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# Melatonin Enhances Object Recognition Memory through Melatonin MT1 and MT2 Receptor-Mediated and Non-Receptor-Mediated Mechanisms in Male Mice

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## Abstract

Melatonin (MEL) has been reported to have acute enhancing effects on some aspects of cognition. Recently, we revealed that N1-acetyl-5-methoxyquinuramine (AMK), a brain metabolite of MEL, is much more potent than MEL in converting short-term memory (STM) to long-term memory (LTM) with a single administration immediately after the acquisition trial of the novel object recognition (NOR) task. These data suggest that the memory-enhancing effects of MEL may be mediated by mechanisms independent of the activation of MEL MT1 and MT2 receptors. In the present study, we examined the contribution of MT1 and MT2 receptor-mediated and non-receptor-mediated mechanisms to the acute memory-enhancing effects of MEL using NOR task. Mice were administered with either MEL, AMK, or a highly selective MT1/MT2 receptor agonist ramelteon (RAM) immediately after the acquisition trial and the effects of varying doses of these drugs on both STM and LTM performance were compared. We found that both AMK and RAM were more potent than MEL in both facilitating STM and promoting LTM formation. We also found that pretreatment with luzindole, a MT1/MT2 receptor antagonist, markedly suppressed only the effects of RAM. These results suggest that acutely administered MEL enhances NOR memory through both MT1 and MT2 receptor-mediated and non-receptor-mediated mechanisms.

## Keywords

Melatonin, N1-Acetyl-5-Methoxykynuramine, Ramelteon, Novel Object Recognition Memory, Melatonin Receptors

## 1. Introduction

Melatonin (MEL; N-acetyl-5-methoxytryptamine) is the main product secreted by the pineal gland, and is involved in various physiological functions including the regulation of body temperature and the cycle of circadian rhythms [1] [2] [3]. In mammals, MEL acts mainly via two high-affinity G protein-coupled membrane receptors (MT1 and MT2) [4] [5]. The MT1 and MT2 receptor sites have been localized to discrete areas of the rodent and human nervous system, including the suprachiasmatic nucleus (SCN) of the hypothalamus, cerebellum, thalamus, hippocampus, as well as peripheral tissues [6] [7] [8] [9].

MEL also exerts strong anti-oxidant, anti-inflammatory, anti-apoptotic effects [10] [11] [12] [13]. MEL is highly permeable to cell membranes and easily crosses the blood-brain barrier [14]. Many studies have reported that MEL and several of its brain metabolites, such as N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), possess strong antioxidant properties because of their chemical properties as effective free radical scavengers [15] [16]. MEL further upregulates the antioxidant defense system by activating MT1 and/or MT2 receptors and increasing the gene expression or activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase [17]. Numerous studies have shown that chronic MEL treatment inhibits apoptosis induced-neurodegeneration [18] [19] [20] [21] and attenuates memory deficits in various animal models of oxidative stress-related neurodegenerative disorders, including Alzheimer-like neurodegeneration [2] [19] [22] [23].

On the other hand, acute MEL treatment has also been found to be beneficial in improving certain aspects of cognitive function. For instance, a single MEL administration in rats enhanced both short-term memory (STM) and long-term memory (LTM) performance in novel object recognition (NOR) task [24] [25] and STM in olfactory social memory test [26]. Our recent study demonstrated that a single post-training administration of AMK promotes the conversion of STM to LTM in the NOR paradigm, with superior effects compared to MEL [27]. Although the mechanisms mediating the memory enhancing effects of acute MEL administration are still elusive, these data suggest that MEL is involved in memory enhancement, at least in part, via an MT1/MT2 independent mechanism after being converted to AMK.

The present study aimed to examine the contribution of MT1/MT2 receptor-mediated and non-receptor-mediated mechanisms to memory enhancement by acute MEL treatment. Here, we compared the dose-dependent effects of a single post-training administration of MEL, AMK, or highly selective MT1/MT2 agonist ramelteon (RAM) on the NOR memory in mice. We also investigated if the effects of these drugs are blocked or attenuated by pretreatment with a MT1/MT2 receptor antagonist luzindole.

## 2. Materials and Methods

### 2.1. Animals

Male ICR mice (8 weeks old) were purchased from Sankyo Labo Service Corpo-

ration Inc. (Tokyo, Japan). In the present study we avoided to use female animals because estrous cycles might cause data variability. The animals were housed individually in cages and provided with food and water ad libitum at 24°C under a 12-h light/12-h dark cycle. After 1 to 2 weeks of acclimation, the animals were subjected to behavioral experiments during the latter half of the 12-h light period. All experimental procedures and animal housing were approved by the Institutional Animal Care and Use Committee of Sophia University and comply with the criteria established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996).

