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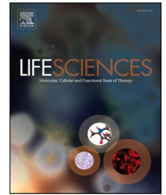

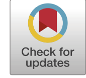

Melatonin reverts methamphetamine-induced learning and memory impairments and hippocampal alterations in mice

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ABSTRACT

Aims: Methamphetamine (METH) has become a major public health problem because of its abuse and profound neurotoxic effects, causing alterations in brain structure and function, and impairing cognitive functions, including attention, decision making, emotional memory, and working memory. This study aimed to determine whether melatonin (MEL), the circadian-control hormone, which has roles beyond circadian rhythm regulation, could restore METH-induced cognitive and neuronal impairment.

Main methods: Mice were treated with either METH (1 mg/kg) or saline for 7 days, followed by MEL (10 mg/kg) or saline for another 14 days. The Morris water maze (MWM) test was performed one day after the last saline or MEL injection. The hippocampal neuronal density, synaptic density, and receptors involved in learning and memory, along with downstream signaling molecules (NMDA receptor subunits GluN2A, GluN2B, and CaMKII) were investigated by immunoblotting.

Key findings: METH administration significantly extended escape latency in learning phase and reduced the number of target crossings in memory test-phase as well as decreased the expression of BDNF, NMDA receptors, TrkB receptors, CaMKII, βIII tubulin, and synaptophysin. MEL treatment significantly ameliorated METH-induced increased escape latency, decreased the number of target crossings and decreased expression of BDNF, NMDA receptors, TrkB receptors, CaMKII, βIII tubulin and synaptophysin.

Significance: METH administration impairs learning and memory in mice, and MEL administration restores METH-induced neuronal impairments which is probably through the changes in BDNF, NMDA receptors, TrkB receptors, CaMKII, βIII tubulin and synaptophysin. Therefore, MEL is potentially an innovative and promising treatment for learning and memory impairment of humans.

1. Introduction

Methamphetamine (METH) is an illicit psychostimulant widely abused and associated with serious physiological and psychological adverse effects, thus, its abuse is a global public health problem [1]. METH abuse strongly induces nigrostriatal dopaminergic neuronal death [2] and highly correlates with Parkinson's disease (PD)-like neurodegeneration [3,4]. Moreover, chronic METH abuse is associated with many structural and functional impairments across most cognitive domains.

Long–term potentiation (LTP) and long–term depression (LTD) are two forms of synaptic plasticity tightly bound to the brain functions

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described above. In addition, two types of glutamate receptors, *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprinonate (AMPA), play crucial roles in the formation of LTP and LTD [5]. The NMDA receptor is categorized into two classes: ionotropic and metabotropic glutamate receptors. The ionotropic receptor was the focus of this study because it responds fast and alters stimuli, which mediates the influx and efflux of Ca^{2+}, Na^{+}, and K^{+} in neurons, and is heterotrimeric, consisting of two subunits, GluN1 and GluN2 [6]. There are four isoforms of the GluN2 subunit: GluN2A/NR2A, GluN2B/NR2B, GluN2C/NR2C, and GluN2D/NR2D. Each isoform is encoded in a different gene. Downstream signaling of activated NMDA receptors is thought to play a critical role in synaptic plasticity, which is a cellular mechanism for learning and memory. Parson reported that GluN2A and GluN2B form a high percentage of NMDA receptors [7]. Many studies have reported that METH induces a direct action on NMDA receptors and excessively releases glutamate, causing neuronal excitotoxicity, oxidative stress, and neuronal death in several brain regions including the cerebral cortex [8], striatum [9], and hippocampus [10]. Therefore, these brain regions may be particularly susceptible to METH-induced abnormalities in behavioral and memory functions. A recent study has reported that repeated METH-induced memory impairment is characterized by decreased NMDA receptor binding in the prefrontal cortex and hippocampus [11].

The brain-derived neurotrophic factor (BDNF) is produced by neurons and astrocytes in the mammalian brain and plays crucial roles in neuronal growth and differentiation, synaptic plasticity, long-term memory formation, neuronal survival, and oxidative DNA repair [12,13]. The expression of BDNF is induced by various endogenous and exogenous factors, such as oxidative and metabolic stress, voluntary exercise, environmental enrichment, and stimulation of NMDA receptors [12,14]. BDNF is suggested as one of the key regulators of LTP and long-term memory [15], neuronal survival [12], neurogenesis [16], and neuronal growth and differentiation [17]. The tropomyosin receptor kinase B/tyrosine receptor kinase B (TrkB) is a neurotrophic receptor that interacts highly with BDNF and neurotrophin-4. The TrkB receptor was activated by BDNF to mediate the multiple effects of BDNF, as described above.

