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Genetic Deletion of MT_1 Melatonin Receptors Alters Spontaneous Behavioral Rhythms in Male and Female C57BL/6 Mice

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Abstract

Behaviors vary over the 24 hr. light/dark cycle and these temporal patterns reflect in part modulation by circadian neural circuits and hormones, such as melatonin. The goal of this study was to investigate if MT_1 melatonin receptors are involved in behavioral regulation by comparing male and female C57 wild type (WT) mice with C57 mice that had a genetic deletion of the MT_1 receptor (MT_1KO). A comprehensive array of fifteen distinct spontaneous behaviors was recorded continuously in the homecage over multiple days using the HomeCageScan system. Behaviors assessed were *activity-like (*i.e. come down, hang, jump, walk), *exploration-like (*i.e. dig, groom, rear up, sniff, stretch), *resting-like (*i.e. awake, remain low, rest, twitch) and *ingestion-like* (i.e. drink, eat). Phenotypic array and temporal distribution analysis revealed distinct behavioral rhythms that differed between WT and MT_1KO mice. The rhythms were consistent from day to day in males and varied with the estrous cycle in females. We also studied the role of MT_1 receptors on depressive and anxiety-like behaviors. Genetic deletion of MT_1 receptors increased immobility time in the forced swim test and decreased the number of marbles buried in the marble burying test in both male and female C57 mice. We conclude that MT_1 melatonin receptors are involved in neural pathways modulating diurnal rhythms of spontaneous behavior in the homecage as well as pathways regulating depressive and anxiolytic-like behaviors.

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RESPECTIVE CONTRIBUTIONS

All authors contributed to the design and/or planning of the experiments. EBAB run all experiments, collected, analyzed and interpreted data in consultation with other authors as appropriated. EBAB prepared all figures and wrote the manuscript which was edited by MLD and RLH. The final version of the manuscript was approved before submission by all authors.

CONFLICT OF INTERESTS

Authors report no conflict of interest.

Keywords

Behavioral Rhythms; C57BL/6 mouse; Homecage; MT_1 Receptor Deletion; Estrous Cycle

B. Introduction

An important aspect of behavior is its temporal expression. Many behavioral activities exhibit daily or other cyclic variations that suggest regulation by biological timing mechanisms. Moreover, disruption of biological rhythms contributes to behavioral and psychiatric dysfunction such as seen with jet lag, circadian sleep disorders and depression (Dubocovich, 2007; Dubocovich and Markowska, 2005; Zhu and Zee, 2012). A better understanding of the mechanisms regulating behavioral rhythms is needed to provide the basis for novel therapeutic targets. The current study focuses on the role of melatonin, a circadian hormone that is released at night by the pineal gland to modulate molecular, cellular, physiological and behavioral rhythms. The melatonin molecule activates two types of G-protein coupled receptors, the MT_1 and MT_2 (Dubocovich et al., 2010; Dubocovich and Markowska, 2005). In this study we used genetic deletion of the MT_1 melatonin receptor in C57 mice (MT_1KO) in order to test whether endogenous melatonin acts via this receptor to regulate spontaneous activity, depressive-like and anxiety-like behaviors.

Although there are no selective MT_1 antagonists available, several studies have used genetic MT_1 deletion to reveal a role for this receptor in melatonin actions. For example, melatonin-mediated MT_1 receptor signaling is necessary to transmit photoperiod information to regulate behavioral, seasonal and reproductive neuroendocrine responses in hamsters (Prendergast, 2010; Weaver et al., 1996). Deletion of MT_1 receptors in C57 and C3H mice abolished the changes in ependymal cell layer deodinase expression that was proposed as a necessary key molecular event upstream of gonadal responses to melatonin and photoperiod (Yasuo et al., 2009). Together these results led to the conclusion that MT_1 receptors are critical for modulation of photoperiodic responses in mammals (Yasuo et al., 2009).

MT_1 melatonin receptors also are important for the effect of melatonin on circadian and behavioral function. Melatonin administration accelerates re-entrainment of wheel running activity after a phase shift in the light-dark cycle; this action requires MT_1 receptors as no effect is observed in mice with genetic deletion of MT_1 receptors (Dubocovich et al., 2005; Dubocovich and Markowska, 2005). Deletion of MT_1 receptors in C57 mice also increased immobility on the forced swim test and impaired pre-pulse inhibition in the acoustic startle/pre-pulse inhibition test (Weil et al., 2006). The heterogeneous distribution of MT_1 receptors in the brain (Adamah-Biassi et al., 2014; Siuciak et al., 1990; Weaver et al., 1989) further suggests that this receptor subtype modulates complex behaviors. However, the role of endogenous melatonin and MT_1 receptors in the regulation of daily spontaneous behaviors remains largely uncharacterized.

Assessment of behavioral rhythms necessitates measuring behavior over extended periods of time, ideally under conditions with minimal interference and stress to the animals. Recently we demonstrated that, with the use of an automated behavioral analysis system to record continuous, single mouse behavioral data in the homecage, we could quantitate multiple

spontaneous behaviors and determine temporal patterns in a phenotypic array (Adamah-Biassi et al., 2013). This analysis revealed distinct patterns of homecage behavior for male C57BL/6 and C3H/HeN mice (Adamah-Biassi et al., 2013), two strains that are commonly used to assess the role of melatonin and its ligands in molecular, physiological and behavioral paradigms (Mexal et al., 2012; Pfeffer et al., 2012; Stehle et al., 2002). These two strains exhibit different gene profiles (Claire et al., 2002; Kasahara et al., 2010; Wade and Daly, 2005), and differ in their ability to produce pineal melatonin (von Gall et al., 2000). The C3H mice produce higher (150 pg/gland) levels of melatonin in the pineal gland at night whereas C57 mice produce lower (8-20 pg/gland) levels (Kennaway et al., 2002; Vivien-Roels et al., 1998; von Gall et al., 2000) because of a point mutation in the melatonin synthetizing enzyme arylalkylamine N-acetyltransferase (AANAT) gene (Roseboom et al., 1998). These differences are reflected in the levels of blood melatonin at night: about 20 pg/ml in C3H mice, and less than 10 pg/ml in C57 mice (Kennaway et al., 2002; Masana et al., 2000). C57 mice also lack the normal circadian rhythm of circulating melatonin with no detectable daily changes in melatonin levels unlike some other mouse strains (e.g., CBA, C3H/HeN) and most other mammals, in which the nocturnal melatonin levels can reach peak values that are ~100 to 300% higher than the basal daytime values (Kennaway et al., 2002; Masana et al., 2000). Administration of exogenous melatonin to C57 mice did not alter the behavioral profile, suggesting that differences in observed behavioral phenotypes between the two strains may not be due to differences in melatonin levels (Adamah-Biassi et al., 2013).

