Journal of Pharmacological Sciences 152 (2023) 128–135

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full Paper

# Ramelteon administration enhances novel object recognition and spatial working memory in mice

Mikuru Kudara [a], Eriko Kato-Ishikura [a], Yuji Ikegaya [a, b, c], Nobuyoshi Matsumoto [a, b, *]

[a] Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan
[b] Institute for AI and Beyond, The University of Tokyo, Tokyo 113-0033, Japan
[c] Center for Information and Neural Networks, National Institute of Information and Communications Technology, Suita City, Osaka, 565-0871, Japan

ARTICLE INFO

*Article history:*
Received 18 November 2022
Received in revised form
17 March 2023
Accepted 10 April 2023
Available online 11 April 2023

*Keywords:*
Ramelteon
Mouse
Memory

ABSTRACT

Ramelteon is used to ameliorate sleep disorders that negatively affect memory performance; however, it remains unknown whether ramelteon strengthens neutral memories, which do not involve reward or punishment. To address this, we monitored behavior of mice treated with vehicle/ramelteon while they performed a novel object recognition task and a spontaneous alternation task. Object memory performance in the novel object recognition task was improved only if ramelteon was injected *before* training, suggesting that ramelteon specifically enhances the acquisition of object recognition memory. Ramelteon also enhanced spatial working memory in the spontaneous alternation task. Altogether, acute ramelteon treatment enhances memory in quasi-natural contexts.



## 1. Introduction

Memory and learning are diminished by age-related dementias. Postmortem histological studies revealed that immunoreactivity of melatonin $MT_1$ and $MT_2$ receptors was increased and decreased, respectively, in the hippocampus of dementia patients,[1–3] implicating the clinical significance of the melatonin receptors. Moreover, memory formation and learning rely on long-term potentiation in the hippocampus, a process by which certain connections between neurons are selectively and cooperatively strengthened by enhanced synaptic activity. The long-term potentiation is impaired in hippocampal slices from mice lacking $MT_2$ receptors,[4] suggesting participation of $MT_2$ receptors in hippocampal synaptic plasticity and memory processes.

Ramelteon, a clinical remedy used to alleviate circadian rhythm sleep disorders,[5] is a selective melatonin receptor agonist with high affinity for $MT_1$ and $MT_2$ receptors.[6] Consistent with the histological and physiological investigations demonstrating a close relationship between memory formation and melatonin receptors,[1,2,4] a previous study concluded that ramelteon treatment did not cause memory impairment in rats.[5] In this previous study, the delayed matching-to-position task, the conditioned place preference task, and the Morris water maze task were used to investigate the influence of ramelteon treatment on memory.[5] However, the delayed matching-to-position and conditioned place preference tasks required animals to undergo operant and Pavlovian conditioning, respectively; that is, the animals learned to associate lever pressing with the delivery of food to them in the delayed matching-to-position task,[7–11] whereas they were forced to be injected with a drug (i.e., an unconditioned stimulus) in a certain compartment in an experimental box and came to prefer the drug-paired compartment, which would eventually serve as a conditioned stimulus in the conditioned place-preference task.[12] Moreover, in the Morris water maze task, animals must be physically capable of swimming satisfactorily to escape from the water.[13] The need to escape repeatedly from the water over days of training is considered an aversive and stressful condition.[14,15] Although these tasks were valid for investigating the effect of ramelteon on memory associated with reward and punishment, it is not fully understood whether the treatment of ramelteon, a melatonin receptor agonist, has an impact on memory in a more natural situation, a context with neither reward nor punishment.

To address this question, we adopted the novel object recognition task in an open field and the spontaneous alternation task in a

* Corresponding author. Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
*E-mail address:* nobuyoshi@matsumoto.ac (N. Matsumoto).
Peer review under responsibility of Japanese Pharmacological Society.

https://doi.org/10.1016/j.jphs.2023.04.002

Y-maze, each of which measures memory performance in animals based on their natural tendency to prefer (relatively) novel objects and places.[15–18] Taking advantage of this tendency, we allowed ramelteon-treated and vehicle-treated mice to perform the novel object recognition task and the spontaneous alternation task to evaluate the effect of ramelteon on object recognition and spatial memory.

## 2. Materials and methods

### 2.1. Ethical approval

Animal experiments were performed with the approval of the Animal Experiment Ethics Committee at the University of Tokyo (approval number: P4-6) and according to the University of Tokyo guidelines for the care and use of laboratory animals. These experimental protocols were carried out in accordance with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 of 2006), the Standards for Breeding and Housing of and Pain Alleviation for Experimental Animals (Ministry of the Environment, Notice No. 88 of 2006) and the Guidelines on the Method of Animal Disposal (Prime Minister's Office, Notice No. 40 of 1995). All efforts were made to minimize animal suffering.

### 2.2. Animals

A total of one hundred fourteen 8- to 10-week-old male ICR mice (Japan SLC, Japan) weighing 35–45 g were housed in groups under conditions of controlled temperature and humidity (22 ± 1 °C, 55 ± 5%) and maintained on a 12:12-h light/dark cycle (lights off from 7:00 a.m. to 7:00 p.m.) with *ad libitum* access to food and water. Mice were acclimated to an experimenter via daily handling for at least 3 days before experiments.

### 2.3. Apparatus

The novel object recognition task took place in an open square field measuring 40 cm in width, 40 cm in depth, and 40 cm in height. The walls and floor of the open field were made of translucent corrugated plastic painted white or black, respectively. Each of the four inner walls was independently colored partially with green and/or blue tape so that mice could recognize their azimuth in the field. The four objects used in this test consisted of two gray plastic cylinders (one solid and one hollow) and two wooden quadrangular prisms (one short and one tall). All objects were also visually highlighted with colored tape. The spontaneous alternation task took place in a Y-shaped maze whose three arms were evenly spaced (120° apart), equal in size (40 cm long × 6 cm wide) and enclosed with opaque white corrugated plastic arms; the maze sat on the floor of the room. The center platform was triangular. For both the novel object recognition task and the spontaneous alternation task, a web camera was placed above the apparatus to monitor the animals' behavior.

### 2.4. Drug

Ramelteon (184-03371, FUJIFILM Wako, Japan) was dissolved in dimethyl sulfoxide at a concentration of 30 mg/ml.[19,20] This solution was diluted with saline solution to final concentrations of 0.1, 0.3, 1.0, and 3.0 mg/ml immediately before use. Saline with 1% dimethyl sulfoxide (i.e., 0 mg/ml ramelteon) was used as the vehicle control solution. Luzindole (L0316, Tokyo Chemical Industry, Japan), a melatonin $MT_1$ and $MT_2$ receptor antagonist, was dissolved in 99.5% ethanol at a concentration of 300 mg/ml. This solution was mixed with Tween 80 at a volume ratio of 1:1. This mixed solution was further diluted with ultrapure water (Milli-Q water) at a volume ratio of 1:49 to yield 3.0 mg/ml luzindole solution.

