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Melatonin MT_1 receptors as a target for the psychopharmacology of bipolar disorder: A translational study

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ABSTRACT

The treatment of bipolar disorder (BD) still remains a challenge. Melatonin (MLT), acting through its two receptors MT_1 and MT_2, plays a key role in regulating circadian rhythms which are dysfunctional in BD. Using a translational approach, we examined the implication and potential of MT_1 receptors in the pathophysiology and psychopharmacology of BD. We employed a murine model of the manic phase of BD (*Clock* mutant (ClockΔ19) mice) to study the activation of MT_1 receptors by UCM871, a selective partial agonist, in behavioral pharmacology tests and in-vivo electrophysiology. We then performed a high-resolution Nuclear Magnetic Resonance study on isolated membranes to characterize the molecular mechanism of interaction of UCM871. Finally, in a cohort of BD patients, we investigated the link between clinical measures of BD and genetic variants located in the MT_1 receptor and *CLOCK* genes. We demonstrated that: 1) UCM871 can revert behavioral and electrophysiological abnormalities of ClockΔ19 mice; 2) UCM871 promotes the activation state of MT_1 receptors; 3) there is a significant association between the number of severe manic episodes and MLT levels, depending on the genetic configuration of the MT_1 rs2165666 variant. Overall, this work lends support to the potentiality of MT_1 receptors as target for the treatment of BD.

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1. Introduction

Bipolar disorder (BD) is a life-long psychiatric condition characterized by recurring episodes of depression and hypomania/mania alternating with intervals of well-being (euthymia) [1]. Several domains, clinical and neuropsychological, tend to be altered during the course of BD and in its prodromal phases [2–4]. The manifestations of bipolar disorder can show considerable variations between and within individuals across their lifespan. The precise biological underpinnings of the wide variety of BD manifestations are still uncertain. Treated BD patients experience substantial residual morbidity resulting in up to about 70 % of follow-up time spent ill, mainly in depression [5]. Therapeutic options are limited, and their safety profile often influences patients' compliance. Thus, the need for more effective and safe treatments is substantial. A key component of BD psychopathology lies in a dysfunction of the circadian system [6] which sees melatonin (MLT) as a major neurochemical modulator. MLT is synthesized mostly at night and regulates the suprachiasmatic nucleus (SCN) via two G-protein coupled receptors, MT_1 and MT_2 [7]. Small cohort studies in BD have revealed that changes in MLT levels might be mood-state specific [8], and that mood stabilizers delay MLT rhythm [9]. Our ongoing research [7,10,11] has shown the importance of selectively targeting one of the two MLT receptors in terms of both disease pathophysiology and drug discovery.

MT_1 receptors (MT_1KO) but not MT_2 receptors knock out (KO) mice display a depressive-like phenotype mostly during the dark phase and symptoms of mania (hyperlocomotion) during the light phase, paired with altered circadian patterns in serotonin (5-HT) and norepinephrine (NE) neurotransmissions [10]. Although on a different time scale, MT_1KO mice have a cyclic behavioral dysregulation similar to that observed in patients with BD.

Clock mutant (ClockΔ19) mice is a validated animal model of the manic phase of BD since it resembles the behavioral symptomatology of BD patients when in manic state, including hyperactivity, disrupted circadian rhythms, decreased anxiety, and hyperhedonia [12]. Most of these manic-like behavioral abnormalities are reversed by the mood stabilizers lithium [12] and valproate [13].

In this translational study, we sought to demonstrate the implication of MLT MT_1 receptors in the pathophysiology of BD and their potential as novel targets for BD treatment by: 1) investigating at preclinical level whether the activation of MT_1 receptors by the selective MT_1 receptor partial agonist UCM871 [14] is able to reverse behavioral and neurophysiological abnormalities of ClockΔ19 mice and its possible underlying neuronal mechanism of action; 2) studying the molecular underpinnings of MT_1 receptor activation by UCM871; and 3) examining in a cohort of BD patients the relationship between the clinical manifestation of the disease and genetic variability within the MT_1 receptor and *CLOCK* genes modulated by MLT levels [15]. Notably, given the well characterized circadian changes in the MLT system, behavioral experiments were conducted during both the light and the dark phases of the light/dark cycle to better identify the time of the day in which the possible treatment with UCM871 should be administered in keeping with previous research with MLTergic compounds [16–18].

2. Methods

2.1. Animals

Adult (2–4 months old) ClockΔ19 male mice and their Wild Type (WT) C57BL/6 J littermates were born and housed in the animal facility of San Raffaele Scientific Institute under standard laboratory conditions (12:12 h light/dark cycle, lights on at 7:30 am; temperature at 20 ± 2 °C) and supplied with food and water ad libitum. Behavioral experiments were performed during both the light/inactive (10 am–3 pm) and the dark/active (10 pm–3 am) phases of the light/dark cycle. Different animals have been used for each behavioral and in-vivo electrophysiology experiment. The number of animals per group and experiment can be found in Supplemental Table 1. Unless otherwise specified or due to the presence of experimental outliers (see the section on statistical analysis for details), for behavioral pharmacology experiments the number of animals per group was ranging between 10 and 14, while for in-vivo electrophysiology between 5 and 8. The study was approved by the institutional Animal Care and Use Committee of the San Raffaele Scientific Institute and by the Italian Ministry of Health (IACUC permission number: 970).

2.2. Drugs

UCM871 (N-(2-{Methyl-[3-(4-phenylbutoxy)phenyl]amino}ethyl) acetamide, referred to as 4a in Rivara et al. [14] was synthesized and contributed by Prof. Bedini, University of Urbino, Italy. The affinity of UCM871 at human MT_1 and MT_2 receptors was the following: pKi 8.93 ± 0.17 (mean±SEM of at least three determinations performed in duplicate) for MT_1 receptors, and pKi 7.04 ± 0.10 for MT_2 receptors [14]. The intrinsic activity of UCM871 at MT_1 receptors relative to that of MLT was 0.68 ± 0.09 [14]. For more details, see Supplemental material.

2.3. Open field test

The animal was left free to explore the arena for 20 min and frequency and total duration of visits in the central zone of the arena and total locomotor activity were calculated as described [19]. The duration of rearing and grooming episodes was measured manually, in a blind manner, by three independent investigators (MTM, LC and SN) to evaluate stereotypic behavior.

2.4. Three-chamber sociability test

This test was conducted following our recent method [19]. For more details, see Supplemental material.

2.5. Rotarod

The test was anticipated by a training session in which the rod was spinning at gradually accelerating speed for 5 min. The training session was performed to determine the mean speed at which mice were falling from the rod and this value was used to set up the rod velocity for the test. During the test, the number of falls from the rod was recorded for each animal at 15, 30, 60 and 120 min after the injection of either VEH or UCM871.

2.6. Single-unit extracellular recordings

In vivo extracellular single-unit recordings of spontaneously active ventral tegmental area (VTA) dopaminergic (DA) neurons were performed following standard methods in our lab [19] to assess if UCM871 was able to modulate DA neurotransmission. For more details, see Supplemental material.

2.7. Local field potential recordings in freely moving animals

LFP recordings were performed as described [20]. For details, see Supplemental material.