In this study we investigated whether endogenous melatonin and specifically, MT_1 melatonin receptors have a role in regulating spontaneous homecage behavioral rhythms as well as depressive-like and anxiety-like behaviors. Wild type (WT) C57 mice were compared with MT_1KO C57 mice that had a genetic deletion of the MT_1 melatonin receptor. An advantage of using C57 mice is that their low production of pineal melatonin is less likely to cause changes in melatonin receptor sensitivity as compared to other strains (Gerdin et al., 2004a; Gerdin et al., 2004b). We tested both males and females, taking into account the different stages of the estrous cycle. Here we report that genetic deletion of MT_1 melatonin receptors, both in male and female mice, results in behavioral profiles that are distinct from C57 WT mice. Thus MT_1 receptors do affect behavior under conditions where melatonin receptors are activated by physiological concentrations of endogenous melatonin (Kennaway et al., 2002).

C. Methods

Animal care and handling

Male and female C57WT (WT) and C57 MT_1KO (MT_1KO) mice were bred in house at the Laboratory Animal Facility at the University at Buffalo, School of Medicine and Biomedical Sciences. The mouse colonies were established from C57 MT_1KO mice donated by Dr. S.M. Reppert (Massachusetts General Hospital, Boston, MA, USA) as described previously (Sumaya et al., 2005). The mice were group-housed by sex (3 t0 4 per cages) and kept in a 14/10 LD with access to food and water *ad libitum*. They were maintained in a temperature (22 ± 1°C) and humidity (20 – 23%) controlled room. The light intensity on the shelf where

cages were placed was 150 to 200 lux. Two weeks before recording in the homecage started, mice were moved to cabinets with LED light (510 nm) intensities between 60-90 lux. All experimental protocols were approved and performed according to the guidelines of the National Institute of Health and the Institutional Animal Care and Use Committee of the University at Buffalo.

Homecage monitoring in male mice

Male C57WT and MT_1KO mice aged 3 to 4 months were separated and placed into individual cages (33 × 12 × 13 cm). The average weight of the male mice was 30 ± 0.63 g (n=16) for WT and 29.21 ± 0.58g (n=16) for MT_1KO mice. The mice were housed in single compartments inside cabinets with constant temperature and humidity and equipped with infrared cameras to monitor behavioral activity continuously over the 14:10 light/dark cycles as previously described (Adamah-Biassi et al., 2013). Mice spent a total period of seven days in the homecage. The first three days served as an acclimatization period to the new environment and during the last four days, mouse behaviors were recorded and analyzed using the HomeCageScan 3.0 software from Cleversys Inc. (Reston, VA).

Vaginal cytology and homecage monitoring in females

Female WT and MT_1KO mice aged 3 months were separated and put into individual cages (33 × 12 × 13 cm). After one week acclimatization, the estrous cycle was monitored by vaginal cytology as described by Byers et al (Byers et al., 2012). A vaginal swab was collected everyday between ZT5 and ZT7 (ZT: zeitgeber; ZT0: Lights ON) time for 12 consecutive days. A cotton tipped swab (Puritan Medical Products Company, LLC Guilford, ME) was dipped in a 1% saline solution and inserted into the vagina of the mouse. The wetted swab was rolled gently against the vaginal wall and the collected cells were placed onto a slide. The slides were air dried and stained with accustain (Sigma-Aldrich, St. Louis, MO) and cells were processed to determine the stage of the estrous cycle.

Mice homecage behaviors were recorded as previously described during the last four days and during vaginal cytology monitoring. The average weight for female mice was 21.14 ± 0.33g (n=16) for WT and 21.00 ± 0.21g (n=16) for MT_1KO mice. Mouse behaviors were analyzed using phenotypic arrays and temporal distribution of behaviors group-based on estrous cycle stage.

Marble Burying Test

The marble burying test was performed, as previously described (Deacon, 2006), three days after the homecage recording in males. For females, the mice used for the marble burying test were different from the groups used for homecage recording. The test was performed in mouse cages (28 cm × 18 cm × 12 cm) filled with 5 cm of corncob bedding on which 20 marbles (1.5 cm in diameter) were placed, aligned and evenly spaced. The corncob bedding was flattened to create an even surface. Female or male C57WT and MT_1KO mice were individually placed in the cage for 30 minutes after an acclimation period of one hour in the experimental room. A new clean cage was used for each animal, and a set of 16 animals was tested at a time. The test was conducted between ZT9 to ZT11. The number of marbles buried at least two thirds deep in the bedding were counted after the 30 minutes period by

two experimenters blind to the genotype of the mice. The amount of marbles buried were averaged per genotype and compared statistically using the Student's T-test.

Forced swim test

Naive groups of male and female WT and MT_1KO mice were subjected to the forced swim test. The forced swim test was performed as described by Porsolt et al (1978) in a glass cylinder (11 cm in diameter and 14 height) filled with water (8.5 cm height) maintained at room temperature (22 to 23°C). For each test session, individual mice (2 WT and 2 MT_1KO) were placed in separate glass cylinders and allow to swim freely. Mice movements (i.e., immobility and swimming) were recorded for a period of 6 minutes using a Sony camera linked to a Dell computer (Dell Precision T3500, W503 @ 2.4 GHz). The last 4 minutes were automatically analyzed with the Forced Swim Scan Software from Cleversys Inc. (Reston, VA 20190). The forced swim test was performed between ZT9 and ZT11. Male and female mice were tested on different days.

Data analysis and statistical analysis

Phenotypic array and temporal distribution analysis were performed as previously described (Adamah-Biassi et al, 2013) and for the data from the four day period of recording. Phenotypic arrays were plotted and analyzed with R.1.2.0 software. The effect of time and genotype (WT and MT_1KO) on the temporal distribution of behaviors were compared using repeated measure two-way ANOVA in GraphPad 6.1 software with genotype and time as factors. IBM SPSS 21 was used to compute the partial eta squared values (η_p^2). Partial eta squared reported in our study is an estimate for genotype effect size which is the ratio of variance accounted for by genotype and this effect plus its associated error variance. For partial eta squared, values of 0.14, 0.06 and 0.01 respectively represent large, medium and small effects (Cohen, 1988). The temporal distribution of a behavior was considered bimodal when it exhibited a peak at the onset (between ZT11 and ZT16) and another one at the offset of darkness (between ZT23 and ZT2), separated by a trough during the middle of the dark period. Each peak had to be at least 6% and the trough no more than 4% of the total amount of time spent in the behavior over 24 hr. Any behavior that did not meet these criteria was considered unimodal except for rest and twitch that were not included in this analysis since they have opposite profiles to that of the other behaviors. These standards were set by: a) first by analyzing the temporal distribution of C57WT mice behaviors in the homecage for all experiments performed over time b) second by calculating the percent of time over total time, mice spent in each behavior at the peak and trough, and c) finally by setting the minimum percent time spent at the peak and the maximum percent time spent at the trough as baseline for defining bimodal temporal distribution.

The effect of genotype on number of marbles buried (Marble Burying Test) and time spent immobile (Force Swim Test) were compared using the student t-test. Direct comparison between sexes was not performed due to the fact that male and female experiments were run separately since smell and sight of female mice can affect the behaviors of male mice and vice versa. Cohen's *d* values for the effect size of genotype were computed using the calculator found at www.cognitiveflexibility.org/effectsize. The calculator evaluates the effect size between two means which is the difference between means divided by standard

deviation. For Cohen's *d* an effect size of 0.2 to 0.3, 0.5 and 0.8 to infinity might be respectively small, medium and large (Cohen, 1988).

