Virtual discovery of melatonin receptor ligands to modulate circadian rhythms

The neuromodulator melatonin synchronizes circadian rhythms and related physiological functions through the actions of two G-protein-coupled receptors: MT_1 and MT_2. Circadian release of melatonin at night from the pineal gland activates melatonin receptors in the suprachiasmatic nucleus of the hypothalamus, synchronizing the physiology and behaviour of animals to the light–dark cycle. The two receptors are established drug targets for aligning circadian phase to this cycle in disorders of sleep and depression. Despite their importance, few in vivo active MT-selective ligands have been reported, hampering both the understanding of circadian biology and the development of targeted therapeutics. Here we docked more than 150 million virtual molecules to an MT crystal structure, prioritizing structural fit and chemical novelty. Of these compounds, 38 high-ranking molecules were synthesized and tested, revealing ligands with potencies ranging from 470 picomolar to 6 micromolar. Structure-based optimization led to two selective MT-inverse agonists—which were topologically unrelated to previously explored chemotypes—that acted as inverse agonists in a mouse model of circadian re-entrainment. Notably, we found that these MT-selective inverse agonists advanced the phase of the mouse circadian clock by 1.3–1.5 h when given at subjective dusk, an agonist-like effect that was eliminated in MT-knockout mice. This study illustrates the opportunities for modulating melatonin receptor biology through MT-selective ligands and for the discovery of previously undescribed, in vivo active chemotypes from structure-based screens of diverse, ultralarge libraries.

The recent determination of the crystal structures of the MT_1 and MT_2 receptors afforded us the opportunity to seek previously undescribed chemotypes with new functions, including MT-selective ligands, by computational docking of an ultralarge make-on-demand library, seeking molecules that complemented the main ligand-binding (orthosteric) site of the receptor. Given the similarity of the MT_1 and MT_2 sites—in which 20 out of 21 residues are identical—and the challenges of docking for selectivity, we sought to prioritize previously undescribed, high-ranking chemotypes from the docking screen that were unrelated to known melatonin receptor ligands and expected that these molecules interacted differentially with the two melatonin receptor types.

We docked more than 150 million ‘lead-like’ molecules, characterized by favourable physical properties from ZINC (http://zinc15.docking.org). These largely make-on-demand molecules have not previously been synthesized, but are usually accessible by two-component reactions. Use of complex building blocks in these reactions biases towards diverse, structurally interesting molecules. Each library molecule was sampled in an average of more than 1.6 million poses (orientations × conformations) in the MT_1 orthosteric site by DOCK3.721, generating more than 72 trillion complexes for the library overall and scoring each pose for physical complementarity to the receptor site. Seeking diversity, the top 300,000 scoring molecules were clustered by topological similarity, resulting in 65,323 clusters, and those that were similar to known MT_1 and MT_2 ligands from ChEMBL were eliminated (Methods, Fig. 1 and Extended Data Table 1).

The best-scoring molecules from each of the top 10,000 clusters were inspected for engagement with residues that recognize ligands in the MT_1 crystal structure and for new polar partners in the MT_2 site. In the docked complexes, these included hydrogen bonds with...
Q181ECL2, N1624.60, T178ECL2 and N2556.52, and hydrogen bonds with the backbone atoms of A1584.56, G1043.29 and F179ECL2. Conformationally strained molecules and those with unsatisfied hydrogen-bond donors were deprioritized. Within the best-scoring clusters, all members were inspected and the one that best fitted these criteria was prioritized. Ultimately, 40 molecules with ranks ranging from 16 to 246,721, or the top 0.00001% to top 0.1% of the more than 150 million docked molecules, were selected for de novo synthesis and testing. Of the 38 molecules that were successfully synthesized (a 95% fulfilment rate), 15 had activity at either or both of the human MT1 and MT2 receptors in functional assays (Fig. 1 and Extended Data Table 1), a hit rate of 39% (calculated as the number of active compounds/number of physically tested compounds).

**Discovery of chemotypes with distinct efficacies**

These active molecules included both agonists and inverse agonists, consistent with the emphasis on chemotype novelty (Fig. 1 and Extended Data Table 1). This novelty is supported quantitatively by their low topological similarity to known melatonin receptor ligands, and visually by comparison of the new ligands to their closest analogues among the known compounds (Extended Data Table 1). The different chemotypes often engaged the same residues that recognize 2-phenylmelatonin in the crystal structures. Examples include the hydrogen-bond interactions with N1624.60 made by the methoxy group of 2-phenylmelatonin that, in the docked models, were made by esters (ZINC92585174), pyridines (ZINC151209032) and benzodioxoles (ZINC301472854).

**Fig. 1 | Large library docking finds novel, potent melatonin receptor ligands.**

a, Docking for new melatonin receptor chemotypes from the make-on-demand library. b, Docked pose of ZINC159050207, an hMT1/hMT2 non-selective agonist with low nanomolar activity. c, Docked pose of ZINC157665999, an MT2-selective inverse agonist. In b and c, the crystallographic geometry of 2-phenylmelatonin is shown in transparent blue, for context. d, The initial 15 docking actives are shown, highlighting groups that correspond to melatonin’s acetamide side chain (blue) and its 5-methoxy-indole (red) in their docked poses and receptor interactions. Boxed molecules are inverse agonists.
Similarly, whereas 2-phenylmelatonin stacks an indole with F179<sup>ECL2</sup> the docked ligands stack benzoxazines (ZINC482850041), thiophenes (ZINC419113878) and furans (ZINC433313647). Whereas 2-phenylmelatonin forms hydrogen bonds with Q81<sup>H</sup> through its acetamide, the docked ligands use esters or pyridines (Fig. 1). The new ligands also dock to interact with new residues, including hydrogen bonds with T179<sup>ECL2</sup>, N255<sup>H2</sup>, A158<sup>433</sup>, G104<sup>393</sup> and F179<sup>ECL2</sup> (Fig. 1b, c and Extended Data Fig. 1a–d). Admittedly, many ligands were just as active at the MT<sub>1</sub> receptor or were even selective for it (Extended Data Table 1 and Extended Data Fig. 1). Thus, although the initial docking against the MT<sub>1</sub> structure found a selective between the receptor types—is a 1 nM MT<sub>1</sub> agonist and is one of up to 30 μM: ZINC419113878, ZINC51209032, ZINC35044322 and ZINC82731037. Notably, ZINC159052027—although it was not selective between the receptor types—is a 1 nM MT<sub>1</sub> agonist and is one of the most-potent molecules found directly from a docking screen<sup>53–54</sup> (Extended Data Table 1 and Extended Data Fig. 1c, d). Indeed, we sought to improve 12 of these chemotype families, selecting analogues from the make-on-demand library. Several thousand such molecules were docked into the MT<sub>1</sub> site (see Methods and Extended Data Table 2). Of the 131 synthesized and tested, 94 analogues had activity at either or both the MT<sub>1</sub> or MT<sub>2</sub> melatonin receptors at concentrations of ≤10 μM (Extended Data Table 2, Extended Data Fig. 2 and Supplementary Data Table 2). Of the 131 synthesized and tested, 94 analogues had activity at either or both the MT<sub>1</sub> or MT<sub>2</sub> melatonin receptors at concentrations of ≤10 μM (Extended Data Table 2, Extended Data Fig. 2 and Supplementary Data Table 1); of the 12 chemotype families, 5 showed improved potency. While this structure-based analogue-finding method could often find more-potent ligands, the efficacy, selectivity and bias of these compounds were sensitive to small structural changes (Extended Data Table 3).