2.8. Cell culture and transfection, Protein extraction and Western blot, and Membrane preparation

MT_1 receptors were expressed on HEK293T cells. For more details, see Supplemental material.

2.9. NMR spectroscopy and Homology modelling and Molecular Docking calculation

NMR experiments were performed using a Bruker AVIII HD 600 MHz spectrometer equipped with a triple resonance Prodigy N2 cryoprobe with z-axis pulse field gradient. The 3D structural model of the MT_1 inactive state was obtained using as template the crystal structure of MT_1 bound to ramelteon (PDB ID: 6ME2) [21]. For the active MT_1 receptor the three-dimensional model was obtained using as template (i) the cryo-EM structure of the human mu-opioid receptor for the residues 21–290 of the transmembrane bundle (TM1-TM7) (PDB ID: 6DDE) [22] and (ii) the crystal structure of the human adenosine A2A receptor (PDB ID: 2YDO) [23] for the region from 291 to 308 comprising ICL4 and helix 8. The UCM871/MT_1 structure was visualized and analyzed by PyMoL [23] and Chimera software [24]. For more details, see Supplemental material.

2.10. Clinical sample

BD individuals were recruited at the Section of Psychiatry of the University of Cagliari, Italy following the study protocol previously described [15]. The study was conducted in accordance with the Declaration of Helsinki and after the ethical approval by the Ethics Committee of the University Hospital Agency of Cagliari (PG/2019/6277). Participants provided written informed consent. For more details, see Supplemental material.

2.11. Genotyping and quality control

Genetic data for 4 variants located in the *CLOCK* gene and 4 in the MT_1 receptor (MTNR1A) gene were available through a previously conducted study (see Supplemental material). We focused on the interaction between SNPs located in the CLOCK and MTNR1A genes given the known link between the CLOCK gene and BD psychopathology and the preliminary preclinical data showing a differential response following the treatment with the selective MT_1 receptor partial agonist UCM871 in Clock mutant animals. We tested the association between each SNP and six outcomes (average severity of mania, number of manic or hypomanic episodes,number of severe manic episodes, average severity of depression, number of depressive episodes, and number of severe depressive episodes) as well as with the Barratt Impulsiveness Scale (BIS-11)the Young Mania Rating Scale (YMRS), and the Hamilton Depression Rating Scale (HDRS) scores under a dominant model using linear regression. For variants showing a significant association, we constructed a more complex model including also MLT levels and the interaction between the genetic variant and MLT levels. This analysis was aimed at assessing whether genetic variants located in the CLOCK or MTNR1A genes might moderate the association between MLT levels and outcome related to severity of mania or depression in patients with BD. For more details, see Supplemental material.

2.12. Determination of plasma levels of melatonin

Plasma levels of MLT were quantified using LC-MS/MS following our standard method [25].

2.13. Statistics

Data analysis was performed with Prism version 8.0 software (GraphPad Prism Software Inc., San Diego, CA, USA). Data distribution was checked using the Shapiro-Wilk test. Depending on the experiment, the following statistical tests have been applied: 1) repeated measures (RM) one-way analysis of Variance (ANOVA) followed by post-hoc analysis using Bonferroni's multiple comparison test; 2) RM and ordinary two-way ANOVA followed by Bonferroni's multiple comparison test; 3) three-way ANOVA followed by Bonferroni's multiple comparison test; 4) unpaired t-test. Possible outliers were identified and when appropriate removed from statistical consideration using the ROUT method. Statistical significance was accepted when $p \leq 0.05$. Detailed statistics for each single experiment are available in Supplemental Table 1.

3. Results

3.1. Manic-like behaviors of ClockΔ19 mice are be reversed by UCM871 treatment

To explore the effects of UCM871 on some of the symptoms of BD, particularly those of the manic phase such as exploratory behavior, stereotypic behavior, locomotor activity and social behavior, we performed the OFT and the three-chamber sociability tests in WT and ClockΔ19 mice (Fig. 1). We studied the dose of 20 mg/kg since, in a preliminary experiment, we found that this was the dose of UCM871 exerting a reduction of locomotor activity (anti-manic effect) in the OFT (Supplemental Fig. 1A-C) in the absence of effects on motor coordination, as measured in the rotarod test (Supplemental Fig. 2D).

First, we characterized the locomotor activity, anxiety-like behavior, and stereotypic behavior of ClockΔ19 mice (Fig. 1A-D). Regarding locomotion (Fig. 1B), we found a significant main effect of genotype, showing an increased distance travelled by ClockΔ19 compared to WT mice, regardless of the phase of the day. Concerning the time spent in the central area of the open field (Fig. 1B), we observed that, independently of genotype, mice spent significantly more time in the center during the light phase. Regarding the number of visits in the central area of the open field (Fig. 1B), ClockΔ19 mice entered the central area significantly more times than WT controls during the light phase and, unlike WT littermates, the number of entries in the center of ClockΔ19 mice was significantly higher during the light than during the dark phase. Taken together, these data confirm the general hyperactivity that was previously observed in ClockΔ19 mice mostly during the light phase [12].

For stereotypic behavior, we observed a significant interaction between genotype and phase of the day for the total duration of rearing (Fig. 1B) but not grooming episodes (Supplemental Fig. 3A). WT mice spent significantly less time rearing during the dark than during the light phase, unlike ClockΔ19 mice that spent significantly less time rearing during the light than during the dark phase. Moreover, during the light phase, we observed a significant reduction in the duration of rearing in ClockΔ19 mice compared with WT mice, while during the dark phase the duration of rearing was significantly higher in ClockΔ19 than in WT mice.

Second, we evaluated the effects of an acute injection of 20 mg/kg of UCM871 in WT and ClockΔ19 mice, during both phases of the light/dark cycle. During the light phase (Fig. 1C), UCM871 significantly reduced the distance travelled in both WT and ClockΔ19, with the latter having a higher distance travelled than WT. After UCM871 treatment, the distance travelled by ClockΔ19 was equal to that travelled by WT mice receiving VEH. Moreover, we observed a main effect of treatment on the time spent in the central area of the arena, which was lower in the animals treated with UCM871 compared to those treated with VEH (Fig. 1C). Regarding the number of visits in the central area (Fig. 1C), during the light phase, both WT and ClockΔ19 mice treated with UCM871 entered the central area significantly less times than the respective VEH controls. Moreover, within the VEH-treated group, ClockΔ19 mice entered the center significantly more times than WT mice. Importantly, ClockΔ19 mice treated with UCM871 entered the center of the arena an equivalent number of times as WT receiving VEH. Finally, the duration of rearing (Fig. 1C) was significantly lower in ClockΔ19 than in WT mice treated with VEH, while UCM871 was increasing the duration of rearing in ClockΔ19 mice up to the level of WT mice receiving VEH. On the contrary, in WT mice the drug was reducing the total duration of rearing. No effects on the duration of