Melatonin (MEL) is a derivative of tryptophan, which is produced mainly by the pineal gland for regulating the circadian rhythm [18]. Studies have reported that MEL not only works on sleep and circadian abnormalities, but also has beneficial effects on neuroprotection, learning and memory, mood disorder, drug abuse, and cancer therapy [19]. The physiological function of melatonin is accomplished by binding to two major types of receptors: melatonin receptor 1 (MT_1) and melatonin receptor 2 (MT_2), which have been observed to be discretely located in different areas of the brain, such as the cerebrum, cerebellum, thalamus, hippocampus, and suprachiasmatic nucleus in the rodent and human central nervous system (CNS) [19]. Neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), are associated with a decline in MEL [20,21]. MEL administration effectively ameliorates the cognitive impairment of AD patients [22,23]. In an animal study, MEL administration effectively improved cognitive impairment and memory deficits [24–26] and increased the regeneration of synapses and cognitive function [27]. However, the effect of MEL on METH-induced cognitive impairment and the molecular mechanism underlying memory formation is elusive. Therefore, this study assessed this. Moreover, to develop necessary treatments, a greater understanding of the drug's mechanism of action and of the way its abuse can affect the user's brain is needed. Therefore, the underlying molecules involved in memory formation, such as BDNF, CaMKII, phosphorylation of GluN2A and GluN2B, were also studied.

2. Materials and methods

2.1. Animal care and drugs administration

Adult male ICR mice (7–8 weeks old) were obtained from the National Laboratory Animals Center of Mahidol University, Salaya Campus, Thailand. All animals were housed in a climate-controlled room (20 ± 2 °C), under a 12 h light/dark cycle with the light on at 6 am (Zeitgeber Times (ZT) 0) and light off at 6 pm (ZT12), and were given free access to water and food. All the care and experimental procedures on animals were approved by the Laboratory Animal Care and Use Committee of Mahidol University (CAO NO. IMB-ACUC 2017/002).

The animals were randomly divided into four groups (n = 12–13/group): saline-treated (NSS), saline-followed by MEL-treatment (MEL), METH-treated (METH), and METH-followed by MEL-treatment (METH+MEL) (Fig. 1). All drugs were administered in a volume of 0.1 ml/10 g body weight. The same volume of saline and METH (1 mg/kg body weight, Lipomed AG, Product Ref# AMP-732-HC) were injected subcutaneously once daily between 9:00 (ZT3) and 11:00 AM (ZT5), for 7 consecutive days. The METH dose utilized was based on previous studies that demonstrated significant effects on recognition memory impairment and cognitive deficits in mice [28,29]. After a period of drugs-induced cognitive impairment, one saline and one METH-treated groups were intraperitoneally injected with MEL (10 mg/kg body weight, Sigma-Aldrich Cat# M5250) once daily, for 14 consecutive days (two bottom groups of Fig. 1). The MEL dose was based on previous reports of significant improving effects on cognitive impairments in mice [29,30]. In clinical trials, a MEL dose of about 3–24 mg, which is comparable to 0.05–0.4 mg/kg for a 60-kg adult, can improve the cognitive function and emotional impact in patients with mild cognitive impairment. Therefore, the 10 mg/kg MEL used in our experiment is ≥25 times higher than the human dose reported [31]. One day after the last saline or MEL injection, the mice were subjected to the Morris water maze (MWM) test performed between ZT3-ZT6. The experimental plan for drug administration is shown in Fig. 1. The NSS group received the same volume of sterile saline, and the effect of MEL injection alone and METH plus MEL were compared with the NSS group.