We were particularly interested in type-selective ligands with in vivo efficacy, as these are unreported in the field. We investigated two MT<sub>1</sub>-selective inverse agonists, ZINC555417447 and ZINC157673384, and a selective MT<sub>1</sub> agonist, ZINC125734226 (from here on referred to as UCSF7447, UCSF3384 and UCSF4226, respectively), for their affinities (Fig. 2 and Supplementary Data 3, 4), in vitro signalling, pharmacokinetics (Extended Data Table 3), selectivity to mouse as well as the human melatonin receptors (Fig. 2 and Supplementary Data 3, 4), and for their efficacies in mouse models of circadian behaviour (Fig. 3 and Extended Data Figs. 4, 5, 7). As expected, UCSF7447 and UCSF3384 competed for 2-[<sup>125</sup>I]iodomelatonin binding with higher affinity for the human MT<sub>1</sub> receptors. Inhibition constant (K<sub>i</sub>) values improving to 7.5 nM and 63 nM, respectively—were improved compared with the human receptors for inverse agonists UCSF7447 (a) and UCSF3384 (b) compared with 2-[<sup>125</sup>I]iodomelatonin for human MT<sub>1</sub> (hMT<sub>1</sub>), human MT<sub>2</sub> (hMT<sub>2</sub>), mouse MT<sub>1</sub> (mMT<sub>1</sub>) and mouse MT<sub>2</sub> (mMT<sub>2</sub>) receptors stably expressed in CHO cells. Competitive binding was measured in the absence or presence of 100 μM GTP, 1 mM Na<sub>2</sub>EDTA and 150 mM NaCl. GTP uncouples G proteins from melatonin receptors, thus promoting the inactive conformations of the receptors<sup>46</sup> and leading to a higher affinity of the receptors for inverse agonists. Therefore, the lighter-coloured bars show higher binding affinities for the drugs compared with the paired darker-coloured bars. Connected symbols represent the pEC<sub>50</sub> values of individual determinations run in parallel. K<sub>i</sub> values were derived from competition binding curves (Supplementary Data 3). Bars represent the mean of five independent determinations. Statistical significance between pEC<sub>50</sub> values was calculated by two-tailed paired Student’s t-test (t, d.f. and P values are described in the ‘Data analysis’ Methods section, and Supplementary Table 5). *P < 0.05; **P < 0.01; ***P < 0.001 compared with the corresponding pEC<sub>50</sub>, averages values derived in the absence of GTP. c-f: Concentration–response curves of human and mouse MT<sub>1</sub> and MT<sub>2</sub> receptors that were transiently expressed in HEK293T cells. Analysis of isoproterenol-stimulated cAMP production combined with UCSF7447 or UCSF3384 treatment. Data for UCSF7447 and UCSF3384 were normalized to the isoproterenol-stimulated basal activity. Insets, data normalized to the maximum effect of UCSF7447 or UCSF3384. Human MT<sub>1</sub> and MT<sub>2</sub> receptors treated with UCSF7447: hMT<sub>1</sub>; pEC<sub>50</sub> = 7.39 ± 0.10, E<sub>max</sub> = −94 ± 5%, n = 8; hMT<sub>2</sub>; pEC<sub>50</sub> = 6.66 ± 0.10, E<sub>max</sub> = −84 ± 5%, n = 8; mMT<sub>1</sub>/hMT<sub>1</sub>; E<sub>max</sub> = 53. d. Human MT<sub>1</sub> and MT<sub>2</sub> receptors treated with UCSF3384: hMT<sub>1</sub>; pEC<sub>50</sub> = 7.68 ± 0.09, E<sub>max</sub> = −47 ± 12%, n = 13; hMT<sub>2</sub>; pEC<sub>50</sub> = 7.39 ± 0.10, E<sub>max</sub> = −56 ± 5%, n = 5; mMT<sub>1</sub>/hMT<sub>1</sub>; E<sub>max</sub> not determined, n = 5; mMT<sub>2</sub>/hMT<sub>1</sub>; E<sub>max</sub> not determined, n = 5; mMT<sub>2</sub>/hMT<sub>2</sub>; E<sub>max</sub> not determined, n = 5; mMT<sub>2</sub>/mMT<sub>1</sub>; E<sub>max</sub> = 158%. e. Mouse MT<sub>1</sub> and MT<sub>2</sub> receptors treated with UCSF3384: mMT<sub>1</sub>; pEC<sub>50</sub> = 7.00 ± 0.22, E<sub>max</sub> = −73 ± 3%, n = 5; mMT<sub>2</sub>/hMT<sub>1</sub>; E<sub>max</sub> not determined, n = 5; mMT<sub>2</sub>/mMT<sub>2</sub>; E<sub>max</sub> = 100%. Data represent mean ± s.e.m. of the indicated number (n) of biologically independent experiments run in triplicate.
for the mouse MT₁ receptor compared with the mouse MT₂ receptor at concentrations of up to 10 μM for either compound (Fig. 2e, f and Supplementary Data 3). Conversely, whereas the agonist UCSF4226 lost little activity against the mouse receptor, its selectivity for the mouse MT₂ receptor was much diminished (Supplementary Data 4). Accordingly, we moved forward to in vivo experiments in mice with the two selective MT₁ inverse agonists.

**In vivo pharmacology reveals new MT₁ activities**

We first examined the in vivo activity of the two MT₁-selective inverse agonists in a mouse model of re-entrainment. In this 'east-bound jet-lag' model, mice were subjected to an abrupt 6-h advance in the light–dark cycle and treated at the new onset of the dark for three consecutive days to assess re-entrainment rate. At 30 μg per mouse, the agonist melatonin accelerates re-entrainment to the new cycle, consistent with its use in the treatment of east-bound jet-lag in humans (Fig. 3b and Extended Data Fig. 4b). Conversely, the prototypical non-selective antagonist/inverse agonist luzindole (administered at 300 μg per mouse) decelerates re-entrainment, measured as the number of days to adapt to the new dark onset (Fig. 3b and Extended Data Fig. 4e), as expected for an inverse agonist. The selective MT₁, inverse agonists UCSF7447 and UCSF3384, at a dose of 30 μg per mouse (about 1 mg kg⁻¹), also decelerated re-entrainment (Fig. 3a, b and Extended Data Fig. 4c, d, f), phenocopying luzindole (encouragingly, at a tenfold lower dose).

Superficially, the shared effect of decelerating re-entrainment by UCSF7447, UCSF3384 (Fig. 3a, b and Extended Data Fig. 4c, d, f) and luzindole might seem expected, as all of the compounds share the same function as melatonin receptor antagonists/inverse agonists. However, luzindole is MT₂-selective, non-selective, in contrast to UCSF7447 and UCSF3384. The phenocopying by the compounds of luzindole suggests that deceleration of re-entrainment by all three molecules—slowing jet-lag accommodation—is mediated through the MT₁ receptor alone. Supporting this, the effect of UCSF7447 was eliminated in MT₂-knockout (MT₂KO) mice (Fig. 3c and Extended Data Fig. 4h, i, m), but not in MT₁-knockout (MT₁KO) mice, in which its effect was actually increased, adding to the deceleration induced by deletion of the MT₁ receptor alone (Fig. 3c and Extended Data Fig. 4j, k, n).

The effect of the MT₁-selective inverse agonists on the circadian phase was even more unexpected. Here, we measured their effects on circadian phase by monitoring the running wheel activity onset of free-running mice in constant darkness and administering the drugs to the mice at subjective dusk (circadian time 10, CT 10). Both inverse agonists induced a phase advance in the onset of the circadian wheel running rhythm, an effect that is characteristic of melatonin—the endogenous, non-selective agonist—and of non-selective agonist drugs such as ramelteon and agomelatine (Fig. 3d and Extended Data Fig. 5b–d, g, h). Whereas MT₁-selective inverse agonists have few if any predecessors in vivo, we would have ordinarily expected the opposite effect of the agonist—that is, delaying rather than advancing the circadian phase. Instead, UCSF7447 advanced the onset of activity by approximately 1 h at 0.9 μg per mouse (about 0.03 mg kg⁻¹), an effect similar to that of melatonin at its half-maximal effective dose (0.72 μg per mouse) (Fig. 3d and Extended Data Fig. 5g, h). At a higher dose (30 μg per mouse, about 1 mg kg⁻¹), both UCSF7447 and UCSF3384 advanced the onset of running wheel activity with an amplitude similar to melatonin at this circadian time (Fig. 3d and Extended Data Fig. 5b–d). Notably, whereas melatonin and ramelteon advance the phase when given at dusk (CT 10), and delay the phase when given at dawn (CT 2) (35–37), UCSF7447 did not affect the circadian phase at dawn (Fig. 3f and Extended Data Fig. 5r–w) and was only effective at dusk (Extended Data Fig. 7a–c).

The phenocopying effect of the non-selective agonist melatonin by the MT₁-selective inverse agonists in shifting circadian phase motivated...
us to investigate the mechanism of action and the role of off-target effects. Accordingly, both molecules, as well as the human MT₁-selective agonist UCSF4226, were tested against a panel of common off-target receptors (Supplementary Data 1). In radioligand competition assays, no activity was seen up to a concentration of 10 μM for the new ligands. Against a panel of 318 G-protein-coupled receptors (GPCRs), activity was observed for only seven receptors when screened at a single concentration, activity against none of these seven receptors was replicated in full concentration–response assays (Supplementary Data 2).

Consistent with activity mediated by the MT₁ receptor, the advance in the onset of running wheel activity at dusk (CT 10) by UCSF7447 was eliminated in MT₁ KO mice but not in MT₂ KO mice (Fig. 3e and Extended Data Fig. S1–q). These observations suggest that the MT₁-selective inverse agonists UCSF7447 and UCSF3384 are not only potent, with effects on phase shift for UCSF7447 at 0.9 μg per mouse (about 0.03 mg kg⁻¹) (Fig. 3d) and efficacies that resemble the long-established reagent luzindole in the jet-lag model at tenfold lower doses, but also that their unexpected activity in the circadian phase paradigm is mediated by the MT₁ receptor. We note that the lack of precedence for this behaviour reflects a lack of MT₁-selective inverse agonists to investigate this effect, something that is addressed by this study.

**Discussion**

We discovered, in a large library docking screen, multiple, previously undescribed chemotypes for melatonin receptors (Fig. 1) that have new signalling mechanisms and pharmacology. Three features of this study are worth emphasising. First, docking a library of more than 150 million diverse, make-on-demand molecules found ligands that are topologically unrelated to known melatonin receptor ligands, with picomolar and nanomolar activity on the melatonin receptors. Second, the chemical novelty of these molecules translated functionally, conferring melatonin-receptor-type selectivity. Whereas the deceleration of dusk, perhaps suggesting previously unknown signalling control for rhythms, which has known time-of-day-dependent receptor-mediated signalling pathways. Third, these suggest to our knowledge, the first MT₁-selective inverse agonists that are active in vivo, with efficacy at doses as low as 0.9 μg per mouse in the circadian phase shift paradigm. Their efficacy in modulating time-dependent circadian entrainment supports their potential as lead molecules for the design of therapeutics for conditions and diseases that are affected by alterations in the circadian phase.

Certain caveats should be noted. Although we sought MT₁-selective ligands, we found ligands for both melatonin receptor types, reflecting their conserved orthosteric sites. Indeed, rather than adopting a structure-based strategy for type selectivity, we simply focused on chemical novelty among the high-ranking docked molecules. Although the 39% docking hit rate was high, and the hits were potent, this probably reflects a site that is unusually well-suited to ligand binding; it is small, solvent-occluded and largely hydrophobic. These high hit rates and potencies may not always translate to other targets.

The key observations of this work should nevertheless be clear. From a structure-based screen of a diverse, 150 million compound virtual library sprang 15 new chemical scaffolds, topologically unrelated to known melatonin receptor ligands, synthesized de novo for this project. From their chemical novelty emerged previously undescribed activities, including inverse agonists and ligands with melatonin-receptor-type selectivity. The potency, brain exposure and selectivity of these new ligands enable the disentanglement of the physiological role of the MT₁ receptor. Accordingly, we are making the MT₁-selective inverse agonist UCSF7447, and the human MT₁-selective agonist UCSF4226, open to the community as probe pairs together with a close analogue that has no measurable activity on the melatonin receptors (Extended Data Table 4). We note that only a small fraction of even the highest-ranking chemotypes from the docking screen were tested here; it is likely that hundreds of thousands of melatonin receptor ligands, representing tens of thousands of new chemotypes, remain to be discovered from the make-on-demand library, which continues to grow (http://zinc15.docking.org). This study suggests that not only potent ligands may be revealed by docking such a library, but also that the new chemotypes explored can illuminate new in vivo pharmacology.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-2027-0.