## 2.2. NOR Test

NOR test was conducted in an open-field polypropylene arena (40 cm width × 30 cm depth × 30 cm height). The floor the arena was divided into 20 equal sections by drawing grid lines. The NOR test comprised three phases: habituation, acquisition and test. During the habituation phase, the animals were allowed to freely explore the empty arena for 5 minutes per day for 3 days. In the acquisition phase, mice were subjected to a single 5-min acquisition trial, where they were allowed to freely explore two identical objects which were symmetrically placed in the arena at a distance of 5 cm from the wall and 15 cm from each other. After a predetermined retention interval (inter-trial interval, ITI) during which animals were kept in their home cages, test phase was initiated. In this phase, mice were subjected to a 5-min test trial which was similar to the acquisition trial, with the exception that one of the familiar objects was replaced with a novel object at the same location. Objects were made of ceramic, which were too heavy to be displaced by animals. Object novelty and location were counterbalanced within experimental groups to eliminate potential biases caused by preference for particular objects or locations. Mice were placed in the middle of the two objects facing the wall at the beginning of each acquisition and test trial, and were recorded using a video camera mounted above the arena. After each trial, the objects were thoroughly cleaned with 70% EtOH to minimize the presence of olfactory trails.

The time spent exploring the objects was quantified by a blinded trained observer using the recordings. Exploratory behavior was defined as the animal directing its nose toward the object at a distance of <1 cm. Animals were excluded from data analyses if they met the following criteria: 1) spent 10 seconds or less exploring objects during any acquisition trial; 2) spent 10 seconds or less exploring objects during the test trial; 3) did not explore both objects during the training and test phases. The discrimination index (DI) was calculated as follows: percent of time spent exploring the novel object divided by the total time spent exploring both objects during the test trial. Object recognition was considered as DIs those were significantly above chance performance (50%).

Off-target drug effects were quantified using spontaneous locomotor activity, defined as the number of times mice crossed the grid lines with all four paws

(grid-crossings), and the total time spent exploring both objects during each phase.

## 2.3. Drugs and Treatments

MEL (Mw: 232.28), RAM (Mw: 259.34), AMK (Mw: 236.27) and luzindole were purchased from Sigma-Aldrich (Tokyo, Japan). MEL, RAM, and AMK were dissolved in dimethyl sulfoxide (DMSO) and then diluted with saline to a final concentration of (1% DMSO) for intraperitoneal administration. The dosage of MEL, RAM, and AMK varied from experiment to experiment. Luzindole was also dissolved in DMSO and then diluted with saline to a final DMSO concentration of 1% for the dosage of 0.1 mg/kg b.w. (Experiment 3) and 10% for the dosage of 1 mg/kg b.w. (Experiment 4). These doses of luzindole were set to more than 1000 times of MEL dose so that the activation of MT1 and MT2 receptors by MEL can be fully antagonized. All drugs were injected intraperitoneally (i.p.) at a volume of 3 ml/kg b.w.

## 2.4. Experimental Procedures

The present study was composed of the following four experiments.

Experiment 1

Untreated mice were tested for their ability to retain STM using NOR task with a 2-h (n = 14) or 3-h ITI (n = 10).

Experiment 2

Mice (n = 7 - 13 per group) were used to investigate the dose-dependent effects of MEL, RAM, and AMK administered immediately after acquisition trial on their STM performance tested after a 3-h ITI.

Experiment 3

Mice (n = 7 - 11 per group) were used to examine if the STM facilitating effects of MEL, RAM, and AMK are inhibited by luzindole. The animals were pretreated with either luzindole or vehicle immediately after acquisition trial and each pretreatment was followed 5 min later by administration of varying doses of either MEL, RAM, or AMK. STM performance of the mice was tested after a 3-h ITI.

Experiment 4

Mice (n = 7 - 13 per group) were used to examine the dose-dependent effects of post training MEL, RAM and AMK on LTM performance, and to examine if LTM formation-promoting effects of these drugs were inhibited by pretreatment with luzindole. The animals were pretreated with either luzindole or vehicle immediately after acquisition trial and each pretreatment was followed 5 min later by administration of varying doses of either MEL, RAM, or AMK. LTM performance of the mice was tested after a 24-h ITI.

## 2.5. Data Analysis

For all test trials, one-sample t-tests were used to analyze whether DIs signifi-

cantly differed from the chance level (50%). Effect size for the one-sample t-tests was given by Cohen's d. One-way analysis of variance (ANOVA) was used to analyze the differences in the total exploration time and the number of grid-crossings among groups administered with varying doses of either MEL, RAM or AMK, including 0 ng (or μg) /kg (vehicle). Post hoc Dunnett's tests were applied where appropriate. Effect size estimates for the one-way ANOVA was computed by partial-Eta squared ($\eta_p^2$). A p value < 0.05 was considered statistically significant.

# 3. Results

## 3.1. Experiment 1

### STM performance in untreated mice

One-sample t-test revealed that the DIs from the untreated mice were significantly greater than the chance level (50%) when tested with a 2-h ITI (t (13) = 2.92, p = 0.0118, d = 1.15), but not with a 3-h ITI (t (9) = 0.19, p = 0.8516, d = 0.09) (Figure 1). These data indicate that the mice were able to retain STM for at least 2 hours, but not for 3 hours or longer in the present experimental conditions.