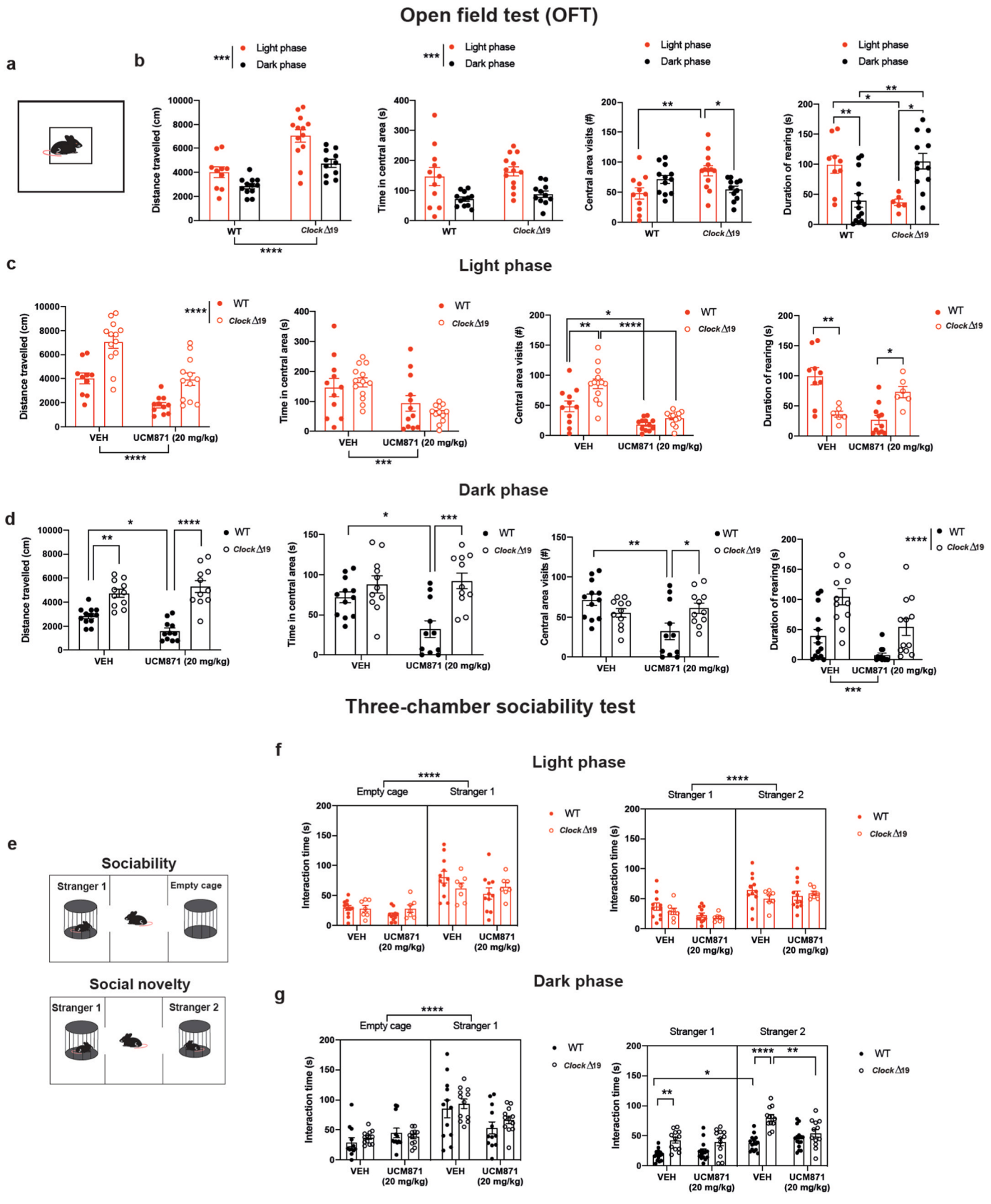

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Fig. 1. ClockΔ19 mice display hyperactivity and altered stereotypic and social behavior that can be reversed by UCM871 treatment. (A) Scheme representing OFT. (B) Results from OFT showing the distance travelled, the time spent and the number of visits in the central area of the open field arena and the duration of rearing episodes in WT and ClockΔ19 mice, during the light and the dark phases of the 12 h:12 h light/dark cycle. (C) UCM871 treatment (20 mg/kg) significantly reduced hyperactivity and increased rearing behavior of ClockΔ19 mice to WT levels during the light phase. (D) UCM871 did not affect locomotor activity of ClockΔ19 mice, but reduced the duration of rearing in both WT and ClockΔ19 mice during the dark phase of the day. (E) Scheme representing the three-chamber sociability and social novelty tests. (F) 20 mg/Kg UCM871 treatment did not affect either sociability or social novelty during the light phase. (G) UCM871 treatment significantly reduced the hyper-social novelty behavior observed in ClockΔ19 mice during the dark phase. Data are reported as mean ± SEM. Each bar represents mean ± SEM and each dot represents a single mouse. *P < 0.05, * *P < 0.01, * **P < 0.001, * ** *P < 0.0001 by (B-D) Two-way ANOVA, considering the effects of the genotype and the phase of the day, followed by Bonferroni's post-hoc multiple comparisons test, or (F G) Three-way ANOVA (treatment x social preference x genotype) followed by Bonferroni's post-hoc multiple comparisons test. Detailed statistics are available in Supplemental Table 1.

grooming were found (Supplemental Fig. 3B).

Concerning the effects of UCM871 in the OFT during the dark phase (Fig. 1D), the distance travelled was significantly higher in ClockΔ19 than in WT mice receiving VEH and following 20 mg/kg UCM871. While a significant reduction of the locomotor activity was induced in WT mice by the drug, no effects on the distance travelled were found in ClockΔ19 mice. Moreover, treatment with UCM871 significantly reduced the time spent and the number of entries in the central area of the open field in WT but not in ClockΔ19 mice. The duration of rearing was significantly higher in ClockΔ19 than in WT mice, and was lower in animals receiving UCM871 compared to those receiving VEH, regardless of the genotype (Fig. 1D). No significant effects were observed for the duration of grooming episodes during the dark phase (Supplemental Fig. 3C).

Finally, we assessed social behavior by performing the three-chamber sociability test (Fig. 1E-G). During the light phase, a three-way ANOVA, considering the effects of genotype, treatment, and social preference, did not reveal any significant interaction between the three factors in both sessions of the test (Fig. 1F). During the first part of the test, we observed that mice spent more time interacting with the stranger mouse rather than with the empty cage regardless of the genotype and the treatment. Similarly, during the second session, mice spent more time interacting with "stranger 2" than with "stranger 1". During the dark phase, a three-way ANOVA test did not detect any significant interaction between genotype, treatment, and social preference in the first part of the test. However, mice of both genotypes spent more time interacting with the stranger mouse rather than with the empty cage (Fig. 1G). In the second part, we detected a significant interaction between the three factors (treatment x social preference x genotype): WT mice treated with VEH spent significantly more time interacting with "stranger 2" than "stranger 1", as expected, and VEH-treated ClockΔ19 mice spent significantly more time interacting with "stranger 1" and with "stranger 2" than VEH-treated WT controls. Treatment with UCM871 attenuated this hyper-sociability, as the time of interaction of ClockΔ19 mice with "stranger 2" was close to that of WT