D. Results

MT_1 receptor deletion alters the behavioral array of male C57 mice

Behaviors were analyzed using phenotypic arrays that allow visualization and comparison of 15 spontaneous homecage behaviors of WT and MT_1KO mice as an ensemble over time (Adamah-Biassi et al., 2013). Figure 1 shows the 24 hr. arrays from WT (Figure 1A) or MT_1KO (Figure 1B) male mice for four consecutive days. The daily phenotypic array for any given genotype was similar from day 1 to day 4. All active behaviors primarily occurred during the dark period and decreased 2 hours after the light onset. As previously reported, WT C57 male mice exhibited a bimodal distribution of homecage behaviors with the first mode of activity at the onset of the dark period (ZT14-17) and the second mode at the onset of the light period (ZT21-2) (Adamah-Biassi et al., 2013). The second mode extended into the light period by 2 to 3 hours. The first and second modes were separated by a resting period of 2 to 3 hours during the middle of the dark period (Figure 1A). A different pattern, however, was seen with MT_1KO mice. They showed a unimodal pattern of activity that extended for two hours after the end of the dark period (Figure 1B) with no clear rest period.

Deletion of the MT_1 receptor alters the temporal distribution of homecage behaviors in male mice

The temporal distribution analysis of individual homecage behaviors revealed distinct diurnal rhythms for WT and MT_1KO male mice (Figures 2) that were generally consistent from day to day over the 4-day recording period (Figure S1-4). The individual activities analyzed were related to four different types of behavior: activity, exploration, ingestion, or resting. Interestingly, the pattern for some types of behaviors were significantly altered by MT_1 deletion, while others such as ingestive behaviors were not affected.

Comparison of *activity-like behaviors* between WT and MT_1KO mice showed a significant effect of genotype in comedown (Figure S1A, E, I, M) and walk (Figure 2A, E, I, M and Figure S1D, H, L, P) behaviors on day 1 [Comedown: $F(1, 30)=8.11$, $\eta_p^2=0.21$; Walk: $F(1, 30)=22.03$, $\eta_p^2=0.18$; $p<0.01$), day 2 [Comedown: $F(1, 30)=9.78$, $\eta_p^2=0.25$; Walk: $F(1, 30)=4.62$, $\eta_p^2=0.13$; $p<0.01$], day 3 [Comedown: $F(1, 30)=6.48$, $\eta_p^2=0.18$; Walk: $F(1, 30)=4.81$, $\eta_p^2=0.14$; $p<0.05$] and day 4 [Comedown: $F(1, 30)=6.65$, $\eta_p^2=0.18$; Walk: $F(1, 30)=4.02$, $\eta_p^2=0.12$, $p<0.05$). Bonferoni posttests showed a decreased in the time spend in comedown and walk behavior at the onset and offset of the dark period in MT_1KO compared to WT mice (Figure S1). No significant effect of genotype was found in jump (Figure S1B, F, J and N) and hang (Figure S1C, G, K and O) behaviors.

For *exploration-like behaviors*, WT mice showed a bimodal temporal distribution pattern except for groom and stretch behaviors (Figure S2). Rear up (Figure 2B, F, J, N and Figure S2C, H, M, R) and sniff (Figure S2D, I, N, and S) showed a significant effect of genotype on day 1 [Rearup: $F(1, 30)=6.36$, $\eta_p^2=0.17$; Sniff: $F(1,30)=14.69$, $\eta_p^2=0.33$; $p<0.01$], day 2 [Rearup: $F(1, 30)=15.90$, $\eta_p^2=0.35$; Sniff: $F(1,30)=16.44$, $\eta_p^2=0.35$; $p<0.0001$], day 3

[Rearup: F(1, 30)=10.01, η_p^2=0.25; Sniff: F(1,30)=10.94, η_p^2=0.24; p<0.05] and day 4 [Rearup: F(1, 30)=13.44, η_p^2=0.31; Sniff: F(1,30)=9.50, η_p^2=0.24; p<0.005] with WT spending more time than MT_1KO at the onset and offset of the dark period. Groom (Figure S2B, G, L and Q), dig (Figure S2A, F, K and P) and stretch (Figure S2E, J, O, T) behaviors did not show any significant effect of genotype.

With regard to *ingestion-like behaviors*, there was no significant effect of genotype on the temporal distribution of drink (Figure S3) and eat (Figure 2C, G, K, O and Figure S3) behaviors during the four days except for eat behavior on day 1 (Figure S3B) [F(1, 30)=24.50, η_p^2=o.45; p<0.01].

For the *resting-like behaviors*, there was no significant effect of genotype from day 1 to day 4 in remain low (Figure S4B, F, J, and N) and twitch (Figure S4D, H, L and P) behaviors. Awake (Figure SA, E, I and M) behavior showed a significant effect of genotype on day 1 [F(1, 30)=7.78, η_p^2=0.21; p<0.005], day 2 [F(1, 30) =6.53, η_p^2=0.16; p<0.01], day 3 [F(1, 30)=7.49, η_p^2=0.2; p<0.01] and day 4 [F(1, 30)=13.82, η_p^2=0.32; p<0.001). Similarly rest behavior (Figure 2D, H, L, P and Figure S4C, G, K, O) showed a significant effect of genotype on day 1 [F(1, 30)=7.54, η_p^2=0.21; p<0.01], day 2 [F(1, 30)=4.37, η_p^2=0.14; p<0.05], day 3 [F(1, 30)=7.55, η_p^2=0.27; p<0.05] and day 4 [F(1, 30)=5.90, η_p^2=0.18; p<0.05].

Effects of MT_1 receptor deletion on the phenotypic profile of female mouse behavior over the light/dark and estrous cycles

For both WT and MT_1KO female mice, the daily behavior patterns were analyzed separately for each stage of the estrous cycle. Using vaginal cytology, the stages of proestrus, estrus, metestrus and diestrus were characterized, respectively by nucleated epithelial, cornified, leucocytes and a majority of leucocytes plus cornified cells (Figure 3A), as described previously (Byers et al., 2012; Caligioni, 2009; Champlin et al., 1973; Koehl et al., 2003; McLean et al., 2012). The length of the estrous cycle of the mice used in this study was 4-5 days. Out of 16 mice, 4 mice in the WT and 2 in the MT_1KO group stopped cycling normally and were excluded from our analysis.

In WT females, the behavioral profile varied depending on the stage of the estrous cycle (Figure 3B). During metestrus and diestrus, when the circulating levels of ovarian hormones are low, the behavioral profile of female WT mice was bimodal, similar to that observed in male WT mice. However during proestrus and estrus, WT females exhibited a unimodal pattern of homecage behaviors that extended for 2 to 3 hours after the onset of light (Figure 3B).

With deletion of the MT_1 receptor, the estrous cycle no longer had an influence on the daily behavioral profile. The pattern in MT_1KO female mice was unimodal at all stages of the cycle (Figure 3C), similar to what was seen in MT_1KO males.

Effect of MT_1 receptor deletion on the daily rhythms of homecage behaviors in female mice at different stages of the estrous cycle

Temporal distribution analysis of individual homecage behaviors in female WT and MT_1KO mice showed distinct effects of MT_1 receptor deletion on the diurnal rhythms.