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in this way, all members of its cluster within the top 300,000 molecules were filtered by the proximity of their polar moieties to N1624.60 or Q181ECL2, and manually inspected for favourable novelty by calculating the ECFP4-based Tanimoto coefficient against the top 300,000 ranked molecules were clustered by ECFP4-based minimizer was used for rigid-body minimization on the best-scored orientation, an average of 485 conformations was sampled. A simplex algorithm was applied for the best-scored docked molecules, 2-phenylmelatonin, were used to seed the matching sphere of the co-crystallized ligand, 2-phenylmelatonin, were used to seed the matching sphere calculation in the orthosteric site; these spheres represent favourable positions for individual ligand atoms to dock; 45 spheres were used in total. DOCK3.7 orients flexibases of pre-calculated ligand conformations into the orthosteric site by overlaying atoms of each library molecule onto these matching spheres. The receptor structure was protonated by REDUCE3 and assigned AMBER united atom charges. For residues N1624.606 and Q181ECL2, the partial atomic charges of the side chain amide were increased without changing the net charge of the residue, as described previously.60 The volume of the low protein dielectric, which defines the boundary between solute and solvent in Poisson–Boltzmann electrostatic calculations, was extended out 1.9 Å from the protein surface using spheres calculated by SPHGEN. Scoring grids were pre-calculated using CHEMGRID for AMBER van der Waals potential, QNIFFT150 for Poisson–Boltzmann-based electrostatic potentials, and SOLVMA151 for ligand desolvation. The resulting potential grids and ligand-matching parameters were evaluated for their ability to enrich known MT1, ligands over property-matched decoys. Decoys share the same physical properties as known ligands but are topologically dissimilar and are therefore unlikely to bind to the receptors. We extracted 31 known MT1, melatonin receptor ligands—both agonists and antagonists—from the IUPHAR database152 and 1,550 property-matched decoys were generated using the DUD-E pipeline. Docking success was judged based on the ability to enrich the known ligands over the decoys by docking rank, using adjusted logAUC values, as is widely done in the field. We also ensured that molecules with extreme physical properties were not enriched, as can happen when only counter-screening against property-matched decoys. In particular, we wanted to ensure that neutral molecules were enriched over charged ones. The docking parameters were also judged on how well they reproduced the expected binding modes of the known ligands as well as their ability to form hydrogen bonds with N1624.60 and Q181ECL2. The 'lead-like' subset of ZINC15 (http://zinc15.docking.org), characterized by favourable physical properties (for example, with calculated octanol-water partition coefficients (cLogP) ≤ 3.5 and with molecular mass ≤ 350 Da), was then docked against the MT1, orthosteric site, using DOCK3.7. This library contained more than 150 million molecules, most of which were make-on-demand compounds from the Enamine REAL set. Of these, more than 135 million molecules successfully docked, with more than 36 million receiving a favourable score (<0 kcal mol−1). An average of 3,445 orientations was calculated for each, and each orientation, an average of 485 conformations was sampled. A simplex minimizer was used for rigid-body minimization on the best-scored pose for each ligand. Overall, about 72 trillion complexes were sampled and scored. The calculation time was 45,020 core hours, or 1.25 weeks, but with a 95% fulfilment rate; in this project the delivery time was 6 weeks, but with a 95% fulfilment rate for the 40 molecules that were prioritized from the initial docking screen. Each reaction is tested for conditions including temperatures, completion time and mixing. Typically, compounds were made in parallel by combining reagents and solvents in a single vial in the appropriate conditions to allow the reaction to proceed to completion. The product-containing vial is filtered by centrifugation into a second vial to remove the precipitate and the solvent is evaporated under reduced pressure; the product is then purified by high-performance liquid chromatography. Identity and purity are assessed by liquid chromatography–mass spectrometry (LC–MS) and, as appropriate, 1H NMR (Supplementary Table 2, Supplementary Data 7). All compounds were shipped with a purity of 90% or better, and the three main compounds UCSF7447, UCSF3384 and UCSF4226 were independently confirmed to be ≥95% pure by LC–MS in secondary confirmation analyses at a second laboratory (Supplementary Data 5). Details regarding the synthesis and analyses of the compounds are provided in Supplementary Data 6, 7.

Structure-based ligand optimization After experimental testing (see ‘cAMP assay’), 12 of the 15 active ligands from docking were prioritized for optimization, representing a range of activities and type selectivity (Extended Data Table 2 and Supplementary Table 1). Several thousand analogues of these ligands—each bearing the same scaffold as the parent molecule and with a Tanimoto coefficient of <0.38 to annotated melatonin receptor ligands—were selected from the ZINC database and docked to the MT1, binding site, again using DOCK3.7. The resulting docked poses were manually evaluated for interactions with N1624.60 and Q181ECL2, and 132 analogues were selected for de novo synthesis at Enamine, in two iterations. Of these, 131 were successfully synthesized, a >99% fulfilment rate.

Cell culture HEK293T cells were maintained with complete Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 U ml−1 penicillin G and 100 μg ml−1 streptomycin. Cells were maintained at 37 °C in the presence of 5% CO2.

Tango arrestin recruitment assay MT1 and MT2 Tango constructs were designed and assays were performed as previously described. In brief, HTLA cells stably expressing TEV-protease-fused β-arrestin (provided by R. Axel) and a tTA-dependant luciferase reporter gene were transfected with the MT1, or MT2, Tango construct. The next day, transfected cells were seeded into poly-1-lysine-coated 384-well white clear-bottom cell-culture plates with DMEM containing 1% dialysed FBS at a density of 20,000 cells per well in 40 μl for another 6 h. The drug-containing solution was prepared in the same medium at a concentration that was 5% of the final concentration and 10 μl per well was added for overnight incubation. The next day, medium and drug solutions were discarded and wells were loaded with 20 μl per well of Bright-Glo reagent (Promega). Plates were incubated for 20 min in the dark, after which luminescence was counted for the cell using a SpectraMax luminescence reader (Molecular Device). Data were analysed using GraphPad Prism 6.0 (GraphPad Software).
cAMP assay
MT and MT2 receptors were tested using split luciferase-based GloSensor cAMP biosensor technology (Promega). HEK293T cells were plated in 15-cm cell-culture dishes (at around 50% cell confluency) with DMEM supplemented with 10% dialysed FBS, 2 mM L-glutamine, 100 μM 3-iodotyrosine, and 100 μg mL⁻¹ streptomycin for 4–6 h. Then, cells were co-transfected with 8 μg of constructs encoding either MT, or MT2 (de-Tango-ized constructs) and 8 μg of GloSensor DNA. The next day, transfected cells were seeded into poly-l-lysine-coated 384-well white clear-bottom cell-culture plates with complete DMEM supplemented with 1% dialysed FBS at a density of 20,000 cells per well for another 24 h. The subsequent day, cell medium was discarded and wells were loaded with 20 μl of assay buffer (1× HBSS, 20 mM HEPES, pH 7.4, 0.1% BSA). To measure agonist activity of MT, or MT2, receptor, 10 μl of test compound solution at 3× final concentration was added for 15 min followed by addition of 10 μl of luciferin/isoproterenol mixture (at a final concentration of 4 mM and 200 nM, respectively) for another 15 min for luminescence quantification. Then, plates were counted using a SpectraMax luminescence reader (Molecular Device). Data were analysed using GraphPad Prism 8.

Calculation of log(Emax/EC50) and quantification of ligand bias
The Δlog(Emax/EC50) was calculated with melatonin as a reference agonist for G protein and β-arrestin pathway, and the ΔΔlog(Emax/EC50) was calculated between two pathways for each ligand, as were corresponding bias plots. The bias factor is unitless and defined as 10^[ΔΔlog(Emax/EC50)].

GPCRome counterscreen
Screening of compounds in the PRESTO-Tango GPCRome was accomplished as previously described with several modifications. First, HTLA cells were plated in DMEM with 10% FBS and 10 μM 3-iodotyrosine. Next, the cells were transfected using an in-plate PEI method. PRESTO-Tango receptor DNAs were resuspended in OptiMEM and hybridized with PEI before dilution and distribution into 384-well plates and subsequent addition to cells. After overnight incubation, drugs were added to wells without replacement of the medium. The remaining steps of the PRESTO-Tango protocol were followed as previously described. For those six receptors for which activity was reduced to less than 0.5-fold of basal levels, assays were repeated as a full dose–response curve. Activity for none of the seven could be confirmed, and we discount the apparent activity seen in the single-point assay.

Inhibition screen
Binding assays were performed by the NIMH Psychoactive Drug Screening program as described previously. Detailed binding assay protocols are available at: https://pdspdb.unc.edu/pdspWeb/content/UNC-CH%20Protocol%20Book.pdf.

BRET recruitment assay
To measure the dissociation of labelled Goα, from the labelled Gβγ complex after the receptor stimulation, HEK293T cells were co-transfected in a 1:1:1:1 ratio of Goα3-RLuc, Gβ3, GFP2-Gγ9, and human MT1 or MT2 (de-Tango-ized constructs), respectively. After 24 h, transfected cells were plated in poly-l-lysine-coated 96-well white clear-bottom cell-culture plates with DMEM containing 1% dialysed FBS, 100 μM 3-iodotyrosine and 100 μg mL⁻¹ streptomycin at a density of 40,000 cells in 200 μl per well and incubated overnight. The following day, the medium was removed and cells were washed once with 100 μl of assay buffer (1× HBSS, 20 mM HEPES, pH 7.4, 0.1% BSA). Then 60 μl of assay buffer was loaded per well followed by addition of 10 μl of the RLuc substrate, coelenterazine 400a (Nanolight), at 5 μM final concentration for 5 min. Drug stimulation was performed with the addition of 30 μl of 3× drug dilution of melatonin or UCSF4226 in assay buffer supplemented with 0.01% (w/v) ascorbic acid per well and incubated at room temperature for another 5 min. Both luminescence (400 nm) and fluorescent GFP2 emission (515 nm) were read for the plate for 1 s per well using Mithras LB940. The ratio of GFP2/RLuc was calculated per well and analysed using log (agonist) vs. response in GraphPad Prism 8.