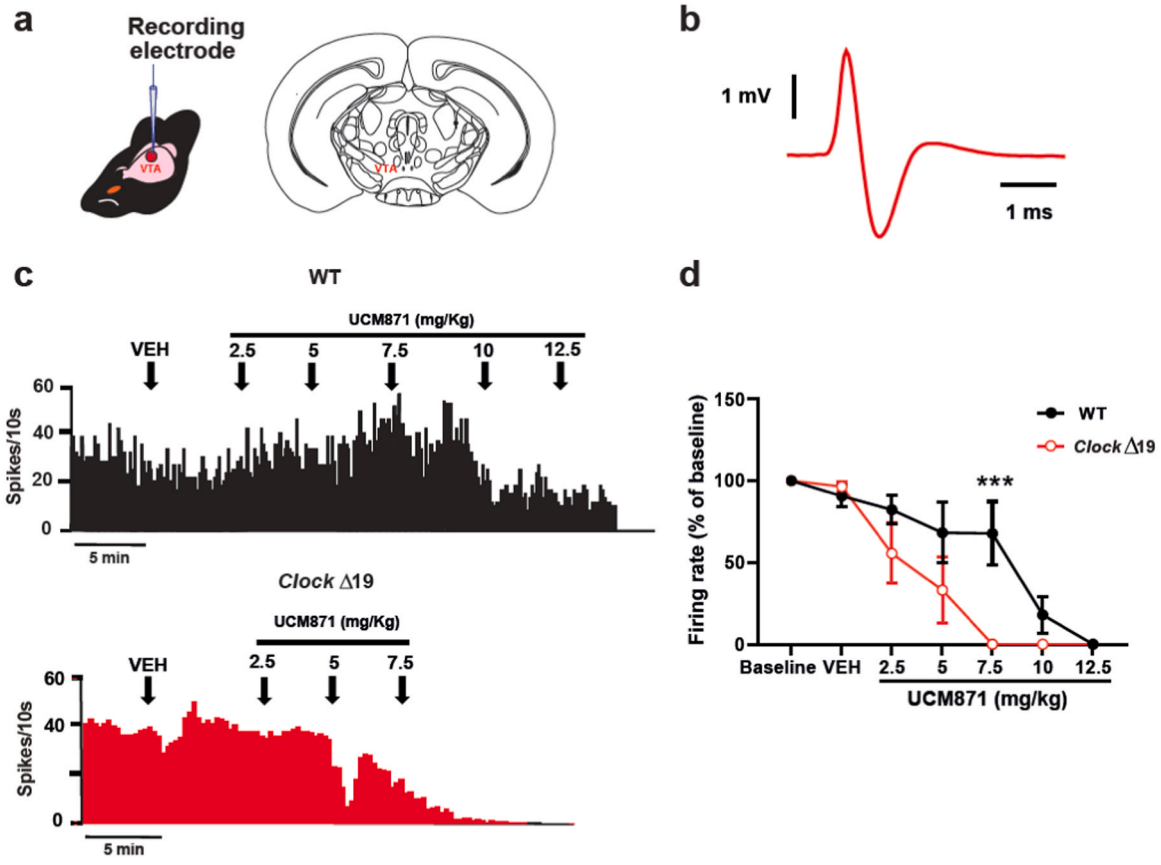

Fig. 2. ClockΔ19 mice are more responsive to UCM871 dose-dependent inhibitory effect on VTA DA firing activity than WT mice. (A) Scheme representing VTA recording site. (B) Typical waveform of spontaneously active VTA DA neurons. (C) Representative firing rate histograms showing the response of single VTA DA neurons to cumulative doses of UCM871 in WT and ClockΔ19 mice. (D) Dose-response curve of UCM871 on VTA DA neurons in WT and ClockΔ19 mice. Repeated measures two-way ANOVA (genotype x treatment) followed by Bonferroni's post-hoc multiple comparisons test. Each point of the curve represents mean ± SEM expressed as percentage of the baseline firing rate. * **P < 0.001. Detailed statistics are available in Supplemental Table 1.

mice treated with UCM871 and significantly lower than that of VEH-treated ClockΔ19 mice.

3.2. ClockΔ19 mice display an increased VTA DA firing activity and higher responsiveness to UCM871 inhibitory effect

Previous findings showed an increased ventral tegmental area (VTA) dopaminergic (DA) neural activity in ClockΔ19 mice correlating with their hyperactive phenotype that was reduced by treatment with lithium [12,26]. We therefore decided to investigate whether UCM871 was affecting VTA DA neural activity (Fig. 2). We confirmed the higher activity of VTA DA neurons in ClockΔ19 with respect to WT mice (Supplemental Fig. 4). We evaluated the effects of cumulative doses of UCM871 on the activity of VTA DA neurons in both WT and ClockΔ19 mice and found that UCM871 induced a dose-dependent inhibition of VTA DA firing activity (Fig. 2D). This experiment was performed during the light phase of the light/dark cycle, the phase of the day in which we observed in ClockΔ19 mice the greater reduction in hyperlocomotion by UCM871 (Fig. 1C). Notably, the inhibition of VTA DA firing activity was significantly greater in ClockΔ19 mice than in WT mice at the dose of 7.5 mg/kg of UCM871 (Fig. 2E). Indeed, we observed that VTA DA neurons were all silenced at the dose of 7.5 mg/kg in ClockΔ19 mice, while they all silenced only at the dose of 12.5 mg/kg in WT mice (Fig. 2E).

3.3. UCM871 reverts the increased hippocampal and prefrontal cortex high-theta activity of ClockΔ19 mice

Using Local Field Potential (LFP) recordings in freely moving animals in their home cage, we evaluated possible baseline differences between WT and ClockΔ19 mice in the total power spectrum and within spectral bands, both in the prefrontal cortex (Fig. 3A) and in the hippocampus (Fig. 3D), two brain regions strongly involved in the control of emotions and mood, and in the pathophysiology of BD [27,28]. In the prefrontal cortex, WT mice displayed a significantly higher power compared to ClockΔ19 mice at the low theta frequencies peak (3–7 Hz), while it was significantly higher in ClockΔ19 than in WT mice at the high theta frequencies peak (7–10 Hz) (Fig. 3A). These results were confirmed by a higher total power of the high theta band in ClockΔ19 than in WT mice (Fig. 3A). Similarly, in the hippocampus, we found a significantly higher power in ClockΔ19 than in WT mice in the frequencies belonging to the high theta band and in the total power of the high theta band (Fig. 3D).

