

# Genetic deletion of MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors differentially abrogates the development and expression of methamphetamine-induced locomotor sensitization during the day and the night in C3H/HeN mice

**Abstract:** This study explored the role of the melatonin receptors in methamphetamine (METH)-induced locomotor sensitization during the light and dark phases in C3H/HeN mice with genetic deletion of the MT<sub>1</sub> and/or MT<sub>2</sub> melatonin receptors. Six daily treatments with METH (1.2 mg/kg, i.p.) in a novel environment during the light phase led to the development of locomotor sensitization in wild-type (WT), MT<sub>1</sub>KO and MT<sub>2</sub>KO mice. Following four full days of abstinence, METH challenge (1.2 mg/kg, i.p.) triggered the expression of locomotor sensitization in METH-pretreated but not in vehicle (VEH)-pretreated mice. In MT<sub>1</sub>/MT<sub>2</sub>KO mice, the development of sensitization during the light phase was significantly reduced and the expression of sensitization was completely abrogated upon METH challenge. During the dark phase the development of locomotor sensitization in METH-pretreated WT, MT<sub>1</sub>KO and MT<sub>2</sub>KO mice was statistically different from VEH-treated controls. However, WT and MT<sub>2</sub>KO, but not MT<sub>1</sub>KO mice receiving repeated VEH pretreatments during the dark phase expressed a sensitized response to METH challenge that is of an identical magnitude to that observed upon 6 days of METH pretreatment. We conclude that exposure to a novel environment during the dark phase, but not during the light phase, facilitated the expression of sensitization to a METH challenge in a manner dependent on MT<sub>1</sub> melatonin receptor activation by endogenous melatonin. We suggest that MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors are potential targets for pharmacotherapeutic intervention in METH abusers.

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## Introduction

Repeated administrations of psychostimulants such as cocaine or methamphetamine (METH) induce a state of heightened sensitivity to subsequent drug exposures. This process, termed sensitization, is thought to reflect neuroadaptations that confer enhanced 'incentive salience' onto a drug and its associated contextual cues, which has implications for the motivational value of drug-associated stimuli and their ability to stimulate craving or drug-seeking behavior [1]. Overt manifestations of psychostimulant-induced sensitization in humans have been demonstrated sparsely, but the process has been proposed to reflect neurological alterations that underlie the transition from casual drug use to the compulsive use that signifies drug addiction [1, 2]. In contrast, sensitization is readily observed in mice as a progressive increase in locomotor activity after repeated intermittent METH pretreatments, culminating in an enhanced locomotor response to a challenge dose of the psychostimulant relative to vehicle-pretreated controls [3–5]. Over the past two decades, a wide variety of receptor-

mediated mechanisms were reported as targets for the modulation of locomotor sensitization induced by drugs of abuse such as cocaine, amphetamine, morphine and methamphetamine. In mice, METH-induced locomotor sensitization is reduced by aripiprazole acting as a partial agonist at the 5HT<sub>1A</sub> and D2 receptors [6], by the  $\mu/\kappa$  opioid receptor antagonist naltrexone [3], and by genetic deletion of the  $\mu$  opioid receptors [7], but is enhanced in mice lacking the corticotrophin-releasing factor gene [8]. Together, these reports identified endogenous targets that regulate METH-induced sensitization and may have potential for treating maladies related to METH abuse.

Melatonin, a hormone secreted into the circulation by the pineal gland, is a candidate for the modulation of psychostimulant responses [9]. The synthesis and release of melatonin from the pineal gland occurs according to a rhythm controlled by the suprachiasmatic nucleus of the hypothalamus, with highest levels measured at night [10, 11]. Melatonin signals the duration of nighttime to virtually all central and peripheral tissues through its interactions with two high-affinity G-protein-coupled receptors termed

MT<sub>1</sub> and MT<sub>2</sub> [12, 13]. These receptors are expressed throughout the brain [13], with mRNA for the MT<sub>1</sub> receptor present in the reward- and motivation-associated sites targeted by METH and other psychostimulants modulating dopaminergic neurotransmission, and include the ventral tegmental area (VTA), nucleus accumbens, and caudate-putamen [14].

By virtue of its importance in circadian physiology, melatonin is a viable candidate for modulating behavioral responses to drugs of abuse, which are well known to vary according to time of day [15]. Kuribara and Takadoro [16] demonstrated that acute METH treatment at 4 hr intervals elicits a diurnal rhythm of locomotor responses in mice with higher levels at night compared to day. Locomotor sensitization in cocaine-treated mice and rats is also subject to time-of-day regulation, with the strongest responses observed during the day in both mice [17–19] and rats [20]. These studies suggested that removal of the pineal gland and hence pineal products abolish the diurnal rhythm of cocaine sensitization in mice [18], leading to the overall conclusion that endogenous melatonin may be counteracting the sensitizing effect of cocaine. Results with exogenous melatonin on sensitization induced by psychostimulants are inconclusive, with no effect of melatonin observed on the expression of sensitization induced by this drug after 2-wk abstinence [21], or the expression of sensitization to METH in mice [22].

Here we demonstrated that both the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors are needed for METH pretreatments to induce the development, and the expression upon METH challenge of locomotor sensitization in the C3H/HeN mice. Melatonin receptors activated by endogenous melatonin through the 24 hr day/night period may participate in the signaling and molecular processes leading to the development and expression of METH-induced locomotor sensitization.

## Materials and methods

### Animals

Male C3H/HeN mice were bred in colonies maintained in the Laboratory Animal Facility at the University at Buffalo. C3H/HeN mice homozygous for the MT<sub>1</sub> melatonin (MT<sub>1</sub>KO) receptor gene deletion and their WT controls were generated by back-crossing C57BL/6J MT<sub>1</sub> knockout mice (donated by Dr. Steven Reppert, University of Massachusetts Medical School, Worcester, MA, USA) with C3H/HeN mice (Harlan, Indianapolis, IN, USA) [23]. Mice were back-crossed for seven generations and were congenic with the C3H/HeN strain expressing the wild-type allele at the arylalkylamine *N*-acetyltransferase (AA-NAT) gene as well as the *rd* (retina degeneration) mutation on the rod photoreceptor cGMP phosphodiesterase gene [24]. C3H/HeN mice homozygous for the MT<sub>2</sub> melatonin receptor gene deletion (MT<sub>2</sub>KO) were also donated by Dr. Steve Reppert. MT<sub>1</sub>/MT<sub>2</sub> double knockout C3H/HeN mice (MT<sub>1</sub>/MT<sub>2</sub>KO) were generated by back-crossing C3H/HeN MT<sub>1</sub>KO with C3H/HeN MT<sub>2</sub>KO. Male mice were bred under a 14:10 hr light/dark cycle, in a temperature- (22 ± 1°C) and humidity-controlled

environment with food and water provided ad libitum. Genotype was confirmed by PCR analysis of DNA samples prepared from tail tips periodically during the tenure of the colony and repeated when animals were sacrificed. All animal procedures were performed in accordance with the guidelines set forth by the National Institutes of Health and the Institutional Animal Care and Use Committee of the University at Buffalo.