Radioligand binding
Reagents and ligands. 2-[125I]iodomelatonin (SA: 2.200 Ci, 81.4 TBq mmol⁻¹) was purchased from Perkin Elmer. Guanosine 5’-triphosphate sodium salt hydrate (GTP), melatonin and all other chemicals and reagents were obtained from Sigma-Aldrich.

Compound preparation. For receptor binding studies, UCSF7447 was dissolved in 50% DMSO/50% ethanol as a 13 mM stock solution, diluted 1/10 in 100% ethanol then 1/10 again in 50% ethanol/50% Tris-HCl buffer, pH 7.4 25°C. Both UCSF3384 and UCSF4226 were dissolved in 100% ethanol as 13 mM stock solutions and then diluted 1/10 in 50% ethanol/50% Tris-HCl buffer, pH 7.4. Further dilutions were done in the same Tris-HCl buffer.

2-[125I]iodomelatonin competition binding. CHO cells stably expressing Flag-tagged recombinant human or mouse MT1 or MT2, melatonin receptors were grown in culture as monolayers in Ham’s F12 medium supplemented with fetal calf serum (10%), penicillin (1%; 10,000 IU mL⁻¹)–streptomycin (5%; 10,000 μg mL⁻¹) in CO2 at 37°C as described. Cells were grown for 4 days to 90–95% confluence, then washed with PBS (potassium phosphate buffer, 10 mM, pH 7.4), detached with PBS containing 0.25% trypsin and 1 mM EDTA, and pelleted by centrifugation (1,700g, 5 min) as described. Cell pellets were suspended and homogenized in control buffer (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4 at 25°C) and washed twice by centrifugation (17,000g, 15 min) in control or inactive conformation buffer (50 mM Tris-HCl, 10 mM MgCl₂, 100 μM GTP, 1 mM EDTA, 150 mM NaCl, pH 7.4 at 25°C) as described. 2-[125I]iodomelatonin binding affinity was determined on membranes from CHO cells expressing human MT1 (9.6 ± 0.3 μg protein per assay; Bmax = 1,154 ± 38 fmol mg⁻¹ protein; n = 3), human MT2 (15 ± 1 μg protein per assay; Bmax = 352 ± 19 fmol mg⁻¹ protein; n = 3), mouse MT1 (6.0 ± 0.022 μg protein per assay; Bmax = 1,705 ± 337 fmol mg⁻¹ protein; n = 3) or mouse MT2 (6.4 ± 0.7 μg protein per assay; Bmax = 725 ± 93 fmol mg⁻¹ protein; n = 3). Ligand competition (10 pM to 100 μM) for 2-[125I]iodomelatonin (104 ± 2 pM, n = 30) binding was performed in control or inactive conformation buffer in a total volume of 0.26 ml as described. Assays were incubated for 1 h at 25°C. Bound radioligand was separated from free by rapid vacuum filtration using glass microfilter fibres (Whatman, Krakeler Scientific) saturated in 0.5% polyethyleneimine solution. Total radioactivity bound to the filters was determined on a gamma counter.

Data analysis. Kᵢ values were calculated from IC₅₀ values using GraphPad Prism 8.0 according to the Cheng–Prusoff equation: Kᵢ = IC₅₀ / (1 + [L]/Kₐ). Where L is the concentration of radioligand, Kᵢ is the dissociation constant of 2-[125I]iodomelatonin in control or inactive conformation buffer for the human MT1 (control Kᵢ = 116 pM; inactive Kᵢ = 280 pM), human MT2 (control Kᵢ = 80 ± 13 pM; GTP Kᵢ = 461 ± 159 pM) and mouse MT1 (control Kᵢ = 87 ± 6 pM; GTP Kᵢ = 201 ± 67 pM) receptors (n = 3). Affinity shifts induced by G-protein uncoupling were measured by subtracting pKi inactive from pKi control (ΔpKi) and normalization to the ΔpKi of melatonin (CHO cells expressing human MT1, L19: CHO cells expressing human MT2, 0.43). Affinity shifts or lack thereof with G-protein uncoupling indicate apparent efficacy as ligands are classified as agonists (ΔpKi percentage of melatonin >20%), antagonists (ΔpKi percentage of melatonin <20%, <−20%) or inverse agonists (ΔpKi percentage of melatonin <−20%) accordingly. Individual data points were
In vivo methods

**Animals and housing.** Male and female C3H/HeN wild-type, MT, KO, and MT, KO mice (average age, 6.28 months) used in this study were raised in our breeding colony at University at Buffalo, C3H/HeN mice homozygous for the MT1 and MT2 melatonin receptor gene deletion and their wild-type controls were generated from breeding pairs donated by S. M. Reppert (University of Massachusetts Medical School) and backcrossed with C3H/HeN mice (Harlan, now Envigo) for at least seven generations as described previously. Genotype was confirmed using tail samples at the end of each experiment and was verified periodically during the tenure of the colony. The strains of mice in our breeding colony were re-derived periodically by backcrossing with wild-type mice to reduce genetic drift.

Mice were group-housed (3–5 per cage) with corncob bedding in polycarbonate translucent cages (30 cm × 19 cm) and maintained in a 14:10 light–dark cycle (Zeitgeber time 0 (ZT 0) corresponds to lights on and ZT 14 to lights off) in temperature- and humidity-controlled rooms with ad libitum access to food and water in the Laboratory Animal Facility at the University at Buffalo. Light levels were 200–300 lx at the level of the cage. Treatments and animal care performed in the dark were carried out under a dim red safelight (15 W, Kodak 1A filter) with illumination of less than 3 lx as described previously. All experimental procedures using mice were conducted in accordance with guidelines set forth by the National Institutes of Health and approved by the University at Buffalo Institutional Animal Care and Use Committee.

**Circadian rhythm measurements.** Circadian rhythm phase was determined for each mouse using the onset of running wheel activity defined as CT 12 (onset of wheel activity). Running wheel activity was measured continuously by magnetic microswitches that detect wheel revolutions using a computer equipped with ClockLab data-collection software (Actimetrics). All actigraphy data were visualized and analysed using ClockLab and MATLAB software. All mice were individually housed in cages (33 cm × 15 cm) equipped with running wheels in light-tight ventilated cabinets with controlled temperature and light–dark cycles (Phenome Technologies). Male and female mice were housed in separate cabinets for all experiments.

**Phase shift.** Changes in circadian phase induced by vehicle or drugs administered at various circadian times were assessed in wild-type, MT, KO and MT, KO male and female C3H/HeN mice (3–8 months of age) using methods and protocols that have been previously described. Following a period of 14 days in a light–dark cycle, mice were placed in constant dark beginning at ZT 12 (dark onset) (ZT 0, lights on). Mice were kept in constant dark (2–3 weeks) until a stable free-running phase of running wheel activity rhythm onset was established. Circadian times of treatment were predicted from best fit lines of running wheel activity onsets for running either before (7–14 days) and after (7–14 days) treatment. Treatment times were within a 2 h window at CT 2 (CT 1–3), CT 6 (CT 5–7) or CT 10 (CT 9–11). Mice were treated (0.1 ml per mouse, subcutaneously) with vehicle (30% ethanol/70% saline) or drugs (melatonin, UCSF3384 or UCSF7447, at 0.9 μg and 30 μg per mouse or luzindole at 300 μg per mouse in vehicle) for 3 consecutive days at the appropriate circadian time under dim red light. Vehicle or drug treatments were repeated for 3 consecutive days at the selected circadian time following the three-pulse treatment protocol described previously. Phase shifts were quantified using the best fit lines for onsets of activity during pre- and post-treatment periods. Differences are characterized as phase delays (pre-treatment ahead of post-treatment best fit line onset) or phase advances (post-treatment ahead of pre-treatment best fit line onset) of running wheel activity onset rhythms.

**Re-entrainment experiments.** Male and female C3H/HeN wild-type, MT, KO and MT, KO mice (3–6 months of age) were maintained under a 12:12 light–dark cycle for at least 2 weeks before experimental manipulations to enable stable entrainment to dark onset before advance of the light–dark cycle. Actigraphy data were recorded as described above and all experimental protocols were performed as described previously. On the first day of treatment, the dark onset was advanced 6 h. This resulted in a short night. Mice were treated (0.1 ml per mouse, subcutaneously) with vehicle (30% ethanol/70% saline) or drugs (melatonin, UCSF3384 or UCSF7447 at 30 μg per mouse, or luzindole 300 μg per mouse, in vehicle) for three consecutive days 10–30 min before the new dark onset. After treatment, mice were given 14–20 days to re-entrain the onset of running wheel activity to the new dark onset. Using exported running wheel activity onsets from actograms, the number of onset hours advanced each day was determined by subtracting this value each day from the average onset of stably entrained running wheel activity for 3 days before treatment for each mouse. Furthermore, using the data from this calculation combined with visualization of actograms, the number of days to reach stable re-entrainment was determined for each mouse.

**In vivo compound preparation.** All compounds were administered in fixed doses of either 0.9 μg or 30 μg subcutaneously in a volume of 0.1 ml per mouse, which are equivalent to doses of 0.03 or 1 mg kg−1 for a 30 g mouse, respectively. Vehicle was 30% ethanol/70% saline for all doses. Melatonin, UCSF7447 and UCSF3384 were prepared as stock solutions of 3 mg ml−1 (100% ethanol) using sonication and vortexing to ensure that each drug was dissolved. Subsequently, stock solutions were diluted to 0.3 mg ml−1 (30 μg per 0.1 ml injection) or 0.009 mg ml−1 (0.9 μg per 0.1 ml injection) in vehicle. Luzindole was prepared similarly, except the starting stock solution was 30 mg ml−1 in 100% ethanol and it was administered from a solution of 3 mg ml−1 (300 μg per 0.1 ml injection) in vehicle. Treatment dilutions were prepared just before use under sonication with intermittent vortexing between steps and used within 5 min of preparation.