Then, we evaluated the effects of cumulative doses of UCM871 on the

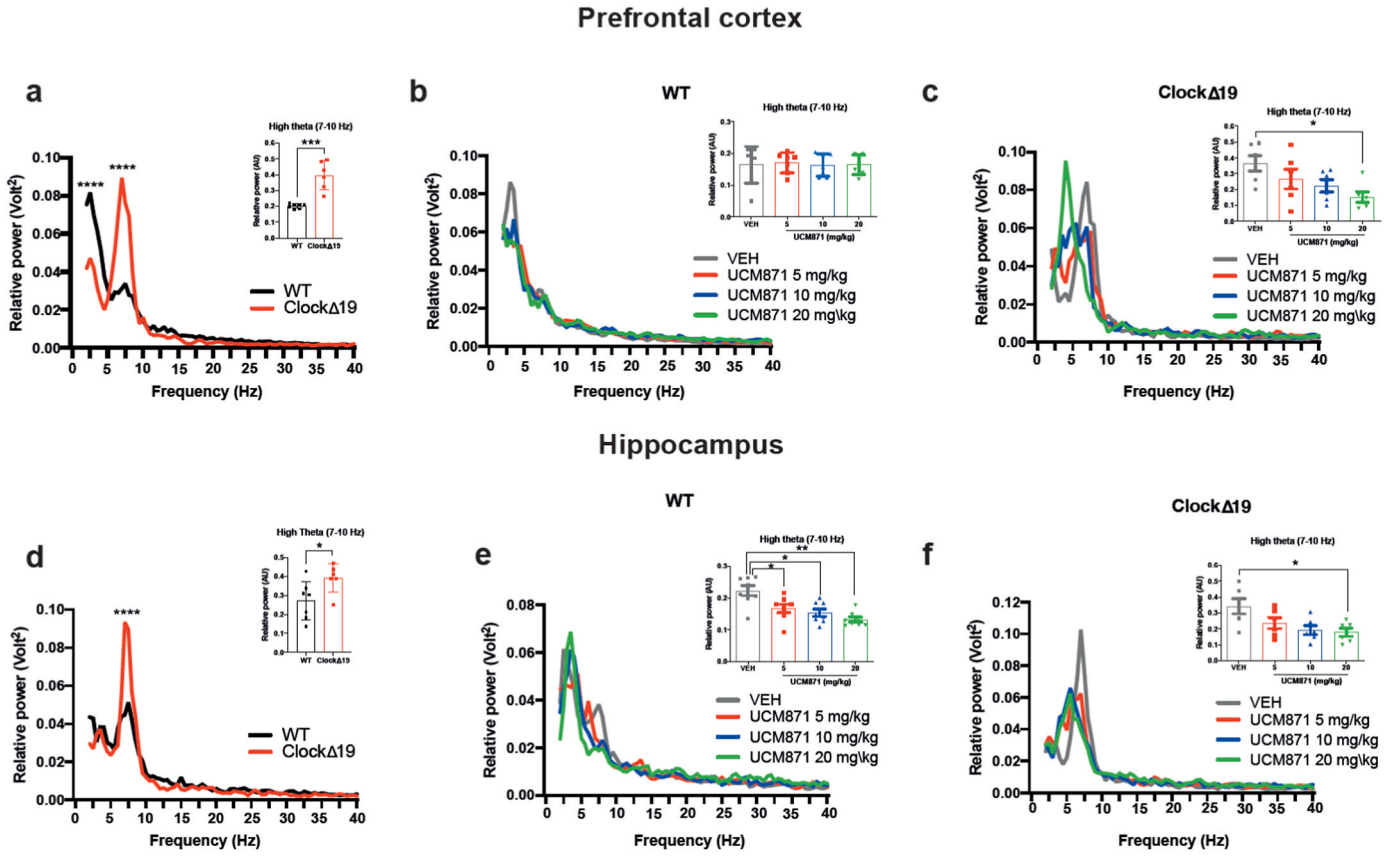

Fig. 3. ClockΔ19 mice display a significantly higher mean power in the high theta band compared to WT controls, both in the prefrontal cortex and the hippocampus during the light phase. UCM871 treatment at the dose of 20 mg/kg was able to significantly reduce the mean power in the high theta bands in the prefrontal cortex of ClockΔ19 mice and in the hippocampus of both WT and ClockΔ19 mice, compared to VEH. (A) LFP baseline 0–40 Hz spectrum recorded in the prefrontal cortex of WT and ClockΔ19 mice. High theta band mean power in the prefrontal cortex of WT and ClockΔ19 mice (apex), where each bar represents mean ± SEM and each dot represents a single recorded mouse. Repeated measures two-way ANOVA followed by Bonferroni's post-hoc multiple comparisons test and unpaired t-test. (B-C) Prefrontal cortex LFP 0–40 Hz spectrum and high theta band mean power (apex) in WT and ClockΔ19 mice treated with cumulative doses of UCM871 (5, 10, 20 mg/kg) or VEH. Repeated measures two-way ANOVA and repeated measures one-way ANOVA followed by Bonferroni's post-hoc multiple comparisons test. (D) LFP baseline 0–25 Hz spectrum and high theta mean power (apex) recorded in the hippocampus of WT and ClockΔ19 mice. Repeated measures two-way ANOVA followed by Bonferroni's post-hoc multiple comparisons test and unpaired t-test. (E-F) Hippocampus LFP 0–40 Hz spectrum and high theta band mean power (apex) in WT and ClockΔ19 mice treated with cumulative doses of UCM871 (5, 10, 20 mg/kg) or VEH. Repeated measures two-way ANOVA and repeated measures one-way ANOVA followed by Bonferroni's post-hoc multiple comparisons test. *P < 0.05, * *P < 0.01, * **P < 0.001, * ** *P < 0.000. Detailed statistics are available in Supplemental Table 1.

power spectra of WT and ClockΔ19 mice, both in the prefrontal cortex and in the hippocampus (Fig. 3B-C, E-F). In the prefrontal cortex of WT animals, there was no significant effect of UCM871 treatment either in the high theta band mean power or in the total power spectrum (0–40 Hz) (Fig. 3B). Interestingly, in ClockΔ19 mice, we found a significant reduction in the high theta band mean power following treatment with 20 mg/kg of UCM871 compared to VEH (Fig. 3C). In the hippocampus, we found a significant decrease in high theta mean power following UCM871 treatment at the doses of 5, 10 and 20 mg/kg compared to VEH in WT mice (Fig. 3E), whereas in ClockΔ19 mice this decrease was significant only at the dose of 20 mg/kg (Fig. 3F).

3.4. Structural model of UCM871 bound to MT_1 GPCR: from inactive to active state

To decipher the molecular mechanism of action of UCM871, which is a key requirement for drug development, we performed a high-resolution NMR investigation using isolated membranes to identify the structural determinants driving MT_1-ligand interactions under native conditions. First, we characterized the structural features of UCM871 in the absence of native membranes by 1D and 2D ^{1}H NMR spectra Supplemental Fig. 5A-C). Yet, STD (Saturation Transfer Difference) and T1ρ-NMR based experiments were acquired as negative controls for avoiding artifacts in the NMR binding studies reported below (Supplemental Fig. 5B,C). (Supplemental Fig. 5C). Then, the UCM871 binding epitope were identified by comparing STD and T1ρ-based NMR experiments acquired in the presence and absence of cell membranes containing MT_1 receptors (Supplemental Fig. 6A-D). STD experiments (Fig. 4A) show that a large number of UCM871 protons receive saturation transfer in the presence of membranes, indicating that the MT_1 binding is modulated by various portions of the ligand. Specifically, weak STD effects (Relative STD percentage<40 %) were observed for the methyl group on the aniline nitrogen: medium STD effects (Relative STD percentage≥40 %) were detected for the methyl group of the acetamide portion; strong STD effects (Relative STD percentage≥80 %) were observed for a few protons of the aniline ring and, for the most part, of the phenylbutil chain. These findings were further confirmed by a recently optimized T1ρ-based NMR methodology (Fig. 4A-E; Supplemental Fig. 5C; Supplemental Fig. 6D). According to STD data, the relative T1ρ BE percentage (Fig. 4A) values clearly indicated that the binding of the ligand to MT_1-receptors is mainly regulated by the aniline ring and the phenylbutyl side chain and is further stabilized by the acetamide group. To deeply describe the molecular basis of MT_1 activation upon binding to UCM871, we applied a combined approach using NMR and Molecular modeling and docking techniques (Fig. 4A-E; Supplemental Fig. 7). We therefore built a 3D model for the inactive and active state of MT_1 receptors (Fig. 4C-E; Supplemental Fig. 8A-C; Supplemental Fig. 1A-B) by homology modeling. Consistent with previous studies [21,29], the 3D structural model of inactive MT_1 indicates that the receptor presents the typical GPCR architecture (Fig. 4C; Supplemental Fig. 8A-B) with the presence of helical kinks in TM5 (Pro^{198}), TM6 (Pro^{253}), and TM7 (Ala^{292}) (Supplemental Fig. 8C), which are essential for coupling ligand binding to conformational changes needed to reach the active state [30,31]. In contrast, TM3 is straight, and forms the structural core of the receptor interacting with many neighboring TMs (Supplemental Fig. 6C).