### 2.5. Behavioral tests

The novel object recognition test consisted of habituation sessions and subsequent training and test sessions on Days 1 and 2, respectively.[21] In the habituation sessions, each mouse was allowed 5 min per day to freely explore the open field arena for 3 days before the training and test sessions. On Day 1, two identical objects were placed in two of the four quadrants of the open field. A mouse was then allowed to freely explore the open field for 5 min. On the next day (i.e., Day 2), one of the two objects was replaced with another novel object in the same location. Once again, the mouse was allowed to freely explore the field for 5 min. Each drug (vehicle or ramelteon) was intraperitoneally injected into mice (i) 20 min before the training session, (ii) immediately after the training session, or (iii) 20 min before the test session to examine different phases of memory: (i) acquisition, (ii) consolidation, and (iii) retrieval.[21] The objects used in each session were randomly predetermined, and their locations were randomized across mice.

The spontaneous alternation task was used to assess spatial working memory and spatial reference memory in mice.[22,23] To assess spatial working memory, a mouse was placed into the distal part of one arm and allowed to freely explore the Y-maze for 5 min. To assess spatial reference memory, one of the arms was closed off with a divider (made of corrugated plastic) to remain unexplored. On Day 1, a mouse was placed on one of the remaining arms (called a starting arm) and allowed to explore the two-arm maze for 15 min, after which the mouse was returned to its home cage. On Day 2 (i.e., one day after the mouse explored the two-arm maze), the divider was removed. The mouse was placed into the same starting arm as is used in the previous run and allowed to explore the Y-maze for 5 min. Note that 3.0 mg/kg of ramelteon was intraperitoneally administered (i) 20 min before exploration to probe spatial working memory and (ii) 20 min before the exploration of the incomplete Y-maze on Day 1 to assess spatial reference memory. The unexplored arm and the starting arm were randomized across mice.

As for the novel object recognition memory and spatial working memory, luzindole (30 mg/kg) was intraperitoneally injected into mice. 120 min after luzindole administration, mice were further injected with ramelteon (3.0 mg/kg). Then, 20 min after ramelteon administration, the mice underwent the training session of the novel object recognition task or the probe trial for spatial working memory.

Behavioral tests were conducted during the nocturnal (dark) period for mice (i.e., from 7:00 a.m. to 7:00 p.m.). Every time each behavioral test was finished, the apparatus was wiped, cleaned, and disinfected using 70% ethanol to eliminate as much of the residual mouse scent as possible before the next mouse was placed in the apparatus.

### 2.6. Immunohistochemistry

Six mice were intraperitoneally injected with saline or ramelteon (3 mg/kg). Twenty min after the injection, they were allowed to explore objects in the open field for 5 min. Approximately 1.5 h after the exploration, they were anesthetized with overdose of urethane. Anesthesia was confirmed by the lack of reflex responses to tail and toe pinches. They were transcardially perfused with chilled 0.01 M phosphate-buffered saline (PBS) followed by 4%

paraformaldehyde in PBS. The animals were then decapitated, and their brains were carefully removed. These brains were postfixed in 4% paraformaldehyde overnight and washed with PBS three times for 10 min each, and coronal sections were prepared using a vibratome at a thickness of 100 μm from the anterior region to the posterior region.

Basic immunohistochemistry procedures have been described previously.[24–28] Sections were blocked with 5% bovine serum albumin and 0.3% Triton X-100 in PBS for 1 h at room temperature and incubated with rabbit primary antibody against c-Fos (1:1000, 226008, Synaptic Systems, Germany) for 16 h at 4 °C. Sections were washed with PBS three times for 10 min each and then incubated with NeuroTrace 435/455 blue fluorescent Nissl stain (1:200, N21479, Thermo Fisher Scientific, USA; hereafter, Nissl) for 4 h at room temperature. The sections were further incubated with Alexa Fluor 488-conjugated goat secondary antibody against rabbit IgG (1:500, A11034, Thermo Fisher Scientific, USA) for 1.5 h at room temperature, followed by another three 10-min washes with PBS. Stained sections were mounted onto microscope slides using aqueous mounting medium.

### 2.7. Image acquisition

Images of stained sections including the medial prefrontal cortex and hippocampus were acquired using a fluorescence microscope (BZ-X810, Keyence, Japan) equipped with a 10×objective.

### 2.8. Data analysis

All data analyses were performed using custom-made routines of MATLAB (MathWorks, USA), Python, and ImageJ. The summarized data are reported as the mean ± the standard error of the mean (SEM). Unless otherwise specified, the null hypothesis was statistically rejected when $P < 0.05$ by Student's *t*-test. When multiple pairwise comparisons between ramelteon-treated and vehicle-treated groups were required, statistical analysis was performed with Dunnett's test (Fig. 1C and D).[29] When multiple pairwise comparisons among arms of the Y-maze were needed, significance levels were adjusted based on the Bonferroni correction after *P* values were computed by Student's *t*-test (Fig. 2B, C, D, F, G, H).

The moment-to-moment positions of the mice were tracked by DeepLabCut, a markerless tracking system,[30,31] or by manual detection with ImageJ (National Institutes of Health, USA). For the novel object recognition test, the discrimination ratio in the test session was calculated as (T2 − T1)/(T2 + T1), where T1 and T2 represented the time spent in an area (~25 $cm^2$) around the familiar and novel objects, respectively. The discrimination ratio in the training session was calculated with the same formula, but T1 and T2 represented the time that mice spent exploring the object that would remain and the object that would be replaced, respectively, in the subsequent test session; ideally, the ratio would be zero in the training session. For the spontaneous alternation task, correct alternations (i.e., α-β-γ (arm α to arm β, then to arm γ), α-γ-β, β-α-γ, β-γ-α, γ-α-β, and γ-β-α) were counted based on triplet combinations of arm entries, and then an alternation index was calculated as 100 × (the number of correct alternations/(the total number of arm entries − 2)).[23] Arm reentries (*e.g.*, α-α) were not excluded from the triplet data, such that the chance level of the alternation index would be 33.3.[32] To quantify sleep onset latency and total sleep time, mice were intraperitoneally injected with vehicle or ramelteon, and their behavior was monitored for up to 90 min since the drug administration. Sleep was behaviorally defined as a state of immobility lasting for more than 2 min based on the video analysis. The moment when the first sleep was started was regarded as the sleep onset (Fig. S1). The total sleep time was represented as the percentage of immobility in the monitoring period (Fig. S1). The density of c-Fos positive cells was quantified using custom-made ImageJ routines (Fig. S2).