At weaning, male mice were group-housed (4 per cage) and maintained in a 14:10 hr light/dark cycle, with 150–200 lux light illumination at the level of the cage. Mice (2–3 months old) were individually housed (cage 30 × 19 cm) and maintained in a 12/12 light/dark cycle inside ventilated and light-tight cabinets for 14 days prior to initiation of the locomotor sensitization experiment. Mice were weighed on the day before the first METH pretreatment (Day –1), after every locomotor activity test (Days 1–6), and 1 day prior to the sensitization test. Occasionally, mice losing more than 10% of their body weight during the course of the experiment were nutritionally supplemented with NutriCal. Only two mice (one WT, one MT<sub>1</sub>/MT<sub>2</sub>KO) were excluded from the experimental procedures after exceeding the weight loss threshold of 15%.

### Drugs

WT, MT<sub>1</sub>KO, MT<sub>2</sub>KO, MT<sub>1</sub>/MT<sub>2</sub>KO mice were randomly assigned to groups receiving either vehicle (saline) or METH (1.2, 2.4 or 4.8 mg/kg in saline). Vehicle or appropriate METH doses were administered via intraperitoneal (i.p.) injections to deliver 0.01 ml solution per gram of mouse weight.

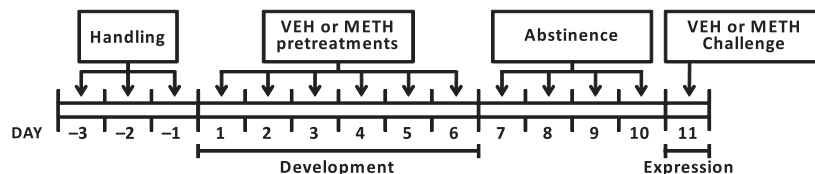
### Chambers and data acquisition

The 16 novel environment chambers consisted of cylindrical (20 cm diameter × 20 cm height) Plexiglas arenas [25]. The walls of the cylindrical arena were lined with black aluminum sheets to reduce shadows and reflections. The tops were open to allow top-view imaging and the floor was transparent to allow illumination from below. Locomotor activity was recorded by 4 overhead-mounted video cameras. Each camera recorded locomotor activity from four arenas simultaneously under white (light phase) or infrared (dark phase) light using the LocoScan behavior analysis system (CleverSys, Inc., Reston, VA, USA). Analog-to-digital conversion of the video data was accomplished by a Dell Precision workstation. Locomotor activity was quantified from the digital video by the TopScan software package running on the same computer.

### Development and expression of METH-induced sensitization

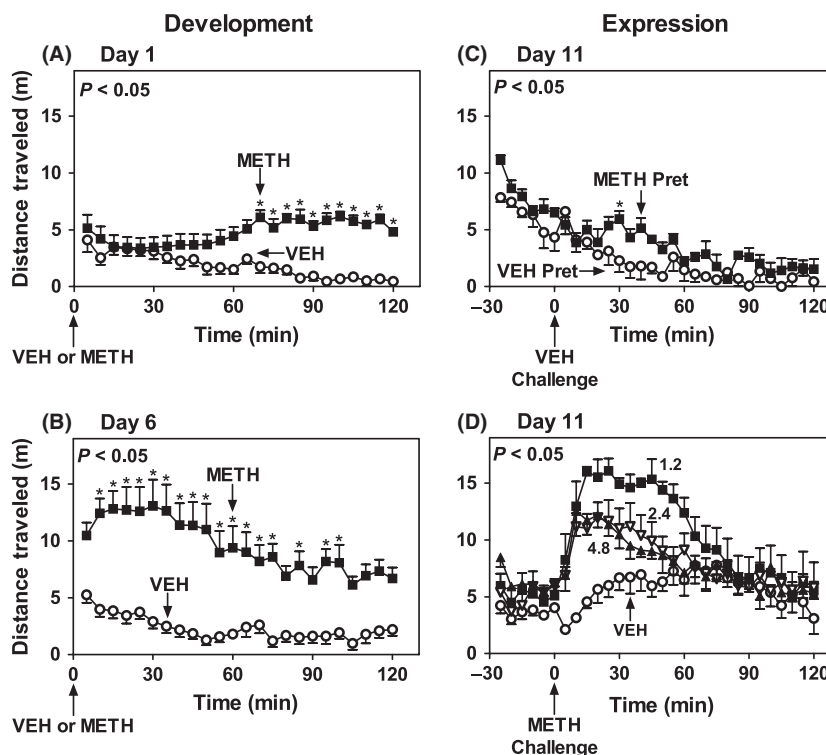
Figure 1(I) shows the experimental protocol used to induce locomotor sensitization by METH during the development and expression phases. Experiments were conducted either under ambient light of approximately 50 lux at the level of the arena floor between Zeitgeber time (ZT) 5 and 7, (ZT 0: lights on), or in complete darkness between ZT 19 and 21, with the experimenter visualizing the work through

# I. Protocol for METH-Mediated Locomotor Sensitization



# II. Locomotor activity

○ VEH    METH: ■ 1.2 mg/kg    ▽ 2.4 mg/kg    ▲ 4.8 mg/kg



**Fig. 1.** Optimization of the METH-induced locomotor sensitization protocol. Part I: protocol for METH induction of locomotor sensitization used during the light (ZT 5–7) or dark (ZT 19–21) phases. Days –3 to –1: mice handling and saline injections. Days 1–6: VEH or METH pretreatments (1.2 mg/kg, i.p.) (2.4 and 4.8 mg/kg, i.p., not shown). Locomotor activity was collected at 5 min intervals for 2 hr after treatment on Days 1, 2, 3 and 6. Day 7–10: abstinence period: no treatments. Day 11: VEH or METH challenge (1.2 mg/kg, i.p.). Locomotor activity was recorded 30 min prior to and 2 hr following treatment. Part II: ordinates represents distance traveled in 5 min intervals as a function of time (abscissae) during *development* (A, B) and *expression* (C, D) of sensitization in VEH (○) and METH (■) pretreated mice. Pretreatments were administered at times indicated by arrows under the abscissae. The main effects of drug treatment were  $F_{(1,14)} = 27.58$ ,  $P < 0.05$  on Day 1 (A) and  $F_{(1,14)} = 32.98$ ,  $P < 0.05$  on Day 6 (B). The main effects of drug pretreatment was  $F_{(1,6)} = 6.36$ ,  $P < 0.05$  in mice challenged with VEH and  $F_{(1,6)} = 6.36$ ,  $P < 0.05$  in mice challenged with METH at times indicated by arrows under the abscissae. Significance values for the main effect of drug pretreatment are noted in the upper left corners of the graphs. \* $P < 0.05$  when compared with the corresponding VEH 5 min bin (Bonferroni test).

night-vision goggles. Mice were moved to the testing room at least 1 hr before the start of the experiment, i.e., at ZT 4 or at ZT 12 for experiments conducted during the light and dark phases, respectively.

