ORIGINAL RESEARCH

Melatonin Improves Short-Term Spatial Memory in a Mouse Model of Alzheimer's Disease

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Introduction: Alzheimer's disease (AD) is a neurodegenerative disease that has become a leading cause of death in recent years. Impairments in spatial learning and memory are an important clinical feature of AD. Melatonin (MLT), the main product secreted by the pineal gland, showed multiple antioxidant, anti-inflammatory, and neuroprotective properties.

Purpose: The present study aimed to explore the possible prophylactic effects of MLT against spatial memory deficits in a sporadic mouse model of AD induced by D-galactose and aluminium chloride ($AlCl_3$).

Methods: Four groups of mice ($n = 10$ per group) were prepared: control, AD (the D-galactose and $AlCl_3$ AD model group), AD+MLT (AD mice treated with 80 mg/kg MLT), and AD+DON (AD mice treated with 3 mg/kg donepezil). We then used the object location and Y-maze tests to assess spatial memory in the four groups. Gene expression levels of brain-derived neurotrophic factor (*Bdnf*) and cAMP-responsive element-binding protein (*Creb1*) were measured using real-time polymerase chain reaction.

Results: We found that MLT improved spatial memory in the sporadic AD mice. MLT ameliorated *Creb1* gene expression and significantly increased *Bdnf* gene expression in the hippocampus of AD model mice compared with the AD group.

Conclusion: MLT could have a substantial potential to alleviate memory impairment in sporadic AD if introduced at early stages.

Keywords: melatonin, Alzheimer's disease, spatial memory, CREB, BDNF

Introduction

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disorder that is characterized by cognitive decline, memory loss, and behavioural abnormalities.[1] Impairments in spatial learning and memory are an important clinical feature of this disease.[2] The accumulation of extracellular β-amyloid (Aβ) plaques and neurofibrillary tangles in the brain are a major pathological feature of AD.[3] Moreover, oxidative stress, neuroinflammation, and cholinergic and mitochondrial dysfunction play crucial roles in the pathophysiology of AD.[3–7]

Melatonin (MLT; N-acetyl-5-methoxytryptamine) is the main product secreted by the pineal gland.[8] It is involved in many physiological functions including the control of circadian rhythms and the regulation of body temperature.[9–11] Many studies have reported that MLT has strong antioxidant and anti-inflammatory effects.[12–14] MLT stimulates certain antioxidant enzymes, including superoxide dismutase 1 and glutathione peroxidase, in the brains of AD mice.[15] Moreover, MLT can protect these antioxidant enzymes from oxidative stress.[16] Chronic MLT administration can enhance synaptogenesis and synaptic function, and can also

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preserve glial cell structure in the AD rat brain.[17,18] In addition, MLT efficiently prevents the formation and accumulation of Aβ and neurofibrillary tangles in the brains of AD model animals.[19,20] MLT also improves performance in novel object recognition and passive avoidance tasks in an adult ICR AD mouse model,[21] and spatial memory assessed by Morris water maze and Y-maze tasks in young and aged AD model mice.[10,22–24] Furthermore, MLT metabolites improve long-term recognition task performance in young, middle aged and old MLT-deficient mice.[25] Protective effects of MLT against learning and memory deficits have also been observed in several other animal studies such as vascular dementia and tau hyperphosphorylation animal models induced by wortmannin, isoproterenol, and calyculin A.[26,27]

Reduction of brain-derived neurotrophic factor (BDNF) has a crucial role in learning, memory, and synaptic plasticity.[28] It is expressed mostly in the hippocampus as well as in the cortex and stimulates long-term potentiation of hippocampal synapses and enhance spatial memory.[28,29] Reduction of BDNF expression has been associated with AD pathogenesis and is reduced in early stages of AD.[30,31] Another study found that BDNF expression level is decreased in the hippocampus and cortex of AD patients.[32] In animal studies, the expression of BDNF was measured in two transgenic AD mouse models and indicated that mRNA is considerably downregulated in cortical tissue when compared to the control.[33] In other studies, the levels of BDNF were significantly reduced in the hippocampus of Aβ-induced AD model and was considerably decreased in the scopolamine-induced AD model when compared to the control.[23,34]

Cyclic AMP response element binding protein (CREB) is a nuclear binding protein that binds to specific DNA sequences, act as a transcription factor for several genes including BDNF, and has an important role in the memory formation and retention.[35,36,38] In addition, CREB is a component of intracellular signalling that control the circadian rhythms of long-term memory effectors in the hippocampus.[36] Previous studies showed that the CREB level is reduced with aging as well as in AD.[36,37] Hence, it is suggested that a correlation exists between the role of BDNF gene expression and its regulation via CREB in reversing cognitive impairments.[38] It has been reported that Aβ and scopolamine induced neurotoxicity down regulates BDNF and its main regulatory molecule CREB in the cortex and hippocampus of AD model.[23,39]

Mouse models of AD induced by D-galactose and/or aluminium chloride ($AlCl_3$) have been used extensively to study the mechanisms of AD and to investigate the effects of drugs.[10,40,41] D-galactose, a widely used to induce brain damage in rodents.[42] It is a reducing sugar that is naturally present in the body in small amounts, however when present in excess quantities, D-galactose lead to increased production of reactive oxygen species and decreased levels of antioxidant enzymes.[43] Other mechanisms have been reported in D-galactose-induced aging in the animal brain, including inflammation, apoptosis, and mammalian target of rapamycin upregulation.[42] $AlCl_3$ is a neurotoxic agent that causes oxidative damage and enhances inflammatory responses in the rodent brain.[41] Chronic administration of $AlCl_3$ promotes the overproduction of extracellular Aβ plaques and intracellular neurofibrillary tangles formation.[44] $AlCl_3$ toxicity disrupts calcium homeostasis, thereby inducing neurodegeneration.[45] The co-administration of D-galactose and $AlCl_3$ has been used to induce a sporadic AD animal model that shows several pathological features of AD.[40,46] In fact, a 10-week co-administration of $AlCl_3$ and D-galactose produced memory impairment, cholinergic system defect, and brain pathology.[40]

No previous studies have investigated the effects of MLT treatment on the BDNF/CREB pathway in the sporadic AD model induced by D-galactose/$AlCl_3$. Therefore, the purpose of this study was to investigate whether treatment with MLT can ameliorate spatial memory impairments induced by D-galactose and $AlCl_3$ compared to Donepezil, a brain-selective acetylcholine esterase inhibitor, which had shown to improve learning and memory tasks in Alzheimer animal models.