For the *Activity-Like Behaviors*, the temporal patterns exhibited by female mice varied over the four days of the estrous cycle. WT behaviors compared to those of MT_1KO showed a significant effect of genotype in come down behavior (Figure S5A, E, I and M) during the metestrus [$F(1, 23)=2.95$, $\eta_p^2=0.10$; $p<0.05$], diestrus [$F(1, 23)=2.82$, $\eta_p^2=0.11$; $p<0.05$] and estrus [$F(1, 23)=4.396$, $\eta_p^2=0.16$; $p<0.05$] but not during proestrus [$F(1, 23)=0.63$, $\eta_p^2=0.03$; n.s.]. A genotype effect was also seen in hang behavior (Figure S5J) during proestrus [$F(1, 23)=15.27$, $\eta_p^2=0.42$; $p<0.001$], in walk behavior (Figure 4E, M and Figure S5H, P) during both diestrus [$F(1, 23)=3.25$, $\eta_p^2=0.13$; $p<0.05$] and estrus [$F(1, 23)=3.38$, $\eta_p^2=0.13$; $p<0.05$] but not the other stages.

For E*xploration-Like Behaviors*, the temporal distribution of dig (Figure S6A, F, K and P), groom (Figure S6B, G, L and Q), rear up (Figure 4B, F, J, N and Figure S6C, H, M, R) and sniff (Figure S6D, I, N and S) showed no significant effect of genotype during the four stages of the estrous cycle. Bonferoni posttest in rearup show significant effect of genotype in the middle of the night (ZT19-10) where MT_1KO female mice spent significant amount of time rearing than WT at all stages of the estrous cycle. MT_1KO females also showed more stretch behavior than WT during metestrus [$F(1, 23)=7.52$, $\eta_p^2=0.20$; $p<0.01$] but not the other stages (Figure S6E, J, O and T).

For *ingestion-Like Behaviors*, drink and eat behaviors were not significantly affected by genotype during the four stages of the estrous cycle. Bonferoni posttest showed an increased time spent eating in MT_1KO female mice during the middle of the night (ZT19-20) in MT_1KO female mice (Figure S7B, D, F and H).

For *Resting Like Behaviors*, MT_1KO females, as compared to WT mice, showed a decreased amount of time spent in awake (Figure S8A, E, I and M) and remain low behaviors (Figure S8B, F, J and N) during metestrus [Awake: $F(1, 23)=17.39$, $\eta_p^2=0.45$; RemainLow: $F(1, 23)=6.18$, $\eta_p^2=0.23$; $p<0.05$], diestrus [Awake: $F(1, 23)=17.64$, $\eta_p^2=0.45$; RemainLow: $F(1, 23)=36.56$, $\eta_p^2=0.62$; $p<0.001$], proestrus [Awake: $F(1, 23)=14.03$, $\eta_p^2=0.40$; RemainLow: $F(1, 23)=24.30$, $\eta_p^2=0.54$; $p<0.001$] and estrus [Awake: $F(1, 23)=11.16$, $\eta_p^2=0.33$; RemainLow: $F(1, 23)=16.12$, $\eta_p^2=0.41$; $p<0.001$]. Rest [$F(1, 23)=16.11$, $\eta_p^2=0.43$; $p<0.001$] and twitch [$F(1, 23)=7.52$, $\eta_p^2=0.26$; $p<0.01$] behaviors only showed a genotype effect during proestrus (Figure 4L and Figure S8K, L).

Effect of MT_1 receptor deletion on the forced swim and marble burying tests in both male and female C57 mice

Male and female WT and MT_1KO mice were evaluated in the forced swim and marble burying tests during a time in the light phase (ZT9-ZT11) when the two strains showed a similar low level of basal activity. In the forced swim test, the amount of time spent immobile is used to assess depressant-like activity while the number of marbles buried in a half-hour session is used to assess anxiogenic-like activity. In the forced swim test, male

MT_1KO mice (202.7 ± 4.71 s, n=14) spent significantly more time immobile as compared to WT males (181.5 ± 4.48 s, n=10; *d*=1.33 and p<0.004) (Figure 5A). Similarly female MT_1KO mice (204.0 ± 6.69s; n=14) remained immobile for a significantly longer period of time than the WT females (173.9 ± 8.00s; n=14; *d*=1.10 and p<0.008) (Figure 5B).

In the marble burying test, WT mice, both male (15.10 ± 0.88 marbles, n=10) and female (12.34 ± 0.38 marbles; n=16), buried significantly more marbles as compared to male MT_1KO (8.308 ± 1.64 marbles; n=13; *d*=1.57 and p<0.002) and female MT_1KO (2.967 ± 0.82 marbles; n=15; *d*=3.97 and p<0.0001) mice (Figure 5C and D). At the time of the test (ZT9-ZT11), basal spontaneous digging behavior was low and similar for males and females of both genotypes (Figures S2 and S6).

E. Discussion

This study shows that deletion of the MT_1 melatonin receptor in C57 mice alters the behavioral rhythms of most activity-, exploratory-, ingestion- and resting-like behaviors in the homecage and transforms a bimodal behavioral profile into a unimodal one. In female mice, the alteration of behavioral rhythms also was influenced by the estrous cycle. Deletion of the MT_1 receptor increased immobility in the forced swim test suggesting a depressant-like activity and decreased the number of marbles buried in the marble burying test indicating an anxiolytic-like activity. Taken together these data suggest that melatonin signaling through the MT_1 receptor plays a role in maintaining the integrity of spontaneous behavioral rhythms and may modulate activity in neuronal pathways mediating depressive-like and anxiety-like behaviors.

Male WT and MT_1KO C57 mice displayed distinct rhythms in their homecage behavior that were consistent from day to day within each genotype. WT male mice exhibited a bimodal pattern of activity, as we found previously (Adamah-Biassi et al., 2013); however MT_1KO mice displayed a unimodal behavioral profile. Thus, deletion of the MT_1 melatonin receptor altered the daily pattern of spontaneous behaviors. Temporal behavioral distribution analysis for males showed that deletion of the MT_1 receptor reduced the peaks of the first and second mode respectively at the onset and offset of the dark period. The rest period that WT C57 mice usually exhibit in the middle of the active phase of the light/dark cycle was eliminated.

In males, the specific behaviors that were affected by MT_1 receptor deletion included activity-like behaviors (come down and walk); resting-like behaviors (awake and rest); and exploration-like behaviors (dig, rear up and sniff). The effect of genotype on the rhythm of these behaviors was consistent from day 1 to day 4 and reflected the overall behavioral profiles for MT_1KO and WT mice. Disruption of behavioral rhythms has been observed following deletion of other genes associated with the circadian system (Kim et al., 2009; Paulus and Mintz, 2012; Yang et al., 2012). Mice lacking the transcription factor Pet-1, which is an early developmental indicator of serotonergic neurons, showed a shift in peak of wheel running behavior toward the late night as compared to wild type and heterozygote mice (Paulus and Mintz, 2012). Furthermore, mice with inducible deletion of peroxisome proliferator-activated receptor gamma (PPARγ) lost rhythms of food and water intake but had normal wheel running activity rhythms (Yang et al., 2012) as compared to controls. In

the present study, the diurnal rhythmicity of some of the behaviors, including ingestion behaviors drink and eat, were not altered by deletion of the MT_1 receptor. This suggests that MT_1 receptors do not just act globally on circadian mechanisms, but they have specific roles in regulating distinct behavioral pathways under basal conditions.