## 3.2. Experiment 2

Dose-dependent effects of a single administration of drugs on STM performance after a 3-h ITI

One-sample t-test revealed that MEL, RAM, and AMK, given at lower doses immediately after the acquisition trial, did not affect DI (p > 0.05) after a 3-h ITI (0 ng/kg (vehicle): t (6) = 0.05, p = 0.9611, d = 0.03; 0.01 and 0.1 ng/kg of MEL: t (9) = 1.25, p = 0.2437, d = 0.59 and t (9) = 0.45, p = 0.6661, d = 0.21, respectively; 0.01 ng/kg of RAM: t (9) = 0.13, p = 0.8987, d = 0.06; 0.01 ng/kg of AMK: t (12) = 0.70, p = 0.5002, d = 0.28). However, higher doses of these drugs all significantly (p < 0.05) increased DI compared to chance level (50%), indicating facilitated STM (Figure 2(a)) (1 and 10 ng/kg of MEL: t (8) = 2.36, p = 0.0458, d = 1.18 and t (7) = 3.66, p = 0.0081, d = 1.96, respectively; 0.1, 1 and 10 ng/kg of
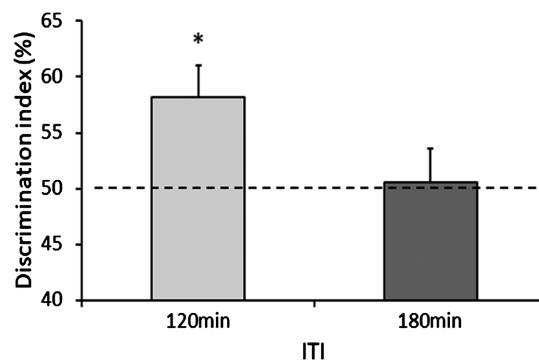


**Figure 1.** Discrimination indices (DIs) for short-term memory (STM) performance of the untreated mice on the test trials of the novel object recognition (NOR) task with a 2-h (n = 10) and 3-h ITIs (n = 13). Results are presented as mean ± SEM. *p < 0.05 vs. chance level (50%) (one-sample t-test).
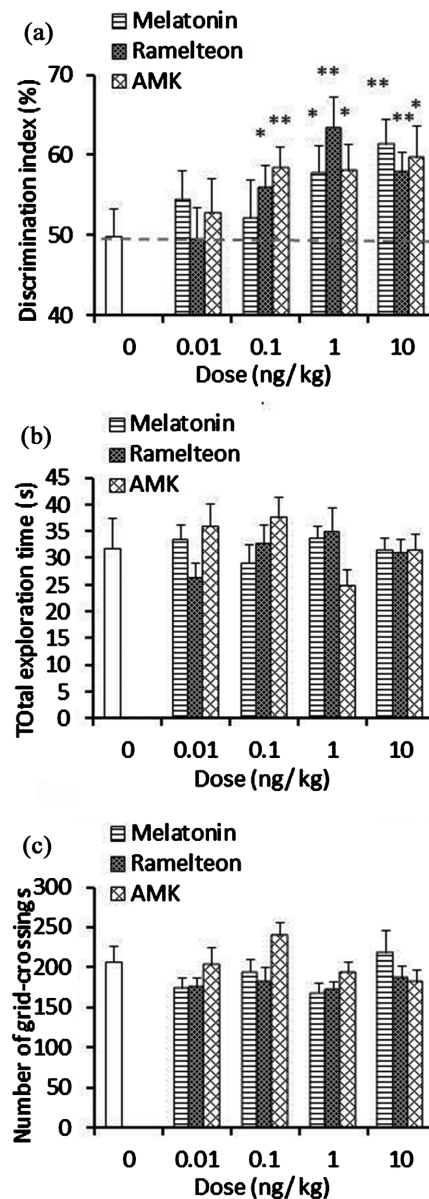
**Figure 2.** DIs for STM performance (a), the total exploration time (b) and the number of grid-crossings (c) on the test trials after a 3-h ITI in the mice received varying doses of either MEL, RAM or AMK, including 0 ng/kg (vehicle), immediately after acquisition trials (n = 7 - 13 per each group). Results are presented as mean ± SEM. *P < 0.05 and **P < 0.01 indicate significant difference in DIs from chance level (50%) (one-sample t-test). There were no significant differences (p > 0.05) in either total exploration time or number of grid-crossings among the groups received any dose of MEL, RAM, or AMK, including 0 ng/kg (vehicle) (one-way ANOVA).

RAM: t (9) = 2.27, p = 0.0497, d = 1.07, t (8) = 3.47, p = 0.0084, d = 1.74, and t (9) = 3.36, p = 0.0084, d = 1.58, respectively; 0.1, 1 and 10 ng/kg of AMK: t (11) = 3.35, p = 0.0065, d = 1.43, t (9) = 2.43, p = 0.0378, d = 1.15, and t (9) = 2.46, p = 0.0364, d = 1.16, respectively). The minimum doses required to facilitate STM were 1 ng /kg for MEL, and 0.1 ng /kg for RAM and AMK.