**Biostatistics and reproducibility.** All statistical analyses as described in further detail for each experiment were conducted using GraphPad Prism 8. For phase shift and re-entrainment experiments, we determined statistical power a priori (error probability, α = 0.05) based on data for a known effect size for melatonin in these paradigms (G-power 3.0.10). Individual actograms of wheel running activity were excluded from analysis based on the exclusion criteria described below, which was completed by at least two individuals who were blind to treatment before data analysis was started. For re-entrainment actograms exclusion criteria included: (1) low running, sporadic activity, substantial missing wheel activity data and/or lack of entrainment before treatment; (2) entrainment of running activity more than 1 h before or after the ‘old’ or ‘new’ dark onset; (3) re-entrainment to the new dark onset before administration of the third injection (entrainment to injection). For phase-shift actograms exclusion criteria included: (1) low running, sporadic activity, missing wheel activity data and/or lack of free running activity rhythms; (2) tau change >0.3 h; (3) at least two out of three injections occurred outside of the target predetermined time range for treatment (CT 1–3, 5–7, 10–12). All datasets were visualized for normality using QQ plots of predicted versus actual residuals. Actigraphy data were generated for visualization blind to treatment before the quantification and statistical analysis stages. Comparisons for Fig. 3a and Extended Data Fig. 4I, m, n were made by mixed-effect two-way repeated-measures ANOVA (treatment × time) with Sidak’s post hoc test (P < 0.05). Number of days to re-entrainment was compared using one-way ANOVA or two-way ANOVA for Fig. 3b, c with a Dunnet’s or Tukey’s post hoc test (P < 0.05), respectively. Group comparisons for phase shift in Fig. 3d (left and centre) and Extended
DataFig. 7a–c were made by one-way ANOVA (P < 0.05) comparing hours shifted of circadian running wheel activity rhythm onsets (Fig. 3d, left), three groups; vehicle, melatonin and UCSF7447; Fig. 3d (centre), four groups vehicle, melatonin, UCSF7447 and UCSF3384; Extended Data Fig. 7a–c, four groups vehicle, melatonin. UCSF7447 and luzuindole) accompanied by post hoc analyses using Dunnet’s test to determine individual group differences compared with vehicle (P < 0.05). Comparisons in Fig. 3d (right) between vehicle and luzindole were made using a two-tailed unpaired Student’s t test (P < 0.05). Data in Fig. 3e, fwere compared using a two-way ANOVA (3 × 2: genotype × treatment) with Tukey’s post hoc analyses (P < 0.05). Values and values for statistical analyses are included in the figure legends or listed in Supplementary Table 4. Either the overall interaction or the main effects are reported and interpreted using two-way ANOVA as appropriate for assumptions of each dataset. No sex differences in treatment effects were evident in any dataset when assessed by two-way ANOVA or three-way ANOVA where appropriate; therefore, data were pooled between male and female mice for the described analyses. The n values represent the number of individual mice per condition or independent biological replicates in each experiment. Each dataset represents 2–4 independent experiments. The n value for each in vivo experiment is listed below (n values with an asterisk indicate values for multiple comparisons ranging from 1 to 2 values less depending on the day of comparison due to missing onset data that are accounted for in statistical models as appropriate). See statistical parameters in Supplementary Table 4. Figure 3a, vehicle (n = 28 mice) versus UCSF7447 (n = 21 mice). Figure 3b, vehicle (n = 28) versus melatonin (n = 21). UCSF7447 (n = 21). UCSF3384 (n = 16) or luzindole (n = 11). Figure 3c. wild-type (n = 28 vehicle: n = 21 UCSF7447), MT,K0 (n = 16 vehicle: n = 16 UCSF7447) and MT,K (n = 20 vehicle: n = 25 UCSF7447). Figure 3d, left: vehicle (n = 8) versus melatonin (n = 8) or UCSF7447 (n = 13); centre: vehicle (n = 15) versus melatonin (n = 10). UCSF3384 (n = 16) or UCSF7447 (n = 15); right: vehicle (n = 6) versus luzindole (n = 3). Figure 3e, wild-type (n = 9; vehicle: n = 10 UCSF7447), MT,K0 (n = 8; vehicle: n = 8 UCSF7447) and MT,K (n = 11 vehicle: n = 9 UCSF7447). Figure 3f, wild-type (n = 8 vehicle: n = 8 UCSF7447), MT,K0 (n = 6 vehicle: n = 7 UCSF7447) and MT,K (n = 10 vehicle: n = 13 UCSF7447).

Extended Data Figure 4l, C3H/HeN wild-type: vehicle (n = 28 mice) versus UCSF3384 (n = 16 mice). Extended Data Figure 4m, C3H/HeN MT,K0: vehicle (n = 16 mice) versus UCSF7447 (n = 16 mice). Extended Data Figure 4n, C3H/HeNMT,K: vehicle (n = 21 mice) versus UCSF7447 (n = 25 mice).

Extended Data Figure 7a, CT 2: vehicle (n = 3), melatonin (n = 3), luzindole (n = 6) and UCSF7447 (n = 3). Extended Data Figure 7b, CT 6: vehicle (n = 8), melatonin (n = 4), luzindole (n = 9) and UCSF7447 (n = 9). Extended Data Figure 7c, CT 10: vehicle (n = 6), melatonin (n = 8), luzindole (n = 3) and UCSF7447 (n = 4).

Pharmacokinetics. Pharmacokinetic experiments were performed by Sai Life Sciences. Plasma pharmacokinetics and brain distribution for UCSF7447, UCSF3384 and UCSF4226 were investigated following a single intravenous dose of 2 mg kg⁻¹ in 9 male C57BL/6 mice. Each compound was formulated in 5% dextrose and administered i.v. to mice via tail vein injection. Blood samples (approximately 60 μl from each of three mice) were collected under light isoflurane anesthesia from the retro-orbital plexus at 0.08, 0.25, 0.51, 2, 4, 8, 12 and 24 h. Immediately after collection, plasma was obtained by centrifugation and stored at −70 °C until analysis. For blood collected at 0.5, 4 and 24 h, animals were euthanized with excess CO₂ asphyxiation and brain samples were collected and homogenized in ice-cold phosphate-buffered saline (pH 7.4). Total homogenate volume was three times the brain weight.

All samples were processed for analysis by protein precipitation using acetonitrile and analysed using a fit-for-purpose LC–MS/MS method (lower limit of quantification, 2.01 ng ml⁻¹ for plasma and 6.03 ng g⁻¹ for brain for UCSF7447. 5.01 ng ml⁻¹ for plasma and 3.00 ng g⁻¹ for brain for UCSF3384. 1.01 ng ml⁻¹ for plasma and 6.09 ng g⁻¹ for brain for UCSF4226). The non-compartmental analysis module in Phoenix WinNonlin (v.7.0) was used to assess the pharmacokinetic parameters. The maximum concentration (Cmax) and time to reach the maximum concentration (Tmax) were measured. The areas under the time curve (AUClast and AUCinf) and elimination half-life were calculated by linear trapezoidal rule. The terminal elimination rate constant, kₑ, was determined by regression analysis of the linear terminal portion of the log plasma concentration–time curve. The terminal half-life (T½) was estimated as 0.693/kₑ.

Reporting summary

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

Data availability

Probe pairs (two similar ligands with and without activity) of inverse agonists selective for MT, and agonists selective for hMT are available by arrangement with Sigma (Extended Data Fig. 3). The identities of the compounds docked in this study are freely available from the ZINC database (http://zinc15.docking.org) and active compounds may be purchased from Enamine. Raw data are available for Fig. 1. Extended Data Tables 1, 2 and Extended Data Figs. 1, 2 in Supplementary Tables 1 (MT, and MT, affinities, MT, DOCK energies and ranks) and 2 (compound purity information). Bias information for Extended Data Fig. 3 is included in Supplementary Table 3. For Fig. 2, data from the GPCRome screens, concentration–response curves, and competition binding and LC–MS experiments are included in Supplementary Data 1–5 and synthesis routes and spectra of compounds in Supplementary Data 6, 7. Further data for Fig. 3 are included in Extended Data Figs. 4, 5, 7 and Supplementary Table 4. Raw data values and transformed data for in vitro cell-based assays as well as in vivo data for phase shift and re-entrainment are available for Figs. 2, 3 and Extended Data Figs. 4 (re-entrainment), 5 (phase shift), 6, 7–c.

Code availability

DOCK3.7 is freely available for non-commercial research (http://dock.compbio.ucsf.edu/DOCK3.7/). A web-based version is freely available all at http://blaster.docking.org/. The ultra-large library used here is freely available at http://zinc15.docking.org/.

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Author contributions B.K.S., B.L.R. and M.L.D. conceived the study. R.M.S. performed the docking and structure-based optimization. J.D.M. and H.J.K. performed the initial binding and functional assays and analyses, assisted by TC. A.J.J. performed the 2-[125I]iodomelatonin and GTP-perturbation assays. S.S. performed the profiling studies. G.C.G. performed the in vivo mouse pharmacology experiments and all animal husbandry. Y.S.M. and O.S. directed the compound synthesis, purification and characterization experiments. B.S., L.C.J., V.C., B.L.R., X.-P.H. and J.D.M. determined and validated the structures of the MT1 and MT2 receptor types, and made them available before publication. T.K. performed signalling bias calculations. J.J.I. created the ultra-large libraries. B.L.R. supervised the pharmacology studies; B.K.S. supervised the docking and compound optimization; M.L.D. supervised the binding studies and the in vivo circadian rhythm experiments in mice. M.L.D. G.C.G. designed all in vivo experiments. R.M.S., B.K.S., M.L.D., G.C.G., J.D.M., H.J.K. and B.L.R. wrote the paper with contributions from other authors.