Many studies have demonstrated that GPCR activation occurs through a conformational selection mechanism in which the agonist induces an allosteric response that shifts the receptor population from the inactive to the active state [30,31]. Therefore, to explore the MT_1 activation mechanism describing the structural rearrangements coupled to the allosteric response upon UCM871 binding, we performed molecular docking studies starting from the 3D structure of the active MT_1 receptor obtained by homology modeling. In agreement with the NMR data, the 3D structure of the UCM871-MT_1 complex showed that the ligand is accommodated into the orthosteric binding site formed primarily by TM3 and TM5- TM7 (Fig. 4A-C).

The MT_1 ligand pocket of UCM871 is lined with His^{195}(TM5), which is highly conserved in melatonin receptors (Fig. 4D). The methyl group of the acetamide portion of UCM871 interacts with Val^{84} (TM2) and Met^{107} (TM3) by hydrophobic interactions, while carbonyl oxygen is hydrogen bonded with Tyr^{281}(TM7) (Fig. 4E). However, the aniline ring of UCM871 forms a hydrophobic interaction with the Trp^{251} (TM6) indole ring. Finally, the UCM871 phenylbutyl side chain is embedded in a hydrophobic sub-pocket formed by Val^{111} and Ile^{112} (TM3); Val^{159} (TM4); Phe^{179} (ECL2); Tyr^{187}, Thr^{188} and Val^{191} (TM5) (Fig. 4E).

To further evaluate the conformational changes of the overall architecture of MT_1 upon activation induced by UCM871, we compared the 3D structural model of the MT_1 receptor in inactive form to the active UCM871-bound MT_1, revealing that the largest structural difference, as observed for other GPCRs, such as the β_2-adrenoreceptor (β_2AR) [32,33], occurs close to the cytoplasmic side of the receptor with the TM6 showing a structural displacement of ~12 Å outward from the TM3 (Supplemental Fig. 8A-B). Overall, our results confirm the importance of an active cytoplasmic conformation for the activity of MT_1 receptor ligands in our in-vitro and in-vivo essays.

3.5. Relationship between CLOCK and MTNR1A genes, MLT levels and BD psychopathology in BD patients

Demographic and clinical characteristics of the study population along with the plasma concentration of MLT are reported in Supplemental Table 2. Briefly, 64 % of the overall study sample was constituted of females. No sex differences emerged in total YMRS and total BIS-11 scores, average severity of mania or depression, average number of hypo- and mania episodes, average number of depressive episodes, average number of severe manic or depressive episodes, and rates of history of suicide ideation or attempts. At the time of the recruitment, 40 individuals were under pharmacological therapy. Characteristics of the investigated SNPs are summarized in Supplemental Table 3. Presence of the G allele of the MNTR1A rs2165666 variant was nominally associated with lower average severity of mania and a lower number of severe manic episodes but not with the total number of manic or hypomanic episodes, with average severity of depression or with the number of depressive or severe depressive episodes (Table 1, Supplemental Table 4). Furthermore, we observed a significant interaction between this variant and MLT levels, in the model with the number of severe manic episodes as the outcome. In stratified analyses, MLT levels were significantly and positively associated with the number of severe manic episodes only in participants without the rs2165666 minor allele. The rs62303689 and rs6832769 SNPs, located in the CLOCK gene, were associated with higher number of severe depressive episodes and lower HDRS score, respectively. For these SNPs, no significant interaction with MLT levels was detected (Supplemental Table 4). No other SNP was significantly associated with the investigated variables. Clinical measures of impulsivity and elation were also significantly modulated by polymorphisms within the CLOCK and MTNR1A genes (Supplemental Table 5). Specifically, we found a statistically significant interaction between the rs6832769 and rs1801260 SNPs of the CLOCK gene and MLT levels in relation to YMRS scores. In addition, the rs62303689 of the CLOCK gene interacted significantly with MLT levels in relation to the BIS11 motor subscale and the BIS11 total score. Interestingly, a similar pattern of interaction was found between MLT levels and the rs2165666 of the MTNR1A gene with the BIS 11 motor subscale and total score, respectively (Supplemental Table 5).

4. Discussion

The treatment of BD still represents a challenge for clinicians and despite progress in the study of the neurobiology and psychopharmacology of the disease, options remain limited, and results are often unsatisfactory. Our findings, obtained following a translational approach,