Mice were handled and injected with sterile saline (0.25 ml, i.p.) during three daily sessions (Days –3 to –1) to habituate them to the experimental procedures and return to the home cage. Next, during the development phase (Days 1–6) mice were treated with either VEH (n = 8) or METH (1.2 mg/kg, i.p.) (n = 8), unless otherwise indicated, and immediately placed in the novel environment arenas (cylindrical chambers). Locomotor

activity was recorded continuously for 2 hr from the time mice were placed in the novel environment and then returned to their home cage. Expression of locomotor sensitization was assessed on Day 11, after four full days of abstinence (Days 7–10). Mice pretreated with VEH (n = 8) or METH (1.2 mg/kg, i.p.) (n = 8) were then challenged with either VEH (VEH-VEH, n = 4; METH-VEH, n = 4) or METH (1.2 mg/kg, i.p.) (METH-VEH, n = 4; METH-METH, n = 4). Mice were moved to the arenas for 30 min of baseline recording at either ZT 4.5 or ZT 18.5 for light or dark phase experiments, respectively. Mice were given either VEH or METH as appropriate, and placed back into

the arenas starting at ZT 5 or ZT 18, respectively for 2 hr of locomotor activity recording. These procedures were conducted during both the light (ZT 5–7) and dark (ZT 19–21) phases in WT, MT<sub>1</sub>KO and MT<sub>2</sub>KO mice. Based on the results observed with the MT<sub>1</sub>/MT<sub>2</sub>KO mice during the light phase and the reduced availability of this genotype after a prolonged interruption in successful breeding, sensitization was conducted only during the light phase.

### Statistical analysis

Locomotor activity was indexed by distance traveled during the development and expression (challenge) phases of sensitization. Sixteen mice were tested in each experiment with VEH or METH administered to 8 mice per group during repeated pretreatments, that is, development of sensitization stage (i.e., VEH [*n* = 8] and METH [*n* = 8]). During the expression of sensitization stage, each group was challenged with either VEH (*n* = 4) or METH (*n* = 4). Experiments were repeated 2–3 times. Time course data were analyzed and then grouped in 5- or 30-min bins and time point averages for each experimental condition expressed as mean ± S.E.M. Time course studies were analyzed by two-way mixed design analysis of variance (ANOVA) having the between-groups factor of pretreatment drug (VEH or METH) and time. The development of sensitization across test day was analyzed by two-way mixed design ANOVA having the between-groups factor of pretreatment drug and the within-groups factor of test day. Treatment X Genotype between-subjects analysis of variance (ANOVA) was conducted on behavioral domains at each time (ZT 5–7 or ZT 19–21) in mice treated with VEH (saline, i.p.) or METH (1.2 mg/kg, i.p., unless otherwise indicated) in the four genotype groups (WT, MT<sub>1</sub>KO, MT<sub>2</sub>KO, MT<sub>1</sub>/MT<sub>2</sub>KO). The Bonferroni correction was used for post hoc comparisons. Analyses were computed using GraphPad Prism v. 5 (GraphPad Software, Inc., LaJolla, CA, USA). For all analysis *P* values < 0.05 were considered statistically significant.

### Results

Figure 1(I) shows the protocol used for inducing the development and expression of locomotor sensitization with METH in male C3H/HeN (WT, MT<sub>1</sub>KO, MT<sub>2</sub>KO, MT<sub>1</sub>/MT<sub>2</sub>KO) mice during the light (ZT 5–7) and dark phases (ZT 19–21). VEH or METH pretreatments and challenge after four full days of abstinence were administered in a novel environment that is, cylindrical arenas 20 cm in diameter [25]. Locomotor activity was recorded continuously for 2 hr after treatment to assess the temporal development of sensitization by METH.

Each experiment included 16 mice, half treated with VEH and the other half treated with METH at the appropriate dose during the 6-day development (pretreatment) stage. Mice treated with METH (1.2 mg/kg, i.p.) during the light phase on Day 1 expressed a gradual increase in locomotor activity compared to VEH controls over the 2 hr recording period (Fig. 1-IIA). Treatment with METH on Day 2–6 gradually increased locomotor activity beginning immediately after treatment (Fig. 1-IIB; Days 2–5 not shown).

Pretreatment with VEH during 6 days did not affect total locomotor activity: 44.6 ± 7.5 (*n* = 8) (Day1) versus 56.0 ± 6.9 (*n* = 8) (Day 6) (*P* > 0.05) (Fig. 1-IIA versus B). Total locomotor activity per 2 hr during repeated pretreatments with METH (1.2 mg/kg, i.p.), on Day 6 was significantly increased over Day 1: 113.7 ± 10.8 m (*n* = 8) versus 229.4 ± 29.4 m (*n* = 8), *P* < 0.05, Fig. 1-IIA versus B. Similar patterns were observed for total locomotor sensitization elicited by the higher doses of METH (2.4 and 4.8 mg/kg, i.p.) (data not shown). Maximal development of sensitization was observed with the lowest dose of METH (1.2 mg/kg, i.p.), with the higher doses producing significant stereotypic behaviors (e.g., rearing, grooming) (data not shown).

The expression of sensitization after the abstinence period (Days 7–10) was assessed by a challenge dose of either VEH or METH (1.2 mg/kg, i.p.) on Day 11 (see Fig. 1-IIC,D). VEH challenge induced a small conditioned-locomotor response in 1.2 mg/kg METH-pretreated mice compared to VEH-pretreated controls (Fig. 1-IIC). By contrast, METH (1.2 mg/kg, i.p.) challenge produced large increases in locomotor activity in mice pretreated with any of the three doses of METH (1.2, 2.4, 4.8 mg/kg), with the greatest expression observed in mice pretreated with 1.2 mg/kg METH (Fig. 1-IID). Thus, a sequence of six doses of METH (1.2 mg/kg, i.p.) induced the development of a state of sensitization expressed by METH challenge and distinct from a conditioned response triggered by the injection (Fig. 1-IIC,D). Thus we selected the 1.2 mg/kg METH dose for both the development and expression of locomotor sensitization in all subsequent experiments conducted during the light (ZT 5–7) or dark (ZT 19–21) periods.

