Dysfunction of serotonergic activity and emotional responses across the light-dark cycle in mice lacking MT_2 receptors.

Running title: MT_2 receptors and emotions

Stefano Comai[1,2], Danilo De Gregorio[1,#], Luca Posa[1,#], Rafael Ochoa-Sanchez[1], Annalida Bedini[3], Gabriella Gobbi[1,*]

[1]Neurobiological Psychiatry Unit, Department of Psychiatry, McGill University and McGill University Health Center, Montréal, QC, Canada

[2]Division of Neuroscience, San Raffaele Scientific Institute and Vita-Salute University, Milan, Italy;

[3]Department of Biomolecular Sciences, University Carlo Bo, 61029 Urbino, Italy.

[#] these authors have equally contributed to this study.

***Address correspondence to:**
Gabriella Gobbi, MD, PhD
Neurobiological Psychiatry Unit, Room 220
Irving Ludmer Psychiatry Research and Training Building
1033 Pine Avenue West, McGill University, Montreal, PQ, Canada H3A 1A1
Tel. No. +1-514-398-1290; Fax No. +1-514-398-4866
Email. gabriella.gobbi@mcgill.ca

Abstract

Melatonin (MLT) levels fluctuate according to the external light/dark cycle in both diurnal and nocturnal mammals. We previously demonstrated that MT_2 receptor knockout ($MT_2^{-/-}$) mice show a decreased non-rapid eye sleep over 24-hr and increased wakefulness during the inactive (light) phase. Here, we investigated the role of MT_2 receptors in physiological light/dark cycle fluctuations in the activity of dorsal raphe nucleus (DRN) serotonin (5-HT) neurons and anxiety- and depression-like behaviour. We found that the 5-HT burst-firing activity was tonically reduced across the whole 24-hr in $MT_2^{-/-}$ mice. Importantly, the physiological changes in the spontaneous firing activity of DRN 5-HT neurons during the light/dark cycle were nullified in $MT_2^{-/-}$ mice, with an higher DRN 5-HT neural firing activity during the light phase in $MT_2^{-/-}$ mice. The role of MT_2 receptors over DRN 5-HT neurons was confirmed by acute pharmacological studies in which the selective MT_2 receptors agonist UCM1014 dose-dependently inhibited DRN 5-HT activity, mostly during the dark phase. Compared with WT, $MT_2^{-/-}$ mice displayed an anxiety-like phenotype in the novelty suppressed feeding and in the light/dark box tests; while anxiety levels in the light/dark box test were lower during the dark than the light phase in WT mice, the opposite was seen in $MT_2^{-/-}$ mice. No differences were observed for depression-like behavior in the forced swim and in the sucrose preference tests in $MT_2^{-/-}$ mice. These results suggest that MT_2 receptor genetic inactivation impacts 5-HT neurotransmission and interferes with anxiety levels by perturbing the light/dark pattern.

Keywords: MT_2 receptors, melatonin, light/dark cycle, serotonin, anxiety, UCM1014.

1 INTRODUCTION

Serotonin (5-HT) is a neurotransmitter strongly implicated in the pathophysiology of several psychiatric disorders (e.g. mood and anxiety disorders) and comorbidities including aggressive behavior and suicide,[1-5] and a direct/indirect target of anxiolytic and antidepressant drugs.[6] 5-HT is not only degraded to 5-hydroxyindoleacetic acid, but by the action of the 5-HT N-acetyltransferase and 5-hydroxyindole-O-methyltransferase can be transformed into melatonin (MLT).[7-9]

MLT, a hormone synthesized by the pineal gland during the dark phase of the light/dark cycle, is involved in the regulation of mood, sleep, pain, immune system, circadian rhythms, and cardiovascular function.[10-12] The effects of MLT are mostly mediated by the activation of two high-affinity G-protein coupled receptors, MT_1 and MT_2.[7,8] Their selective role in brain function is still matter of active research.[10,13-15] Using knockout mice for MT_1, MT_2 and both MT_1/MT_2 receptors, and selective MT_2 receptor agonists/antagonists, during the last decade we have demonstrated that: 1) MT_1 and MT_2 receptors have opposite roles in the regulation of the non-rapid movement (NREM) sleep[15-17] and temperature;[18] 2) the blockage of MT_1 receptors is involved in the pathophysiology of depression,[13] and in REM sleep regulation;[15] 3) activation of MT_2 receptors by selective MT_2 partial agonists produces -at high doses- a hypnotic effect,[16,17] while -at low doses- analgesic[19,20] and anxiolytic[21] effects. Other groups have suggested that the two receptors have opposite role in blood vessel function[22] and that MT_2 receptors are likely involved in depression,[23,24] depression and anxiety,[14] and hippocampal LTP maintenance.[25,26] The circulating levels of MLT peak at night and are low during the day,[12] and the expression of MLT receptors, particularly MT_2 receptors,[27] varies according to the time of the day. The activity of 5-HT neurons, which originate in the dorsal raphe nucleus (DRN), also undergoes daily fluctuations

according to the light/dark cycle,[28] and interestingly, MLT is one of the modulators[28] due to the presence of MT_1 receptors directly on DRN 5-HT neurons, and of MT_2 receptors in other neuronal populations that project to DRN 5-HT neurons.[29,30] Indeed, daily fluctuations in the DRN 5-HT activity is abolished in MT_1 receptor knockout ($MT_1^{-/-}$) mice, and in these mice, 5-HT burst-firing activity is overall reduced during the entire 12:12 hr light/dark cycle.[13] These light/dark dysfunctions in 5-HT activity in $MT_1^{-/-}$ mice were associated with abnormal light/dark phase fluctuations in depression and anxiety levels,[13] and in the duration of REM sleep.[15] Pharmacological responses upon selective activation of MT_2 receptors are also dependent on the time of the day.[10,11,16,17] Indeed, the selective MT_2 partial agonists UCM765 and UCM924 enhance NREM sleep only during the light phase.

To date, an extensive neurophysiological characterization of MT_2 receptor knockout ($MT_2^{-/-}$) mice, accounting for the possible effect of the light/dark cycle along with the behavioral consequences, has not yet been performed. To fill this gap, here we therefore studied in $MT_2^{-/-}$ mice, during both the light and the dark phases of the 12:12 hr light/dark cycle: 1) the 5-HT neurotransmission by using *in-vivo* electrophysiology, and 2) their behavioral phenotype in animal paradigms of anxiety and depression. This comprehensive analysis will allow us to gain further insights on how MT_2 receptors regulate brain function across 24-hr, and the possible consequences for future drug discovery projects in neuropsychopharmacology.