## 3. Results

We first examined whether ramelteon treatment affected the acquisition of memory regarding object recognition in mice.[21] We intraperitoneally injected 0, 0.1, 0.3, 1.0, and 3.0 mg/kg ramelteon into mice 20 min before the training session for the novel object recognition test (i.e., acquisition group) and then began the training (Fig. 1A). On the next day, we monitored mouse behavior and assessed behavioral and cognitive performance (Fig. 1B). None of the tested concentrations of ramelteon significantly affected the locomotor activity or cognitive performance of mice in the acquisition group on Day 1 (Fig. 1C and D). However, we found that compared with a control treatment, 3.0 mg/kg ramelteon significantly improved memory acquisition (based on the discrimination ratio) without any effects on locomotor activity, whereas 0.1, 0.3, and 1.0 mg/kg ramelteon failed to have significant effects on memory acquisition (Fig. 1C and D, Table S1). Intraperitoneal pretreatment of luzindole, a melatonin receptor antagonist, blocked the ramelteon-mediated improvement of memory acquisition indicated by the discrimination ratio (0.02 ± 0.06, $n = 6$ mice, Fig. 1D), and did not affect locomotion of mice (28.6 ± 1.9 m, $n = 6$ mice, Fig. 1C). To assess the acute effects of ramelteon treatment on memory consolidation or retrieval, we also injected other cohorts of mice with 3.0 mg/kg ramelteon after the training session or before the test session, confirming that there was no significant difference in locomotion or memory performance between vehicle-treated and ramelteon-treated mice in the consolidation phase (Fig. 1E and F, Table S2) or the retrieval phase (Fig. 1G and H, Table S3). As ramelteon is known to shorten the sleep onset latency and increase the total sleep time,[19,33] we quantified the sleep latency and total sleep time of mice after administration of vehicle or 3.0 mg/kg ramelteon and examined whether the effects on sleep were involved in the enhancement of memory acquisition. Video analysis confirmed that there was no significant difference in the sleep latency (Fig. S1A, Table S4) or total sleep time (Fig. S1B, Table S4) between the two groups, indicating that at least in the current experimental paradigm, sleep-promoting effects of ramelteon were not involved in the enhancement of memory acquisition. To examine which brain region was activated in the training session, we immunostained brain sections of mice treated with vehicle or ramelteon for c-Fos protein (Fig. S2A). We found that there were significantly more c-Fos positive cells in the hippocampal CA1, CA2/CA3 areas, and dentate gyrus in the ramelteon-treated mice than vehicle-treated mice (Fig. S2B, Table S5), whereas we failed to find any difference in the density of the c-Fos positive cells in the medial prefrontal cortex between the two groups (Fig. S2B, Table S5). These results confirmed that ramelteon treatment facilitated the acquisition phase of object recognition memory through hippocampal plasticity.

Next, to assess spatial memory, we used a Y-maze to perform a spontaneous alternation task in mice (Fig. 2).[22,23] To determine the effects of ramelteon on spatial working memory, we intraperitoneally injected 3.0 mg/kg ramelteon or vehicle into mice before exploration and monitored their spontaneous exploratory behavior (Fig. 2A, E). Neither the vehicle-treated mice nor the ramelteon-treated mice exhibited any preference among the arms of the maze (Fig. 2A–H, Table S6, 7). Neither the total distance traveled by mice nor the total number of arm entries was significantly different between the two groups of mice (Fig. 2I and J, Table S8). However, we found that spontaneous alternation was significantly improved