One-way ANOVA revealed that there were no significant differences (p >

0.05) in either total exploration time (**Figure 2(b)**) (F (12, 115) = 1.24, p = 0.2630, $\eta_p^2$ = 0.11), or number of grid-crossings (**Figure 2(c)**) (F (12, 115) = 1.73, p = 0.0696, $\eta_p^2$ = 0.15) among groups received varying doses of MEL, RAM, or AMK, including 0 ng/kg (vehicle). These observations indicate that at the doses used in this experiment, these drugs did not significantly affect the factors required for exploratory behavior, such as motivation and motor function.

### 3.3. Experiment 3

Effect of luzindole pretreatment on STM performance in mice received varying doses of either MEL, RAM, or AMK effective to facilitate STM.

In this experiment, the mice were pretreated with either luzindole (0.1 mg/kg) (n = 7 - 11 per group) or its vehicle (1% DMSO saline) (n = 7 - 10 per group) immediately after the acquisition trial, and each pretreatment was followed 5 min later by a single administration of either MEL, RAM, or AMK at two different doses, 10 and 100 ng/kg. Here, we selected these doses as those that would facilitate STM, based on the results of Experiment 2. As expected, in the vehicle-pretreated control mice (**Figure 3(a)**), one-sample t-test revealed that all drugs (MEL, RAM and AMK) significantly (p < 0.05) increased DIs compared with chance performance (50%) after a 3-h ITI, indicating facilitated STM (10 and 100 ng/kg of MEL: t (9) = 2.57, p = 0.0301, d = 1.21 and t (9) = 2.36, p = 0.0428, d = 1.11, respectively; 10 and 100 ng/kg of RAM: t (8) = 3.65, p = 0.0065, d = 1.83 and t (8) = 3.01, p = 0.0169, d = 1.50, respectively; 10 and 100 ng/kg of AMK: t (8) = 3.43, p = 0.0090, d = 1.71 and t (7) = 2.92, p = 0.0224, d = 1.56, respectively; cf. 0 ng/kg (vehicle): t (6) = 0.69, p = 0.5167, d = 0.40).

In contrast, as shown in **Figure 3(b)**, pretreatment with luzindole readily suppressed the STM-facilitating effects produced by both 10 ng/kg and 100 ng/kg of RAM (t (10) = 0.78, p = 0.4530, d = 0.35 and t (8) = 0.03, p = 0.974, d = 0.02, respectively), but not those produced by either dose of AMK (10 and 100 ng/kg of AMK: t (9) = 2.36, p = 0.0425, d = 1.11 and t (9) = 2.50, p = 0.0340, d = 1.18, respectively; cf. 0 ng/kg (vehicle): t (6) = 0.15, p = 0.8879, d = 0.08). Luzindole also failed to suppress the STM-facilitating effects produced by 100 ng/kg of MEL (t (7) = 3.33, p = 0.0126, d = 1.78), although it could suppress these effects produced by10 ng/kg of MEL (t (7) = 0.76, p = 0.4781, d = 0.40).

For both vehicle- (**Figure 3(c)** and **Figure 3(e)**) and luzindole-pretreated mice (**Figure 3(d)** and **Figure 3(f)**), one-way ANOVA revealed that there were no significant differences (p > 0.05) in either total exploration time (**Figure 3(c)** and 3D) (F (6, 55) = 0.86, p = 0.5273 $\eta_p^2$ = 0.09 and F (6, 56) = 0.25, p = 0.9565, $\eta_p^2$ = 0.03, respectively) or number of grid-crossings (**Figure 3(e)**) and **Figure 3(f)**)) (F (6, 55) = 0.76, p = 0.6074, $\eta_p^2$ = 0.08 and F (6, 56) = 0.77, p = 0.5954, $\eta_p^2$ = 0.10, respectively) among groups received varying doses of MEL, RAM, or AMK, including 0 ng/kg (vehicle).