Behavior in female mice is known to vary over the estrous cycle (Basterfield et al., 2009; Hyde and Sawyer, 1977; Meziane et al., 2007). Thus we determined phenotypic arrays in female C57 WT and MT_1KO mice at each of the four stages of the estrous cycle, as assessed by vaginal cytology (Byers et al., 2012). The phenotypic profile of female WT mice was bimodal for most behaviors during metestrus (i.e., come down, hang, jump, walk, dig, rear up, sniff, eat and remain low) and several behaviors during diestrus (i.e., come down, hang, jump, walk, dig, rear up and sniff). The bimodal pattern is similar to what we found for male WT mice in the present study and as previously reported (Adamah-Biassi et al., 2013). During proestrus and estrus, however, the phenotypic profile for WT females was unimodal, suggesting a high level of spontaneous activity during the two stages that are associated with elevated levels of ovarian hormones, ovulation and sexual receptivity. Activated behaviors also have been reported to vary over the estrous cycle. Despair-like behaviors of female C57BL/6 and BALBc mice in the tail suspension test were increased during metestrus and estrus, respectively (Meziane et al., 2007). Running wheel activity (Basterfield et al., 2009) and aggressiveness (Hyde and Sawyer, 1977) in female C57BL/6 mice increased at the onset of proestrus and decreased immediately after estrus. Our study shows for the first time that non-activated, spontaneous behaviors during the dark period increase in WT mice during proestrus and to a lesser extent, during estrus, followed by lower levels during metestrus and diestrus.

For female MT_1KO mice, the behavioral profile was unimodal during all four days of the estrous cycle, similar to the profile of male MT_1KO mice. This suggests that deletion of the MT_1 receptor had similar consequences on the behavioral profile of male and female mice, particularly during the stages of metestrus and diestrus. Interpreting effects of MT_1 receptor deletion on female behavior during proestrus and estrus is confounded by the fact that WT females also display a unimodal profile during these stages. Estrogen is elevated during this part of the cycle, and it may act to physiologically suppress MT_1 receptor function in WT mice to produce a shift from bimodal to unimodal behavior, similar to what occurs when MT_1 receptors are suppressed by genetic deletion in males and females in metestrus and diestrus. MT_1 receptor function has been shown to be modulated by estrogen and affected during proestrus (Chuffa et al., 2011; Soares et al., 2003; Zhao et al., 2002). Conversely, melatonin can also affect estrogen function (Chuffa et al., 2011; Soares et al., 2003; Zhao et al., 2002). Thus interactions between melatonin and estrogen may influence the behavioral profile in WT but not MT_1KO female. Our study accentuates the importance of correlating behavioral outcomes with the stages of the estrous cycle when assessing behavioral patterns in female mice. Patterns may vary from day-to-day, and variable expression of behaviors may be due to hormonal modulation of receptor expression and function (Fink et al., 1996; Goel and Bale, 2009; Palanza, 2001).

In addition to spontaneous behaviors, we studied the consequence of MT_1 receptor deletion on depressive-like and anxiety-like behaviors. Genetic deletion of MT_1 melatonin receptors

increased the immobility time in both male and female C57 mice, suggesting the involvement of this receptor in neuronal brain pathways modulating depressive behaviors. Our data support previous research that showed that both male and female C57 mice lacking the MT_1 receptor exhibit behavioral depression and sensorimotor gating respectively in the forced swim and acoustic startle/prepulse inhibition tests (Weil et al., 2006). Deletion of the MT_1 melatonin receptor also produced anxiolytic-like effects, as assessed using the marble burying test. MT_1KO mice buried fewer marbles than WT mice indicating that MT_1 melatonin receptors are involved in pathways modulating anxiety-like behaviors. This result is in contrast with previous findings showing an anxiogenic-like effects of the MT_1 receptor deletion on the open field test and the prepulse inhibition test (Weil et al., 2006). It is important to point out that these tests assess different aspects of anxiety-like behaviors. The marble burying test measures a repetitive and perseverative behavior that is sensitive to anxiolytics rather than an isomorphic model of anxiety (Njung'e and Handley, 1991; Thomas et al., 2009). In contrast, the open field test portrays a novelty-induced anxiety termed “state anxiety” (Crawley, 1985; Prut and Belzung, 2003).

Our results here suggest that even in the absence of high pineal melatonin production in the C57 mouse (Ebihara et al., 1987; Kennaway et al., 2002; Roseboom et al., 1998), MT_1 melatonin receptor signaling appears to play a role in the maintenance of spontaneous behavioral rhythms in the homecage as well as in depressive-like and anxiety-like behaviors. Ebihara et al., (1987) originally found no melatonin in the C57 mice (Ebihara et al., 1987), probably due to limitations in the techniques used to measure melatonin levels. Subsequent studies using antibodies with higher limits of sensitivity for melatonin detection found low levels of melatonin in the pineal (10-12 pg/gland) and the blood (~43 pM or 10 pg/ml) during the dark period in C57 mice (Kennaway et al., 2002; Vivien-Roels et al., 1998; von Gall et al., 2000). This small amount of melatonin would be sufficient to physiologically activate high affinity MT_1 melatonin receptors to modulate behavioral functions (Dubocovich et al., 2010). It is also possible that MT_1 melatonin receptors expressed in a constitutively active form (Browning et al., 2000; Devavry et al., 2012; Soares et al., 2003) may signal in the absence of melatonin to regulate pathways modulating behaviors. In summary genetic deletion of MT_1 melatonin receptors may impair activation of melatonin receptor signaling by circulating melatonin and/or by the absence of functional constitutively active receptors. In conclusion, we suggest that the MT_1 melatonin receptor signaling is required for normal brain functioning as its lack produced behavioral rhythm disruption as well as depressant and anxiolytic-like activities in mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MT_1 receptors activation appear to modulate diurnal rhythms of homecage behaviors in C57BL/6 mice
- Diurnal behavioral profiles of female C57/BL6 mice are dependent on the estrous cycle
- MT_1 receptors may modulate activity in pathways mediating depressive and anxiety-like behaviors