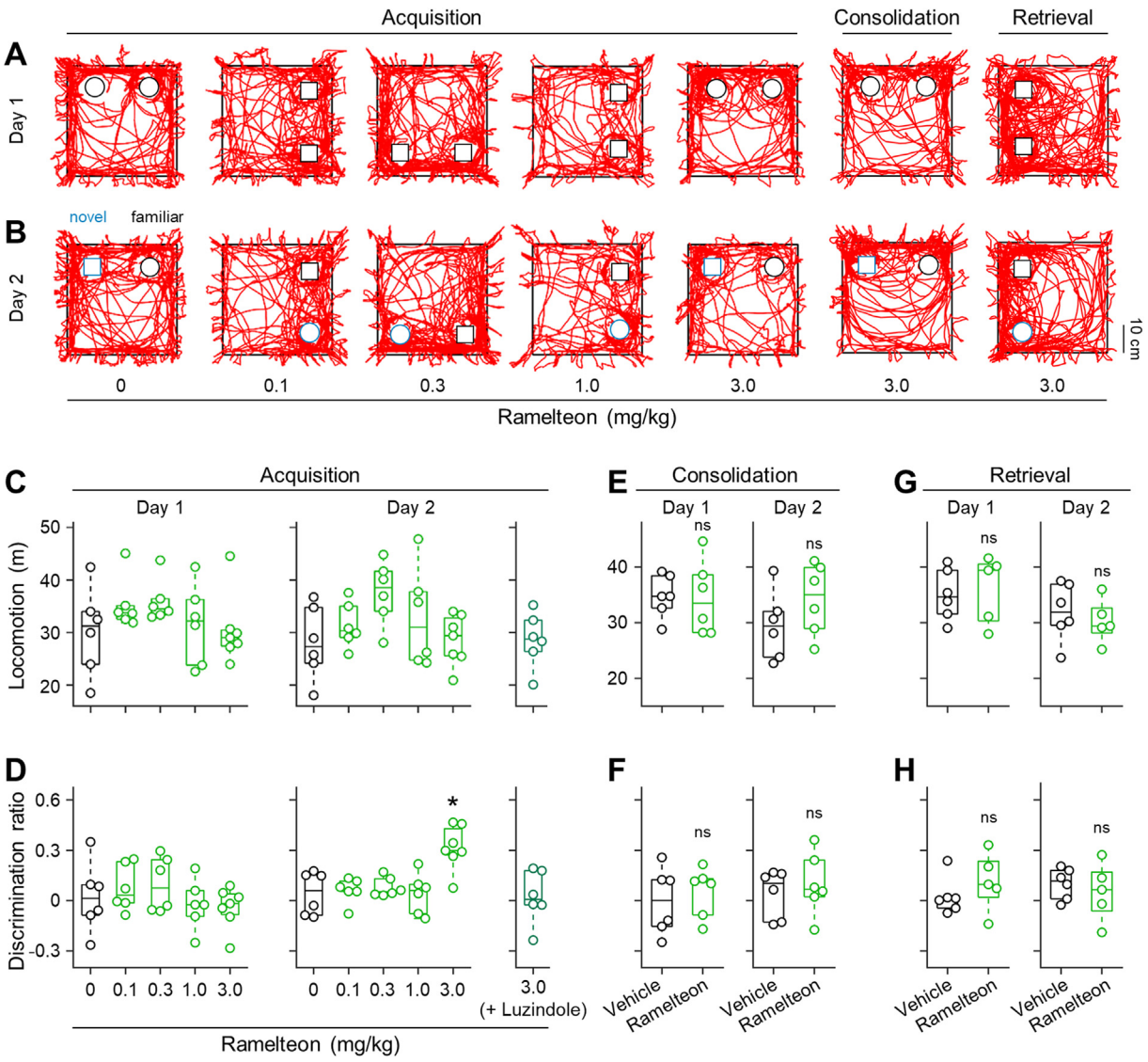


**Fig. 1.** Ramelteon enhances memory acquisition but not consolidation or retrieval in the novel object recognition task. A, Trajectories (*red*) of mice in the acquisition group during the training session on Day 1. The trajectories were taken by mice intraperitoneally injected with 0 (*first* (*leftmost*), vehicle), 0.1 (*second*), 0.3 (*third*), 1.0 (*fourth*), and 3.0 (*fifth*) mg/kg ramelteon before the training, 3.0 mg/kg ramelteon after the training (*sixth*), and 3.0 mg/kg ramelteon before the test (*seventh*). B, The same as A, but during the test session on Day 2. C, Distance traveled by mice in the acquisition cohort on Day 1 (*left*) and Day 2 (*middle* and *right*). The mice were treated with vehicle (*black*) or different concentrations of ramelteon (*green*) before the training session. The other mice were first treated with luzindole, and then treated with 3.0 mg/kg ramelteon before the training session (*jade*). D, The same as C, but for the discrimination ratio. E, Distance traveled by mice in the consolidation cohort on Day 1 (*left*) and Day 2 (*right*). The mice were treated with vehicle (*black*) or 3.0 mg/kg ramelteon (*green*) after the training session. F, The same as E, but for the discrimination ratio. G, The same as E, but for mice in the retrieval cohort that were treated with drugs before the test session. H, The same as G, but for the discrimination ratio.

in the ramelteon-treated mice compared with the vehicle-treated mice (Fig. 2K, Table S8). This ramelteon-induced improvement of spatial working memory was blocked by luzindole-pretreatment as indicated by an alternation index (0.57 ± 0.02; Fig. 2L). To further evaluate the performance of spatial reference memory, we administered 3.0 mg/kg ramelteon before the exploration of the two-arm maze on Day 1 and conducted probe trials using a complete Y-maze on Day 2 (Fig. 3); however, on Day 2, ramelteon-treated mice did not make significantly more entries into or spent significantly more time in the novel arm than vehicle-treated mice. Neither the total distance traveled by mice nor the total number of arm entries significantly differed between the two groups (Fig. 3C and D, Table S9). We also did not find any significant difference in the amount of time spent in the novel arm or the entries into the novel arm between the two groups (Fig. 3E and F, Table S9). Together, these results suggest that acute ramelteon treatment enhanced working memory but not reference memory for spatial novelty.

## 4. Discussion

In this study, we found that ramelteon treatment enhanced memory acquisition but not consolidation or retrieval in the novel object recognition task. Moreover, we demonstrated that ramelteon improved spatial working memory in the spontaneous alternation task. In contrast to novel object recognition memory, we should be cautious with interpretation of spatial 'novelty' in this study; by a strict definition, the spatial working memory assessed here is for less recently experienced (but still familiar) stimuli, not for truly novel stimuli, as mice alternate arms many times in a