Materials and Methods
Animals
Forty adult (4–8 weeks old) male Swiss mice (SWR/J) weighing 18–22 g were obtained from the animal facilities of King Fahd Medical Research Center, King Abdulaziz University, Jeddah. The mice were kept in the experimental area for 2 weeks (4–6 weeks old) for habituation phase. The experiment started by administration of prophylactic doses of MLT at age of 6–8 weeks old for two weeks followed by AD induction at age of 8–10 weeks old for 8 weeks. The mice were housed at five mice per cage at an appropriate temperature (22 ± 2 °C) and humidity (60% ± 10%), with a standard 12-hour light/

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dark cycle and free access to water and standard food. The mice were not mixed between procedures and all experiments were performed with one batch of mice. All mice were cared for following the guidelines of the Animal Care and Use Committee at King Fahd Medical Research Center All experiments were performed according to the guidelines of the biomedical ethics research committee (reference no. 102-19) of King Abdulaziz University, which comply with the guidelines of the "System of ethics of research on living creatures", prepared by the King Abdulaziz City for Science and Technology, approved by Royal Decree No. M/59 on 24 August 2010.

Treatment Preparation

D-galactose extra pure powder (Loba Chemie Pvt Ltd, Mumbai, India) was dissolved in normal saline. AlCl$_3$ (Techno PharmChem, Haryana, India) was dissolved in distilled water. D-galactose and AlCl$_3$ were freshly prepared twice per week and stored at 4 °C. MLT crystalline powder (M5250; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 20% ethanol and then diluted with saline; the final ethanol concentration was 1%. MLT was freshly prepared before administration in dark bottles to avoid oxidation of the solution by light. Donepezil hydrochloride, tablets (5 mg) were purchased from a local pharmacy, ground to a fine powder then dissolved in distilled water. All treatments were given to mice in volumes of 0.2 mL. Doses were adjusted weekly according to body weight.

Treatment Groups

Forty mice were randomly divided into the following four groups (n = 10 per group): control (treated with the drug vehicles; mice received saline subcutaneously, distilled water orally, and 1% ethanol in saline intraperitoneally (IP), AD (the AD model; mice received 120 mg/kg D-galactose subcutaneously, 20 mg/kg AlCl$_3$ orally, and 1% ethanol in saline IP), AD+MLT (the MLT treatment group; mice received 120 mg/kg D-galactose subcutaneously, 20 mg/kg AlCl$_3$ orally, and 80 mg/kg MLT IP), and AD+DON (a positive control group; mice received 120 mg/kg D-galactose subcutaneously, 20 mg/kg AlCl$_3$ and 3 mg/kg donepezil orally, and 1% ethanol in saline IP). We used the highest prophylactic dose of MLT (80 mg/kg), which does not produce any signs of toxicity in mice.[14,18,47]

Experimental Design

The drug administration regimen lasted for 10 weeks. MLT administration started on week −1, whereas D-galactose, AlCl$_3$, and donepezil administration was started on week 1. Each treatment was given once per day. The animals received MLT doses from 8:00 to 9:00 a.m. and received D-galactose and AlCl$_3$ doses from 10:00 to 12:00 p.m. The behavioural tests were performed during week 9 (Figure 1).

Open Field Test

To assess the locomotor activity of mice, the OF test was used to measure velocity and total distance moved (TDM). The locomotor activity of mice was monitored using an EthoVision XT8A system (Noldus Information Technology, Wageningen, Netherlands). Mice were placed individually in the arena, which was a transparent square box (45 × 45 cm) and allowed to move freely for 3 minutes.[48] The EthoVision XT8A system was used to calculate velocity and TDM.

Object Location Test (OLT)