2 MATERIALS AND METHOD

2.1 Animals

C3H/HeN $MT_2^{-/-}$ mice[31] (*Mtnr1b*^{tm1Drw}) were purchased from The Jackson Laboratory (Bar Harbor, Maine, US), and the colony was maintained in our animal housing facility at McGill University. C3H/HeN $MT_2^{-/-}$ mice were crossed with C3H/HeN mice and a colony with $MT_2^{+/+}$, $MT_2^{+/-}$, $MT_2^{+/+}$ mice was obtained. Experiments were conducted in adult (2-4-month-old) male C3H/HeN $MT_2^{-/-}$ mice (mean weight (n=10): 30.1± 2.7 g, mean±SD) and their wild-type littermates (mean weight (n=10): 31.2± 2.2 g) at the 3-4 generation. Mice were subjected to a 12-h light/dark cycle (lights ON at 07:30AM and OFF at 07:30PM) and housed in groups of 4-5 animals per cage at a temperature of 20±2°C with food and water ad libitum. Experiments were performed during both light (2:00-5:00 PM) and dark (2:00-5:00 AM) phases. The experiments were approved by the Animal Care Committee of McGill University and were performed following the guidelines of the Canadian Council on Animal Care.

2.2 Electrophysiology

In vivo single-unit extracellular recordings of DRN 5-HT neurons were performed following a well-validated procedure in our lab.[13,32-35] Mice used for electrophysiology experiments did not undergo any previous behavioral experiment. Recordings were performed in seven WT and four $MT_2^{-/-}$ mice during the light phase and four WT and four $MT_2^{-/-}$ mice during the dark phase. Mice were anesthetised with chloral hydrate (400 mg/kg, 2% solution, i.p.), and the anaesthesia was confirmed by the absence of nociceptive reflex reaction to a tail or paw pinch and lack of an eye

blink response to pressure. Additional doses of chloral hydrate (100 mg/kg, i.p.) were used during the experiment to maintain anaesthesia. DRN 5-HT firing activity was measured with a single-barreled glass micropipette (Harvard Apparatus, Quebec, Canada) pulled on a Narashige (Tokyo, Japan) PE-2 pipette puller to reach an impedance ranged from 4 to 8 MΩ, and filled with 2% Pontamine Sky Blue solution in 2 M NaCl. The stereotaxic brain coordinates of DRN were chosen according with the Paxinos and Franklin mouse brain atlas:[36] 0.5–1.0 mm posterior to the interaural line on the midline, 2–3 mm from the brain surface. After the mouse was anesthetized, it was placed into a stereotaxic frame and an hole drilled through the skull above the DRN. Using a hydraulic micropositioner (David Kopf Instruments, USA), the electrode was then inserted slowly into the brain. The spontaneous firing activity of single DRN 5-HT neurons was recorded using an MDA-3 amplifier system (Bak Electronics, MD, USA). The analog signal was converted into a digital signal via a 1401 Plus interface (CED, Cambridge, UK). The analysis was conducted off-line using the Spike2 software version 5.20 (CED, Cambridge, UK). DRN 5-HT neurons were recognized according to their slow (0.1–4 Hz) and prominently regular firing rate (coefficient of variation (COV) range: 0.12-0.87), and their broad biphasic (positive–negative) or triphasic waveforms (0.8-3.5 ms; 1.4 ms first positive and negative deflections). We have determined DRN 5-HT mean-firing (Hz) and burst-firing activities per genotype and phase of the day. 5-HT burst-firing activity was defined as a train of at least two spikes with an initial interspike interval (ISI)≤20 ms and a maximum ISI of 40 ms, and analyzed using a Spike2 script.[13,32] The site of recording was histologically verified by injecting iontophoretically, after the recording of the last neuron, a constant negative current of 27μA for 5 min through the recording pipette.

2.4 Pharmacological treatment

During both light and dark phases, we tested in WT mice the acute pharmacological effects of MT_2 receptors activation or inhibition upon DRN 5-HT firing activity by performing dose-response curves with cumulative doses (intraperitoneal (i.p.) injections) of either the potent and selective MT_2 receptors full agonist UCM1014 (5-30 mg/kg; 1 neuron per mouse with a total of 5 mice/phase of the day)[37] or the selective MT_2 receptors antagonist 4-phenyl-2-propionamidotetralin (4P-PDOT; 5-20 mg/kg; 1 neuron per mouse with a total of 3-4 mice/phase of the day). The drugs were dissolved in 70% DMSO/30% 0.9%NaCl solution and injected in a volume of 0.1 mL. The doses of both compounds were chosen according to previous experiments.[16-21] 8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT, 10 μg/kg) and WAY100,635 maleate (WAY, 0.3 mg/kg)(Sigma-Aldrich, Oakville, Canada) were dissolved in a 0.9%NaCl solution and used to 5-HT_{1A} receptor responsiveness of DRN 5-HT neurons.

2.3 Behavior

For each genotype and phase of the day, 10 mice per group were used. One group of animals per phase of the day and genotype underwent the forced swim test, a second group was used in the novelty suppressed feeding test, and finally a third group was used in the sucrose preference and the light-dark box tests. In the third group, the light/dark box test was performed 7 days after the sucrose preference test to minimize the stress and mood effects of multiple testing. The animals were habituated to the behavioral room by moving them in 1-h before the behavioral testing. After each mouse completed the behavioral session, the apparatus was cleaned with a 70% alcohol solution and carefully dried with paper towel. Behavioral experiments were recorded and analyzed using an automated tracking system (Videotrack, View Point Life Science, Montreal, Canada)

equipped with infrared lighting-sensitive CCD cameras. Concerning the lighting, we used the same conditions previously employed for light/dark experiments in $MT_1^{-/-}$ mice[13] (light phase: standard room lighting (350 lx) and a white lamp (100 W); dark phase: infrared light-emitting diodes and a red light bulb lamp (8 lux)).

2.4 Forced swim test (FST)

Mice were individually placed for 6-min in a Plexiglas cylinder (20 cm diameter, 50 cm high) filled with 20 cm of water at 25°C. We then calculated the duration of immobility during the last 4-min of the test.[38]

2.5 Novelty-suppressed feeding test (NSFT)

After 48-h of food-deprivation, mice were individually placed in the corner of an open arena (40×40×30 cm) they never explored before, in which 3 pellets of lab chow were present in the center. We then measured the latency to initiate feeding and afterwards, the mouse was returned to its home cage in which we put 3 pellets of food the center. The latency to feed in the home cage as well as the amount of food eaten in 5 min were also measured.[13,33]

2.6 Sucrose preference test

The sucrose preference test was performed according to the protocol we used in $MT_1^{-/-}$ mice.[13] Each mouse was singularly housed for 3 days and then trained for 3 days to drink water from two

bottles. During these 3 days (habituation phase), we changed the two bottles with water with two bottles containing a 2% (w/v) sucrose solution for 1-h a day. Next, the experimental phase lasting 48-h took place. During this 48-hr period, mice were left free to drink from 2 bottles, one containing water and the other one the 2% sucrose solution. The position of these 2 bottles was inverted at the middle of the light and dark phases to prevent conditioned place preference learning. Moreover, at the beginning of the light and dark phases, we weighed the two bottles to determine the sucrose preference during each phase of the light/dark cycle. The sucrose preference (%) was determined as sucrose solution intake (g)/total fluid intake (g, water+sucrose solution)×100.