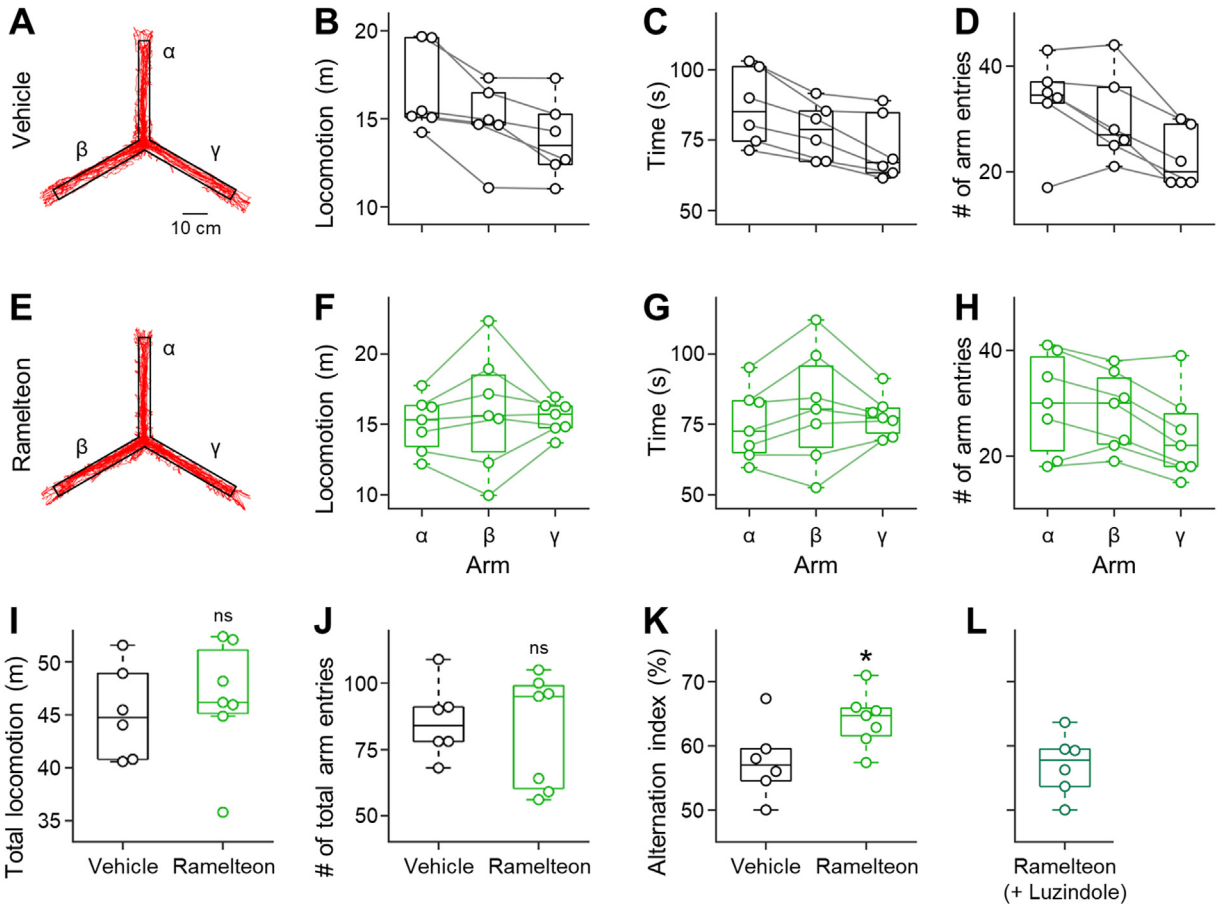


**Fig. 2.** Ramelteon enhances spatial working memory as assessed by the spontaneous alternation task in a Y-maze. A, Trajectories (*red*) followed during the spontaneous alternation task by mice pretreated with vehicle. B, The distance traveled by mice in the vehicle group was not significantly different between arms. C, The time spent by vehicle-treated mice was not significantly different between arms. D, The number of arm entries was almost the same among arms for the vehicle-treated mice. E–H, The same as A–D, respectively, but for mice treated with 3.0 mg/kg ramelteon. I, The total distance traveled in the Y-maze was not significantly different between the vehicle-treated (*black*) and ramelteon-treated (*green*) mice. J, The total number of arm entries was not significantly different between the two groups. K, Spontaneous alternation was significantly higher in the ramelteon group (*green*) than in the vehicle group (*black*). $n = 6$ vehicle-treated mice and 7 ramelteon-treated mice. L, The same as K, but for the luzindole-pretreatment group (*jade*). $n = 6$ mice treated with luzindole before ramelteon administration.

session. In any case, however, we speculate that the arm visited least recently is relatively novel in terms of the capacity of mice to store information about the temporal order of visits to the arms. Taken together, our results suggest that ramelteon administration enhances memory for novel environmental stimuli.

To assess memory performance, we chose the novel object recognition task for nonspatial memory and the spontaneous alternation task for spatial memory. A number of tasks to probe memory have been proposed thus far,[34] but among them, neither the novel object recognition task nor the spontaneous alternation task bestows rewards or inflicts punishment[35]; rather, animals instinctively explore novel objects or enter recently unvisited locations due to their natural propensity for novelty.[16,22] In this light, both tasks are able to measure more 'natural' memory at a rudimentary level than other behavioral tasks that require remuneration or punishment. Thus, our findings highlight the contribution of ramelteon to memory performance in a more natural and realistic situation than explored in previous studies.

Although we did not identify the mechanisms underlying the modulatory role of ramelteon in memory performance, the most plausible mechanism would be an impact of ramelteon on melatonin $MT_2$ receptors[36] expressed in memory-related brain regions across various species.[37,38] In particular, $MT_2$ receptors are expressed in the hippocampus in mice[39] and rats.[40–42] Moreover, electrophysiological studies have suggested that melatonin *per se* or a melatonin receptor agonist has the potential to depolarize neurons partly through $MT_2$ receptors.[42] Nonspatial object memory as assessed by the novel object recognition task has been attributed to the perirhinal cortex according to previous compelling studies of rodents;[43,44]; it should be noted that lesions of the rodent perirhinal cortex spared spatial memory.[43] However, a recent study has demonstrated that a longer period (more than approximately 40 s) of exploration makes object memory dependent on the hippocampus and has concluded that the hippocampus is also responsible for object recognition memory.[45] Indeed, we selected 5 min as the exploration time in the training session in our experimental paradigm using the novel object recognition task (Fig. 1). Therefore, memory for the novel object evaluated in our task was dependent on the hippocampus and could have been modulated by ramelteon-mediated activation of neurons through $MT_2$ receptors. These notions are indeed experimentally supported by increased expression patterns of c-Fos protein in the hippocampus of the ramelteon-treated mice (Fig. S2) and consistent with a previous literature that discussed a role of immediate early genes in synaptic plasticity that serve as the underpinning of the memory trace.[46]

In contrast to nonspatial memory, we examined the spatial memory of ramelteon-treated mice using the spontaneous alternation task (Figs. 2 and 3). Previous studies have demonstrated that

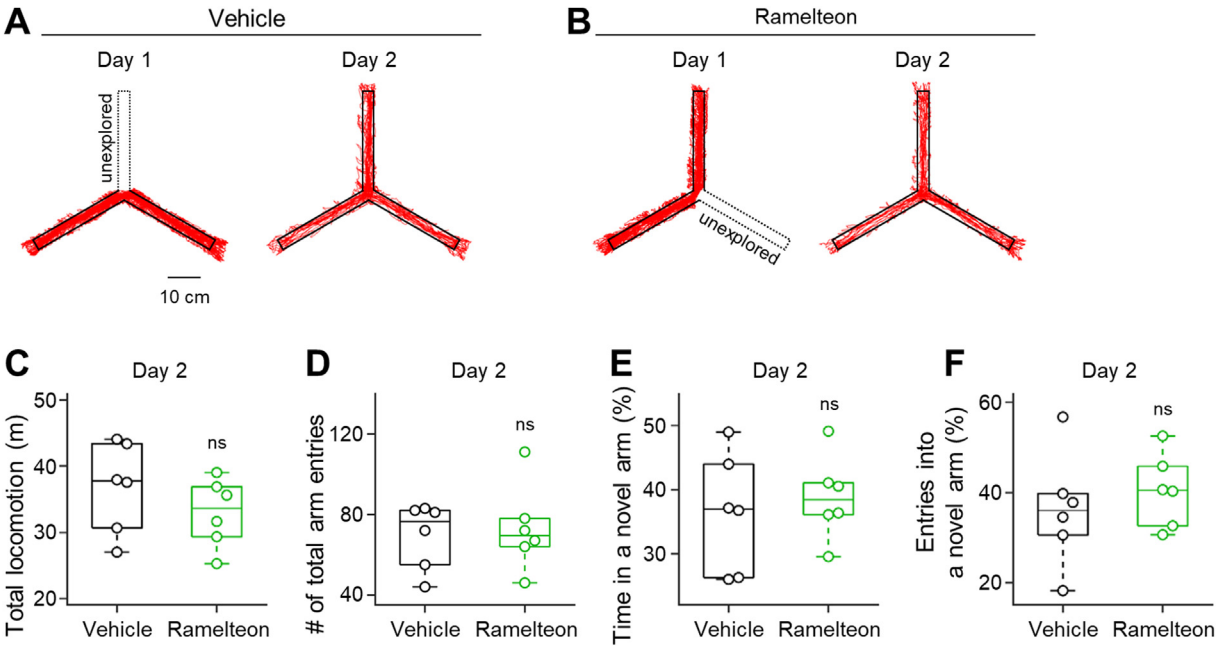


**Fig. 3.** Ramelteon does not affect the acquisition of spatial reference memory as assessed by the spontaneous alternation task. A, Mice treated with vehicle before the spontaneous alternation task on Day 1 took the trajectories shown in *red*. One of the arms (marked with dotted lines) was closed so that the mice could not visit it on Day 1, but the closed arm was opened and regarded as the novel arm on Day 2. B, The same as A, but for mice treated with 3.0 mg/kg ramelteon. C, The total distance traveled by mice was not significantly different between the vehicle-treated (*black*) and ramelteon-treated (*green*) mice on Day 2. D, The total number of arm entries was not different between the two groups on Day 2. E, The time spent in the novel arm was not different between the two groups. F, The percentage of entries into the novel arm out of the total number of arm entries was not different between the two groups. $n = 6$ vehicle-treated mice and 6 ramelteon-treated mice.