The OLT is a widely used behavioural test for assessing spatial memory deficits in mice.[49] Each mouse was habituated for 15 minutes the day before the test. The aim of habituation was to introduce the mice to the arena (without objects) to minimize confounding anxiety and novelty factors, which can negatively affect interaction with and discrimination of objects. On the day of the OLT, we first conducted a familiarisation trial (Figure 2), in which two identical objects (eg, two metal cylinders) were placed in a particular location within the arena and the animals were allowed to explore these two objects for 3 minutes (Supplementary Figure S1). The zones are defined as 1 inch around each object to ensure detection of exploration behaviour around each object. The objects used in this experiment were selected according to particular properties (ie, cleanable and not easily moved).[50] After 10 minutes, we conducted the test trial, in which the location of one of the familiar objects was changed (Figure 2) and object exploration was recorded for 3 minutes (Supplementary Figure S2). Parameters were recorded and analysed using a video tracking system (EthoVision XT8A). The frequency of bringing the nose close to both objects (sniffing) was assessed in both the familiarisation and test trials. Mouse memory was also evaluated by the time spent in the familiar and novel zones during the test trial. The discrimination index (DI) was
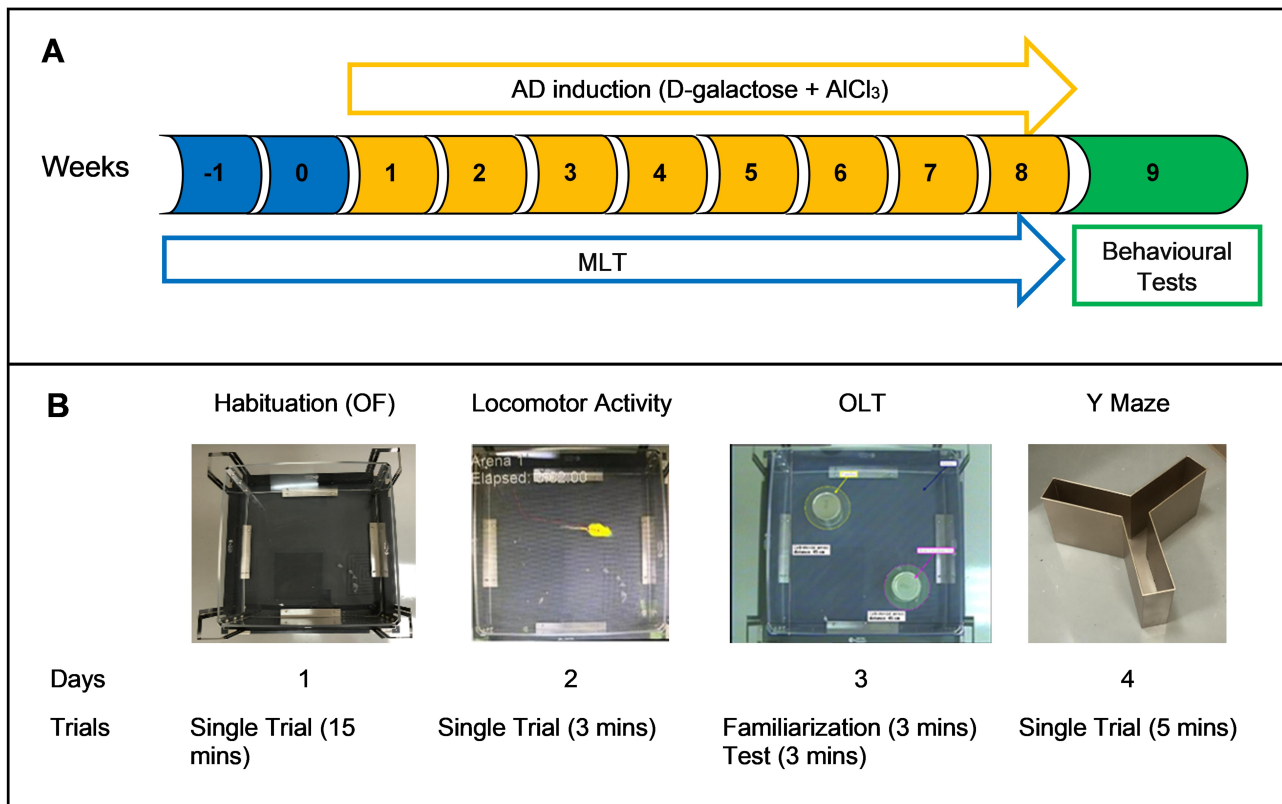
Figure 1 (**A**) Timeline of the experiment. (**B**) Sequence of the behavioural tests.
Abbreviations:AD, Alzheimer's disease; AlCl$_3$, aluminium chloride; MLT, melatonin; OF, open field; OLT, object location test.
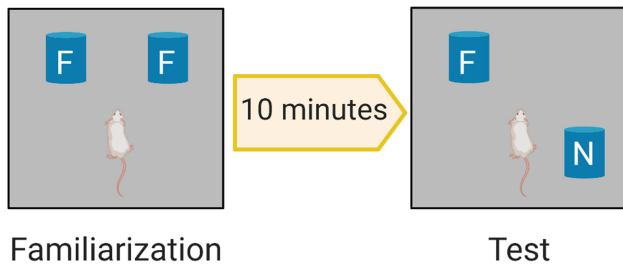

Figure 2 OLT Phases. Familiarization phase allows the rodent to explore two identical familiar objects F. The test phase evaluates memory by investigating the ability of the rodent to discriminate the displaced new location; N object from the previously explored F object.

calculated according to the following equation: DI = (TN–TF)/(TN+TF) where TN is the time spent in the novel (displaced object in the new location) zone and TF is the time spent in the familiar zone.[48]

Spontaneous Alternation Test (Y-Maze)

Spontaneous alternation is another test of spatial memory and was performed using a Y-maze with symmetrical arms. Each arm of the maze was 60 cm long, 10 cm wide, and 15 cm high.[51] This test consisted of only one

5-minute trial and was conducted in a sound-insulated room. The mice were individually placed at the end of one arm and allowed to explore all arms of the maze freely. Between each trial, the maze arms were wiped with 70% alcohol to avoid odour cues.[52] The maze arms were labelled A, B, and C. The spontaneous alternation score was defined as sequential entry into the three different arms (ie, ABC, CBA, or BAC). Re-entry into the same arm such as ABA or ACA was considered an entry error. The spontaneous alternation percentage (SAP) was calculated by SAP = number of alternations/(total arm entries − 2) × 100.[51]

Brain Tissue Collection

After behavioural testing, mice were anaesthetized with isoflurane and euthanized by decapitation following the rules and regulations of King Fahd Medical Research Center. Whole brains were carefully removed and washed in saline. The hippocampus of each brain was dissected, and the left hippocampus was submerged in RNAlater solution (Invitrogen) to be used for gene expression analysis.

Gene Expression

Expression levels of the genes encoding cAMP-responsive element-binding protein (*Creb1*) and brain-derived neurotrophic factor (*Bdnf*) in the hippocampus of mice were measured using real-time polymerase chain reaction (PCR) analysis.

RNA Extraction and Real-Time PCR Analysis

Total pure RNA was extracted from hippocampal tissue using a PureLink™ RNA Mini Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Complementary DNA synthesis was performed using a SuperScript™ IV VILO™ Master Mix Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase gene (*Gapdh*) was used as a housekeeping gene. Details of the primers used are shown in Table 1.