2.7 Light/dark box

For the light-dark box test we used an apparatus consisting of a light, open topped, opaque, plexiglas box (27×27×30 cm) connected to a dark, closed topped, opaque, plexiglas box (18×27×30 cm). The animals were placed in the illuminated box and left free to move from the light to the dark compartment through a connecting door (12×5 cm) for 5 min. The light box was illuminated by a 100 W desk lamp. A mouse was considered in the light compartment if the four paws were all in the light box. The following measures were collected: 1) latency to move to the dark compartment, 2) time spent in the light compartment of the apparatus as a measure of anxiety-like behavior, and 2) number of transitions between the light and the dark compartments, a measure related with activity-exploration.[39] The number of transitions in the light/dark box test is an index of activity-exploration but it may be strongly influenced by the levels of anxiety meaning that an anxious animal will spent most of the time in the dark/"safer" compartment without going into the light/more aversive compartment.

2.8 Locomotor activity

Mice were singularly placed in an open arena (40×40×30 cm) and left free to explore it for 30 min. The total distance travelled during 30 min was measured.

2.9 Statistical analysis

Statistical analysis was conducted using SigmaPlot 11 (San Jose, California) and IBM SPSS Statistics 24.0 (IBM, Armonk, NY, USA). Data were indicated as mean±S.E.M. We performed two-way ANOVA or two-way ANOVA for repeated measures (factors genotype and phase of the day) followed by Bonferroni post hoc comparisons. Differences between WT and $MT_2^{-/-}$ mice in the number of DRN 5-HT burst-firing neurons were calculated using Pearson's chi-square test. Similarly to our previous studies,[13,33] we used *K*-means cluster analysis to determine the clusters of DRN 5-HT neurons with low and high firing activities in WT and $MT_2^{-/-}$ mice during the light and the dark phases. Statistically significant difference was accepted for $P<0.05$.

3 RESULTS

3.1 In-vivo electrophysiology

3.1.1 DRN 5-HT firing activity in $MT_2^{-/-}$ mice across the light/dark cycle

As indicated in Figure 1A, spontaneous firing activity of DRN 5-HT neurons was significantly higher in $MT_2^{-/-}$ than in WT mice (P=0.023; interaction phase of the day x genotype: ($F_{1,238}$)=4.79, P=0.030; genotype: ($F_{1,238}$)=0.42, P= 0.518; phase of the day: ($F_{1,238}$)=0.51, P=0.476) during the light phase, while no difference was present during the dark phase. Of interest, while as expected, the activity of DRN 5-HT was higher during the dark than during the light phase in WT mice (P=0.042), no phase difference was found in the 5-HT firing activity in $MT_2^{-/-}$ mice. The comparisons between the number of DRN 5-HT burst- and non-burst-firing neurons among genotypes and phase of the day revealed a significant overall difference (Figure 1B; chi-square=8.13, P=0.043). During the dark phase, the number of 5-HT burst-firing compared with that of 5-HT non burst-firing neurons was lower in $MT_2^{-/-}$ than in WT mice (chi-square=4.59, P=0.032). During the light phase, a tendency to a reduced number of 5-HT burst-firing versus non burst-firing neurons was seen in $MT_2^{-/-}$ than in WT mice (chi-square=3.36, P=0.067). No analysis of the burst-firing parameters could be performed due to the low number of burst-firing neurons recorded in $MT_2^{-/-}$ mice. The global decrease in the burst activity suggest a decrease in 5-HT release in post-synaptic areas such as the prefrontal cortex or hippocampus.[6,40]

Figure 1C reports the example of an integrated histogram of spontaneous firing rate of one neuron recorded in a WT (left side) and a $MT_2^{-/-}$ (right side) mouse during the light phase. Figure 1D shows the typical wave-form of a DRN 5-HT neuron, and Figures 1E-F report the illustrative

depiction of the recording site of the two neurons shown in Figure 1C. Figure 1G shows the photomicrograph of the recording site of the WT DRN 5-HT neuron represented in Figure 1C. According to our previous study in $MT_1^{-/-}$[13] and in $FAHH^{-/-}$[33] mice, we could detect the clusters of DRN 5-HT neurons with low- and high-firing activities for each genotype and phase of the day using a *K*-means cluster analysis (Fig. 1 H,I). Concerning the subgroup of DRN 5-HT neurons with low-firing activity, we did not find any difference in their firing activity (Fig. 1H; interaction phase of the day x genotype: ($F_{1,164}$)=1.21, P=0.274; genotype: ($F_{1,164}$)=2.16, P=0.144; phase of the day: ($F_{1,164}$)=3.48, P=0.064). In contrast, the neural activity of DRN 5-HT neurons belonging to the high-firing subgroup (Fig. 1I; interaction phase of the day x genotype: ($F_{1,70}$)=21.41, P<0.001; genotype: ($F_{1,70}$)=3.25, P=0.076; phase of the day: ($F_{1,70}$)=2.77, P=0.101) was greater during the dark than during the light phase in WT mice (P<0.001), whereas the opposite was seen in $MT_2^{-/-}$ mice with higher activity during the light than during the dark phases (P=0.018; Fig. 1I). Moreover, the neural activity of DRN 5-HT neurons belonging to the high-firing subgroup is higher in $MT_2^{-/-}$ than in WT mice during the light phase (P=0.022), whereas is lower in $MT_2^{-/-}$ than in WT mice during the dark phase (P<0.001).

3.1.2 Acute selective MT_2 receptors activation inhibits DRN 5-HT firing activity in a phase of the day dependent manner

UCM1014 induces a dose-dependent inhibitory effect on DRN 5-HT neurons (Figure 2A-C; interaction phase of the day x treatment: ($F_{5,40}$)=2.45, P=0.0497; treatment: ($F_{5,40}$)=31.35, P<0.0001; phase of the day: ($F_{1,8}$)=6.98, P=0.0296), meaning that the MT_2 agonist induces a phasic inhibition of the DRN 5-HT neurons. The inhibitory effect upon DRN 5-HT activity of 10 mg/kg UCM1014 is greater during the dark than during the light phase (Figure 2B, P<0.01). Finally,

UCM1014 at the dose of 20 mg/kg during the dark phase and of 30 mg/kg during the light phase completely silenced DRN 5-HT firing activity (Figure 2B). The injections of WAY following 5-HT silencing by _UCM1014 induced a slight recovery of DRN 5-HT firing activity (Figure 2C).

3.1.3 Acute selective inhibition of MT_2 receptors does not affect DRN 5-HT firing activity

Fig. 2E illustrates the integrated histogram of a DRN 5-HT neuron upon injections of 4P-PDOT during the light and the dark phase, respectively. Acute inhibition of MT_2 receptors using the potent and selective MT_2 receptor antagonist 4P-PDOT did not influence the activity of DRN 5-HT neurons during both light and dark phases (Figure 2D; 4P-PDOT treatment: ($F_{4,16}$)=1.42, P=0.272; phase of the day: ($F_{1,16}$)=0.230, P=0.651), meaning that in physiological conditions there is not a MT_2 receptor tonic activity into the DRN. As expected, the subsequent injection of the 5-HT_{1A} agonist 8-OH-DPAT (10 μg/kg) led to the inhibition of DRN 5-HT firing activity (Figure 2D,E).