### 3.4. Experiment 4

Effects of pretreatment with luzindole on LTM performance in the mice received
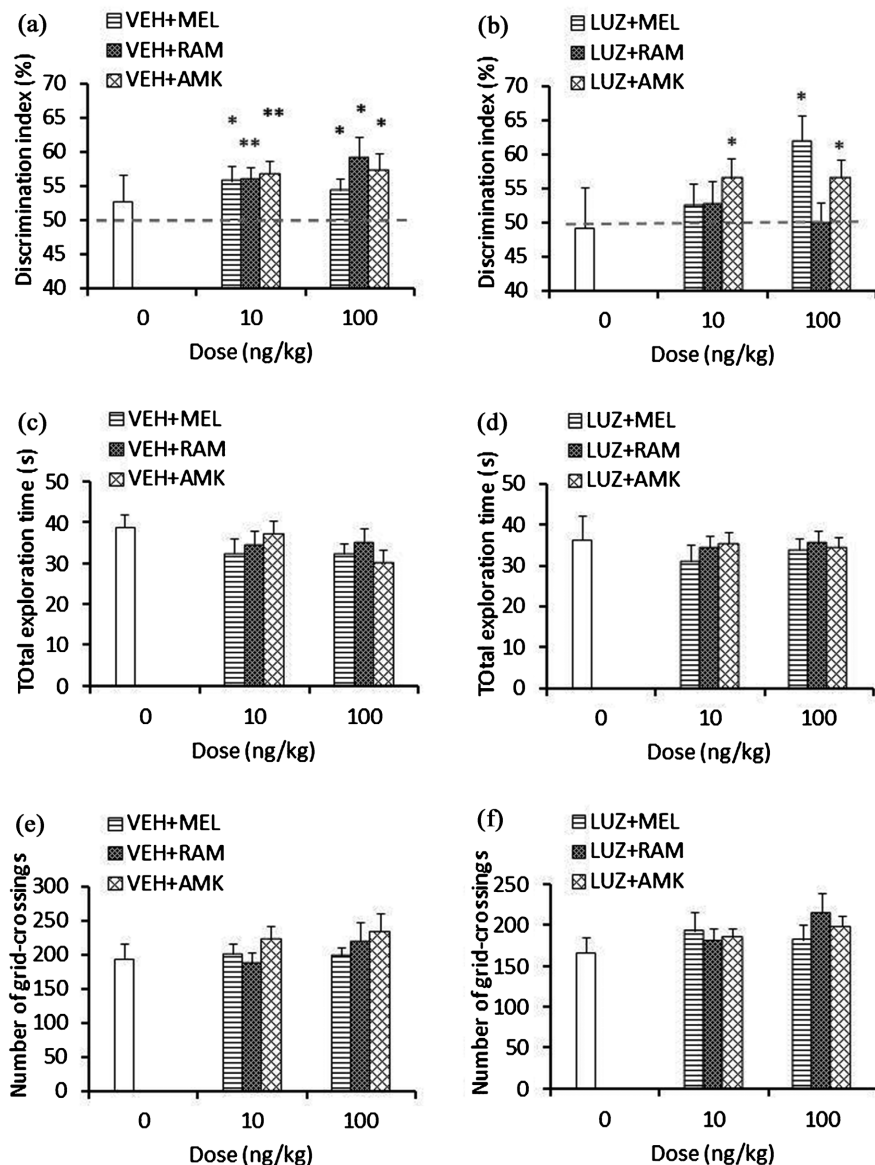
**Figure 3.** Effects of pretreatment with luzindole on DIs for STM performance ((a), (b)), the total exploration time ((c), (d)), and the number of grid-crossings ((e), (f)) on the test trials after a 3-h ITI in the mice received varying doses of either MEL, RAM, or AMK. The mice were pretreated with either vehicle (VEH) ((a), (c), (e)) (n = 7 - 10 per group) or 0.1 mg/kg of luzindole (LUZ) ((b), (d), (f)) (n = 7 - 11 per group), immediately after acquisition trials, and each pretreatment was followed 5 min later by administration of varying doses of either MEL, RAM, or AMK. Values are presented as mean ± SEM. *P < 0.05 and **P < 0.01 indicate significant difference in DIs from chance level (50%) ((a), (b)) (one-sample t-test). For both vehicle- and luzindole-pretreated mice, there were no significant differences (p > 0.05) in either total exploration time ((c), (d)) or the number of grid-crossings ((e), (f)) among groups received any doses of MEL, RAM, or AMK, including 0 ng/kg (vehicle) (one-way ANOVA).

varying doses of either MEL, RAM or AMK.

In this experiment, the mice were pretreated with either luzindole (1 mg/kg) (n = 7 - 13 per group) or its vehicle (10% DMSO saline) (n = 7 - 11 per group)

immediately after the acquisition trial, and each pretreatment was followed 5 min later by a single administration of MEL, RAM and AMK. Test trials were performed after a 24-h ITI to assess LTM performance. First, we investigated the doses of MEL, RAM and AMK required to promote LTM formation using the control mice pretreated with vehicle. We used here varying doses of MEL (0.1 or 1 μg/kg), RAM (0.01, 0.1, or 1 μg/kg) and AMK (0.001, 0.01, 0.1 or 1 μg/kg), speculating that promotion of LTM formation requires higher doses than STM facilitation. As shown in Figure 4(a), one-sample t-tests revealed that the minimum doses of MEL, RAM and AMK required to significantly ($p < 0.05$) promote LTM formation were 1 μg/kg, 0.1 μg/kg, and 0.01 μg/kg, respectively (0.1 and 1 ug/kg of MEL: $t_{(7)} = 0.37$, $p = 0.7228$, $d = 0.20$ and $t_{(7)} = 2.79$, $p = 0.0271$, $d = 1.49$, respectively; 0.01, 0.1 and 1 ug/kg of RAM: $t_{(9)} = 1.31$, $p = 0.2217$, $d = 0.62$, $t_{(10)} = 2.51$, $p = 0.0308$, $d = 1.12$ and $t_{(8)} = 2.41$, $p = 0.0422$, $d = 1.21$, respectively; 0.001, 0.01, 0.1 and 1 μg/kg of AMK: $t_{(9)} = 0.76$, $p = 0.4670$, $d = 0.36$, $t_{(10)} = 2.38$, $p = 0.0383$, $d = 1.07$, $t_{(6)} = 2.68$, $p = 0.0366$, $d = 1.55$ and $t_{(8)} = 2.83$, $p = 0.0221$, $d = 1.41$, respectively; cf. 0 μg/kg (vehicle): $t_{(7)} = 0.60$, $p = 0.5703$, $d = 0.32$).