During the light phase (ZT 5–7), METH (1.2 mg/kg, i.p.) increased locomotor activity on Day 1 to a similar extent in WT, MT<sub>1</sub>KO, MT<sub>2</sub>KO and MT<sub>1</sub>/MT<sub>2</sub>KO when compared with VEH-treated controls (Fig. 2A–D). Mice exhibited little change in total locomotor activity in a 2 hr period across pretreatment days in response to VEH injections (Fig. 3A), with the MT<sub>1</sub>/MT<sub>2</sub>KO exhibiting a small but significant decrease in distance traveled on Days 2, 3 and 6 compared to Day 1. In contrast, METH-treated WT, MT<sub>1</sub>KO and MT<sub>2</sub>KO mice expressed larger total locomotor activity responses after successive daily treatments (Day 1 to Day 6) with a maximal effect at Day 6 when compared with VEH pretreatment (Figs 2E–G and 3B). However, the magnitude of the METH pretreatment-induced development of sensitization of MT<sub>1</sub>/MT<sub>2</sub>KO mice during Days 2–6 was significantly attenuated (Figs 2H and 3B). On Day 11, METH challenge (1.2 mg/kg, i.p.) induced a robust expression of locomotor sensitization in METH-pretreated WT, MT<sub>1</sub>KO and MT<sub>2</sub>KO (Fig. 2I–K), but not in MT<sub>1</sub>/MT<sub>2</sub>KO mice compared to their VEH-pretreated counterparts (Fig. 2L).

During the dark phase, when circulating melatonin peaks in C3H mice [22, 25], METH pretreatment on Night 1 significantly increased locomotor activity in WT (Fig. 4A), MT<sub>1</sub>KO (Fig. 4B) and MT<sub>2</sub>KO (Fig. 4C) mice relative to VEH controls. No change in total locomotor responses was observed in VEH-pretreated mice across six consecutive test nights (Fig. 3C). In METH-pretreated WT, MT<sub>1</sub>KO and