3.2 Behavior

3.2.1 Depressive-like behavior and sucrose preference in $MT_2^{-/-}$ mice across the light/dark cycle

In the FST, there was no difference between WT and $MT_2^{-/-}$ mice in the time the animals spent immobile (Figure 3A) or swimming (data not shown). The duration of immobility was greater during the light than during the dark phase in both WT and $MT_2^{-/-}$ mice (interaction phase of the

day x genotype: ($F_{1,36}$)=2.07, P=0.159; genotype: ($F_{1,36}$)=1.38, P=0.248; phase of the day: ($F_{1,36}$)=77.42, P<0.001). In the SPT (Figure 3B), we found no interaction between genotype and phase of the day ($F_{1,15}$)=0.01, P=0.921, no effect of genotype ($F_{1,15}$)=0.26, P=0.620) and an effect of the phase of the day ($F_{1,15}$)=48.29, P<0.001, with mice having an higher preference for the sucrose solution during the dark phase. Altogether, this data suggests that the genetic inactivation of the MT_2 receptors does not produce any change in the depressive-like behavior.

3.2.2 $MT_2^{-/-}$ mice show increased anxiety-like behavior across the light/dark cycle

In the NSFT (Figure 4A), a paradigm used to assess anxiety-like behavior but also sensitive to chronic antidepressant treatment,[41] $MT_2^{-/-}$ mice compared to WT controls displayed a longer latency to feed in the novel environment (P=0.010), and the latencies were shorter in the dark than in the light phase (P<0.001) (interaction phase of the day x genotype: ($F_{1,36}$)=2.61, P=0.115; genotype: ($F_{1,36}$)=7.44, P=0.010; phase of the day: ($F_{1,36}$)=16.75, P<0.001). No difference between genotypes was detected in the latency to eat in the familiar environment (Figure 4B) as well as in the amount of food eaten during 5 min (e.g. during the light phase: 0.28±0.02 g for WT vs 0.29±0.02 g for $MT_2^{-/-}$). In the light/dark box test (Figure 4C), the number of transitions between the light and the dark compartment was higher in $MT_2^{-/-}$ than in WT mice during the dark phase (P<0.001), and no differences were observed during the light phase (interaction phase of the day x genotype: ($F_{1,36}$)=12.60, P=0.001; genotype: ($F_{1,36}$)=11.26, P=0.002; phase of the day: ($F_{1,36}$)=0.85, P=0.362). The number of transitions was higher during the light than during the dark phase in WT mice (P=0.003), and no phase of the day difference was seen in $MT_2^{-/-}$ mice. Concerning the duration spent by the animals in the light compartment (Figure 4D; interaction

phase of the day x genotype: ($F_{1,36}$)=20.39, P<0.001; genotype: ($F_{1,36}$)=7.67, P=0.009; phase of the day: ($F_{1,36}$)=0.34, P=0.562), we found that WT animals spent more time in the light compartment during the dark than during the light phase (*P=0.009*) while in contrast, $MT_2^{-/-}$ mice displayed an opposite behavior with longer time in the light compartment during the light than during the dark phase (*P<0.001*). In addition, no difference between genotypes was found during the light phase, but during the dark phase, $MT_2^{-/-}$ mice were in the light compartment less than WT (P<0.001). Finally, the latency to enter the dark/"safer" compartment (Figure 4E; interaction phase of the day x genotype: ($F_{1,36}$)=6.99, P=0.012; genotype: ($F_{1,36}$)=53.19, P<0.001; phase of the day: ($F_{1,36}$)=15.29, P<0.001) was shorter in $MT_2^{-/-}$ than in WT mice during both light (*P=0.002*) and dark (*P<0.001*) phase, and while the latency was longer in WT animals during the dark than during the light phase (*P<0.001*), no light/dark differences were seen in $MT_2^{-/-}$ mice. We also measured the distance travelled by WT and $MT_2^{-/-}$ mice during both light and dark phases and we did not find any difference between genotypes but, as expected, mice covered a longer distance during the dark than during the light phase (Figure 4F; interaction phase of the day x genotype: ($F_{1,36}$)=0.46, P=0.50; genotype: ($F_{1,36}$)=0.01., P=0.932; phase of the day: ($F_{1,36}$)=28.07, P<0.001). These data suggest that MT_2 inactivation induces an anxiety like-behavior by disrupting the light-dark cycle of the physiological levels of anxiety.

4 DISCUSSION

The DRN 5-HT neural activity and the behavioral phenotype of $MT_2^{-/-}$ mice were examined here across the light-dark cycle given the physiological changes occurring within the MLT system during the 24-hr light-dark cycle.[11,12,27] We found that the 5-HT burst-firing activity is globally reduced across the 24-hr, suggesting a decrease in the release of 5-HT and likely in the efficiency of the 5-HT signal transmission in putative areas controlling emotions such as the prefrontal cortex and the hippocampus.[6,40] Moreover, the light-dark activity fluctuations of the DRN 5-HT neurons are abolished in $MT_2^{-/-}$ mice, especially in the DRN 5-HT spontaneous firing activity in $MT_2^{-/-}$ mice and when considering their low- or high-firing activity, with the latter more sensitive to the light/dark cycle. We have previously showed that the response of DRN 5-HT neurons to different pharmacological challenges may vary according to their high- or low-firing activity.[33] 5-HT firing, especially the burst activity, represents a common neurobiological substrate in depression and anxiety,[42] and it is a biomarker of depressive-anxiety behavior in knockout mice and humans.[43] Interesting, our data show that at night (active phase) $MT_2^{-/-}$ mice have less 5-HT burst activity and increase anxiety at the light/dark box test. Interestingly, we had previously demonstrated that genetic inactivation of MT_1 receptor also reduces 5-HT burst-firing activity while leaving 5-HT mean firing activity unchanged.[13] Consequently, one may hypothesize that both MT_1 and MT_2 receptors have a control over the 5-HT release. Unlike MT_1 receptors, we did not find the presence of MT_2 receptors on DRN 5-HT neurons.[30] Consequently, the modulatory effects of MT_2 receptors upon 5-HT neurons are probably indirect and mediated by the presence of this MLT receptor subtype on neural pathways projecting to the DRN as example from the prefrontal cortex and/or subcortical regions.[30] DRN 5-HT activity varies according to the stage of the sleep-wake cycle being higher during wakefulness, low throughout NREM sleep, and silent in the course of REM

sleep (for a review see Atkin et al.[44]). Accordingly, we found that $MT_2^{-/-}$ mice are more awake than WT during the light phase[15] in which they also display higher 5-HT activity than WT.

The important modulatory activity of MT_2 receptors over DRN 5-HT neurons was also demonstrated by experiment with selective MT_2 receptors ligands namely the selective MT_2 agonist UCM1014 and the antagonist 4P-PDOT. Acute activation of MT_2 receptors led a dose-dependent inhibitory activity of DRN 5-HT neurons similar to the acute effect elicited by currently used antidepressants/anxiolytic drugs including selective serotonin reuptake inhibitors (SSRIs).[6] Importantly, the sensitivity of 5-HT neurons to the effects of the drug was phase of the day dependent, with a higher inhibitory potency of UCM1014 during the dark phase. This novel finding thus suggests that the anxiolytic effects of the MT_2 partial agonist UCM765 and of MLT through MT_2 receptors[21] are at least in part occurring through the indirect modulation of 5-HT neuronal activity. Since $MT_2^{-/-}$ mice displayed increased DRN 5-HT activity during the light phase, we have hypothesized a similar effect upon the treatment with the MT_2 receptors antagonist 4P-PDOT. In contrast, we found that 4P-PDOT does not alter 5-HT firing activity indicating that, in physiological conditions, there is not a tonic control of the MT_2 receptors over the DRN, but the MT_2 agonist may exert a phasic/acute inhibition of the 5-HT neurons.