Competing interests B.K.S. and J.J.I. are founders of a company, BlueDolphin LLC, that works in the area of molecular docking. All other authors declare no competing interests.

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Extended Data Fig. 1 | Concentration–response curves of the initial 15 compounds in cAMP assays. a–f, Inhibition of isoproterenol-stimulated cAMP mediated by hMT1 (a, c, e) or hMT2 (b, d, f) in HEK293T cells by melatonin and the 15 initial compounds. Data are normalized to the melatonin response. The 15 initial compounds were split into three graphs for clarity, melatonin response curves are the same across graphs in a, c, e and b, d, f. Data are mean ± s.e.m. of four biologically independent experiments (n = 4) run in triplicate, unless otherwise indicated, in which case the number of biologically independent experiments is indicated in parentheses next to the compound name.
Extended Data Fig. 2 | Concentration–response curves of notable analogues based on initial hits in cAMP assays.

a–f, Inhibition of isoproterenol-stimulated cAMP mediated by hMT1 (a, c, e) or hMT2 (b, d, f) in HEK293T cells by melatonin and select analogues. Data are normalized to the melatonin response. The compounds were split into three graphs for clarity, melatonin response curves are the same across graphs in a, c, e and b, d, f. Data are mean ± s.e.m. of four biologically independent experiments (n = 4) run in triplicate, unless otherwise indicated, in which case the number of biologically independent experiments is indicated in parentheses next to the compound name.
Extended Data Fig. 3 | Small changes in the ligand structure have large effects on the activity and selectivity of the melatonin receptor. **a**, Docked pose of ZINC151209032, an MT₁-selective direct-docking hit. **b**, Docked pose of ZINC497291360, a close analogue of ZINC151209032 that has twofold selectivity for MT₂ over MT₁. **c**, Docked pose of ZINC151192780, an analogue for which the MT₂ selectivity increases to 89-fold over MT₁. **d**, Docked pose of ZINC485552623, which adds a bulkier 2-chloro-3-methylthiophene into a proposed MT₂-selective hydrophobic cleft, resulting in a fully MT₂-selective agonist without detectable MT₁ activity. All docked poses are overlaid onto the crystallographic pose of 2-phenylmelatonin in transparent blue. **e**, Concentration–response curves of the four analogues binding to MT₁ and MT₂. Data are normalized to the melatonin response and are mean ± s.e.m. of four biologically independent experiments (n = 4) run in triplicate. **f**, Bias plots of ZINC482850041 and ZINC608506688 relative to melatonin signalling. Mean values (Supplementary Table 3) are presented as solid lines and the shading indicates the 95% confidence interval. Data in **f** are normalized to the melatonin response and represent mean ± s.e.m. of three biologically independent experiments (n = 3) run in triplicate, except for ZINC608506688 for G, activation (n = 4).
Extended Data Fig. 4 | MT₁-selective inverse agonists decelerate the re-entrainment rate in vivo via MT₁ receptors. Data are an extension of Fig. 3a–c. 

**a–e.** Representative actograms of running wheel activity in wild-type (WT) C3H/HeN mice treated with vehicle (VEH) (a), 30 μg melatonin (MLT) per mouse (b), UCSF7447 (7447) (c), UCSF3384 (3384) (d) or 300 μg luzindole (LUZ) per mouse (e) immediately before the new dark onset (black dots) after an abrupt advance in the dark onset of 6 h in a 12:12 light:dark cycle (grey, dark phase; white, light phase). Compounds were administered once a day for 3 days (see Methods for additional details).

**f–k.** Representative actograms of running wheel activity for C3H/HeN mice treated with vehicle (wild-type (f), MT1KO (h), MT2KO (j)) or inverse agonist UCSF7447 (wild-type (g), MT1KO (i), MT2KO (k)) after a 6-h advance in dark onset. Mice were kept on a 12:12 light:dark cycle. UCSF7447 (30 μg per mouse) was administered for three consecutive days immediately before the new dark onset (black dots). 

**l.** The inverse agonist UCSF3384 decelerates the rate of re-entrainment of the rhythm of running wheel activity onset in C3H/HeN wild-type mice. Data are expressed in hours advanced each day for wild-type mice treated with vehicle or UCSF3384 (two-way repeated-measures ANOVA; treatment × time interaction: \(F_{16,647} = 1.99, P = 0.0122\)).

**m.** The inverse agonist UCSF7447 does not modulate the rate of re-entrainment of the onset of a running wheel activity rhythm in C3H/HeN MT1KO mice. Data are expressed in hours advanced each day for MT1KO mice treated with vehicle or UCSF7447 (mixed-effect two-way repeated-measures ANOVA; treatment × time interaction: \(F_{16,474} = 1.44, P = 0.117\)).

**n.** The inverse agonist UCSF7447 decelerates the rate of re-entrainment of the onset of a running wheel activity rhythm in C3H/HeN MT2KO mice. Data are expressed in hours advanced each day for MT2KO mice treated with vehicle or UCSF7447 (mixed-effect two-way repeated-measures ANOVA; treatment × time interaction: \(F_{16,683} = 2.57, P = 0.000686\)). Data are mean ± s.e.m. *P < 0.05, **P < 0.01; multiple comparisons were corrected using Tukey’s post-test (\(P < 0.05\)). The dotted line in l–n indicates the new dark onset. Additional details of all statistical analyses as well as n numbers for each condition can be found in the Methods, ‘Statistics and reproducibility’. All treatments were given as a subcutaneous injection.
**Extended Data Fig. 5 | MT₁-selective inverse agonists induce a phase advance in circadian activity at CT 10 that is mediated by MT₁ in vivo.** Data are an extension of Fig. 3d–f. a–e, Representative actograms of running wheel activity from individual C3H/HeN wild-type mice kept in constant dark (grey bars) treated with vehicle (a), melatonin (b), UCSF7447 (c), UCSF3384 (d) or luzindole (e). All treatments were 30 μg per mouse except for luzindole, which was 300 μg per mouse as described in the Methods. Mice were treated at dusk (CT 10; 2 h before the onset of running wheel activity) for three consecutive days (black dots). Red lines indicate the best-fit line of pre-treatment onsets and blue lines indicate the best-fit line of post-treatment onsets, which were both used for phase shift determinations (see Methods for more details). The corresponding quantification can be found in Fig. 3d.
f–h, Representative actograms of running wheel activity from individual C3H/HeN wild-type mice kept in the constant dark treated with melatonin (i) at CT 2 (10 h before the onset of running wheel activity) or vehicle (j) compared with UCSF7447 (k; all treatments were 30 μg per mouse) at CT 6 (6 h before the onset of running wheel activity). The corresponding quantification can be found in Extended Data Fig. 7.
i–k, Representative actograms of running wheel activity from individual C3H/HeN wild-type mice kept in the constant dark treated with vehicle (l), melatonin (m) or UCSF7447 (n; all treatments were 30 μg per mouse) at CT 10. The corresponding quantification can be found in Fig. 3d.
l–q, Representative actograms of running wheel activity from individual C3H/HeN wild-type (r), MT₁KO (t, u) and MT₂KO (v, w) mice kept in constant dark treated with vehicle (white; r, t, v) or UCSF7447 (blue; s, u, w; 30 μg per mouse) at CT 2. The corresponding quantification can be found in Fig. 3f. All treatments were given by subcutaneous injection.
Extended Data Fig. 6 | Concentration-response curves and Schild plots of the inverse agonists UCSF7447 and UCSF3384 in cAMP assays. a, b, d, e. Modulation of hMT1-mediated (a, d) or hMT2-mediated (b, e) inhibition of isoproterenol-stimulated cAMP in HEK293T cells by melatonin in the presence of UCSF7447 (a, b) or UCSF3384 (d, e) for a range of concentrations. Data are normalized to the effect of isoproterenol alone and are mean ± s.e.m. of three biologically independent experiments (n = 3) run in triplicate. c, f. Schild plots depicting the competitive antagonism of melatonin by UCSF7447 (c) and UCSF3384 (f). Schild analysis of hMT1 (purple) and hMT2 (teal) show the competitive antagonism of UCSF7447 (hMT1, pKB = 7.4 ± 0.1, slope = 0.98 ± 0.03; hMT2, pKB = 6.2 ± 0.1, slope = 1.3 ± 0.4) (c) and UCSF3384 (hMT1, pA2 = 7.9 ± 0.1, slope = 0.80 ± 0.04; hMT2, pKB = 6.7 ± 0.1, slope = 1.0 ± 0.1) (f). Data are mean ± s.e.m. of three biologically independent experiments (n = 3) run in triplicate.
Extended Data Fig. 7 | Differential phase shift profile for the inverse agonist UCSF7447 compared to the agonist melatonin and a prototype antagonist (luzindole) across the circadian cycle. a–c, C3H/HeN mice were kept in constant dark and treated with vehicle, melatonin, luzindole or UCSF7447 (all treatments were 30 μg per mouse except for luzindole, which was 300 μg per mouse, subcutaneously). Mice were treated at CT 2, 6 or 10 (10, 6 or 2 h before the onset of running wheel activity) for three consecutive days (Methods). a, CT 2 phase shift data were compared using one-way ANOVA (F_{3,11} = 28.16, P = 1.85 × 10^{-5}). b, CT 6 phase shift data were compared using one-way ANOVA (F_{3,26} = 0.61, P = 0.61). c, CT 10 phase shift data were compared using one-way ANOVA (F_{3,17} = 35.13, P = 1.66 × 10^{-7}). All multiple comparisons were made compared with vehicle using a Dunnet’s post hoc test (P < 0.05). Values for melatonin and UCSF7447 at CT 10 were pooled from previous data for comparison to luzindole. Data are mean ± s.e.m. ****P < 0.0001 for comparisons with vehicle. All treatments were given by subcutaneous injection.
## Extended Data Table 1 | Active molecules from the initial docking screen

