.460 [4] (9.34±0.15)	148±6 (100)	-1.05	0.008 [4] (11.12±0.11)	153±7 (94±9)	+0.56	0.008 [3] (11.11±0.09)	153±11 (114±11)	+0.48	0.015 [3] (10.83±0.20)	118±11 (143±12)	-0.15
Y308S (7.53)	<i>No activity</i>											

Data were acquired with MT₂ mutants by using GloSensor to measure $G_{i/o}$ -mediated cAMP inhibition via isoproterenol stimulation. Data represent mean ± s.e.m. for *n* independent experiments as indicated in square brackets. %E_{MAX} is relative to wild-type receptor (in columns), and %E_{MAX}* is relative to melatonin (in rows). Mutant effects were calculated by the change in relative activity, or log(E_{MAX}/EC₅₀) subtracting wild-type from mutant. ND, not determined. Dose-response curves are shown in Supplementary Fig. 5.

Extended Data Table 5 | Thermostability data for MT₂ mutants

Mutant	apo T _m , °C	mlt T _m , °C	2-pmt T _m , °C	mlt ΔT _m , °C	2-pmt ΔT _m , °C
MT ₂ -CC	63.6±0.3	73.4±0.1	79.9±0.4	+9.8	+16.3
W129F (3.41)	59.0±0.2	72.4±0.1	78.9±0.3	+13.4	+19.9
N175A (4.60)	64.6±0.3	70.6±0.1	78.1±0.2	+6	+13.5
F192A (ECL2)	57.1±0.5	66.5±0.1	75.4±0.1	+9.4	+18.3
Q194A (ECL2)	65.5±0.3	69.9±0.3	77.3±0.2	+4.4	+11.8
H208A (5.46)	58.7±0.6	72.6±0.4	78.9±0.3	+13.9	+20.2
N268A (6.52)	63.3±0.3	66.1±0.2	74.6±0.3	+2.8	+11.3
Q194A (ECL2)/N268A (6.52)	67.9±0.3	67.9±0.1	71.4±0.3	0	+3.5
Y308S (7.53)	ND	65.5±0.2	75.8±0.3	ND	ND

Melting temperature T_m determined using the CPM assay²⁸ (mean ± s.d. for $n = 3$ independent experiments) for the crystallized construct (MT₂-CC), and indicated mutants (in the MT₂-CC background), purified in the absence (apo) or presence (100 μM) of ligand (mlt, melatonin and 2-PMT, 2-phenylmelatonin). ND, not determined. W129F refers to MT₂-CC but without the F129³⁴¹W mutation. Melting curves are shown in Supplementary Fig. 7.

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- | n/a | Confirmed |
|-------------------------------------|--|
| ☐ | ☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☐ | ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☒ | ☐ The statistical test(s) used AND whether they are one- or two-sided
<i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i> |
| ☒ | ☐ A description of all covariates tested |
| ☒ | ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐ | ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☒ | ☐ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted
<i>Give P values as exact values whenever suitable.</i> |
| ☒ | ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☒ | ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☒ | ☐ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	XFEL data were collected at the CXI instrument at LCLS using in-house DAQ software.
Data analysis	Prodrgr server (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrgr/submit.html ; queried in 2017-2018) CHARMM-GUI web-server (http://www.charmm-gui.org/ ; queried in January 2018) GPCRdb (http://gpcrdb.org/ ; queried in 2015-2018) MolProbity server (v4.4; http://molprobity.biochem.duke.edu/ ; queried in 2018) QC Check server (v3.1; https://smb.slac.stanford.edu/jcsg/QC/ ; queried in 2018) OPM database (http://opm.phar.umich.edu/ ; queried in January 2018) ChEMBL24 database (https://www.ebi.ac.uk/chembl/ ; queried in 2018) HHpred server (https://toolkit.tuebingen.mpg.de/#/tools/hhpred ; queried in 2017) UniProt data resource (https://www.uniprot.org/ ; queried in July 2018) Cheetah v.2017.3; CrystFEL v.0.6.2; Prism v.7.0; CCP4 v.7.0; Phaser v.2.1; COOT v.0.8.8; BUSTER v.2.10.2; CAVAR analyst v.2.0; ICM-PRO v.3.8-6; Pymol v.1.5; MOSFLM v.7.2.1; DirAx v. 1.17; XDS BUILT 20170601; Sculptor v. 2.1; Phenix v. 1.14; Refmac5 v. 5.5

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Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Structure factors and coordinates were deposited in the Protein Data Bank under the following accession codes: 6ME6 (MT2-CC-2-pmt), 6ME7(MT2-CC-H208A-2-pmt), 6ME8 (MT2-CC-N86D-2-pmt), 6ME9 (MT2-CC--ramelteon).

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to predetermine sample size. Thermostability and pharmacological characterization were conducted at least in n=3 independent experiments and are comparable to other published studies. Wild-type constructs were used as internal controls, resulting in larger number of independent repeats. Diffraction data from thousands of protein crystals were integrated and scaled to ensure 100% completeness of the dataset.
Data exclusions	No data were excluded.
Replication	All measurements were done in triplicate and all attempts at replication were successful and presented.
Randomization	This study did not allocate experimental groups thus no randomization was required for the reported experiments.
Blinding	The researchers were not blinded to allocation during experiments and outcome assessment. Blinding was not required for the reported experiments because all functional and structural data were analyzed using the same methods, and results are not subjective.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	Cell lines were purchased from the American Type Culture Collection (ATCC). Sf9: ATCC-CRL-1711. HEK293T cells: ATCC CRL-11268.
Authentication	The cell lines were authenticated by the supplier (ATCC) using morphology and growth characteristics (for Sf9 and HEK293T), and STR profiling (for HEK293T).
Mycoplasma contamination	Both Sf9 and HEK293T cells have been tested and shown to be free from mycoplasma (Hoechst DNA stain and Direct Culture methods employed).

Commonly misidentified lines
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No commonly misidentified cell lines were used.