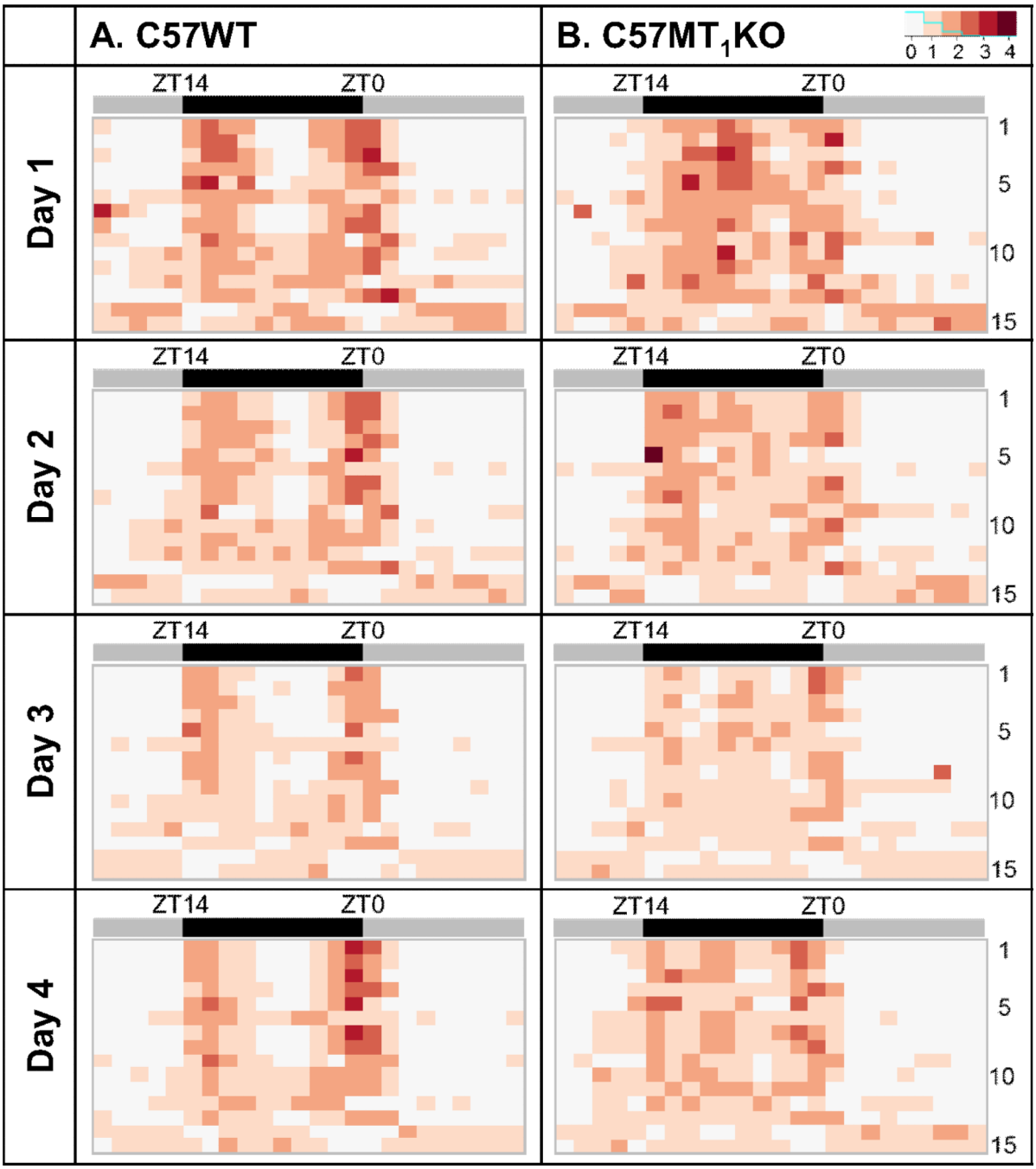

Figure 1. Phenotypic arrays of daily behavior for male WT and MT_1KO mice

Phenotypic arrays illustrate the overall daily pattern of 15 spontaneous behaviors measured in the homecage over a four-day recording period for WT (A) and MT_1KO (B) male mice. Hourly mean values for each behavior (1-15) of WT (n=16) and MT_1KO (n=16) were scaled and plotted over a 24 hr. period; fold increase is indicated by the increase in color intensity from the baseline. The 14/10 light/dark cycle is indicated by the shading of the bar at the top of the arrays with the onset of dark at ZT14 and the onset of light at ZT0. Numbers 1

through 15 indicate, respectively, the behaviors of come down, hang, jump, walk, dig, groom, rear up, sniff, stretch, drink, eat, awake, remain low, rest and twitch.

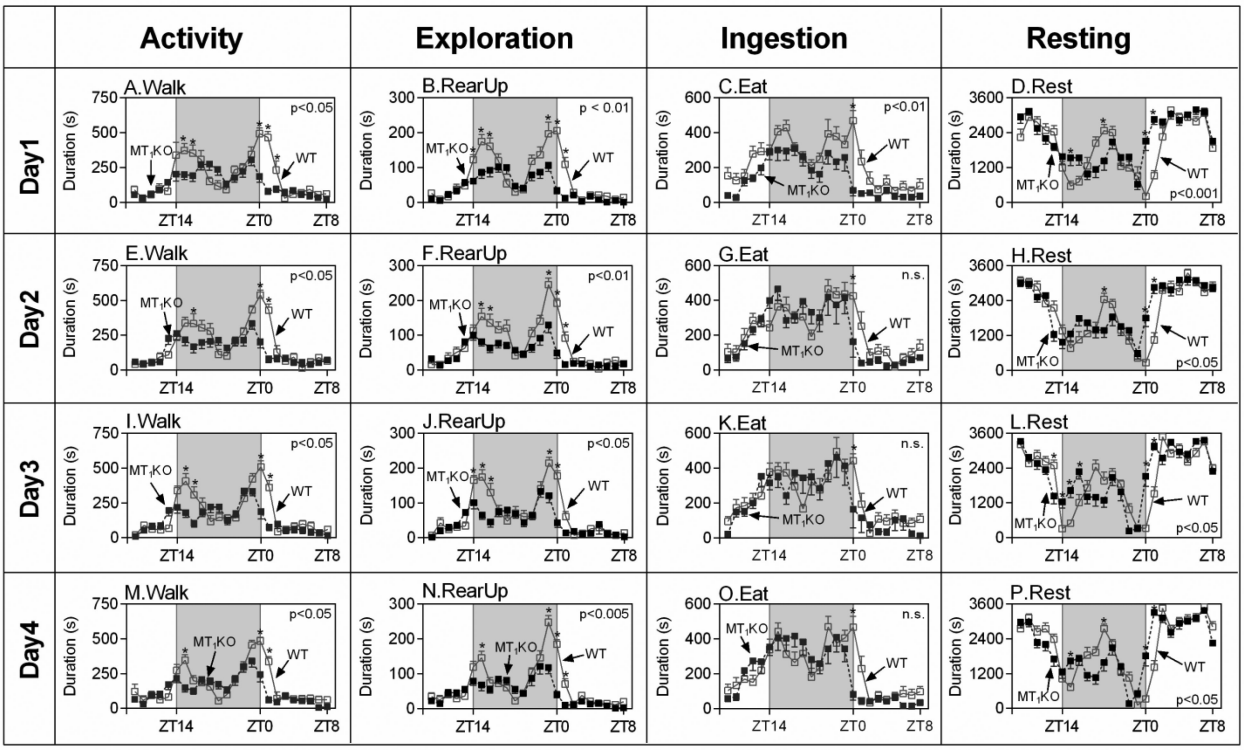

Figure 2. Comparison of individual homecage behaviors of WT and MT_1KO male mice over the light/dark cycle

Behaviors indicative of Activity (A, E, I, M), Exploration (B, F, J, N), Ingestion (C, G, K, O) and Resting (D, H, L, P) are plotted individually over the light/dark cycle to compare WT (□) and MT_1KO (■) genotypes. The abscissae represents zeitgeber time (ZT) with ZT14 and ZT0 representing respectively the onset of the dark and the light periods. The shaded gray area represents the dark period. Hours spent in each behavior were plotted as a function of ZT and compared using repeated measure two-way ANOVA with genotype and time as main factors. The overall genotype effect on total magnitude of behavior is indicated by the p-value in the upper right corner of each graph. n.s. non-significant. The genotype effect at each ZT was determined by Bonferoni post-test. *p<0.05.