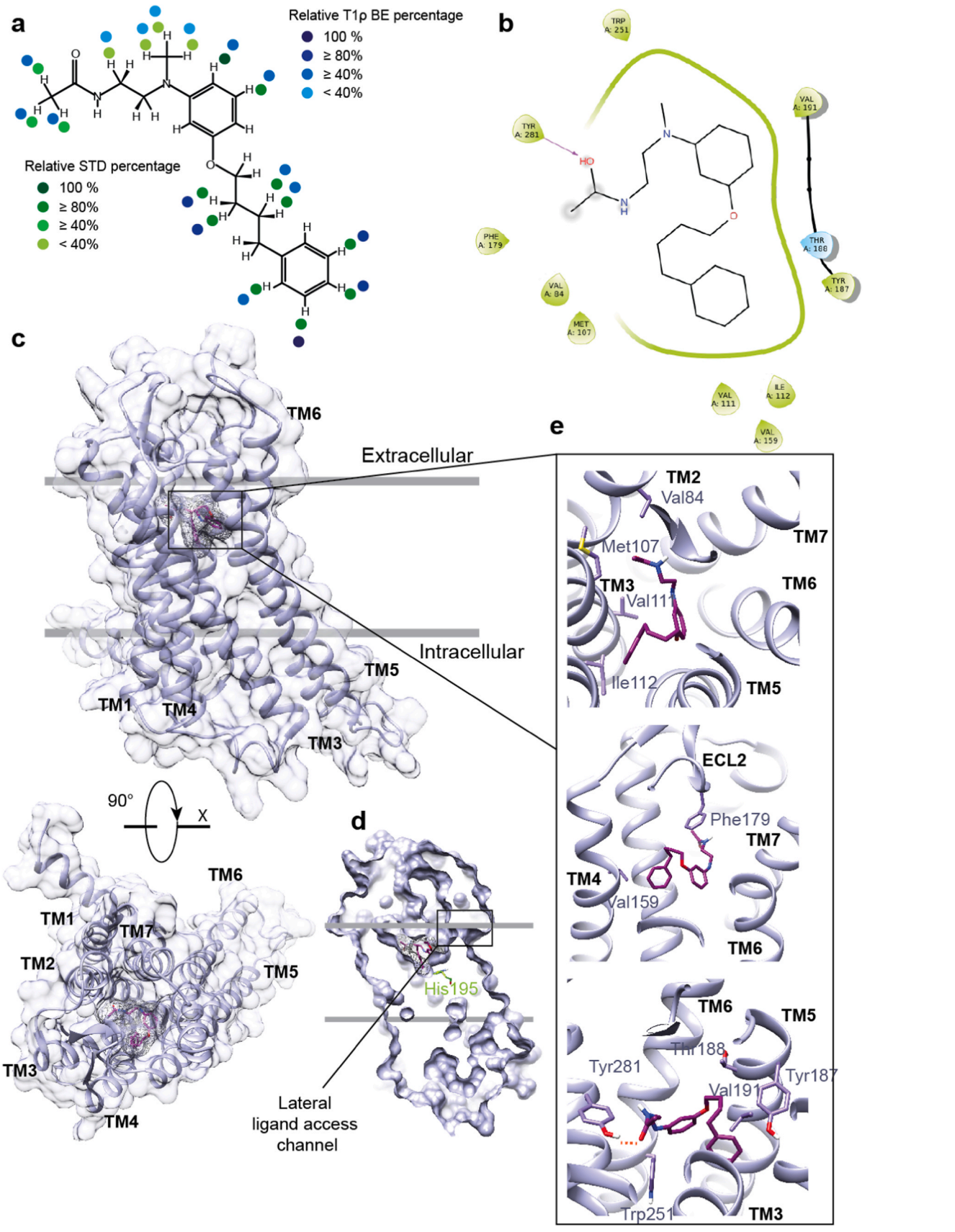

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Fig. 4. UCM871 recognition mechanism at the MT_1 receptor. (A) UCM871 epitope mapping. Relative STD percentages, calculated with respect to the most intense STD signal, are reported by color code: dark green dots (100 % relative STD), forest green dots over 80 %, sea green dots over 40 %, and lime green dots under 40 %. Relative STD percentages. Relative T1ρ Binding Effect (BE) percentage values are illustrated by the following color code: navy dots 100 % relative T1ρ-BE; medium blue dots over 80 %, deep blue over 40 %, deep sky blue under 40 %. (B) 2D interaction scheme of the UCM871 binding by MT_1 receptor. (C) Docking structural model of the UCM871/MT_1 complex. The receptor is reported as ribbon drawing (medium purple) whereas UCM871 is reported as magenta sticks. The structural model is reported in two orientations rotated of 90° around x axis. (D) Section through the receptor showing the His^{195} located close to the orthosteric binding site. (E) Close-up view of the UCM871/MT_1 complex. The side chains of the MT_1 residues playing a crucial role in the recognition mechanism are illustrated as medium purple sticks; whereas UCM871 is depicted in magenta. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Association between genetic variants located in the CLOCK and MTNR1A genes and clinical variables.

			Average severity of mania		Number of hypomanic or manic episodes			Number of severe manic episodes
	A1/A2	MAF	Beta (se)	p	Beta (se)	p	Beta (se)	p
Melatonin levels	/	/	0.00 (0.02)	0.73	0.06 (0.28)	0.84	0.05 (0.05)	0.31
CLOCK								
rs62303689	A/C	0.20	-0.36 (0.19)	0.07	0.16 (3.44)	0.96	-0.33 (0.61)	0.60
rs6832769	G/A	0.31	0.25 (0.18)	0.19	2.38 (3.28)	0.47	-0.29 (0.59)	0.63
rs1801260	C/T	0.39	-0.12 (0.19)	0.53	-3.00 (3.33)	0.37	-0.45 (0.60)	0.45
rs114621641	T/C	0.07	-0.30 (0.29)	0.31	-2.83 (5.07)	0.58	-0.84 (0.90)	0.35
MTNR1A								
rs11943499	T/C	0.07	-0.47 (0.26)	0.08	3.00 (4.69)	0.53	-1.14 (0.82)	0.17
rs2165666	G/A	0.19	**-0.39 (0.19)**	**0.049**[1]	-0.14 (3.50)	0.97	**-1.29 (0.59)**	**0.035**[2]
rs6820205	T/C	0.08	0.02 (0.27)	0.95	-1.86 (4.71)	0.70	-0.75 (0.83)	0.37
rs13113549	G/A	0.42	-0.15 (0.20)	0.44	0.06 (0.28)	0.84	0.53 (0.61)	0.39

A1, minor allele; A2, other allele; MAF, minor allele frequency.
[1]The interaction between rs2165666 and melatonin levels was not significant
[2]Significant interaction between rs2165666 and melatonin levels.

showcase the proof-of-concept for targeting MT_1 receptors to advance the neurobiological understanding and increase the psychopharmacological armamentarium of BD.

First, using a well-validated animal model of the manic phase of BD, the ClockΔ19 mice, we demonstrated that the selective MT_1 receptors partial agonist UCM871 reverted behavioral and electrophysiological abnormalities of ClockΔ19 mice. Second, we showed that the pharmacological modulation with UCM871 promoted the shift of MT_1 receptors from the inactive to the active state. Finally, in a group of BD patients we found a statistically significant association between clinical measures related to episodicity and circadian cyclicity (number of severe manic episodes) and MLT levels depending on the genetic configuration of the rs2165666 variant of the MTNR1A gene.

BD involves disruption of intrinsic biological rhythms, including the sleep-wake cycle, hormonal rhythms and temperature regulation [34] and therefore it is not surprising that old studies demonstrated changes in MLT levels associated with BD [8]. Low serum levels of MLT were shown to be a trait marker in BD, even though MLT levels did not show state-dependent changes across mania or depression states [8]. A shift in the nocturnal MLT peak was also observed in BD patients [35] and the suppression of MLT synthesis by light is greater in BD than in control subjects [36].

However, whether MLT alone or as adjunct therapy may have pharmacological efficacy for BD or BD comorbidities is still to be demonstrated as a very limited number of trials has thoroughly investigated this issue [37]. Our research over the past decade on the functioning of the MLT system in the brain has highlighted the importance of a selective modulation of the MLT receptor subtypes, since two receptors may mediate complementary or even opposite functions [7,11]. This selectivity translates into a higher pharmacological efficacy than non-selectivity as in the case of MLT that has comparable affinity for MT_1 and MT_2 receptors [16,18,38]. In keeping, this work shows that targeting selectively MT_1 receptors may ameliorate mania-like symptoms. The MLT system is a relatively safe system [39] and thus we may expect limited adverse effects if moving MT_1 agonists toward clinical trials in humans. Supporting the low predictive toxicity of MT_1 agonism, we found that the dose of UCM871 reducing the hyperactivity of mice affected neither their locomotor coordination (no effects in the rotarod test) nor social behavior (three-chamber test), working memory (data not shown) or anxiety levels (data not shown).