Statistical Analysis

All data are presented as the mean ± SEM. The D'Agostino-Pearson normality test and Q-Q plots were used to test the normality assumption of parametric statistics. One-way analysis of variance (ANOVA) followed by post hoc Tukey's test was used to analyse locomotor activity, DI, Y-maze, and gene expression data. In addition, we used two-way ANOVA followed by post hoc Bonferroni multiple comparison test to compare differences within groups of percentages of mice sniffing and exploration time spent in familiar and novel objects during the familiarization and test phases of the experiment. Differences between groups were considered statistically significant if *P* was less than 0.05. GraphPad Prism 8.3.8 was used for the statistical analysis.

Results

Effects of MLT on Locomotor Activity

There was no significant difference in velocity between study groups [$F (3, 36) = 2.19$, $P = 0.106$; Figure 3A]. Moreover, there was no significant difference in TDM among groups [$F (3, 36) = 2.12$, $P = 0.114$; Figure 3B].

Effects of MLT on the OLT

There was no significant difference between the percentage of sniffs of each object (familiar vs to be changed) in all groups in the familiarisation phase [$F (1, 72) = 0.002$, $P = 0.963$; Figure 4A]. However, in the test phase, the percentage of sniffs of the familiar object was significantly different from that of the novel object in all groups [$F (1, 72) = 11.27$, $P = 0.001$; Figure 4B]. For instance, there was a significantly higher percentage of sniffs of the novel object than of the familiar object in the control group ($P = 0.0002$). In contrast, in the AD group, the percentage of sniffs was significantly lower for the novel object than for the familiar object ($P = 0.0006$). The AD+MLT and AD+DON groups both had a significantly higher percentage of sniffs of the novel object than of the familiar object ($P = 0.007$ and $P = 0.010$, respectively).

There was no significant difference between the total exploration time of each object zone (familiar vs to be changed) in all groups in the familiarisation phase [$F (1, 72) = 0.7168$, $P = 0.400$; Figure 5A]. However, in the test phase, the time spent in the familiar object zone was significantly different from that of the novel object zone in all groups [$F (1, 72) = 10.75$, $P=0.001$; Figure 5B]. For instance, there was a significantly higher exploration time to the novel object than of the familiar object in the control group ($P = 0.006$). However, in the AD group, the total exploration time was not significantly different between objects ($P = 0.101$). The AD+MLT and AD+DON groups

Table 1 Sequences of PCR Primers

Gene	Accession Number	Oligo	Sequence	Amplicon Length
Gapdh	NM_001289726.1	Forward primer	5′-GTGAACGGATTTGGCCGTATT-3′	70
		Reverse primer	5′-CAATCTCCACTTTGCCACTGC-3′	
Creb1	NM_001037726.1	Forward primer	5′-ACCCACGAGCACCATTGC-3′	120
		Reverse primer	5′-TGCCTCCCTGTTCTTCATTAG-3′A	
Bdnf	NM_001048139.1	Forward primer	5′-AAGGGCCAGGTCTGTTAATCG-3′	70
		Reverse primer	5′-ATGGCTCTATGAAACTGTTCTGGT-3′	

Abbreviations: *Bdnf*, brain-derived neurotrophic factor; *Creb1*, cAMP-responsive element-binding protein; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.
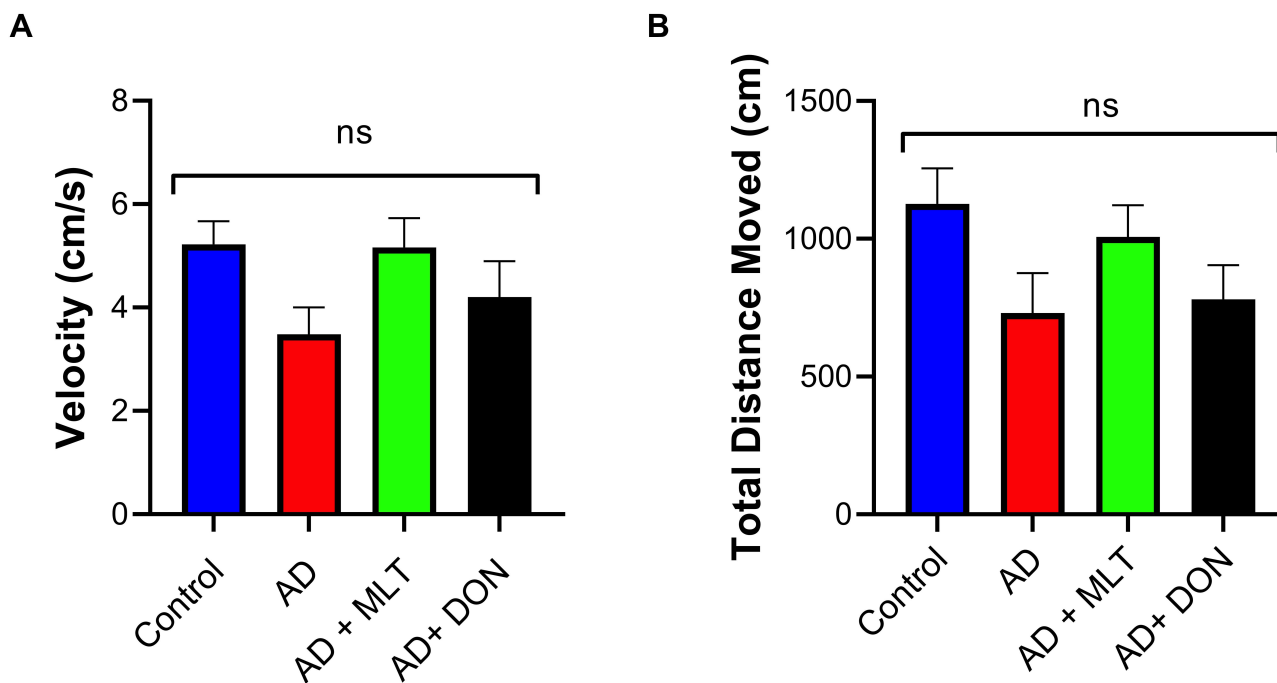
A

B

Figure 3 Locomotor activity. Data are presented as the mean ± SEM and one-way ANOVA was used to compare differences between groups ($P > 0.05$). (**A**) There were no significant differences in mouse velocity among any of the treatment groups. (**B**) There were no significant differences in the TDM among groups. N=10. Non-significant (ns).

both had a significantly higher exploration time in the novel object zone than of the familiar object ($P = 0.029$ and $P = 0.025$, respectively).