Based on these results, we then selected the following doses of MEL, RAM, and AMK from a range that included the doses effective in promoting LTM formation: 0.1 and 1 μg/kg for MEL and RAM, and 0.01, 0.1, and 1 μg/kg for AMK. We examined the effects of luzindole pretreatment on LTM performance of the mice received these doses of drugs. As shown in Figure 4(b), one-sample t-tests revealed that pretreatment with luzindole readily suppressed the promoting effects of LTM formation produced by both 0.1 and 1 μg/kg of RAM ($t_{(12)} = 1.47$, $p = 0.1662$, $d = 0.60$ and $t_{(6)} = 0.78$, $p = 0.4642$, $d = 0.45$, respectively), but not those produced by any dose of AMK including 0.01 μg/kg (0.01, 0,1 and 1 ug/kg of AMK: $t_{(10)} = 2.32$, $p = 0.0430$, $d = 1.04$, $t_{(11)} = 2.43$, $p = 0.0332$, $d = 1.04$, and $t_{(11)} = 2.37$, $p = 0.0369$, $d = 1.01$, respectively; cf. 0 ug/kg (vehicle) control: $t_{(11)} = 1.09$, $p = 0.2974$, $d = 0.47$). Luzindole also failed to suppress the LTM formation-promoting effects produced by 1 μg/kg of MEL ($t_{(8)} = 2.65$, $p = 0.0293$, $d = 1.32$).

One-way ANOVA revealed that there were no significant differences ($p > 0.05$) in either total exploration time or number of grid-crossings among vehicle-pretreated groups received varying doses of MEL, RAM, or AMK, including 0 μg/kg (vehicle) (Figure 4(c) and Figure 4(e)) ($F_{(9, 81)} = 0.67$, $p = 0.7100$, $\eta_p^2 = 0.07$ and $F_{(9, 81)} = 0.76$, $p = 0.6536$, $\eta_p^2 = 0.07$, respectively). For luzindole-pretreated mice, however, there were significant differences ($p < 0.05$) in both total exploration time and the number of grid-crossings (Figure 4(d) and Figure 4(f)) ($F_{(7, 77)} = 2.39$, $p = 0.0289$, $\eta_p^2 = 0.18$ and $F_{(7, 77)} = 3.19$, $p = 0.0051$, $\eta_p^2 = 0.23$, respectively). Post hoc Dunnett tests showed no significant difference ($p > 0.05$) in both total exploration time and number of grid crossings between the groups received any dose of MEL, RAM, or AMK and the group receiving vehicle (0 μg/kg).
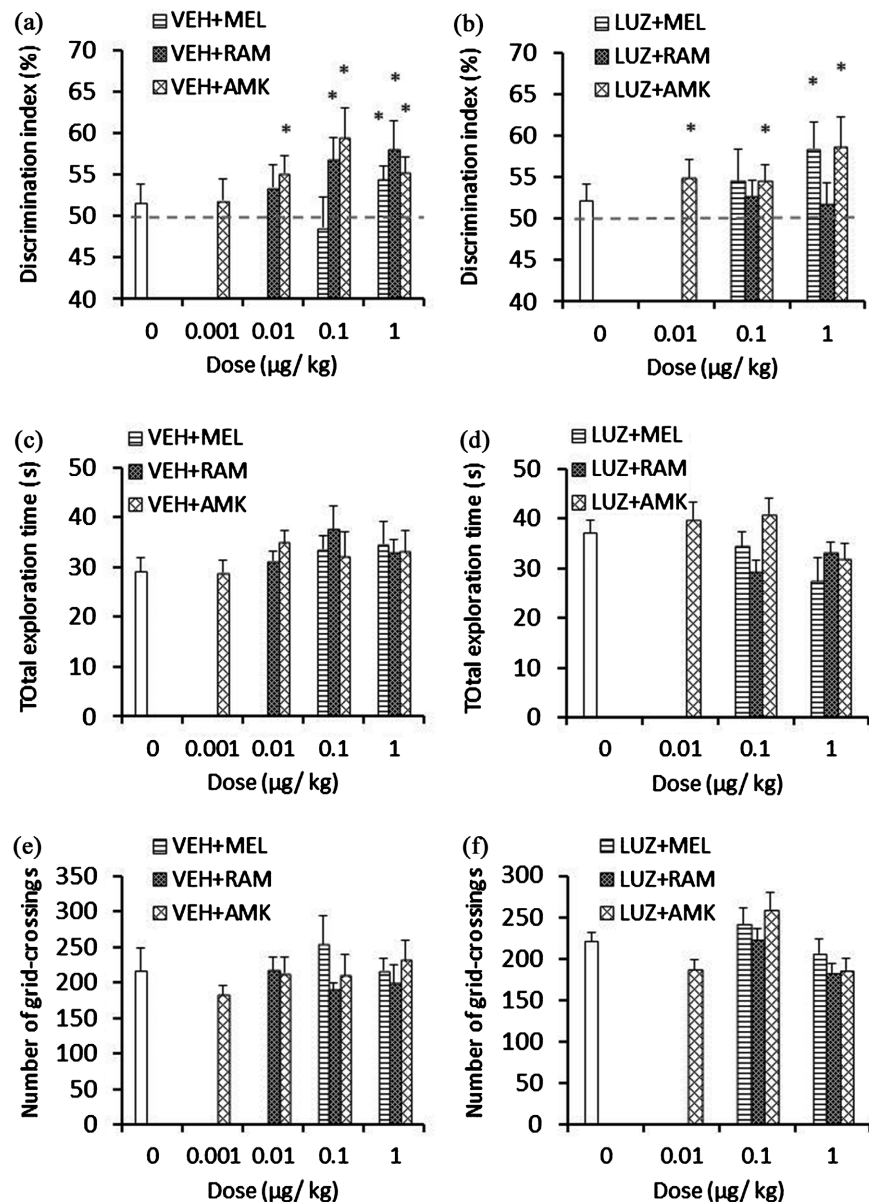
**Figure 4.** Effects of pretreatment with luzindole on DIs for LTM performance ((a), (b)), the total exploration time ((c), (d)), and the number of grid-crossings ((e), (f)) on the test trials after a 24-h ITI in the mice received either MEL, RAM, or AMK. The mice were pretreated with either vehicle (VEH) ((a), (c), (e)) (n = 7 - 11 per each group) or 1 mg/kg of luzindole (LUZ) ((b), (d), (f)) (n = 7 - 13 per each group) immediately after acquisition trials and each pretreatment was followed 5 min later by administration of varying doses of either MEL, RAM, or AMK. Values are presented as mean ± SEM. *$P < 0.05$ and **$P < 0.01$ indicates significant difference in DIs from chance level (50%) ((a), (b)) (one-sample t-test). No significant difference ($p > 0.05$) in both total exploration time and number of grid crossings between the groups received any dose of MEL, RAM, or AMK and the group received vehicle (0 μg/kg) for both luzindole-pretreated (one-way ANOVA followed by Post hoc Dunnett's test) and vechicle-pretreated groups (one-way ANOVA).