The hyperactivity of ClockΔ19 mice was shown to be in part related to a higher neuronal activity of VTA DA neurons which could be reverted by lithium [12]. Here, we showed that a higher activity also of the prefrontal cortex and hippocampus in the high theta band was likely involved in the phenotype of ClockΔ19 mice, and treatment with UCM871 was able to revert behavioral and electrophysiological dysfunctions to control levels.

A previous report in rats [40] found that MLT produced a moderate inhibition of VTA DA neuronal activity but did not explore whether that effect was MT_1 or MT_2 receptor mediated. In addition, in another study it was found that acute injection of MLT in rats inhibited the activity of serotonergic neurons in the dorsal raphe nucleusvia MT_1 receptors, similarly to acute injection of classical antidepressants such as selective serotonin reuptake inhibitors (SSRIs) [41]. Of note, selected SSRIs can also inhibit VTA DA firing activity [42]. The presence of MT_1 receptor mRNA and protein has been reported in the VTA [43,44]. The VTA receives inputs from many different brain areas containing MT_1 receptors [44] including other midbrain nuclei (such as the dorsal raphe nucleus) as well as the prefrontal cortex. The observed inhibitory effect of UCM871 upon VTA DA neurons could thus be the result of 1) the activation of MT_1 receptors present on VTA DA neurons (direct effect), 2) the activation of MT_1 receptors present in brain regions modulating the activity of the VTA DA neurons (indirect effect), or very likely 3) both direct and indirect effects. Future experiments, e.g. involving selective knockdown of MT_1 receptors on VTA DA neurons or lesions of the neural pathways connecting the VTA with other brain regions, will help addressing this point. In the perspective of moving further the drug discovery process, the identification of the binding site on the receptor is essential, as it may drive the development of other ligands with better affinity and efficacy. In our study, NMR structural data clearly indicate that the binding of UCM871 to MT_1 receptors is principally modulated by the aniline ring and the phenylbutyl side chain and is further stabilized by the acetamide group. Accordingly, the three-dimensional docking model shows that the ligand is placed in the orthosteric

binding site formed by TM3, TM5, TM6, and TM7. UCM871 binding to MT_1 receptors is further stabilized by additional contacts between the ligand and the MT_1 residues located in the TM2 and TM4 helices and in the extracellular loop. Overall, these data show that the MT_1 activation mechanism upon binding to UCM871 is driven by structural changes involving mainly the cytoplasmatic side of the receptor. As reviewed by Comai et al. [45], MT_1 activation may initiate a cascade of intracellular signaling events, including the stimulation of adenylyl cyclase with subsequent increase in the production of cyclic adenosine monophosphate (cAMP), activation of protein kinase A (PKA), and thus phosphorylation of various downstream targets, stimulation of the phospholipase C (PLC) pathway, leading to inositol 1,4,5-trisphosphate (IP3) formation triggering the release of intracellular Ca^{2+} stores, and to diacylglycerol (DAG) which activates protein kinase C (PKC), further influencing cellular responses. Future works should further clarify the intracellular mechanisms modulated by UCM871 that may be involved in its antimanic-like activity, as some of the signaling pathways downstream of MT_1 receptors including the Akt and the MAPK/ERK signaling pathways were shown to be dysregulated in individuals with bipolar disorder [46–48].

The finding that MLT levels in carriers of the wild type allele of rs2165666 of the MTNR1A gene were associated with the number of severe manic but not depressive episodes deserves some comments, although in general both candidate genes [49] and genome-wide studies [50] did not find statistically significant associations between polymorphisms within the MTNR1A gene and the risk of BD. However, there is evidence suggesting that patients with an increased number of hospitalizations have a higher genetic loading for BD [51]. Even if it was not possible to extrapolate data on genetic variants of MTNR1A in this polygenic risk score associated with higher severity of clinical course, it is important to note that hospitalizations might occur also during severe depressive phases. Indeed, several data point to an involvement of MTNR1A genetic and epigenetic variation in this mood polarity. First, the genome-wide association study of Demirkan et al. [52] showed that the rs713224 SNP, located near MTNR1A gene, was associated with the somatic complaints domain of depression symptoms although with borderline genome-wide significance. Furthermore, Lesicka et al. [53] showed that depressed patients with BD, as well as with unipolar depression, had a significantly low methylation index of MTNR1A and that this pattern was associated also with familial loading. Finally, levels of salivary MLT assessed with dim light MLT onset procedure, moderated with total sleep time and sleep onset latency the relationship between impulsivity and mood symptoms in a cohort of patients at risk for BD. The latter finding is intriguing given our results of an association between MLT levels, impulsivity, and genetic variation within the MTNR1A gene.

In summary, while preclinical data are straightforward in showing the link between MT_1 receptors and BD treatment, clinical data appear still preliminary but point toward alterations of the MLT pathway in severe mood phases of BD. However, future research should demonstrate more in detail the mechanisms underlying the effect of UCM871 in BD, and more in general the link between MT_1 receptors and BD. As previously mentioned, a key factor for BD psychopathology is a dysfunction of the circadian system [6] which is regulated by the SCN under the influence of MLT via MT_1 and MT_2 receptors [11]. Notably, improving biological rhythm disturbances seems to be promising for the treatment of BD [54].

The present findings in ClockΔ19 mice do not allow establishing whether the reversal of the manic phenotype of the animals may be driven by/secondary to a physiological reinstatement of their altered circadian rhythms, but given the important role of MT_1 receptors in modulating the activity of the SCN [55], future work should explore this possibility. It is conceivable that subgroups of patients with BD more genetically predisposed to circadian disruptions, possibly of genetic origin, exist. In such subgroups, the pharmacological modulation of MT_1 receptors might be particularly effective. Should the present findings be replicated in independent cohorts and new sets of experiments, they would prove the validity of such a precision psychiatry approach in BD.

Several limitations should be considered in interpreting our findings. The clinical arm of the study was performed in a small Sardinian sample of patients diagnosed with BD. However, this cohort has been deep-phenotyped and data were collected toward a systematic prospective longitudinal follow up as detailed elsewhere [15].

Collectively, this work paves the way for the drug discovery process of MT_1 receptors ligands for the treatment of BD.

Ethics approval

Experiments in animals were conducted in compliance with the guidelines of and approved by the institutional Animal Care and Use Committee of the San Raffaele Scientific Institute and by the Italian Ministry of Health (IACUC permission number: 970). The study in patients with BD was approved by the Ethics Committee of the University Hospital Agency of Cagliari: PG/2019/6277) in compliance with the current revision of the Declaration of Helsinki and the current EU regulations for the protection of privacy.

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CRediT authorship contribution statement

MM and SC had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: MM, SC. Acquisition, analysis, or interpretation of data: MTM, CP, LR, CA, MC, SDG, AS, LC, EM, FS, MG, BG, VP, MNI, IP, GS, LA,CC, DA, DDG, SN, SDA, SS, FS, PP, FP. Drafting of the manuscript: MTM, CP, LR, MM, SC. Critical revision of the manuscript for important intellectual content: MTM, CP, LR, MC, GG, FV, BC, MM, SC. Statistical analysis: MTM, CP. Obtained funding: SC, LR, MM. Supervision: MM and SC.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

All data associated with this study are available upon reasonable request to the corresponding authors (SC: stefano.comai@unipd.it; MM: mirkomanchia@unica.it). A material transfer agreement will be required for the sharing of materials.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.106993.

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