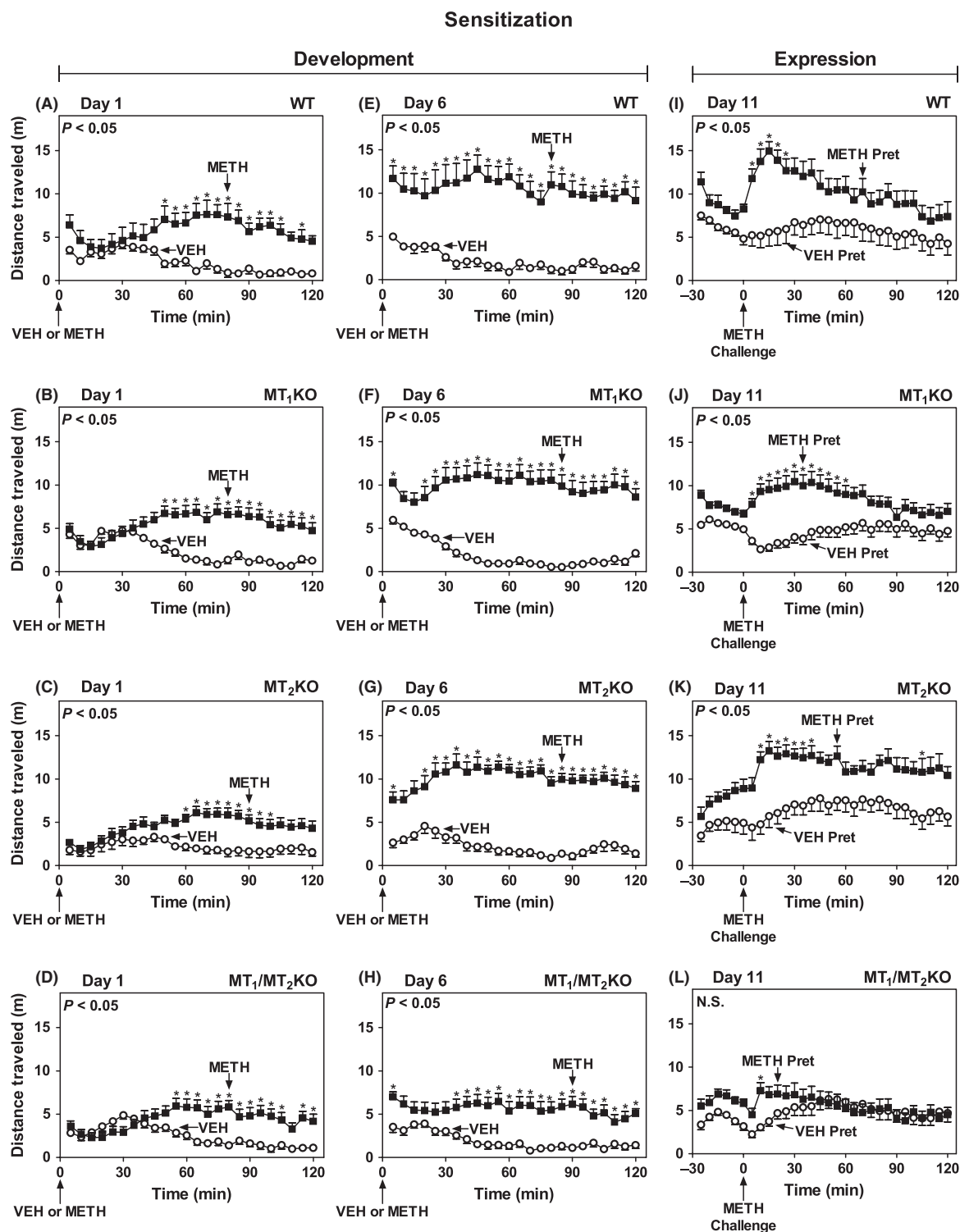


Fig. 2. Development and expression of locomotor sensitization during the light phase (ZT 5–7) in mice lacking MT<sub>1</sub> and/or MT<sub>2</sub> melatonin receptors. Ordinates represent distance traveled in 5 min intervals as a function of time (abscissae) during the development (Day 1: A, B, C, D; Day 6: E, F, G, H) and expression (Day 11: I, J, K, L) of sensitization, in mice pretreated with VEH (○) or METH (1.2 mg/kg, i.p.) (■) (A–H) and challenged with METH (1.2 mg/kg, i.p.) (I–L) at times indicated by arrows under the abscissae. The main effects of drug treatment for each genotype were as follows: Day 1: WT:  $F_{(1,14)} = 16.73$ ,  $P < 0.05$  (A); MT<sub>1</sub>KO:  $F_{(1,29)} = 24.88$ ,  $P < 0.05$  (B); MT<sub>2</sub>KO:  $F_{(1,19)} = 22.33$ ,  $P < 0.05$  (C); and MT<sub>1</sub>/MT<sub>2</sub>KO:  $F_{(1,23)} = 15.40$ ,  $P < 0.05$  (D). Day 6: WT:  $F_{(1,14)} = 37.69$ ,  $P < 0.05$  (E); MT<sub>1</sub>KO:  $F_{(1,29)} = 52.71$ ,  $P < 0.05$  (F); MT<sub>2</sub>KO:  $F_{(1,19)} = 112.40$ ,  $P < 0.05$  (G); and MT<sub>1</sub>/MT<sub>2</sub>KO:  $F_{(1,23)} = 25.08$ ,  $P < 0.05$  (H). Day 11: WT:  $F_{(1,14)} = 7.98$ ,  $P < 0.05$  (I); MT<sub>1</sub>KO:  $F_{(1,29)} = 17.18$ ,  $P < 0.05$  (J); MT<sub>2</sub>KO:  $F_{(1,19)} = 14.38$ ,  $P < 0.05$  (K); MT<sub>1</sub>/MT<sub>2</sub>KO:  $F_{(1,23)} = 0.79$ ,  $P > 0.05$  (L). Significance of the main effect of drug (A–H) or drug pretreatment (I–L) is noted in the upper left of each graph. NS, not significant. \* $P < 0.05$  when compared with the corresponding 5 min VEH interval (Bonferroni test).

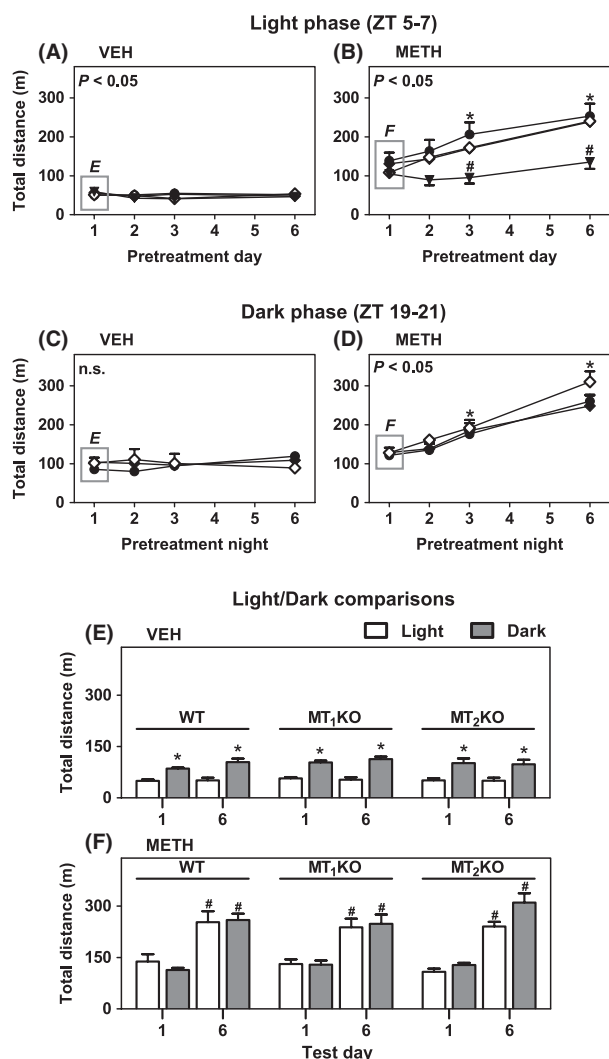


Fig. 3. Development of locomotor sensitization during the light (ZT 5–7) and dark (ZT 19–21) phases across pretreatment days. Panels A–D: ordinates represent total distance traveled in 2 hr as a function of pretreatment in Day 1 to Day 6 (abscissae) during development of sensitization in the light phase (ZT 5–7) (A, B) and the dark phase (ZT 19–21) (C–D) in WT (●), MT<sub>1</sub>KO (◆), MT<sub>2</sub>KO (▼) and MT<sub>1</sub>/MT<sub>2</sub>KO (▼) C3H mice. Significance of the main effect of test day (A:  $F_{(3,123)} = 3.15$ ,  $P < 0.05$ ; B:  $F_{(3,132)} = 47.24$ ,  $P < 0.05$ ) and test night (C:  $F_{(3,72)} = 0.74$ ,  $P > 0.05$ ; D:  $F_{(3,75)} = 77.85$ ,  $P < 0.05$ ) is noted in the upper left of the graphs. Panel B: \* $P < 0.05$  when compared to Day 1 in each genotype; # $P < 0.05$  when compared to corresponding values in WT, MT<sub>1</sub>KO and MT<sub>2</sub>KO mice. Panel D: \* $P < 0.05$  when compared to Day 1 in each genotype. Panels E and F: ordinates represent total locomotor distance traveled in 2 hr during the light (ZT 5–7) (open columns) and dark (ZT 19–21) (gray columns) phases at Day/Night 1 and Day/Night 6 of pretreatment with VEH or METH (1.2 mg/kg, i.p.). Panel E: \* $P < 0.05$  when compared with corresponding light phase values. Panel F: # $P < 0.05$  when compared with corresponding Day 1 values.

MT<sub>2</sub>KO mice the increases in locomotor responses were statistically different from VEH-pretreated and became progressively higher with the successive nightly treatments, with a maximal development of sensitization on Night 6 (Figs 3D and 4D–F). The magnitude of locomotor

responses during the development phase in each test night was not significantly different among either VEH- or METH-pretreated WT, MT<sub>1</sub>KO and MT<sub>2</sub>KO mice, suggesting that deletion of either MT<sub>1</sub> or MT<sub>2</sub> receptors has no effect on the development of sensitization at night. On Night 11, however, METH challenge triggered statistically significant expression of behavioral sensitization when compared with vehicle only in the MT<sub>1</sub>KO (Fig. 4H versus G and I). Expression of locomotor sensitization in METH-pretreated WT (Fig. 4G) and MT<sub>2</sub>KO mice (Fig. 4I) was of similar magnitude as in VEH-pretreated controls.

The total distance traveled by WT, MT<sub>1</sub>KO and MT<sub>2</sub>KO mice pretreated with VEH were significantly higher at night compared to day, with no significant differences observed between Day 1 and Day 6 at either time point (Fig. 3E). The converse was true for METH-treated mice, which exhibited no significant difference between night and day but did express larger locomotor responses on Day 6 relative to Day 1 at both time points (Fig. 3F).