In the OLT, the DIs for all groups were significantly greater than the chance level of zero [control: $t(9) = 11.05$, $P < 0.0001$; AD: $t(9) = 3.96$ $P = 0.003$; AD+MLT: $t(9) = 2.83$, $P = 0.019$; AD+DON $t(9) = 2.62$, $P = 0.027$]. Also, there were statistically significant differences in the DI between study groups [$F (3, 36) = 14.54$, $P < 0.0001$; Figure 6]. For instance, the post hoc analysis showed that the control ($P < 0.0001$), AD+MLT ($P = 0.0002$) and AD+DON ($P < 0.0001$) groups had significantly higher DIs than the AD group. However, there was no statistically significant difference between the control and AD+MLT or AD+DON groups ($P = 0.636$ and $P = 0.827$, respectively).

Effects of MLT on the Y-Maze Test

The SAP results of all study groups are shown in Figure 7A. There were statistically significant differences between the study groups [$F (3, 36) = 8.427$, $P = 0.0002$]. For instance, there was a lower SAP in the AD group compared with the control group ($P = 0.0001$). In contrast, the SAPs in the AD+MLT and AD+DON groups were not significantly different compared with the control group ($P = 0.328$ and $P = 0.432$, respectively). The SAPs

in the AD+MLT and AD+DON groups were significantly higher than that of the AD group ($P = 0.015$ and $P = 0.009$, respectively). Additionally, there was no significant difference in the number of arm entries between groups [$F (3, 36) = 0.91$, $P = 0.447$; Figure 7B].

Effects of MLT on *Bdnf* and *Creb1* Expression in the Hippocampus of AD Mice

There were statistically significant differences in *Bdnf* expression among groups [$F (3, 35) = 4.221$, $P = 0.014$; Figure 8A]. For instance, Tukey's post hoc analysis showed significantly lower *Bdnf* expression in the AD group (M = 0.78, SEM = 0.05) compared with the control group (M =1.12, SEM= 0.07, $P =0.013$). In addition, *Bdnf* expression was significantly higher in the AD+MLT group (M = 1.07, SEM = 0.09) compared with the AD group ($P = 0.045$). Furthermore, there was no statistically significant difference between the control group and AD +MLT and AD+DON groups ($P = 0.958$ and $P = 0.447$, respectively). There were significant differences in *Creb1* expression among groups [$F (3, 28) = 4.40$, $P = 0.003$; Figure 8B]. Post hoc analysis showed a significant reduction in *Creb1* expression in the AD group (M = 0.76, SEM = 0.02) compared with the control group (M = 1.13, SEM

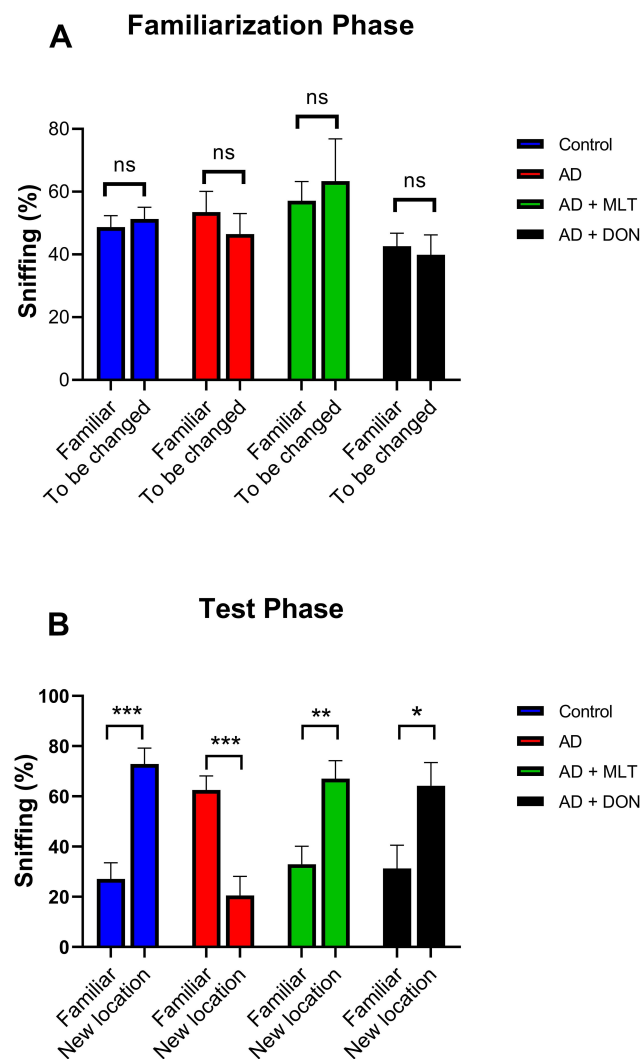**A** **Familiarization Phase**

B **Test Phase**

Figure 4 Percentages of sniffs in both phases of the object location test. Data are presented as the mean ± SEM. Two-way ANOVA was used, followed by the Bonferroni multiple comparisons test. (**A**) There were no significant differences among the groups in the percentages of sniffs of each object (familiar vs to be changed) in the familiarisation phase. (**B**) There were significant differences among groups in the percentages of sniffs of each object (familiar vs novel) in the test phase. N=10. Non-significant (ns). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

= 0.07; $P = 0.041$). There was also a significant increase in *Creb1* expression between AD and AD+DON groups (M =1.3, SEM=0.16, $P = 0.008$). However, there was only a marginal increase in the expression of *Creb1* in the AD +MLT group (M = 1.043, SEM = 0.04) compared with the AD group.