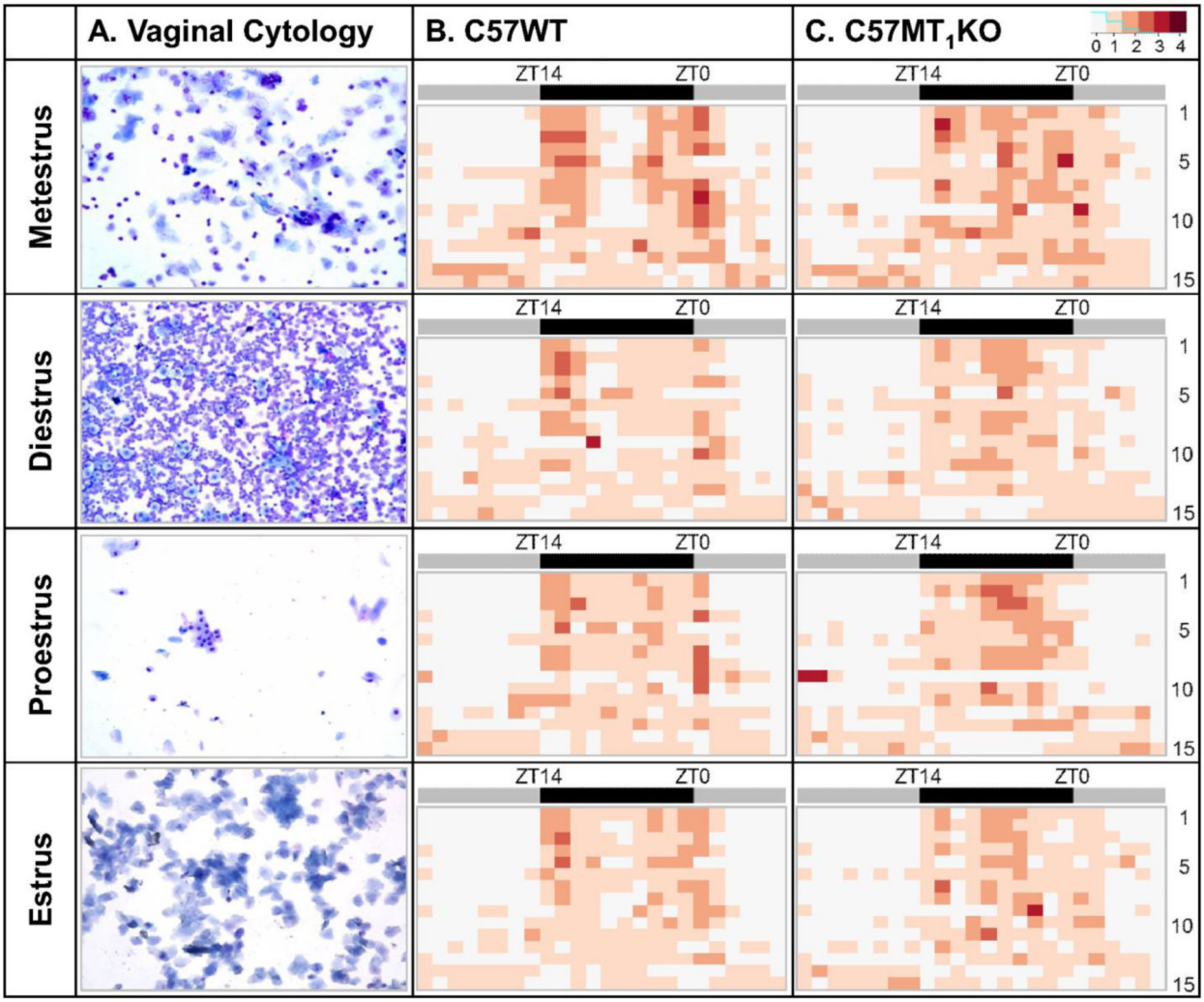

Figure 3. Phenotypic arrays of daily behavior for female WT and MT_1KO over the estrous cycle

Phenotypic arrays illustrate the overall daily pattern of 15 spontaneous behaviors measured in the homecage over a four-day recording period for WT (A) and MT_1KO (B) female mice. For each day, the stage of the estrous cycle was determined using vaginal cytology (A). Proestrus, estrus, metestrus and diestrus stages were characterized respectively by nucleated epithelial, cornified, leucocytes and cornified and leucocytes cells. Mice were grouped by genotype and estrous cycle stage and their behaviors analyzed. Hourly mean values for each behavior (1-15) were scaled and plotted over a 24 hr. period; fold increase is indicated by the increase in color intensity from the baseline. The 14/10 light/dark cycle is indicated by the shading of the bar at the top of the arrays with the onset of dark at ZT14 and the onset of light at ZT0. Numbers 1 through 15 indicate, respectively, the behaviors of come down, hang, jump, walk, dig, groom, rear up, sniff, stretch, drink, eat, awake, remain low, rest, and twitch.