Locomotor responses triggered by METH challenge during the light and dark phases on Day 11 are summarized in Fig. 5A,B. In VEH-pretreated WT and MT<sub>2</sub>KO mice (Fig. 5A), the expression of locomotor sensitization was significantly higher after METH challenge during the dark phase (ZT 19–21) than the light phase (ZT 5–7). By contrast, mice lacking the MT<sub>1</sub> receptors and pretreated with VEH show the same magnitude of locomotor sensitization upon a METH challenge on Day 11 during the light and dark phase (Fig. 5A). The magnitude of locomotor sensitization induced by METH challenge in METH-pretreated WT, MT<sub>1</sub>KO, or MT<sub>2</sub>KO mice was identical during the light and dark periods (Fig. 5B). VEH-pretreated mice challenged with VEH showed no difference between the light and dark phases (Fig. 5C).

VEH-pretreated WT and MT<sub>2</sub>KO mice became sensitized to the effects of METH on Day 11 uniquely at night. Interestingly, the dramatic locomotor sensitization to METH challenge was also observed during the dark period in mice not pretreated with VEH during the development of sensitization stage (Fig. 5D). These mice were subjected to an identical locomotor testing protocol, i.e., for example, placement in novel arenas for six consecutive days. Moreover, this response to the novel arenas was comparable to the sensitized response observed in METH-pretreated WT mice during the light (Fig. 2I) and dark (Fig. 4G) phases.

## Discussion

This study reports the involvement of the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors in modulating the development and expression of METH-induced locomotor sensitization in melatonin proficient C3H/HeN mice. In C3H/HeN WT mice, treatment with consecutive daily doses of METH during the light phase (ZT 5–7) when endogenous melatonin levels are low induced a gradual and statistically significant increase in the development of locomotor sensitization from Day 1 to Day 6 when compared with vehicle-treated controls. A METH challenge dose after abstinence during the light phase induced a robust expression of sensitization in mice pretreated with METH as compared with those pretreated with vehicle. Mice