### Compound | Cluster rank \(^a\) (global rank) | hMT-I\(^b\) pEC50 (% Emax) \(n\) | hMT-II\(^c\) pEC50 (% Emax) \(n\) | Tc\(^d\) | Nearest ChEMBL23\(^e\) MT-I/MT-II Ligand
--- | --- | --- | --- | --- | ---
ZINC157665999 | 167 (197) | 4.89±0.38 (82±6) \(n=3\) | Inverse 7.29±0.16 (Inverse 90±16) \(n=3\) | 0.33 | CHEMBL398017
ZINC419113878 | 396 (522) | 5.20±0.08 (84±4) \(n=4\) | < 4.5 \(n=4\) | 0.22 | CHEMBL494566
ZINC433313647 | 875 (1242) | 6.81±0.32 (42±2) \(n=3\) | 7.77±0.02 (96±5) \(n=3\) | 0.19 | CHEMBL125226
ZINC159050207 | 1559 (2474) | 9.00±0.15 (99±1) \(n=4\) | 8.70±0.25 (83±3) \(n=4\) | 0.24 | CHEMBL1223128
ZINC151209032 | 1981 (3583) | 5.70±0.11 (88±4) \(n=4\) | < 4.5 \(n=4\) | 0.31 | CHEMBL394676
ZINC448250041 | 4123 (7672) | 7.91±0.04 (99±3) \(n=3\) | 9.33±0.33 (97±2) \(n=3\) | 0.29 | CHEMBL344242
ZINC353044322 | 5764 (28,258) | 5.48±0.05 (87±6) \(n=4\) | < 4.5 \(n=4\) | 0.33 | CHEMBL218225
ZINC603324490 | 7612 (53,767) | Inverse 5.92±0.29 | Inverse 6.20±0.08 | 0.27 | CHEMBL3260982
ZINC182731037 | 7640 (17,095) | 5.30±0.09 (82±2) \(n=4\) | < 4.5 \(n=4\) | 0.29 | CHEMBL3612457
ZINC92585174 | 1836 (3010) | 7.80±0.17 (98±1) \(n=4\) | 7.68±0.14 (74±8) \(n=4\) | 0.23 | CHEMBL1760949
ZINC432154404 | 1849 (3035) | 6.63±0.17 (95±2) \(n=4\) | 7.00±0.17 (74±4) \(n=4\) | 0.27 | CHEMBL1760956
ZINC664088238 | 2248 (3816) | < 5 \(n=4\) | 5.85±0.06 (75±8) \(n=4\) | 0.20 | CHEMBL435032
ZINC576887661 | 4161 (14,292) | 7.10±0.19 (83±6) \(n=4\) | 7.28±0.36 (68±5) \(n=4\) | 0.27 | CHEMBL491605
ZINC301472854 | 5033 (10,022) | 6.03±0.10 (95±5) \(n=4\) | 7.00±0.21 (88±6) \(n=4\) | 0.26 | CHEMBL115444
ZINC580731466 | 8503 (19,003) | 5.70±0.13 (71±3) \(n=4\) | 7.55±0.10 (96±5) \(n=4\) | 0.26 | CHEMBL115444

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\(^a\)Cluster rank and global rank are shown; the global rank is shown in brackets (Methods).

\(^b\)The log-transformed half-maximal concentration (pEC50) of the inhibition of isoproterenol-stimulated cAMP production on hMT1 or hMT2 melatonin receptors transiently expressed in HEK293T cells. Values in parentheses represent the percentage of the maximal inhibition normalized to the percentage of the melatonin response, except for inverse agonists, which are indicated by (Inverse), for which the data are normalized to percentage of basal response induced by isoproterenol. Data represent mean ± s.e.m. of the indicated number (n) of biologically independent experiments run in triplicate.

\(^c\)ECFP4 Tanimoto coefficient (Tc) to the most similar known MT1 or MT2 ligand in ChEMBL23.

\(^d\)The MT1/MT2 ligand in ChEMBL23 that is most similar to the docking active molecule.
## Extended Data Table 2 | Some of the potent analogues from initial hits

| Initial Hit$^a$ | Analog$^b$ | hMT$^c$ | hMT$^d$ |
|-----------------|------------|---------|---------|
|                 |            | pEC50  | pEC50  |
|                 |            | (% Emax) | (% Emax) | n | n |
| ZINC1576659999  | ZINC864032792 | 7.49 ± 0.04 | Inverse 6.66 ± 0.08 | 3 | 3 |
|                 |             | (57 ± 3)  | (Inverse 62 ± 13) | 8 | 8 |
| ZINC1576659999  | ZINC555417447 | 7.68 ± 0.09 | Inverse 6.18 ± 0.04 | 13 | 12 |
| ZINC1576659999  | ZINC157673384 | 6.81 ± 0.72 | 8.07 ± 0.15 | 3 | 4 |
| ZINC1576659999  | ZINC5586789  | (37 ± 6) | (51 ± 3) | 4 | 4 |
| ZINC1576659999  | ZINC128734226 | 6.83 ± 0.17 | 8.15 ± 0.09 | 3 | 4 |
| ZINC1576659999  | ZINC602421874 | 7.75 ± 0.22 | 8.23 ± 0.11 | 4 | 4 |
| ZINC159050207   | ZINC713465976 | 7.05 ± 0.02 | 7.48 ± 0.05 | 4 | 4 |
| ZINC151209032   | ZINC497291360 | 5.18 ± 0.22 | 7.13 ± 0.12 | 4 | 4 |
| ZINC151209032   | ZINC151192780 | < 5      | 5.80 ± 0.06 | 4 | 4 |
| ZINC151209032   | ZINC485552623 | 9.78 ± 0.13 | 8.60 ± 0.10 | 4 | 4 |
| ZINC151209032   | ZINC6808506688 | 6.40 ± 0.18 | 6.45 ± 0.20 | 4 | 4 |

$^a$Compounds were selected directly from the primary docking screen and were found to be active after in vitro testing.

$^b$Analogue of the initial hit.

$^c$The pEC$_{50}$ of the inhibition of isoproteenol-stimulated cAMP production on hMT$_1$ or hMT$_2$ melatonin receptors transiently expressed in HEK293T cells. Values in parentheses represent the percentage of the maximal inhibition normalized to the percentage of the melatonin response, except for inverse agonists, indicated by (Inverse), for which the data are normalized to the percentage of the basal response induced by isoproteenol. Data are mean ± s.e.m. of the indicated number ($n$) of biologically independent experiments run in triplicate.
Extended Data Table 3 | Pharmacokinetics of three melatonin receptor type-selective ligands

| Compound          | pEC<sub>50</sub> (Emax %) | C<sub>max</sub><sup>a</sup> (ng/mL) | AUC<sup>b</sup> (h*ng/mL) | T<sub>1/2</sub><sup>c</sup> (hr) | CL<sup>d</sup> (mL/min/kg) | Vss<sup>e</sup> | Brain/Plasma ratio |
|-------------------|--------------------------|-------------------------------|------------------------|--------------------------|---------------------------|-------------|-------------------|
| ZINC128734226     | 1922.8                   | 282.1                         | 0.29                   | 117.9                    | 1.11                      | 1.58        | (30')            |
| MT<sub>2</sub>-selective agonist | MT<sub>1</sub> = 6.8 (48%) | MT<sub>2</sub> = 8.2 (80%)     |                        |                          |                           |             |                   |
| ZINC555417447     | 1948.6                   | 494.5                         | 0.27                   | 67.11                    | 1.11                      | 3.03        | (30')            |
| MT<sub>1</sub>-selective inverse agonist | MT<sub>1</sub> = 7.4 (IA) | MT<sub>2</sub> = 5.8 (IA)     |                        |                          |                           |             |                   |
| ZINC157673384     | 1299.6                   | 563.8                         | 0.32                   | 58.48                    | 1.38                      | 1.43        | (30')            |
| MT<sub>2</sub>-selective inverse agonist | MT<sub>1</sub> = 7.7 (IA) | MT<sub>2</sub> = 6.2 (IA)     |                        |                          |                           |             |                   |

<sup>a</sup>C<sub>max</sub>, maximum concentration reached in blood plasma of mice.

<sup>b</sup>AUC, area under the plasma concentration–time curve.

<sup>c</sup>T<sub>1/2</sub>, half-life of the compound.

<sup>d</sup>CL, clearance from blood plasma of mice.

<sup>e</sup>Vss, volume of the distribution at steady-state.
Extended Data Table 4 | Probe pairs of in vivo tested molecules

| Active Selective Probe (Sigma RefCode) | hMT<sub>1</sub> pEC<sub>50</sub><sup>a</sup> (% Emax) | hMT<sub>2</sub> pEC<sub>50</sub><sup>b</sup> (% Emax) | Inactive analog (Sigma RefCode) | hMT<sub>1</sub> pEC<sub>50</sub><sup>a</sup> | hMT<sub>2</sub> pEC<sub>50</sub><sup>b</sup> |
|----------------------------------------|-----------------------------------------------|-----------------------------------------------|---------------------------------|--------------------------------|--------------------------------|
| ZINC555417447 (SML2751)              | Inverse 7.4 ± 0.10 (Inverse 62 ± 13)          | Inverse 5.7 ± 0.10 (Inverse 84 ± 9)          | ZINC37791618 (SML2752)          | < 4.5                         | < 4.5                         |
| ZINC128754226 (SML2753)              | 6.8 ± 0.2 (79 ± 3)                            | 8.2 ± 0.1 (89 ± 3)                           | Z3670677764 (SML2754)          | < 4.5                         | < 4.5                         |

<sup>a</sup>The pEC<sub>50</sub> of the inhibition of isoproterenol-stimulated cAMP production on hMT<sub>1</sub> or hMT<sub>2</sub> melatonin receptors transiently expressed in HEK293T cells. Values in parentheses represent the percentage of the maximal inhibition normalized to the percentage of the melatonin response for UCSF4226 and to the percentage of the basal activity for UCSF7447. Compounds were tested at concentrations up to 30 μM. Data are mean ± s.e.m. of the indicated number (n) of biologically independent experiments run in triplicate.
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Probe pairs are available from Sigma with the identifiers SML2751 (ZINC555417447), SML2752 (ZINC37781618), SML2753 (ZINC128734226), and SML2754 (Z3670677764). The identities of the compounds docked in this study are freely available from the ZINC database, http://zinc15.docking.org, and all active compounds may be purchased from Enamine. Figures with associated raw data include: Extended Data Figs. 1&2, Extended Data Table 1&2 for which further data are included in Supplementary Tables 1 (MT1 and MT2 affinities, MT1 DOCK energies/ranks) and 2 (compound purity information); Extended Data Fig. 3, for which bias information is included in Supplementary Table 3; Fig. 2, for which GPCRome screening, concentration-response curves, competition binding, and LC/MS data is included in Supplementary Data 1-5, Extended Data Fig 6, and statistical parameters in Supplementary Table 5; Supplementary Data 6&7 (synthesis routes and spectra of compounds); Fig. 3, for which further data is included in Extended Data 4-5, Supplementary Table 4, Extended Data Fig 7.