The neurophysiological dysfunctions in the 5-HT neurotransmission across the 24-hr in $MT_2^{-/-}$ mice were paralleled by no altered levels of depression in the forced swim test, anhedonia in the sucrose preference test, and locomotor behavior. In contrast, an increase in the levels of anxiety was observed in $MT_2^{-/-}$ mice compared with WT controls in the NSFT and in the light/dark box test, in particular during the dark/active phase.

In keeping, Liu et al.[14] analyzed the behavioral correlates of $MT_1^{-/-}$, $MT_2^{-/-}$, and $MT_1^{-/-}/MT_2^{-/-}$ mice during the light phase only, and found that $MT_2^{-/-}$ mice displayed increased levels of anxiety in the

NSFT and obsessive-compulsive features in the Marble burying test. They also found increased social avoidance, a depressive-like phenotype, and a reduced preference for sucrose. Noseda et al.[23] reported that a substantial antidepressant effect is promoted by the blockade of striatal MT_2 receptors, while Gałecka et al.[45] showed that variability within *MTNR1B*, the gene encoding the MT_2 receptor, is associated with a risk for recurrent depressive disorders. Although further studies are necessary, these results may highlight a possible involvement of MT_2 receptors in depression-like behavior.

We previously demonstrated that the acute and selective pharmacological activation of MT_2 receptors during the light phase with UCM765 yielded to anxiolytic effects as measured by a decreased latency to feed in the NSFT.[21] In agreement, here, we found that in the NSFT and in the light/dark box test, the genetic inactivation of MT_2 receptors induced an anxiogenic effect.

Similarly, we found that $MT_2^{-/-}$ mice display reduced NREM sleep duration during the light phase and across 24-hr,[15,16] and that, on the other hand, the pharmacological activation of MT_2 receptors increases NREM sleep duration during the light phase.[16,17] The increased anxiety paired to the decreased duration of sleep and increased wakefulness during the inactive (light) phase observed in $MT_2^{-/-}$ mice suggest an increased arousal phenotype of these animals in keeping with the high comorbidity between anxiety and sleep disorders observed in humans.[46,47] Therefore, MT_2 receptors might be seen as a common neurobiological denominator of anxiety and insomnia disorder.

In conclusion, our results demonstrate that MT_2 receptors regulate 5-HT activity and are implicated in the control of anxiety rather than depression in a phase of the day-dependent manner. Of importance, these findings provide further evidence on the opposite and/or complementary

function of the two MLT receptor subtypes in brain function[10,11] which probably relies on their different localization within brain circuits regulating emotions.[30]

Overall, MT_2 receptors are thus endowed with great potential for drug development in the psychopharmacology of anxiety.

Acknowledgments

S.C. was supported by a postdoctoral fellowship from the McGill University Health Center. S.C. is supported in part by a 2017 NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation. L.P. was recipient of the Alan Edward Pain Centre scholarship and a fellowship from the FRQS. D.D.G. was supported by a post-doctoral fellowship from the Fonds de la recherché du Quebec en Santé (FRQS). G.G. was supported by FRSQ (clinical scientist salary program), CIHR (MOP130285), CFI (23381), and MERST (PSR-SIIRI-855). G.G. is inventor and assignee in patents regarding novel melatonin MT_2 receptors ligands. Authors thank Mr Matthew Kaba Aboud for assistance in mice colony care and breeding.

Authorship statement

S.C., D.D.G., L.P., R.O.S., G.G. performed the research. S.C. and G.G. designed the research study. S.C., D.D.G., L.P. and R.O.S. analysed the data. AB synthetized the UCM1014, and S.C. and G.G. wrote the paper.

References

1. Manchia M, Carpiniello B, Valtorta F, Comai S. Serotonin Dysfunction, Aggressive Behavior, and Mental Illness: Exploring the Link Using a Dimensional Approach. *ACS chemical neuroscience.* 2017;8(5):961-972.
2. Mann JJ. The serotonergic system in mood disorders and suicidal behaviour. *Philosophical transactions of the Royal Society of London Series B, Biological sciences.* 2013;368(1615):20120537.
3. Lucki I. The spectrum of behaviors influenced by serotonin. *Biol Psychiatry.* 1998;44(3):151-162.
4. Graeff FG, Guimaraes FS, De Andrade TG, Deakin JF. Role of 5-HT in stress, anxiety, and depression. *Pharmacology, biochemistry, and behavior.* 1996;54(1):129-141.
5. Padovan CM. Serotonin in Anxiety and Depression. *Journal of Psychopharmacology.* 2013;27(12):1083-1083.
6. Gobbi G, Blier P. Effect of neurokinin-1 receptor antagonists on serotoninergic, noradrenergic and hippocampal neurons: comparison with antidepressant drugs. *Peptides.* 2005;26(8):1383-1393.
7. Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J. International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, Classification, and Pharmacology of G Protein-Coupled Melatonin Receptors. *Pharmacological Reviews.* 2010;62(3):343-380.
8. Jockers R, Delagrange P, Dubocovich ML, et al. Update on melatonin receptors: IUPHAR Review 20. *Br J Pharmacol.* 2016;173(18):2702-2725.
9. Cecon E, Liu L, Jockers R. Melatonin receptor structures shed new light on melatonin research. *J Pineal Res.* 2019:e12606.
10. Comai S, Gobbi G. Unveiling the role of melatonin MT2 receptors in sleep, anxiety and other neuropsychiatric diseases: a novel target in psychopharmacology. *J Psychiatry Neurosci.* 2014;39(1):6-21.
11. Posa L, De Gregorio D, Gobbi G, Comai S. Targeting melatonin MT2 receptors: A novel pharmacological avenue for inflammatory and neuropathic pain. *Current medicinal chemistry.* 2017.
12. Reiter RJ. Melatonin: clinical relevance. *Best practice & research Clinical endocrinology & metabolism.* 2003;17(2):273-285.
13. Comai S, Ochoa-Sanchez R, Dominguez-Lopez S, Bambico FR, Gobbi G. Melancholic-Like behaviors and circadian neurobiological abnormalities in melatonin MT1 receptor knockout mice. *Int J Neuropsychopharmacol.* 2015;18(3).
14. Liu J, Clough SJ, Dubocovich ML. Role of the MT1 and MT2 melatonin receptors in mediating depressive- and anxiety-like behaviors in C3H/HeN mice. *Genes Brain Behav.* 2017;16(5):546-553.
15. Comai S, Ochoa-Sanchez R, Gobbi G. Sleep-wake characterization of double MT(1)/MT(2) receptor knockout mice and comparison with MT(1) and MT(2) receptor knockout mice. *Behavioural brain research.* 2013;243:231-238.
16. Ochoa-Sanchez R, Comai S, Lacoste B, et al. Promotion of Non-Rapid Eye Movement Sleep and Activation of Reticular Thalamic Neurons by a Novel MT2 Melatonin Receptor Ligand. *JNeurosci.* 2011;31(50):18439-18452.
17. Ochoa-Sanchez R, Comai S, Spadoni G, Bedini A, Tarzia G, Gobbi G. Melatonin, selective and non-selective MT1/MT2 receptors agonists: differential effects on the 24-h vigilance states. *Neurosci Lett.* 2014;561:156-161.