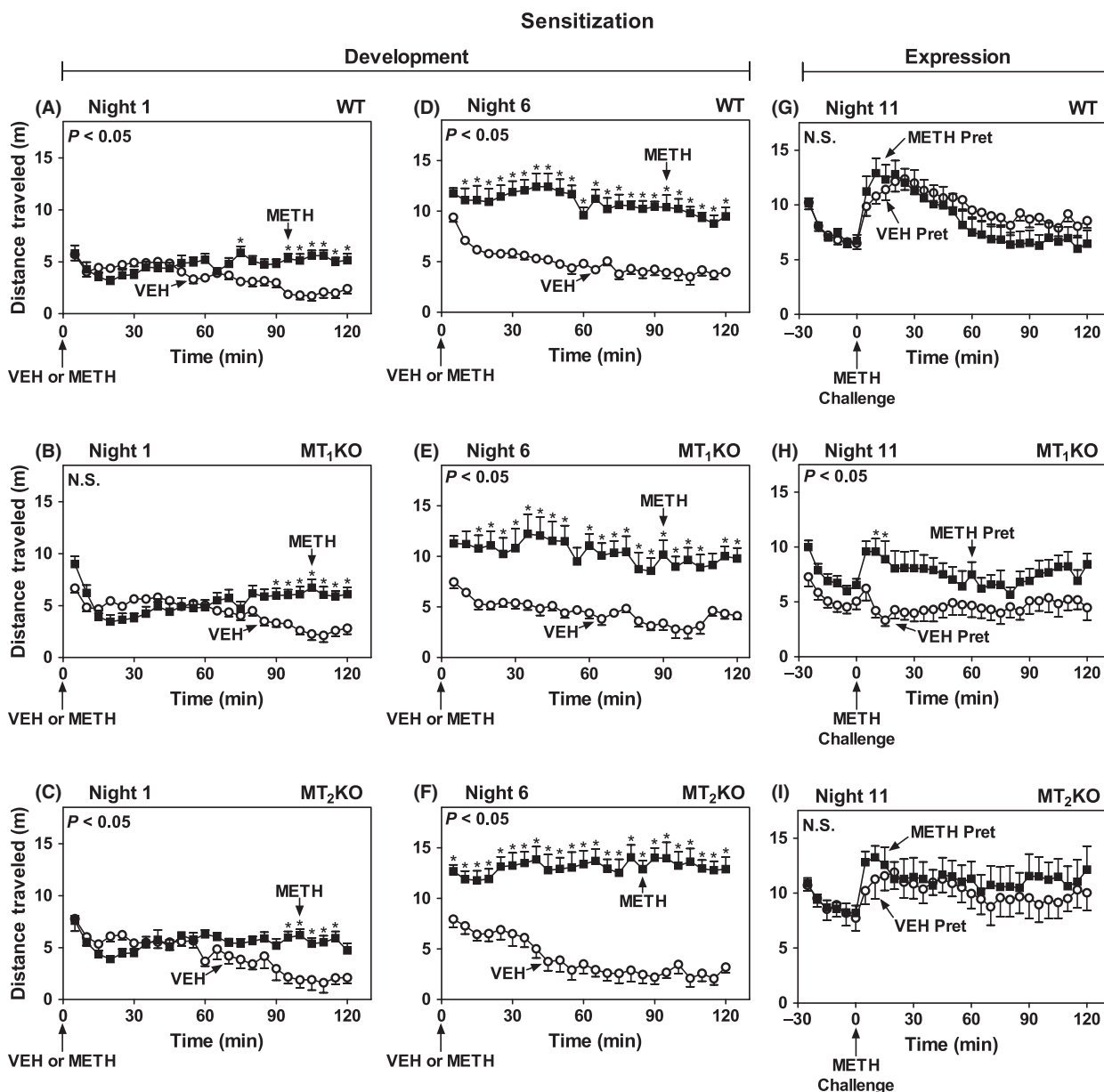


Fig. 4. Development and expression of locomotor sensitization during the dark phase (ZT 19–21) in mice lacking  $MT_1$  or  $MT_2$  melatonin receptors. Ordinates represent distance traveled in 5 min intervals as a function of time (abscissae) during the development (Night 1: A, B, C; Night 6: D, E, F) and expression (Night 11: G, H, I) of sensitization, in mice pretreated VEH (○) or METH (1.2 mg/kg, i.p.) (■) (A–F) and challenged with METH (1.2 mg/kg, i.p.) (G–I) at times indicated by arrows under the abscissae. The main effects of drug treatment for each genotype were as follows: Night 1: WT:  $F_{(1,19)} = 17.63$ ,  $P < 0.05$  (A);  $MT_1KO$ :  $F_{(1,15)} = 3.17$ ,  $P > 0.05$  (B); and  $MT_2KO$ :  $F_{(1,17)} = 4.75$ ,  $P < 0.05$  (C). Night 6: WT:  $F_{(1,19)} = 54.45$ ,  $P < 0.05$  (D);  $MT_1KO$ :  $F_{(1,15)} = 21.55$ ,  $P < 0.05$  (E); and  $MT_2KO$ :  $F_{(1,17)} = 63.73$ ,  $P < 0.05$  (F). Night 11: WT:  $F_{(1,19)} = 0.72$ ,  $P > 0.05$  (G);  $MT_1KO$ :  $F_{(1,15)} = 5.98$ ,  $P < 0.05$  (H);  $MT_2KO$ :  $F_{(1,17)} = 0.30$ ,  $P > 0.05$  (I). Significance of the main effect of drug (A–F) or drug pretreatment (G–I) is noted in the upper left of each graph. NS, not significant. \* $P < 0.05$  when compared with the corresponding VEH 5 min interval (Bonferroni test).

with genetic deletion of the  $MT_1$  ( $MT_1KO$ ) or the  $MT_2$  ( $MT_2KO$ ) melatonin receptors exhibited similar development and expression sensitization after METH pretreatments during the light phase, but these responses were significantly attenuated in  $MT_1/MT_2KO$  mice. During the dark phase (ZT 19–21), when endogenous melatonin levels are elevated [26], the locomotor responses to METH challenge were identical in VEH- and METH-pretreated mice in both WT and  $MT_2KO$  groups. This sensitization-

like response to METH challenge was observed in mice exposed to novel environments only during the dark phase, with or without VEH pretreatments. This nocturnal enhancement of the response to METH challenge after repeated VEH pretreatments was not seen in  $MT_1KO$  mice. The data suggest that repeated exposure to a novel environment during the dark phase triggers sensitization to METH via endogenous melatonin activation of  $MT_1$  receptor-mediated signaling and/or other circadian factors.

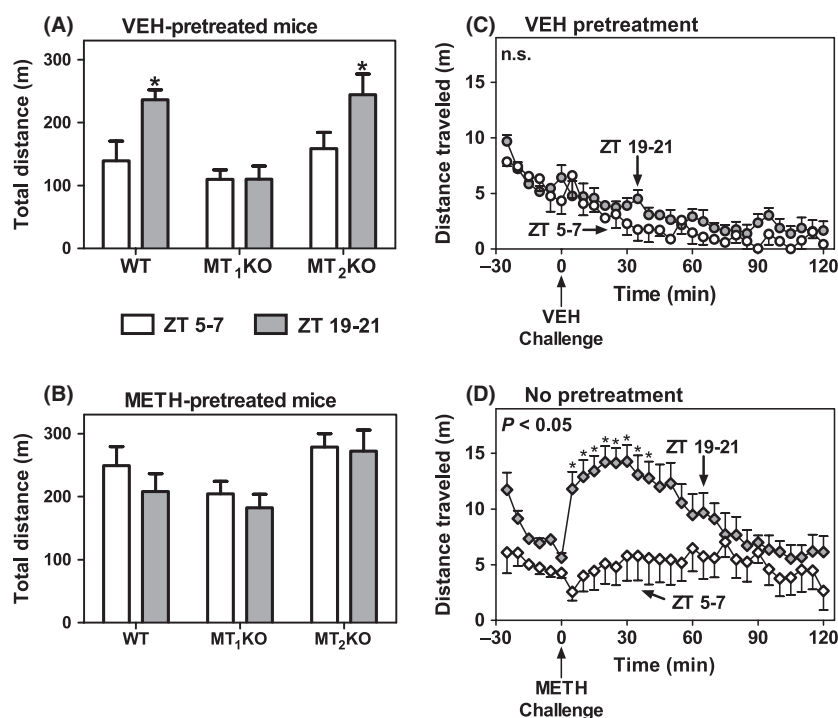


Fig. 5. Time-of-day differences in the expression of locomotor sensitization in VEH-pretreated mice. Panels A and B: ordinates represent total distance traveled for 2 hr after METH challenge on Day/Night 11 during the light (ZT 5–7) (open columns) and dark (ZT 19–21) (gray columns) phases following pretreatments with VEH (A) or METH (1.2 mg/kg, i.p.) (B). \* $P < 0.05$  when compared to corresponding ZT 5–7 values in VEH-pretreated WT and MT<sub>2</sub>KO mice (Bonferroni test). Panels C and D: ordinate represents distance traveled in 5 min intervals as a function of time before and after VEH (C) or METH (1.2 mg/kg, i.p.) (D) challenge at the times indicated by arrows under the abscissae. The main effect of *time of day* was in C:  $F_{(1,17)} = 2.45$ ,  $P > 0.05$  and in D:  $F_{(1,6)} = 7.21$ ,  $P < 0.05$ . \* $P < 0.05$  when compared with the corresponding 5 min VEH bin (Bonferroni test).

Melatonin or opioid receptor activation modulates METH-induced locomotor sensitization as this response was significantly attenuated by deletion of both the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors or  $\mu$  opioid receptors [7]. The  $\mu/\kappa$  opioid receptor antagonist naltrexone, as well as the 5HT<sub>1A</sub>/D<sub>2</sub> receptor partial agonist aripiprazole, blocked METH-induced development and expression of sensitization in mice [3, 6]. In the present studies, genetic deletion of both the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors was necessary to abrogate locomotor sensitization, which is consistent with other reports aiming to block G-protein coupled receptor functional responses. Genetic deletion of both the presynaptic  $\alpha 2a$  and  $\alpha 2c$  adrenergic inhibitory autoreceptors on noradrenergic neurons was necessary to enhance norepinephrine release from cultured mouse atrium and cortical slices [27]. Further, deletion of both alpha adrenergic receptors was necessary to increase the circulating levels of norepinephrine. With the caveats associated with the use of global knockout mice [28], our findings in the MT<sub>1</sub>/MT<sub>2</sub>KO mice could reflect redundancy or compensation in the function of the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors.