## 4. Discussion

The current results show that not only MEL but also RAM and AMK are capable

of both increasing STM retention (Figure 2 and Figure 3(a)) and promoting conversion from STM to LTM (Figure 4(a)). These data indicate that acute MEL administration produces these memory-enhancing effects both via MT1 and MT2 receptor-mediated and non-receptor-mediated mechanisms.

RAM and AMK can facilitate STM at doses an order of magnitude lower than MEL (Figure 2(a)), suggesting that both of these drugs are considerably more potent in facilitating STM compared to MEL. As shown in Figure 3(b), pretreatment with luzindole inhibited the STM-facilitating effects induced by both 10 and 100 ng/kg of RAM. On the other hand, the STM-facilitation induced by AMK at any dose examined (1, 10, or 100 ng/kg) were not suppressed by the luzindole pretreatment, confirming that AMK facilitates STM through MT1/MT2 receptor-independent mechanisms. Luzindole pretreatment inhibited the STM-facilitating effects induced by 10 ng/kg of MEL, but not those induced by 100 ng/kg of MEL. It is unlikely that this result simply suggests that the antagonism of MT1/MT2 receptors by luzindole was reversed by increasing doses of MEL, because the same dose (100 ng/kg) of RAM, despite its greater potency in facilitating STM than MEL, remained suppressed by luzindole. Probably, MEL, at doses of 100 ng/kg, reaches the minimum effective dose sufficient to cause STM facilitation solely through MT1/MT2 receptor-independent mechanisms, mainly via its brain metabolite AMK. The facilitation of STM by 10 ng/kg of MEL, on the other hand, may be caused primarily by MT1/MT2 receptor-mediated mechanisms, and non-receptor-mediated mechanisms, if any, may not contribute substantially.

Figure 4(a) shows that, for all drugs, MEL, RAM, and AMK, higher doses are required for promotion of LTM formation compared to facilitation of STM (see Figure 2); *i.e.*, even doses that were not effective in promoting LTM formation (*i.e.*, 0.1 µg/kg for MEL, 0.01 µg/kg for RAM, and 0.001 µg/kg for AMK) were sufficient to facilitate STM. Figure 4(a) also showed that AMK is about two orders of magnitude more effective than MEL in promoting LTM formation, confirming our recent findings [27]. These data may suggest that the MT1/MT2 receptor-independent mechanisms contribute more to the promotion of LTM formation than facilitation of STM. In fact, pretreatment with luzindole failed to suppress even the LTM formation-promoting effects of 1 µg/kg (the minimum effective dose) of MEL, but did suppress the effects of both 0.1 µg/kg (the minimum effective dose) and 1 µg/kg of RAM (Figure 4(b)). This may suggest that even at the minimum effective dose of MEL, the amount of AMK converted from MEL may have already reached the level sufficient to promote LTM formation solely through MT1/MT2 receptor-independent mechanisms. Although MEL itself may be at least partially involved in these mechanisms, AMK is likely to be the major contributor to the promoting effects of MEL on LTM formation. Indeed, our recent study [27] has reported that the effect of acute administration of 10 µg/kg MEL in promoting LTM formation in the NOR paradigm is completely suppressed when MEL to AMK metabolic pathway was inhibited by pretreat-

ment with norharman, an indoleamine 2,3-dioxygenase (IDO) inhibitor.