Discussion

In the present study, we aimed to investigate the possible prophylactic effects of MLT against memory deficits in the D-galactose/AlCl₃-induced sporadic AD mouse model, focusing on the impacts of MLT on spatial memory.

Several animal models of AD have been used to investigate the effectiveness of different treatments targeting AD.[53,54] These models vary in their cognitive and behavioural performances, and these differences in exploratory, activity, and anxiety behaviours are reflected in several learning and memory tests.[55,56] It has been noted that the D-galactose/AlCl₃-induced mouse model mimics the cognitive deficits and pathological alterations of clinical AD.[57,58] Although many studies have been performed using different sporadic AD models, cognitive and behavioural data from the D-galactose/AlCl₃ mouse model are limited.[54] In the present study, we therefore used a combined administration of D-galactose and AlCl₃ to induce an AD mouse model. We first assessed the motor activity of mice to ensure that there were no motor limitations that would affect memory test outcomes.[59] The open field test demonstrated that there were no significant differences in motor activity between the AD group and any other group. This is consistent with previous studies that also reported no significant differences in motor activity between the D-galactose/AlCl₃ AD model and control groups.[57,60] Based on this finding, any learning and memory improvements caused by the treatments in this study are likely to be mnemonic, rather than sensorimotor.

In many AD models, spatial memory is severely affected because of neuronal damage, especially in hippocampal areas.[61–63] Consistent with previous studies, our results revealed that spatial memory was affected in D-galactose/AlCl₃ AD mice.[51,65] We assessed spatial memory using the OLT and the Y-maze place recognition tasks. A decline in spatial memory is associated with disrupted hippocampus-dependent functions, and these features are a primary characteristic of the cognitive impairments that are observed in AD patients.[64]

Several studies have indicated that MLT prevents the progression of AD and improves the cognitive impairment associated with the disease via several mechanisms.[10,26,27] For these effects, MLT doses need to be approximately twice those required to influence sleep and circadian rhythmicity.[19] Clinical studies that use MLT in the range of 50–100 mg/day are needed to investigate the therapeutic validity of MLT treatment in AD.[19] Interestingly, no previous studies have used high doses of MLT (eg, 80 mg/kg) to ameliorate memory decline in AD animal models.[47] In addition, no previous studies have investigated the potential prophylactic effects of MLT on an AD model induced by the