The progressive increase in locomotor activity induced by daily doses of psychostimulants correlates with increases in dopamine release from presynaptic terminals in the nucleus accumbens [29, 30]. Presynaptic melatonin heteroreceptors modulate calcium-dependent release of dopamine in the retina and brain, playing an important role in modulating

dopaminergic transmission [31–34]. Melatonin attenuates the excitation of striatal neurons induced by glutamatergic cortical inputs, which is counteracted by the D<sub>2</sub> dopamine receptor antagonist sulpiride [35]. Activation of somadendritic MT<sub>1</sub> receptors inhibiting neuronal firing, and/or presynaptic MT<sub>1</sub> or MT<sub>2</sub> heteroreceptors inhibiting dopamine release on dopaminergic neurons originating in the VTA and/or substantia nigra pars compacta provides inhibitory and modulatory inputs [14, 30, 36, 37]. It follows that the circadian release of endogenous melatonin through activation of receptors on somadendritic and/or axon terminals may exert a net diurnal inhibitory effect on basal dopaminergic activity, thereby establishing a relatively low, primed, basal level of dopaminergic activity in areas where METH exerts its effects. The sensitizing properties of METH may require this primed state which could be precluded by removal of the MT<sub>1</sub> and MT<sub>2</sub> receptors leading to an abnormally elevated basal level of dopamine signaling and the obscuring of sensitized activity after repeated METH.

Removal of presynaptic melatonin heteroreceptors on dopaminergic neurons may lead to persistent increases in dopamine release. In MT<sub>1</sub>/MT<sub>2</sub>KO mice persistent increases in extracellular dopamine could promote oxidative stress leading to programmed cell death [38]. The neurotoxic effects of METH are partially mediated by programmed cell death leading to increases in dopamine release. METH-mediated increases in dopamine release are known to cause apoptosis through activation of D<sub>1</sub>



receptor-mediated mitochondrial dysfunction [39] and autophagy through inhibition of the mammalian target of rapamycin [40]. Neuroprotective effects of melatonin on multiple pathways coupling METH to apoptotic and autophagic cell death have been reported [40–44]. Removal of presynaptic inhibitory MT<sub>1</sub> and MT<sub>2</sub> melatonin heteroreceptors on dopaminergic neuron receptors could potentially increase the apoptotic effects of METH via increases in dopamine release [31, 33]. Further, genetic deletion of MT<sub>1</sub> mitochondrial receptors may potentiate METH-induced programmed cell death as activation of this receptor reduces cytochrome c and caspase activation [45]. Activation of the MT<sub>1</sub> receptors blocks mitochondrial dysfunction and apoptosis in a mouse model of Huntington's disease [45], and melatonin reverses the effects of METH on mTOR-controlled autophagy and activation of pro-apoptotic proteins including Bax, calpain and caspases [40–42]. It is then conceivable that in dopaminergic neurons expressing melatonin receptors, activation of the MT<sub>1</sub> and perhaps the MT<sub>2</sub> melatonin receptors may attenuate the neurotoxic effect of METH, by reducing dopamine release [31, 33] and/or mitochondrial mediated apoptosis [45]; hence preserving the health and proper function of dopaminergic pathways throughout the repeated treatments with METH. Deletion of the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors in dopaminergic neurons since birth may lead to neuronal dysfunction by depriving the neuron of the circadian modulation of dopaminergic activity via inhibition of dopamine release and apoptosis.

The mechanism of novelty-induced expression of sensitization to METH challenge during the dark period is not known. Exposure to a novel environment stimulates the transient release of dopamine in the nucleus accumbens as measured by voltammetry [46], suggesting a mechanism by which METH could facilitate sensitization under conditions in which dopaminergic transmission via MT<sub>1</sub> receptors is inhibited by endogenous melatonin. Notably, the magnitude of this putative novelty-induced sensitized response to single METH challenge is identical to that induced by repeated METH pretreatments, in mice endowed with MT<sub>1</sub> receptors, indicating that novelty may have a greater role in determining the magnitude of sensitization at night than does METH. This phenomenon may have its roots in an association between novelty and stress, as environmental novelty up-regulates stress-related hormones (e.g., corticosterone) [47] and repeated mild stressors (e.g., tail pressure) induce locomotor sensitization to amphetamine [48]. Activation of signaling pathways involves circadian molecules such as Per1, which is linked to MT<sub>1</sub> melatonin receptor activation [18, 49] and necessary for METH-mediated sensitization [50].

The nocturnal nature of the effect of novelty in our studies is likely explained by intracellular signaling through the MT<sub>1</sub> receptor. Signaling targets for this receptor in C3H/HeN mice includes Period 1 (Per1) [51], a clock gene whose protein oscillates in the caudate-putamen and nucleus accumbens with high levels during the day (ZT 5) and low levels at night (ZT 17–24) in a manner contingent on the presence of the pineal gland [18]. In the pars tuberalis, a similar rhythmic pattern of Per1 expression requires the MT<sub>1</sub> receptor [49]. METH treatment leads to

the acute induction of Per1 expression in the striatum through activation of D<sub>1</sub>-like dopamine and *N*-methyl-D-aspartate (NMDA) receptors. Further, METH challenge triggers a sensitized response in Per1 induction [50]. Interactions between METH (acting through dopamine and its receptors) and the MT<sub>1</sub> receptor at the level of oscillating Per1 protein may account for the appearance of a sensitization-like state during the dark phase in mice exposed to novel environments. Specifically, MT<sub>1</sub> receptor activation by endogenous melatonin in a setting of low Per1 protein abundance may render cells in the dopaminergic pathway to be more responsive to the dopamine release events triggered by exposure to the locomotor arenas. Consistent with this hypothesis, deletion of CLOCK (circadian locomotor output cycles kaput) protein, a transcriptional initiator of Per1 expression, has been shown to induce hyperactivity in the dopaminergic pathways and increased responsiveness to cocaine in behavioral assays of reward in mice [51, 52].

Our results demonstrate that deletion of the MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors decreases the development and expression of METH-induced locomotor sensitization during the light phase and that the MT<sub>1</sub> receptor uniquely confers enhanced susceptibility to the effect of novelty on METH-induced locomotor responses during the dark phase. The effect of novelty on the enhancement of METH-induced locomotor activity exclusively at night has important implications for understanding the factors that regulate sensitivity to METH in humans, that is, the time of day and the novelty of the context surrounding drug use. Circadian changes in MT<sub>1</sub> melatonin receptor signaling together with changes in circadian molecules might play an important role in mediating the expression of sensitization by a single dose of METH to mice exposed in consecutive days to a novel environment at night. Sensitization is widely considered a viable model for the neuroadaptations that underlie the advancement of drug craving; therefore, the melatonin receptors may be a target worthy of additional investigations for their potential in treating METH abusers.

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## Author contributions

All authors contributed to the conception and design, acquisition of data, or analysis and interpretation of data (AJH, MLD, RLH). AJH drafted the manuscript, which was substantially edited by MLD. All authors approved the manuscript before submission.

## Conflict of interest

The authors declare that this work was funded by R01DA021870 to MLD. The authors declare that over the last 3 yr MLD was a consultant for and received compensation from Adolor, and from Takeda Pharmaceutical North America Inc.

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