the success of spontaneous alternation requires integrity of the medial prefrontal cortex,[47–49] hippocampus,[50,51] and other regions.[52,53] Among these regions, we consider it possible that the hippocampus may account for the ramelteon-mediated enhancement of spatial working memory. Molecular studies demonstrated that melatonin $MT_2$ receptors were enriched in the hippocampal CA2/CA3 subregions in mice[39] and rats.[41,42] Furthermore, a previous behavioral investigation in combination with optogenetic silencing of neural activity suggested a significant contribution of the CA3 area to short-term spatial working memory in the spontaneous alternation task in mice.[54,55] Hence, we speculate that ramelteon enhanced spatial working memory in the Y-maze through the $MT_2$ receptors in the hippocampus, although this type of memory enhancement cannot be accounted for by the melatonin receptors in the hippocampus alone.

There are few papers reporting the behavioral effects of $MT_1$/$MT_2$ receptor deficiency, but one literature described such effects using $MT_1$ receptor- or $MT_2$ receptor-deficient knockout mice.[56] Pistono et al. launched a 16-day chronic treatment on the knockout mice with melatonin in drinking water (or pure water; that is, control) and performed the novel object recognition test. They demonstrated that the lack of $MT_2$ receptors, not $MT_1$ receptors, precluded memory-enhancing effect of melatonin in the object recognition task. In fact, they chronically administered melatonin to mice and insisted that the memory 'retention' was enhanced by melatonin treatment. Compared with the context of our behavioral test, such chronic administration made it difficult to understand which phase (i.e., acquisition, consolidation, or recall) was affected; that is, we cannot exclude the possibility that the acquisition phase of memory was modulated by melatonin receptor activation. In this sense, their conclusion is (partially) consistent with our current finding. Moreover, hippocampal long-term potentiation was impaired in melatonin $MT_2$ receptor-deficient mice,[4] suggesting that $MT_2$ receptors are required for hippocampal long-tern potentiation. This discovery is also consistent with our speculation that $MT_2$ receptors are involved in memory acquisition. On the other hand, to the best of our knowledge, the involvement of $MT_1$ and $MT_2$ receptors in spatial working memory has not been reported thus far.

We examined the pharmacological effects of the exogenous factor, ramelteon, on cognitive performance, but it is unknown whether endogenous melatonin is released from the pineal gland of naïve mice. This issue could be addressed by chronic biochemical sample collection[57] from the brain region downstream of the pineal gland. Moreover, whether melatonin receptors in the hippocampus and other memory-related regions are activated during the acquisition of memory is of interest, although it is technically difficult to experimentally address this question. Since the melatonin receptor is one of G protein-coupled receptors (GPCRs),[3] direct evidence of the activation of GPCRs could be secured by investigating the conformational changes of the melatonin receptors (with the ligands).[36,58] However, such demonstration of GPCR activation during behavior has not been performed thus far. Instead, we examined c-Fos expression in the hippocampus and medial prefrontal cortex (Fig. S2), and demonstrated that luzindole pretreatment blocked the memory acquisition improved by ramelteon (Figs. 1D and 2L). We thus consider that these results provide indirect evidence of the activation of melatonin receptors.

Currently, ramelteon is suggested to induce quasi-natural sleep[59,60] and is used as a remedy for insomnia,[61,62] a condition that sometimes negatively affects memory.[63,64] Various neural activity patterns during sleep are crucial for memory,[65] such as sharp-wave ripple complexes[24,66–68] and theta oscillations[69] emerging in the hippocampus and slow oscillations and spindles in the neocortex.[70] Accordingly, the ramelteon-mediated improvement in object recognition memory in the present study may be attributable to preceding and subsequent sleep and associated with miscellaneous neural activity. Moreover, spatial working memory is

related to theta oscillations in the hippocampus and neocortex.[71,72] Chronic electrophysiological recordings from the hippocampus and neocortex of ramelteon-treated animals during behavioral tasks and sleep will allow us to precisely probe physiological evidence of memory modulation by melatonin receptors and possible nootropic therapeutics.

## Author contributions

Y.I. and N.M. conceived the project and designed the study; M.K., E. K-I., and N.M. performed the experiments; M.K. and N.M. analyzed the data; Y.I. and N.M. oversaw and managed the project; M.K., Y.I., N.M. wrote the manuscript and approved its final version.

## Declaration of competing interest

The authors have no conflicts of interest to disclose with respect to this research.

## Acknowledgments

The authors thank Jun Ogasawara and Ayako Ishigaki for their technical assistance. This work was supported by JST ERATO (JPMJER1801 (Y.I.)), JSPS Grants-in-Aid for Scientific Research (18H05525 (Y.I.), 20K15926 (N.M.)), the Institute for AI and Beyond (Y.I.), and Tokyo Foundation for Pharmaceutical Sciences (N.M.).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2023.04.002.