18. Lopez-Canul M, Min SH, Posa L, et al. Melatonin MT1 and MT2 Receptors Exhibit Distinct Effects in the Modulation of Body Temperature across the Light/Dark Cycle. *International journal of molecular sciences.* 2019;20(10).
19. Lopez-Canul M, Comai S, Dominguez-Lopez S, Granados-Soto V, Gobbi G. Antinociceptive properties of selective MT2 melatonin receptor partial agonists. *Eur J Pharmacol.* 2015;764:424-432.
20. Lopez-Canul M, Palazzo E, Dominguez-Lopez S, et al. Selective melatonin MT2 receptor ligands relieve neuropathic pain through modulation of brainstem descending antinociceptive pathways. *Pain.* 2015;156(2):305-317.
21. Ochoa-Sanchez R, Rainer Q, Comai S, et al. Anxiolytic effects of the melatonin MT(2) receptor partial agonist UCM765: Comparison with melatonin and diazepam. *Progress in neuro-psychopharmacology & biological psychiatry.* 2012;39(2):318-325.
22. Doolen S, Krause DN, Dubocovich ML, Duckles SP. Melatonin mediates two distinct responses in vascular smooth muscle. *European Journal of Pharmacology.* 1998;345(1):67-69.
23. Noseda AC, Rodrigues LS, Targa AD, et al. Putative role of monoamines in the antidepressant-like mechanism induced by striatal MT2 blockade. *Behavioural brain research.* 2014;275:136-145.
24. Sumaya IC, Masana MI, Dubocovich ML. The antidepressant-like effect of the melatonin receptor ligand luzindole in mice during forced swimming requires expression of MT2 but not MT1 melatonin receptors. *J Pineal Res.* 2005;39(2):170-177.
25. Larson J, Jessen RE, Uz T, et al. Impaired hippocampal long-term potentiation in melatonin MT2 receptor-deficient mice. *Neuroscience Letters.* 2006;393(1):23-26.
26. Wang LM, Suthana NA, Chaudhury D, Weaver DR, Colwell CS. Melatonin inhibits hippocampal long-term potentiation. *The European journal of neuroscience.* 2005;22(9):2231-2237.
27. Witt-Enderby PA, Bennett J, Jarzynka MJ, Firestine S, Melan MA. Melatonin receptors and their regulation: biochemical and structural mechanisms. *Life Sciences.* 2003;72(20):2183-2198.
28. Dominguez-Lopez S, Mahar I, Bambico FR, et al. Short term effects of melatonin and pinealectomy on serotonergic neuronal activity across the light-dark cycle. *JPsychopharmacol.* 2011.
29. Comai S, Lopez-Canul M, De Gregorio D, et al. Melatonin MT1 receptor as a novel target in neuropsychopharmacology: MT1 ligands, pathophysiological and therapeutic implications, and perspectives. *Pharmacological research : the official journal of the Italian Pharmacological Society.* 2019;144:343-356.
30. Lacoste B, Angeloni D, Dominguez-Lopez S, et al. Anatomical and cellular localization of melatonin MT1 and MT2 receptors in the adult rat brain. *J Pineal Res.* 2015;58(4):397-417.
31. Jin X, von GC, Pieschl RL, et al. Targeted disruption of the mouse Mel(1b) melatonin receptor. *MolCell Biol.* 2003;23(3):1054-1060.
32. Gobbi G, Bambico FR, Mangieri R, et al. Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proceedings of the National Academy of Sciences of the United States of America.* 2005;102(51):18620-18625.
33. Bambico FR, Cassano T, Dominguez-Lopez S, et al. Genetic deletion of fatty acid amide hydrolase alters emotional behavior and serotonergic transmission in the dorsal raphe, prefrontal cortex, and hippocampus. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology.* 2010;35(10):2083-2100.
34. Bambico FR, Nguyen NT, Katz N, Gobbi G. Chronic exposure to cannabinoids during adolescence but not during adulthood impairs emotional behaviour and monoaminergic neurotransmission. *Neurobiology of disease.* 2010;37(3):641-655.

35. Labonte B, McLaughlin RJ, Dominguez-Lopez S, et al. Adolescent amphetamine exposure elicits dose-specific effects on monoaminergic neurotransmission and behaviour in adulthood. *Int J Neuropsychopharmacol.* 2012;15(9):1319-1330.
36. Paxinos G, Franklin K. *The Mouse Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press; 2001.
37. Spadoni G, Bedini A, Lucarini S, et al. Highly Potent and Selective MT2 Melatonin Receptor Full Agonists from Conformational Analysis of 1-Benzyl-2-acylaminomethyl-tetrahydroquinolines. *Journal of medicinal chemistry.* 2015;58(18):7512-7525.
38. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. *Archives internationales de pharmacodynamie et de therapie.* 1977;229(2):327-336.
39. Hascoët M, Bourin M, Dhonnchadha BÁN. The mouse ligth-dark paradigm: a review. *Prog Neuropsychopharmacol Biol Psychiatry.* 2001;25(1):141-166.
40. Gartside SE, Hajos-Korcsok E, Bagdy E, Harsing LG, Jr., Sharp T, Hajos M. Neurochemical and electrophysiological studies on the functional significance of burst firing in serotonergic neurons. *Neuroscience.* 2000;98(2):295-300.
41. Dulawa SC, Hen R. Recent advances in animal models of chronic antidepressant effects: The novelty-induced hypophagia test. *Neuroscience & Biobehavioral Reviews.* 2005;29(4–5):771-783.
42. Blier P, De Montigny C. Current advances and trends in the treatment of depression. *Trends in pharmacological sciences.* 1994;15(7):220-226.
43. Dominguez-Lopez S, Howell R, Gobbi G. Characterization of serotonin neurotransmission in knockout mice: implications for major depression. *Reviews in the neurosciences.* 2012;23(4):429-443.
44. Atkin T, Comai S, Gobbi G. Drugs for Insomnia beyond Benzodiazepines: Pharmacology, Clinical Applications, and Discovery. *Pharmacol Rev.* 2018;70(2):197-245.
45. Galecka E, Szemraj J, Florkowski A, et al. Single nucleotide polymorphisms and mRNA expression for melatonin MT(2) receptor in depression. *Psychiatry research.* 2011;189(3):472-474.
46. Neckelmann D, Mykletun A, Dahl AA. Chronic insomnia as a risk factor for developing anxiety and depression. *Sleep.* 2007;30(7):873-880.
47. van Mill JG, Hoogendijk WJ, Vogelzangs N, van Dyck R, Penninx BW. Insomnia and sleep duration in a large cohort of patients with major depressive disorder and anxiety disorders. *The Journal of clinical psychiatry.* 2010;71(3):239-246.