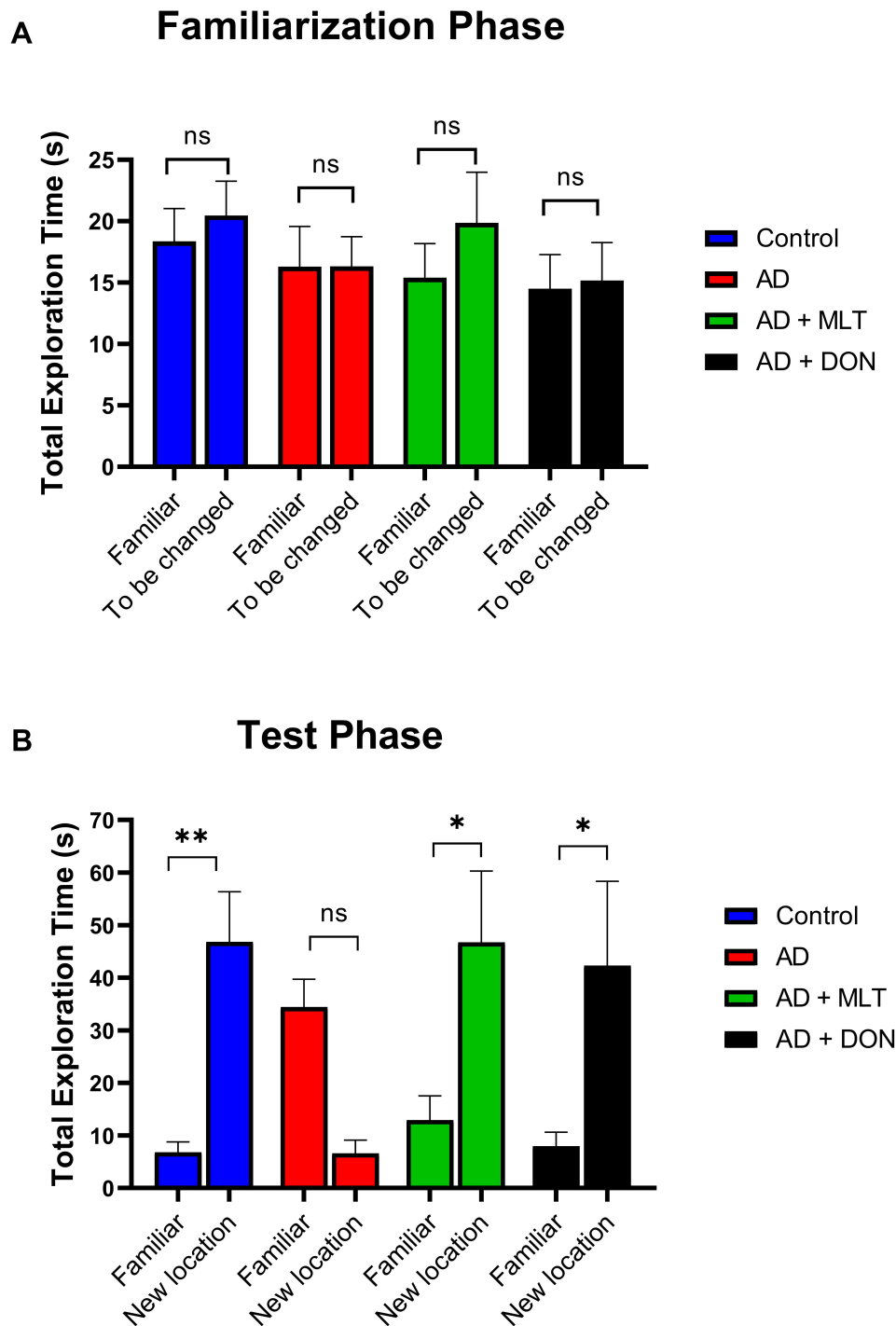## A **Familiarization Phase**

B **Test Phase**

Figure 5 Total exploration time in both phases of the object location test. Data are presented as the mean ± SEM. Two-way ANOVA was used, followed by the Bonferroni multiple comparisons test. (**A**) There were no significant differences among the groups in the total exploration time spent in each object zone (familiar vs to be changed) in the familiarisation phase. (**B**) There were significant differences among groups in the total exploration time spent in each object zone (familiar vs novel) in the test phase. N=10. Non-significant (ns). *$P < 0.05$, **$P < 0.01$.

co-administration of D-galactose and AlCl₃. In our study, we thus used the highest prophylactic dose of MLT (80 mg/kg), which does not produce any signs of toxicity in mice.[14,18,66] This high dose of MLT is found to be a potent antioxidant and anti-inflammatory which improved memory outcomes and locomotor activity in multiple sclerosis animal model.[14,18] Moreover, this high dose of MLT altered the T effector/regulatory balance which therefore protect against experimental autoimmune encephalomyelitis animal model.[66]
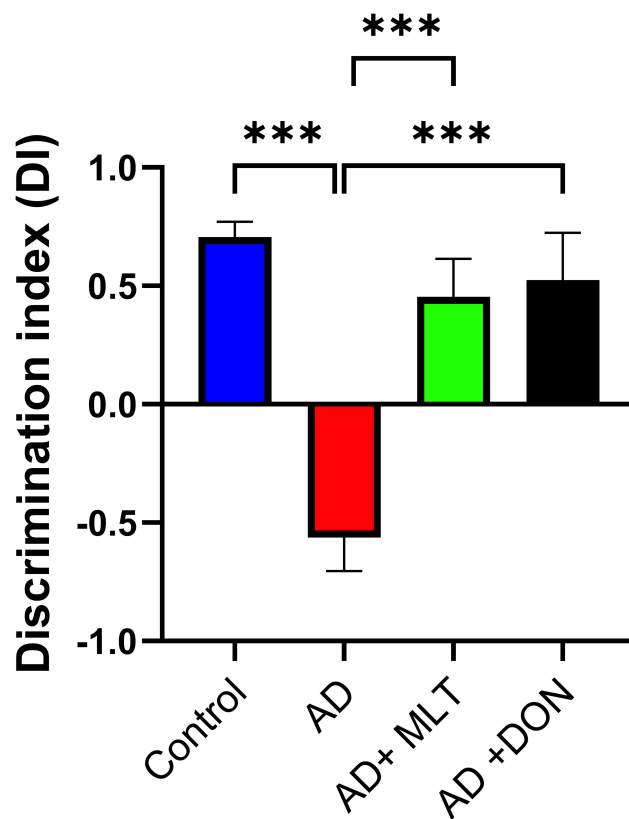
Figure 6 Discrimination index in the object location test. Data are presented as the mean ± SEM. One-way ANOVA was used, followed by Tukey's multiple comparisons test. The DI was significantly lower in the AD group vs the control group. DIs were significantly higher in both the AD+MLT and AD+DON groups vs the AD group. N=10. ***$P < 0.001$.

In the current study, spatial memory-dependent tasks (the OLT and Y-maze) revealed an improvement in spatial memory performance by MLT-treated mice compared with the AD model mice. In the OLT, we measured the DI, exploring time, and sniff frequency for each group. By calculating the frequency that each object was sniffed and the exploration time for each object we found that each group took similar chances to explore both objects in the familiarisation phase. However, there were significant differences in the frequency of sniffing and the exploration time between the familiarisation and test phases among the groups. Our results indicate that MLT and DON treatment improved the spatial memory outcomes measured by the OLT and Y-maze tasks, compared with the AD group, which is consistent with previous studies.[10,23,24]

BDNF is a crucial molecule involved in synaptic plasticity, learning and memory, and neurogenesis.[28] BDNF signalling can stimulate long-term potentiation of hippocampal synapses and enhance spatial memory.[28] Reduction in BDNF expression levels have been reported in AD, especially in brain areas important for learning and memory, such as the hippocampus.[30] BDNF levels are also reduced in the early stages of AD.[31] In our study, *Bdnf* expression was significantly depleted in the hippocampus of the AD model, as has been previously reported.[31,36,66] However, MLT increased *Bdnf* expression levels and DON ameliorated *Bdnf* expression levels in the AD model. This finding is consistent with those of previous studies, which indicated that MLT treatment can reverse the decreases in BDNF protein levels in the hippocampus of AD models.[24,67,68] This beneficial effect of MLT in reversing BDNF level in AD hippocampus is
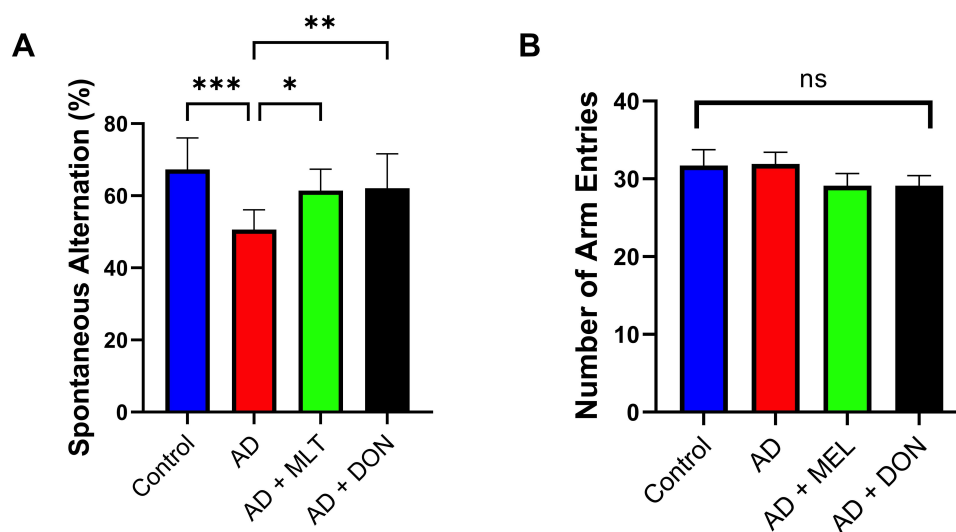