## References

1. Savaskan E, Ayoub MA, Ravid R, et al. Reduced hippocampal MT2 melatonin receptor expression in Alzheimer's disease. *J Pineal Res*. 2005;38(1):10–16.
2. Savaskan E, Olivieri G, Meier F, et al. Increased melatonin 1a-receptor immunoreactivity in the hippocampus of Alzheimer's disease patients. *J Pineal Res*. 2002;32(1):59–62.
3. Liu J, Clough SJ, Hutchinson AJ, Adamah-Biassi EB, Popovska-Gorevski M, Dubocovich ML. MT1 and MT2 melatonin receptors: a therapeutic perspective. *Annu Rev Pharmacol Toxicol*. 2016;56:361–383.
4. Larson J, Jessen RE, Uz T, et al. Impaired hippocampal long-term potentiation in melatonin MT2 receptor-deficient mice. *Neurosci Lett*. 2006;393(1):23–26.
5. Hirai K, Kita M, Ohta H, et al. Ramelteon (TAK-375) accelerates reentrainment of circadian rhythm after a phase advance of the light-dark cycle in rats. *J Biol Rhythm*. 2005;20(1):27–37.
6. Uchikawa O, Fukatsu K, Tokunoh R, et al. Synthesis of a novel series of tricyclic indan derivatives as melatonin receptor agonists. *J Med Chem*. 2002;45(19):4222–4239.
7. Brimberg L, Flaisher-Grinberg S, Schilman EA, Joel D. Strain differences in "compulsive" lever-pressing. *Behav Brain Res*. 2007;179(1):141–151.
8. Correa M, Mingote S, Betz A, Wisniecki A, Salamone JD. Substantia nigra pars reticulata GABA is involved in the regulation of operant lever pressing: pharmacological and microdialysis studies. *Neuroscience*. 2003;119(3):759–766.
9. Shapiro MM. Respondent salivary conditioning during operant lever pressing in dogs. *Science*. 1960;132(3427):619–620.
10. Kilonzo K, van der Veen B, Teutsch J, et al. Delayed-matching-to-position working memory in mice relies on NMDA-receptors in prefrontal pyramidal cells. *Sci Rep*. 2021;11(1):8788.
11. Panlilio LV, Yasar S, Thorndike EB, Goldberg SR, Schindler CW. Automatic recording of mediating behavior in delayed matching- and nonmatching-to-position procedures in rats. *Psychopharmacology*. 2011;214(2):495–504.
12. Prus AJ, James JR, Rosecrans JA. Conditioned place preference. In: Buccafusco JJ, ed. *Methods of Behavior Analysis in Neuroscience*. CRC Press/Taylor & Francis; 2011.
13. Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*. 1984;11(1):47–60.
14. Sharma S, Rakoczy S, Brown-Borg H. Assessment of spatial memory in mice. *Life Sci*. 2010;87(17-18):521–536.
15. Lissner LJ, Wartchow KM, Toniazzo AP, Gonçalves CA, Rodrigues L. Object recognition and Morris water maze to detect cognitive impairment from mild hippocampal damage in rats: a reflection based on the literature and experience. *Pharmacol Biochem Behav*. 2021;210, 173273.
16. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn Process*. 2012;13(2):93–110.
17. Hughes RN. The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. *Neurosci Biobehav Rev*. 2004;28(5):497–505.
18. Miedel CJ, Patton JM, Miedel AN, Miedel ES, Levenson JM. Assessment of spontaneous alternation, novel object recognition and limb clasping in transgenic mouse models of amyloid-β and tau neuropathology. *J Vis Exp*. 2017;123. https://doi.org/10.3791/55523.
19. Yoshimoto A, Yamashiro K, Suzuki T, Ikegaya Y, Matsumoto N. Ramelteon modulates gamma oscillations in the rat primary motor cortex during non-REM sleep. *J Pharmacol Sci*. 2021;145(1):97–104.
20. Yoshimoto A, Yamashiro K, Ikegaya Y, Matsumoto N. Acute ramelteon treatment maintains the cardiac rhythms of rats during non-REM sleep. *Biol Pharm Bull*. 2021;44(6):789–797.
21. Lueptow LM. Novel object recognition test for the investigation of learning and memory in mice. *J Vis Exp*. 2017;126. https://doi.org/10.3791/55718.
22. Prieur EAK, Jadavji NM. Assessing spatial working memory using the spontaneous alternation Y-maze test in aged male mice. *Bio Protoc*. 2019;9(3), e3162.
23. Kraeuter AK, Guest PC, Sarnyai Z. The Y-maze for assessment of spatial working and reference memory in mice. *Methods Mol Biol*. 2019;1916:105–111.
24. Matsumoto N, Okamoto K, Takagi Y, Ikegaya Y. 3-Hz subthreshold oscillations of CA2 neurons in vivo. *Hippocampus*. 2016;26(12):1570–1578.
25. Noguchi A, Matsumoto N, Morikawa S, Tamura H, Ikegaya Y. Juvenile hippocampal CA2 region expresses aggrecan. *Front Neuroanat*. 2017;11:41.
26. Kashima T, Noguchi A, Ikegaya Y, Matsumoto N. Heterogeneous expression patterns of fibronectin in the mouse subiculum. *J Chem Neuroanat*. 2019;98:131–138.
27. Liu J, Kashima T, Morikawa S, Noguchi A, Ikegaya Y, Matsumoto N. Molecular characterization of superficial layers of the presubiculum during development. *Front Neuroanat*. 2021;15, 662724.
28. Yamashiro K, Liu J, Matsumoto N, Ikegaya Y. Deep learning-based classification of GAD67-positive neurons without the immunosignal. *Front Neuroanat*. 2021;15, 643067.
29. Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc*. 1955;50(272):1096–1121.
30. Yamashiro K, Aoki M, Matsumoto N, Ikegaya Y. Polyherbal formulation enhancing cerebral slow waves in sleeping rats. *Biol Pharm Bull*. 2020;43(9):1356–1360.
31. Mathis A, Mamidanna P, Cury KM, et al. DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. *Nat Neurosci*. 2018;21(9):1281–1289.
32. Plucińska K, Mody N, Dekeryte R, et al. High-fat diet exacerbates cognitive and metabolic abnormalities in neuronal BACE1 knock-in mice – partial prevention by Fenretinide. *Nutr Neurosci*. 2022;25(4):719–736.
33. Roth T, Seiden D, Sainati S, Wang-Weigand S, Zhang J, Zee P. Effects of ramelteon on patient-reported sleep latency in older adults with chronic insomnia. *Sleep Med*. 2006;7(4):312–318.
34. Dudchenko PA. An overview of the tasks used to test working memory in rodents. *Neurosci Biobehav Rev*. 2004;28(7):699–709.
35. Baxter MG. "I've seen it all before": explaining age-related impairments in object recognition. Theoretical comment on Burke et al. (2010). *Behav Neurosci*. 2010;124(5):706–709.
36. Wang Q, Lu Q, Guo Q, et al. Structural basis of the ligand binding and signaling mechanism of melatonin receptors. *Nat Commun*. 2022;13(1):1–10.
37. Ng KY, Leong MK, Liang H, Paxinos G. Melatonin receptors: distribution in mammalian brain and their respective putative functions. *Brain Struct Funct*. 2017;222(7):2921–2939.
38. Tosini G, Owino S, Guillaume JL, Jockers R. Understanding melatonin receptor pharmacology: latest insights from mouse models, and their relevance to human disease. *Bioessays*. 2014;36(8):778–787.
39. Klosen P, Lapmanee S, Schuster C, et al. MT1 and MT2 melatonin receptors are expressed in nonoverlapping neuronal populations. *J Pineal Res*. 2019;67(1), e12575.
40. Ishii H, Tanaka N, Kobayashi M, Kato M, Sakuma Y. Gene structures, biochemical characterization and distribution of rat melatonin receptors. *J Physiol Sci*. 2009;59(1):37–47.
41. 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.
42. Musshoff U, Riewenherm D, Berger E, Fauteck JD, Speckmann EJ. Melatonin receptors in rat hippocampus: molecular and functional investigations. *Hippocampus*. 2002;12(2):165–173.
43. Winters BD, Forwood SE, Cowell RA, Saksida LM, Bussey TJ. Double dissociation between the effects of peri-postrhinal cortex and hippocampal lesions on tests of object recognition and spatial memory: heterogeneity of function within the temporal lobe. *J Neurosci*. 2004;24(26):5901–5908.
44. Winters BD, Bussey TJ. Transient inactivation of perirhinal cortex disrupts encoding, retrieval, and consolidation of object recognition memory. *J Neurosci*. 2005;25(1):52–61.