Figure Legends

Figure 1 Dorsal raphe serotonergic firing activity in WT and $MT_2^{-/-}$ mice across the 12:12 hr light/dark cycle. (A) Mean DRN 5-HT firing activity. Results are given as mean ± S.E.M. **P*<0.05, $MT_2^{-/-}$ vs. WT mice; #*P*<0.05 light vs. dark phase, two-way ANOVA followed by Bonferroni post hoc comparisons. (B) Contingency pie chart showing the number (#) of recorded burst-firing and non-burst-firing DRN 5-HT neurons in WT and $MT_2^{-/-}$ mice during the light and dark phases of the light/dark cycle. (C) Representative histogram of DRN 5-HT spontaneous firing rate of one neuron in WT (left trace) and in $MT_2^{-/-}$ mice (right trace). The time scale (1 min) applies to both traces. (D) Typical wave-form of a DRN 5-HT neuron. (E, F) Representation of coronal sections of the mouse brain[36] with the magnification of DRN; circle indicates the site of registration of the 5-HT neurons in the WT mouse illustrated in Figure 1(C); cross indicates the site of registration of the 5-HT neuron in the $MT_2^{-/-}$ mouse illustrated in Figure 1(C). Aq = Sylvian aqueduct; DRD = dorsal raphe nucleus, dorsal part; DRVL = dorsal raphe nucleus, ventrolateral part, DRV = dorsal raphe nucleus, ventral part, DRI = dorsal raphe nucleus, interfascicular part; mlf = medial longitudinal fasciculus; Me5 = mesencephalic trigeminal nucleus. (G) Representative photomicrograph of the recording site in the DRN of the WT mouse in Figure 1(C). Aq = Sylvian aqueduct. The black arrow indicates the site of the electrode recording labeled with pontamine sky blue dye. (H) Mean firing activity of the DRN 5-HT low firing subgroup. Results are given as mean ± S.E.M. (I) Mean firing activity of the DRN 5-HT high firing subgroup. Results are given as mean ± S.E.M. **P*<0.05, ****P*<0.001, $MT_2^{-/-}$ vs. WT mice; #*P*<0.05, ###*P*<0.001, light vs. dark phase, two-way ANOVA followed by Bonferroni post hoc comparisons.

Figure 2 Effects of MT_2 receptors activation and inhibition on dorsal raphe serotonergic firing activity across the 12:12 hr light/dark cycle.

(A) Representative section of the recording site in the DRN. (B) UCM1014 inhibits DRN 5-HT firing activity in a dose- and phase of the day-dependent manner. Each point of the line represents mean±SEM expressed as percentage of firing rate of baseline. (C) Representative histogram of the effects of UCM1014 on DRN 5-HT spontaneous firing rate of one neuron in the light phase (top trace) and one in the dark phase (bottom trace). WAY partially restores 5-HT firing activity. ***P<0.001 vs. baseline; ##P<0.01, light vs. dark phase, two-way ANOVA followed by Bonferroni post hoc comparisons. (D) 4P-PDOT does not affect DRN 5-HT firing activity. 8-OH-DPAT injected after cumulative doses of 4P-PDOT inhibits 5-HT neural activity. Each point of the line represents mean±SEM expressed as percentage of firing rate of baseline. (E) Representative histogram of the effects of 4P-PDOT on DRN 5-HT spontaneous firing rate of one neuron in the light phase (top trace) and one in the dark phase (bottom trace). 8-OH-DPAT inhibits 5-HT firing activity.

Figure 3 Behavioral phenotype of $MT_2^{-/-}$ mice in the forced swim and sucrose preference tests across the 12:12 hr light/dark cycle. (A) Time of immobility (s) in the FST. (B) Preference for the sucrose over the water solution (%) in the SPT. Results are given as mean ± S.E.M (n= 10 in the FST for both genotypes and phase of the day; n= 16 in WT and n= 15 in $MT_2^{-/-}$ mice in the SPT). **P < 0.01 $MT_2^{-/-}$ vs. WT mice; ### P < 0.001 light vs. dark phase, two-way ANOVA for repeated measures followed by Bonferroni post hoc comparisons.

Figure 4 Phenotype of $MT_2^{-/-}$ mice in the novelty suppressed feeding (NFST) and light/dark box tests across the 12:12 hr light/dark cycle. (A) Latency to feed (s) in the novel and (B) familiar environments in the NSFT. (C) Number of transition between the light and the dark compartment, (D) time spent in the light compartment and (E) latency to enter the dark compartment of the light/dark box test. Results are given as mean ± S.E.M (n= 10). * $P < 0.05$, ***$P < 0.001$ $MT_2^{-/-}$ vs. WT mice; ## $P < 0.01$, ### $P < 0.001$ light vs. dark phase, two-way ANOVA followed by Bonferroni post hoc comparisons.

Figure 1

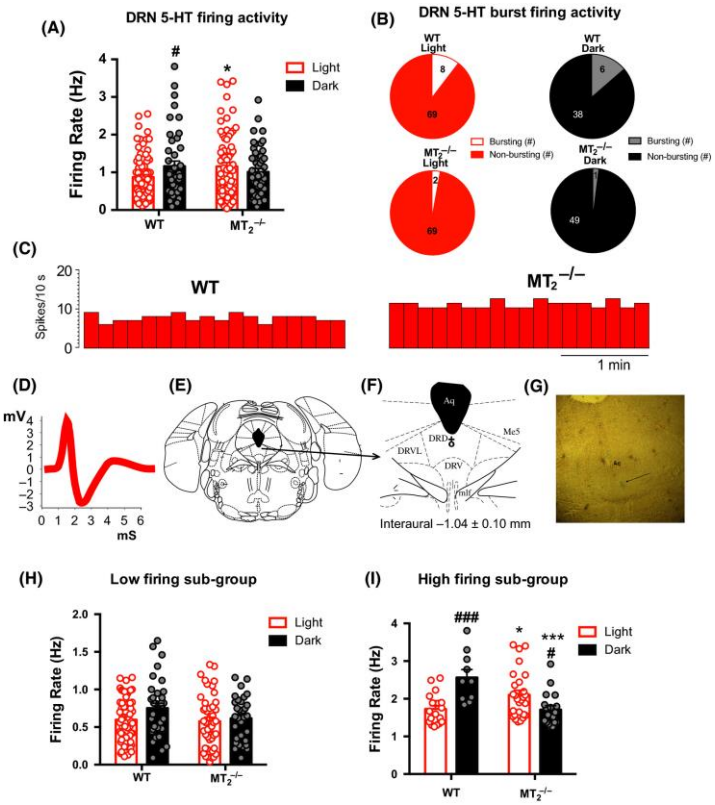


Figure 2.