It has been reported that RAM has a 3 - 16 fold greater affinity for MT1 and MT2 receptors [28] and a considerably longer half-life (1.0 - 2.6 h) than MEL (<30 min) [29]. M-II, the major metabolite of RAM, has also an affinity to MT1 and MT2 of about one-tenth of the parent compound and has a longer half-life (2 - 5 hours) than RAM [30] [31]. M-II had no significant affinities for other receptors or various enzyme activities, suggesting that M-II, as well as RAM, is an MT1/MT2 receptor selective agonist [32] [33]. Therefore, the strong potency of RAM on memory-enhancing effects may be due to the strong effect of RAM and its metabolite M-II on MT1/MT2 receptors. [29] [30]. Contrary to RAM, AMK has been reported to have about two orders of magnitude weaker affinity for MT1/MT2 receptors than MEL [34] [35]. This supports the notion that AMK induces strong memory enhancing action through non-receptor-mediated mechanisms.

Activation of the extracellular signal-regulated protein kinase (ERK) pathway and phosphorylation of cAMP response element-binding protein (CREB) have been shown to be required for the formation and storage of memories in the hippocampus [36] [37]. Recent in vitro studies using the HT-22 mouse hippocampal neuron cell line showed that MEL mediates the Raf-ERK-CREB cascade via MT1 receptors [38]. In this study, expression levels of p-CREB and BDNF were significantly increased in HT-22 cells treated with either MEL or RAM for 2 hours. These data support previous in vitro studies suggesting that activation of G protein-coupled MEL receptors by MEL increased BDNF in rat midbrain neural stem cells [39]. Imbesi *et al.* [40] also demonstrated that RAM increased BDNF in all primary mouse cerebellar granule cells prepared from wild-type, MT1 KO, and MT2 KO mice, suggesting that activation of either MT1 or MT2 receptors is involved in this effect of RAM. CREB is a major transcription factor that regulates the synthesis of new proteins required for LTM formation [41]. BDNF has been shown to be essential for promoting memory persistence in LTM [42] [43]. It was demonstrated that BDNF mRNA expression in the hippocampus increases after learning and memory [44] [45]. ERK signaling is involved in many learning tasks, including associative fear conditioning [46], spatial learning [47], and conditioned place preference [48]. Specifically, ERK is involved in both early and late stages of long-term potentiation (E-LTP and L-LTP, respectively) [49] [50] [51]. E-LTP lasts from minutes to hours, depends on activation of kinases and phosphatases [52] and is thought to be the basis for STM formation [53] [54]. L-LTP, on the other hand, lasts days and is mRNA and protein synthesis-dependent process involved in LTM formation [55]. Taken together, these data suggest that activation of the Raf-ERK-CREB cascade via MT1 and/or MT2 receptors may be one of the mechanisms of memory enhancement, including both STM facilitation and LTM formation-promotion, by a single administration of MEL.

MT1/MT2 receptor-independent memory enhancing mechanisms by acute MEL administration remains largely unknown. The results of the present study

and our recent study [27], however, suggest the possibility that these mechanisms are mediated mainly via a brain MEL metabolite AMK. MEL is a pleiotropic signal molecule that reaches multiple intracellular targets. Among them, MEL directly binds $Ca^{2+}$-activated calmodulin (CaM) with high affinity [56], and thus it may modulates the $Ca^{2+}$/CaM signaling pathways which are closely involved in memory formation. $Ca^{2+}$/CaM activates CaMKII directly or activates CaMK-kinase (CaMKK), which in turn phosphorylates CaMKIV [57] [58] [59] [60] [61]. Recent evidence suggests that $Ca^{2+}$-CaM-MEL increased the activity of CaMKII in some experimental conditions [62]. Compared to MEL, AMK has been reported to have a higher affinity for Ca2+-CaM [63]. Given the role of CaMKII and CaMKIV, these data may be related to the fact that AMK has stronger STM-facilitating and LTM-formation promoting effects than MEL. CaMKII regulates numerous neuronal functions, including phosphorylation of the AMPA-type glutamate receptor, resulting in increased conductance during E-LTP [64] [65]. CaMKIV is primarily restricted to the nucleus [66] [67], in which it stimulates gene transcription required for L-LTP through phosphorylation of transcription factors such as CREB [68] [69] [70].

## 5. Conclusion

In summary, the present study demonstrated that acute administration of MEL facilitates STM and further promotes conversion of STM into LTM, via both MT1/MT2 receptor-mediated and non-mediated mechanisms. For the latter ones, at least AMK, and possibly MEL itself, they may directly influence some intracellular signaling pathways involved in memory formation. Further elucidation of these mechanisms at the cellular and molecular levels remains for future research.

## Acknowledgements

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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