Raw data is available for Fig 2 and Fig 3. We have also included raw data for Supplementary Data 3a-h and Supplemental Data 4a-d. Also included is raw data for Fig 3a,b,c (re-entrainment) and Extended Data Fig 4 (re-entrainment), Fig 3d,e,f (phase shift) and Extended Data Fig 7.
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Sample size

For phase shift and re-entrainment experiments we determined statistical power a-priori (α error probability = 0.05) based on data for a known effect size for melatonin in these paradigms (Dubocovich et al. 1998; Dubocovich et al. 2005; G-power 3.0.10).

Data exclusions

Individual actograms of wheel running activity were excluded from analysis based on the exclusion criteria described below, which was completed by at least two individuals blind to treatment before data analysis was started. For re-entrainment actograms exclusion criteria includes: a) low running, sporadic activity, missing wheel activity data and/or lack of entrainment; b) entrainment of running activity more than 1 h before or after the "old" or "new dark" onset; c) re-entrainment to new dark onset after administration of the third injection (entrainment to injection). For phase shift actograms exclusion criteria includes: a) low running, sporadic activity, missing wheel activity data and/or lack of free running activity rhythms; b) tau change > 0.3 h; c) at least 2 out of 3 injections occurred outside of the target pre-determined time-range for treatment (CT 1 – 3, 5 – 7, 10 – 12).

Individual points were excluded from cell based data sets when meeting the exclusion criteria for the outliers Grubbs test.

Replication

Fig. 2(a-b): The data are the mean ± s.e.m. from five independent assays
Fig 2(c-f): Data represent mean ± s.e.m. from the indicated number (n) of biologically independent experiments run in triplicate.
Fig 3a: The data are the mean ± s.e.m. of independent measurements in 21 (vehicle) and 28 (7447) individual mice from 2-3 independent experiments.
Fig 3b: The data are the mean ± s.e.m. of independent measurements in vehicle (n = 28) vs. melatonin (n = 21), UCSF7447 (n = 21), UCSF3384 (n = 16), or luzindole (n = 11) individual mice from 2-3 independent experiments.
Fig 3c: The data are the mean ± s.e.m. of independent measurements in WT (n = 28 vehicle; n = 21 UCSF7447), MT1KO (n = 16 vehicle; n = 16 UCSF7447), and MT2KO (n = 20 vehicle; n = 25 UCSF7447) individual mice from 2-3 independent experiments.
Fig 3d: The data are the mean ± s.e.m. of independent measurements in (left panel) vehicle (n = 8) vs. melatonin (n = 8) or UCSF7447 (n = 13); (center panel) - vehicle (n = 15) vs. melatonin (n = 10), UCSF3384 (n = 16), or UCSF7447 (n = 15); (right panel) - vehicle (n = 6) vs luzindole (n = 3) from 2-3 independent experiments.
Fig 3e: The data are the mean ± s.e.m. of independent measurements in WT (n = 9 vehicle; n = 10 UCSF7447), MT1KO (n = 8 vehicle; n = 8 UCSF7447), and MT2KO (n = 11 vehicle; n = 9 UCSF7447) from 2-3 independent experiments.
Fig 3f: The data are the mean ± s.e.m. of independent measurements in WT (n = 8 vehicle; n = 8 UCSF7447), MT1KO (n = 6 vehicle; n = 7 UCSF7447), and MT2-KO (n = 10 vehicle; n = 13 UCSF7447) from 2-3 independent experiments.

Extended Data Fig 4h-j (re-entrainment): The data are the mean ± s.e.m. in 4h) C3H WT - vehicle (n = 28 mice) vs. UCSF3384 (n = 16 mice); 4i) C3H WT - vehicle (n = 21 mice) vs. UCSF7447 (n = 16 mice); 4j) C3H MT1KO - vehicle (n = 21 mice) vs. UCSF7447 (n = 25 mice) from 2-3 independent experiments.

Ext Data Fig 6: The data represent mean ± s.e.m. of three biologically independent experiments (n=3) run in triplicate
Ext Data Fig 7 a-c: The data are the mean ± SEM (n value listed in Fig legend) of independent measurements in individual mice derived from two independent experiments.

Extended Data Fig 7a: Data represent the mean ± s.e.m. in 7a) CT 2 - vehicle (n = 3), melatonin (n = 3), luzindole (n = 6), or UCSF7447 (n = 3); 7b) CT 6 - vehicle (n = 8), melatonin (n = 4), luzindole (n = 9), or UCSF7447 (n = 9); 7c) CT 10 - vehicle (n = 6), melatonin (n = 8), luzindole (n = 3), or UCSF7447 (n = 4) from 2-3 independent experiments.

Extended Data Table 1: The data are the mean ± s.e.m. from three to four biologically independent experiments run in triplicate.

Ext. Data Table 2: The data are the mean ± s.e.m. from three to four biologically independent experiments run in triplicate.

Ext. Data Table 3: The data are the mean ± s.e.m. from nine animals for each compound.

Ext. Data Fig 1 and Fig. 2: The data are the mean ± s.e.m. from four biologically independent experiments run in triplicate, unless otherwise, which is indicated in parenthesis next to each compound name.

Ext. Data Table 4: These data are the mean ± s.e.m. from the indicated number of biologically independent experiments run in triplicate.

Ext. Data Fig 3: The data are the mean ± s.e.m. of four biologically independent experiments (n=4) run in triplicate.

Ext. Data Fig 3e,f: The data are the mean ± s.e.m. of four (panel e) and three (panel f) biologically independent experiments run in triplicate.

Supplementary Data 1: Data represent mean ± s.e.m. of a single representative biological replicate using technical quadruplicates, and a second confirmatory biological replicate (again using technical quadruplicates) was also run for each compound. For the primary binding assay, data represent mean ± s.e.m. of 4 biologically independent experiments. Full concentration response curves represent mean ± s.e.m. of 3 biologically independent experiments run in triplicate.

Supplementary Data 2: Data represent the mean ± s.e.m. of three biologically independent experiments run in triplicate.

Supplemental Data 3 (a-h): Data represent mean ± s.e.m. of five independent assays, except for panel G with six independent assays.

Supplemental Data 4 a-d: The data are the mean ± s.e.m. from five independent assays.

Supplemental Data 4 e-f: The data represent mean ± s.e.m. of indicated number (n) of biologically independent experiments run in triplicate.

Supplemental Data 4 g: Data represent mean ± s.e.m. of three biologically independent experiments run in triplicate.

Randomization

We did not apply random assignment by using a specific method like a number generator, but animals were randomly put into treatment groups. We did not use criteria to assign groups.
Blinding

Experimental treatments were not done blind, but scoring of phase shift or re-entrainment was done blind to treatment and genotype when possible.

### 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 | Methods |
|----------------------------------|---------|
| n/a | Involved in the study |
| - Antibodies | n/a |
| - Eukaryotic cell lines | - ChIP-seq |
| - Palaeontology | - Flow cytometry |
| - Animals and other organisms | - MRI-based neuroimaging |
| - Human research participants | |
| - Clinical data | |

#### Eukaryotic cell lines

**Policy information about** [cell lines](#)

- **Cell line source(s)**
  - HEK293 T cells (ATCC CRL-11268)

- **Authentication**
  All cells used in this study are commercial and were obtained from vendors as indicated in the manuscript. HEK293T were certified mycoplasma free and authenticated by ATCC. Cells were also validated by analysis of short tandem repeat (STR) DNA profiles and these profiles showed 100% match at the STR database from ATCC.

- **Mycoplasma contamination**
  - HEK293T (ATCC CRL-11268; 59587035) were certified mycoplasma free by ATCC.
  - CHO-hMT1, CHO-hMT2, CHO-mMT1 and CHO-mMT2 cell lines are mycoplasma free determined using the LookOut® Mycoplasma PCR Detection Kit (Sigma-Aldrich).

- **Commonly misidentified lines**
  - (See [ICLAC register](#))
  - No commonly misidentified cell lines were used.

#### Animals and other organisms

**Policy information about** [studies involving animals](#) [ARRIVE guidelines](#) recommended for reporting animal research

- **Laboratory animals**
  - For circadian rhythm measurements, male and female C3H/HeN (C3H) wild-type (WT), MT1 knockout (MT1KO), and MT2 knockout (MT2KO) mice (average 6.28 months) used in this study were raised in our breeding colony at University at Buffalo. For pharmacokinetic experiments, healthy male C57BL/6 mice (8-12 weeks old) weighing between 20-35 g were procured from Global, India.

- **Wild animals**
  - Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

- **Field-collected samples**
  - For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

- **Ethics oversight**
  - For animals used in circadian rhythm experiments, all experimental procedures were conducted in accordance with guidelines set forth by the National Institutes of Health and approved by the University of Buffalo Institutional Animal Care and Use Committee. For animals used in pharmacokinetic experiments, the study was performed with approval of the Institutional Animal Ethics Committee (IAEC) in accordance with requirement of The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Note that full information on the approval of the study protocol must also be provided in the manuscript.