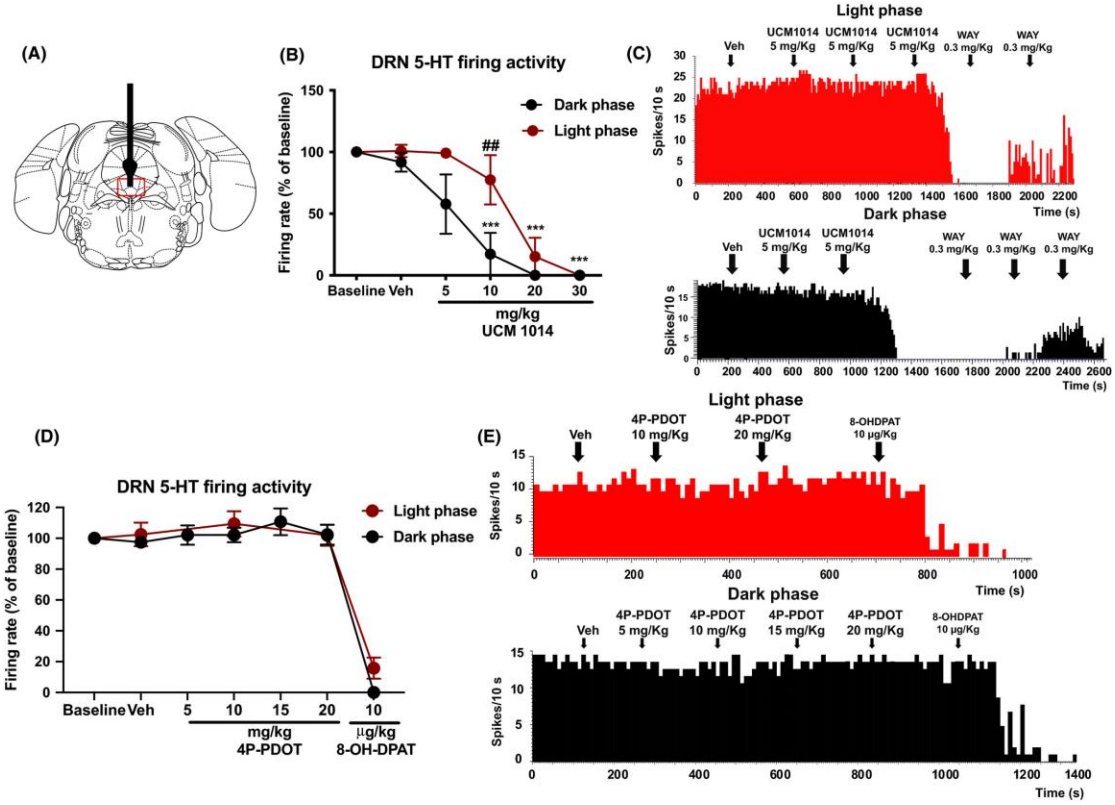


Figure 3.

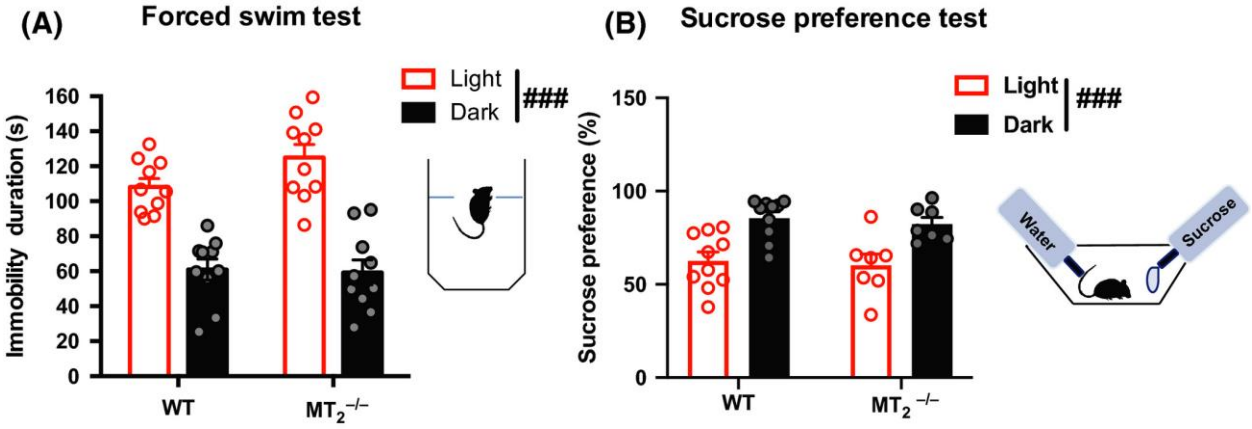
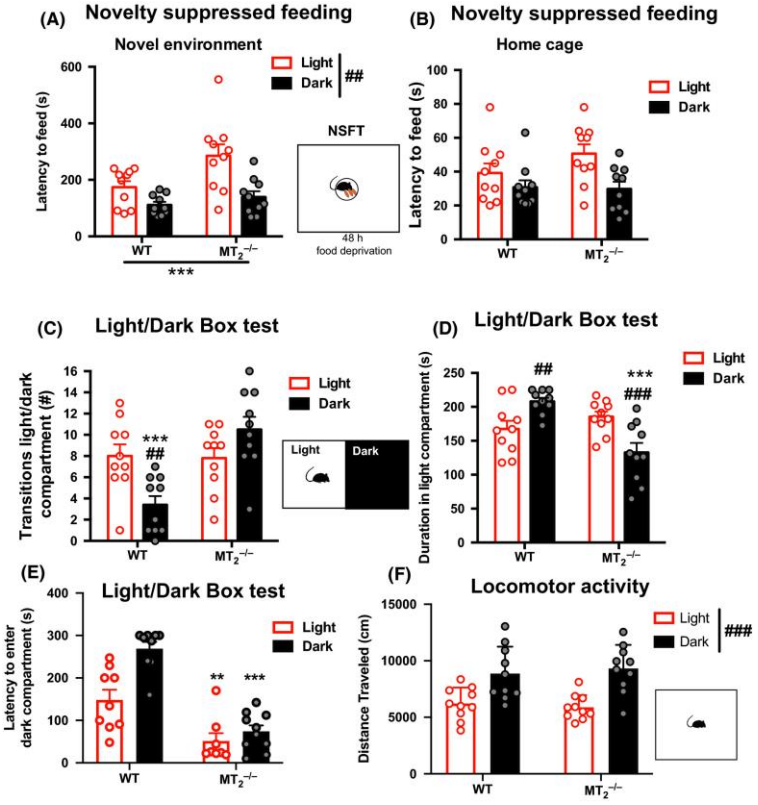


Figure 4.


(A)
Novelty suppressed feeding
Novel environment
Latency to feed (s)
Light
Dark
##
NSFT
48 h
food deprivation
WT
MT2−/−
***
(B)
Novelty suppressed feeding
Home cage
Latency to feed (s)
Light
Dark
WT
MT2−/−
(C)
Light/Dark Box test
Transitions light/dark compartment (#)
***
##
Light
Dark
WT
MT2−/−
(D)
Light/Dark Box test
Duration in light compartment (s)
##
***
###
Light
Dark
WT
MT2−/−
(E)
Light/Dark Box test
Latency to enter dark compartment (s)
**
***
Light
Dark
WT
MT2−/−
(F)
Locomotor activity
Distance Traveled (cm)
Light
Dark
###
WT
MT2−/−