Figure 7 Spontaneous alternation and number of arm entries in the Y-maze task. Data are presented as the mean ± SEM. One-way ANOVA was used, followed by Tukey's multiple comparisons test. (**A**) SAP was significantly lower in the AD group vs the control group. SAPs were significantly higher in the AD+MLT and AD+DON groups vs the AD group. (**B**) There were no significant differences in the number of arm entries among the groups. N=10. Non-significant (ns). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
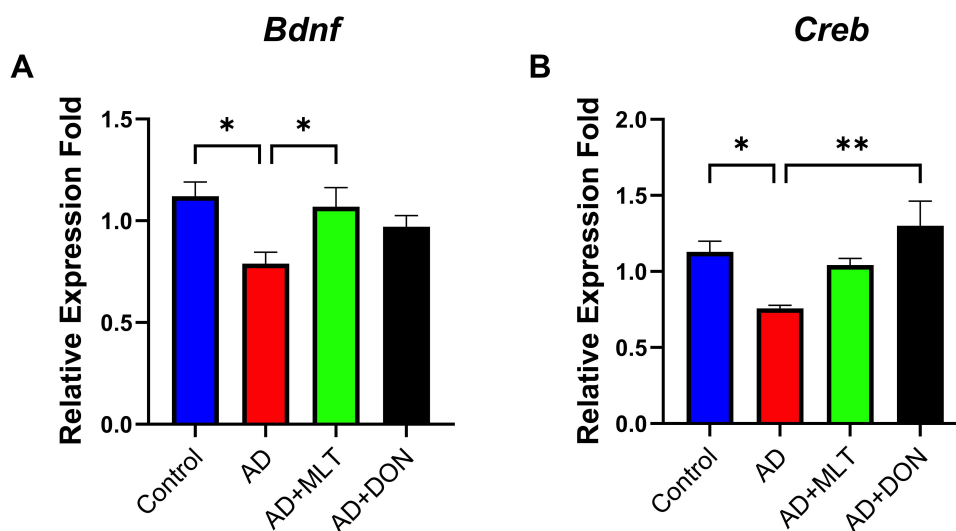
Figure 8 Expression of *Bdnf* and *Creb1* in the hippocampus. Data are presented as the mean ± SEM. One-way ANOVA was used, followed by Tukey's multiple comparisons test. (**A**) *Bdnf* expression was significantly lower in the AD group vs the control group. *Bdnf* expression was significantly higher in the AD+MLT group vs the AD group. (**B**) *Creb1* expression was significantly lower in the AD group vs the control group. *Creb1* expression was significantly higher in the AD+DON group vs the AD group. N=10. *$P < 0.05$, **$P < 0.01$.

reflected by improving the spatial memory in AD model in our study.

CREB is a crucial nuclear transcription factor that plays a key role in BDNF transcription.[35] A reduction in CREB phosphorylation leads to the downregulation of BDNF levels, which ultimately results in working memory dysfunction.[69] In addition, CREB is a component of the intracellular signalling that controls the circadian rhythms of long-term memory effectors in the hippocampus.[36] However, CREB expression levels decrease with age as well as in some neurodegenerative diseases such as AD.[36,37] In the present study, *Creb1* expression levels were markedly reduced in the hippocampus of D-galactose/AlCl$_3$ AD model mice. This finding is consistent with the results of previous studies, which reported that CREB expression levels are significantly reduced in the hippocampus of AD animal models.[17,35,70] In addition, DON increases *Creb1* expression levels and MLT amelio-rated the reduction in *Creb1* expression in the present study, similar to the results of previous reports.[71–73]

Conclusions

To the best of our knowledge, no previous studies have investigated the effects of MLT treatment on the BDNF/CREB pathway in the D-galactose/AlCl$_3$ AD model. Our results indicate that MLT reversed the spatial memory decline in a sporadic D-galactose/AlCl$_3$ AD model. This could be due to its role in improving *Bdnf* and *Creb1* gene expression in the AD mouse hippocampus. Therefore,

MLT could have a substantial potential to alleviate memory impairment in AD if introduced at early stages. However, further biochemical, genetic, and immunohisto-chemical analyses are needed to provide more information about the mechanisms underlying the effects of MLT in a sporadic AD model.

Abbreviations

AD, Alzheimer's disease; AlCl$_3$, aluminium chloride; MLT, melatonin; OLT, object location test; TDM, total distance moved; DI, discrimination index; SAP, spontaneous alternation percentage; DON, donepezil; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; BDNF, brain-derived neurotrophic factor; CREB, cAMP-responsive element-binding protein; Aβ, β-amyloid; ANOVA, analysis of variance.

Data Sharing Statement

The datasets used and/or analysed are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

All experimental protocols and procedures were approved by the Animal Care and Use Committee of KFMRC and performed according to the guidelines of the biomedical ethics research committee (reference no. 102-19) of King Abdulaziz University.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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