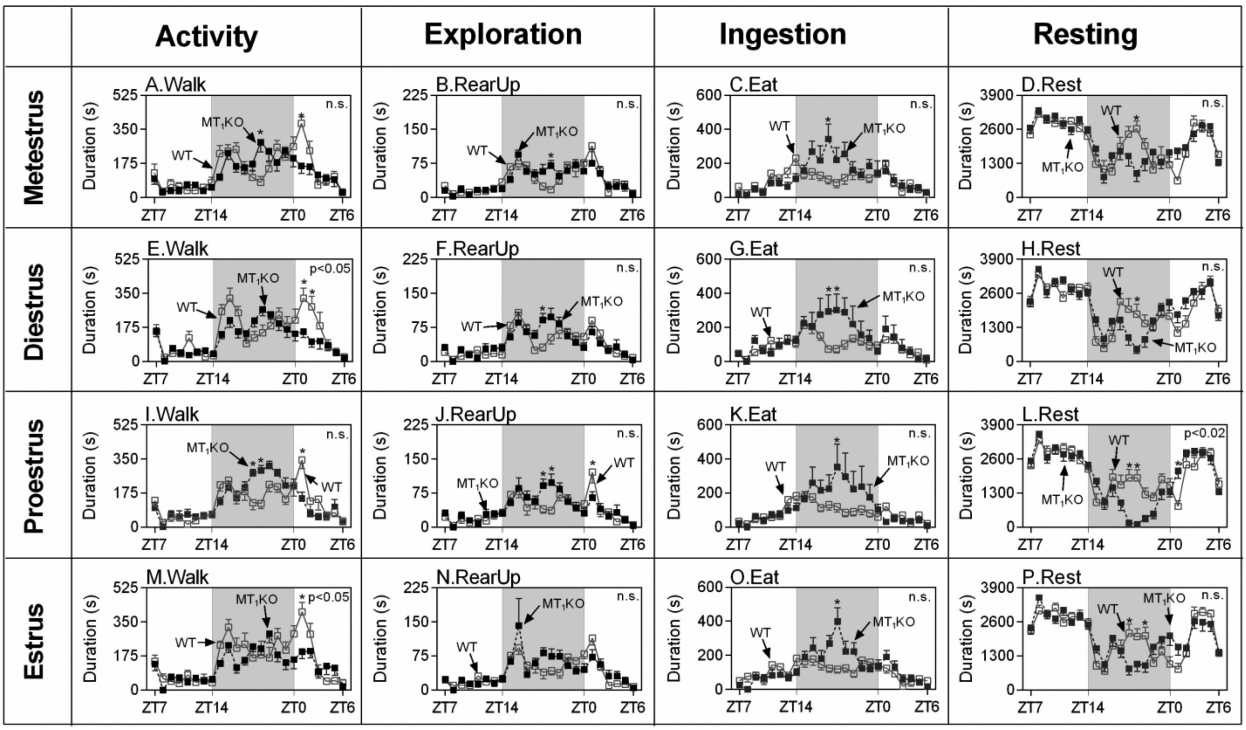

Figure 4. Comparison of temporal variations in individual homecage behaviors of WT and MT_1KO female mice

Behaviors indicative of Activity (A, E, I, M), Exploration (B, F, J, N), Ingestion (C, G, K, O) and Resting (D, H, L, P) are plotted individually over the light/dark cycle at each stage of the estrous cycle. Each plot compares the pattern for WT (□) and MT_1KO (■) genotypes. The abscissae represents zeitgeber time (ZT) with ZT14 and ZT0 representing respectively the onset of the dark period and the light period. The shaded gray area represent the dark period. Hours spent in each behavior were plotted as a function of ZT and compared using repeated measure two-way ANOVA with genotype and time as main factors. The overall genotype effect on total magnitude of behavior is indicated by the p-value in the upper right corner of each graph. n.s. non-significant. The genotype effect at each ZT was determined by Bonferoni post-test. *p<0.05.

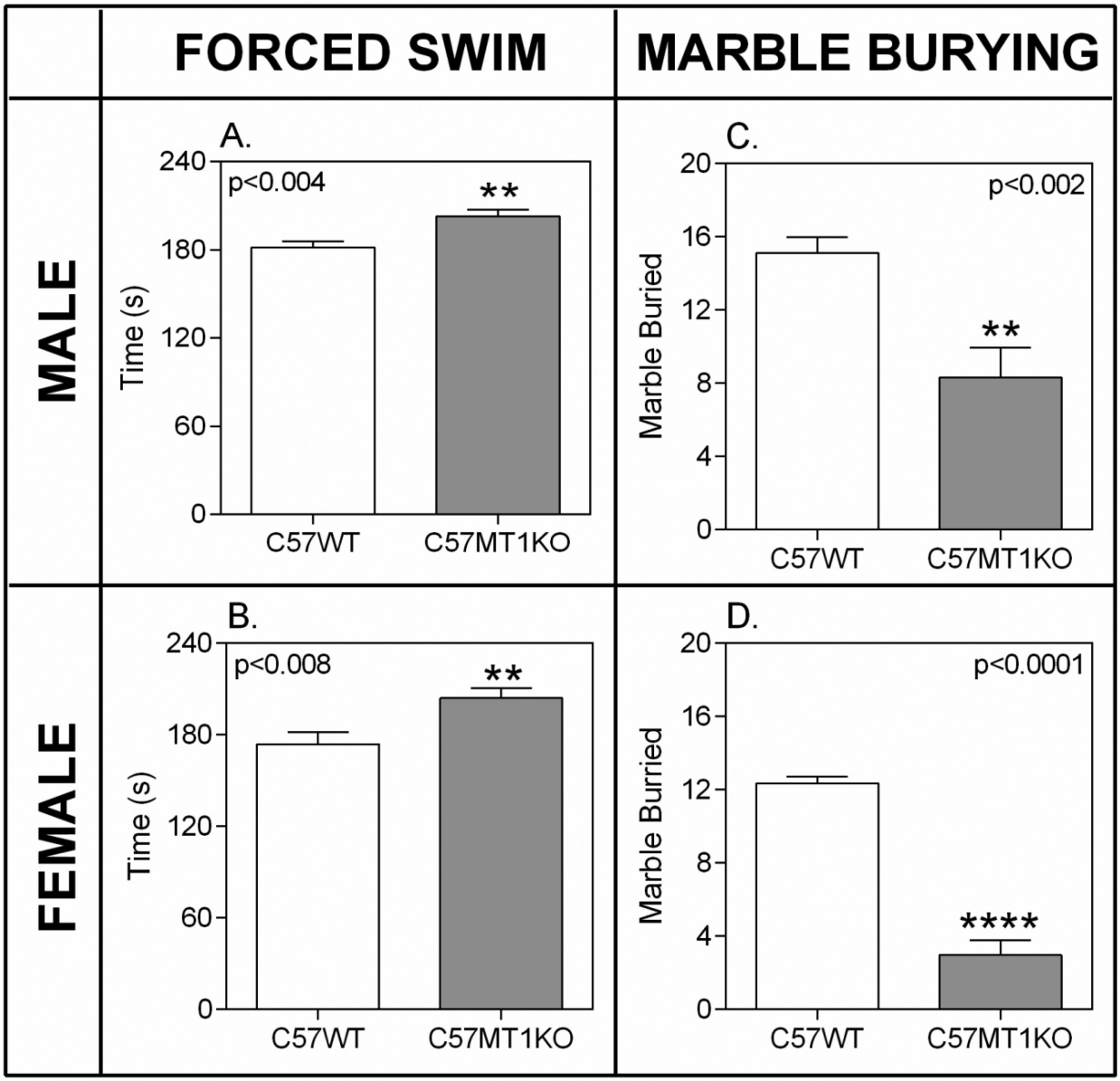

Figure 5. Effects of MT_1 receptor deletion on the forced swim test and the marble burying test in male and female mice

Male (A) and female (B) WT and MT_1KO mice were recorded during the Porsolt forced swim test to assess antidepressant-like activity. Bars indicate the amount of time that mice were immobile over a 4 min period for males (WT: 181.5 ± 4.48 s, n=10 and MT_1KO: 202.7 ± 4.71 s, n=14; *d*=1.33) and females (WT: 173.9 ± 8.00 s, n=14 and MT_1KO: 204.0 ± 6.69s, n=14; *d*=1.10). The marble burying test was used to assess anxiolytic-like activity in male (C) and female (D) WT and MT_1KO mice. The number of marbles buried in the bedding during a 30 min period is shown for males (WT: 15.10 ± 0.88 marbles, n=10 and MT_1KO: 8.308 ± 1.64 marbles, n=13; *d*=1.57).and females (WT: 12.34 ± 0.38 marbles, n=16 and

MT_1KO: 2.967 ± 0.82 marbles, n=15; *d*=3.97). All tests were performed during the light period between ZT9 and ZT11,