45. Cinalli Jr DA, Cohen SJ, Guthrie K, Stackman Jr RW. Object recognition memory: distinct yet complementary roles of the mouse CA1 and perirhinal cortex. *Front Mol Neurosci*. 2020;13, 527543.
46. Minatohara K, Akiyoshi M, Okuno H. Role of immediate-early genes in synaptic plasticity and neuronal ensembles underlying the memory trace. *Front Mol Neurosci*. 2015;8:78.
47. Wikmark RG, Divac I, Weiss R. Retention of spatial delayed alternation in rats with lesions in the frontal lobes. Implications for a comparative neuropsychology of the prefrontal system. *Brain Behav Evol*. 1973;8(5):329–339.
48. Divac I, Wikmark RGE, Gade A. Spontaneous alternation in rats with lesions in the frontal lobes: an extension of the frontal lobe syndrome. *Physiol Psychol*. 1975;3(1):39–42.
49. Delatour B, Gisquet-Verrier P. Prelimbic cortex specific lesions disrupt delayed-variable response tasks in the rat. *Behav Neurosci*. 1996;110(6):1282–1298.
50. Kirkby RJ, Stein DG, Kimble RJ, Kimble DP. Effects of hippocampal lesions and duration of sensory input on spontaneous alternation. *J Comp Physiol Psychol*. 1967;64(2):342–345.
51. Johnson CT, Olton DS, Gage 3rd FH, Jenko PG. Damage to hippocampus and hippocampal connections: effects on DRL and spontaneous alternation. *J Comp Physiol Psychol*. 1977;91(3):508–522.
52. Bizon JL, Foster TC, Alexander GE, Glisky EL. Characterizing cognitive aging of working memory and executive function in animal models. *Front Aging Neurosci*. 2012;4:19.
53. Lalonde R. The neurobiological basis of spontaneous alternation. *Neurosci Biobehav Rev*. 2002;26(1):91–104.
54. Shipton OA, El-Gaby M, Apergis-Schoute J, et al. Left-right dissociation of hippocampal memory processes in mice. *Proc Natl Acad Sci U S A*. 2014;111(42):15238–15243.
55. Myhrer T. Locomotor, avoidance,and maze behavior in rats with selective disruption of hippocampal output. *J Comp Physiol Psychol*. 1975;89(7):759–777.
56. Pistono C, Zimmermann A, Morel C, et al. Major role of MT2 receptors in the beneficial effect of melatonin on long-term recognition memory in C57BL/6J male mice. *Horm Behav*. 2021;136, 105076.
57. Konno D, Nakayama R, Tsunoda M, Funatsu T, Ikegaya Y, Sasaki T. Collection of biochemical samples with brain-wide electrophysiological recordings from a freely moving rodent. *J Pharmacol Sci*. 2019;139(4):346–351.
58. Okamoto HH, Miyauchi H, Inoue A, et al. Cryo-EM structure of the human MT1-Gi signaling complex. *Nat Struct Mol Biol*. 2021;28(8):694–701.
59. Yukuhiro N, Kimura H, Nishikawa H, Ohkawa S, Yoshikubo SI, Miyamoto M. Effects of ramelteon (TAK-375) on nocturnal sleep in freely moving monkeys. *Brain Res*. 2004;1027(1-2):59–66.
60. Naono-Nagatomo K, Abe H, Araki R, et al. A survey of the effects of ramelteon on benzodiazepine-dependence: comparison between a ramelteon add-on group and a continuous benzodiazepine administration group. *Asian J Psychiatr*. 2018;36:20–24.
61. Atkin T, Comai S, Gobbi G. Drugs for insomnia beyond benzodiazepines: pharmacology, clinical applications, and discovery. *Pharmacol Rev*. 2018;70(2):197–245.
62. Kuriyama A, Honda M, Hayashino Y. Ramelteon for the treatment of insomnia in adults: a systematic review and meta-analysis. *Sleep Med*. 2014;15(4):385–392.
63. McKenna JT, Gamble MC, Anderson-Chernishof MB, Shah SR, McCoy JG, Strecker RE. A rodent cage change insomnia model disrupts memory consolidation. *J Sleep Res*. 2019;28(2), e12792.
64. Brownlow JA, Miller KE, Gehrman PR. Insomnia and cognitive performance. *Sleep Med Clin*. 2020;15(1):71–76.
65. Girardeau G, Lopes-Dos-Santos V. Brain neural patterns and the memory function of sleep. *Science*. 2021;374(6567):560–564.
66. Noguchi A, Huszár R, Morikawa S, Buzsáki G, Ikegaya Y. Inhibition allocates spikes during hippocampal ripples. *Nat Commun*. 2022;13(1):1280.
67. Liu AA, Henin S, Abbaspoor S, et al. A consensus statement on detection of hippocampal sharp wave ripples and differentiation from other fast oscillations. *Nat Commun*. 2022;13(1):6000.
68. Buzsáki G. Hippocampal sharp wave-ripple: a cognitive biomarker for episodic memory and planning. *Hippocampus*. 2015;25(10):1073–1188.
69. Buzsáki G. Theta oscillations in the hippocampus. *Neuron*. 2002;33(3):325–340.
70. Ikegaya Y, Matsumoto N. Spikes in the sleeping brain. *Science*. 2019;366(6463):306–307.
71. Wirt RA, Crew LA, Ortiz AA, et al. Altered theta rhythm and hippocampal-cortical interactions underlie working memory deficits in a hyperglycemia risk factor model of Alzheimer's disease. *Commun Biol*. 2021;4(1):1036.
72. Tamura M, Spellman TJ, Rosen AM, Gogos JA, Gordon JA. Hippocampal-prefrontal theta-gamma coupling during performance of a spatial working memory task. *Nat Commun*. 2017;8(1):2182.