2.2. Animal behavioral task

The MWM is a tool for spatial learning and memory analysis, consisting of two training procedures, including an acquisition and probe trial. The water maze procedures were performed according to those described in previous studies [25,29]. The Morris water maze consisted of a circular pool (140 m in diameter and 60 cm in depth), and a platform (5 cm in diameter and 40 cm in height). In habituation sessions, mice were randomly placed at each of the four starting positions: north (N), east (E), south (S), and west (W), facing the pool wall. Mice were allowed to swim for 60 s per training session without platform. In the visible platform test, mice were randomly placed at each of the four starting positions (N, S, E, and W), facing the pool wall. The platform protruded 1 cm over the water surface. Mice were allowed to swim for 60 s per training session. If mice could not find the visible platform within 60 s, mice were guided and left on the platform for 10 s. The acquisition training trials were conducted on the next day with an invisible platform, four trials per day, for 5 days consecutively. The platform was hidden 1 cm below the water surface. The escape latency and swimming path of each animal were recorded and analyzed to determine the spatial learning and memory performance. The probe trial was performed on the fifth day of the MWM task with the platform removed. Mice were randomly placed at each of the four starting positions (N, S, E, and W) facing the pool wall, and allowed to swim for 60 s

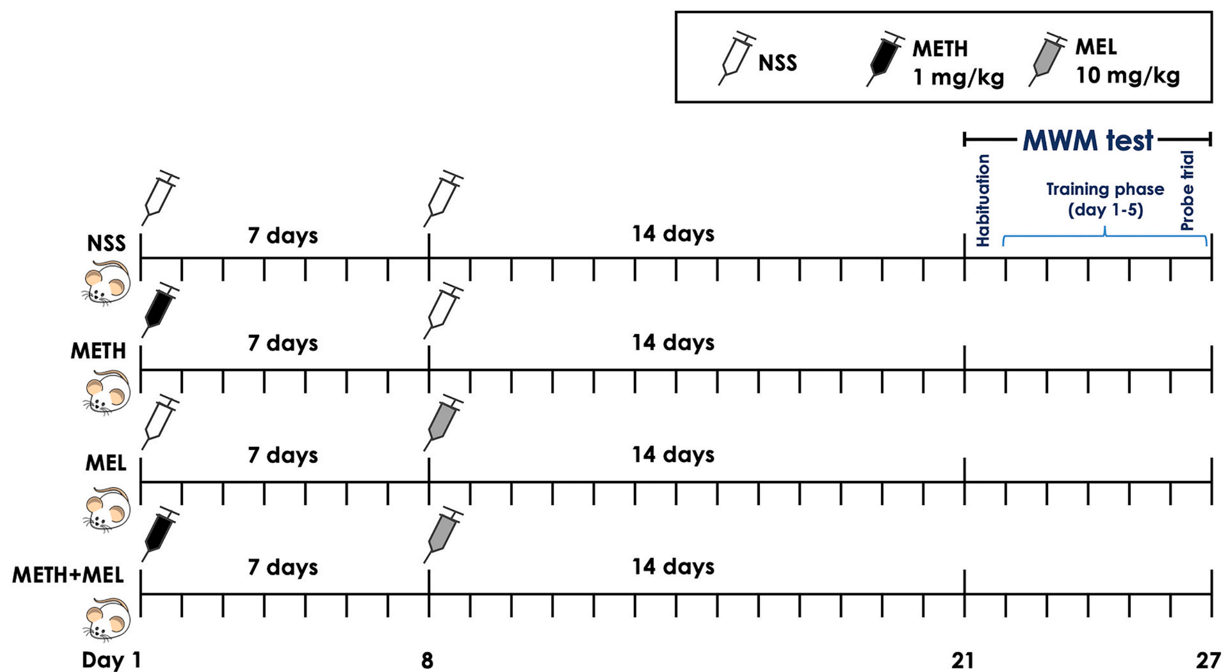

Fig. 1. Schematic diagram indicating the time point of each experimental treatment and behavioral analysis. Four groups of animals (n = 12–13/group) were subcutaneously administered normal saline solution (NSS), methamphetamine (METH, 1 mg/kg body weight/day), melatonin (MEL, 10 mg/kg body weight/day), and METH+MEL, respectively for 7 consecutive days. After 14 days, the NSS and METH groups received only normal saline; the MEL and the METH+MEL groups received MEL (10 mg/kg body weight/day). MWM training procedures were performed for 5 consecutive days starting one day after the last dose. The probe trial was performed during the last day of the MWM task. NSS: normal saline solution (blank-filled syringe); METH: methamphetamine (black-filled syringe); MEL: melatonin; MWM: Morris water maze (gray-filled syringe).

without the escape platform. The swimming path to the target quadrant, the time spent in the target quadrant, and the number of target quadrant crossings were analyzed to determine the memory retained in the animals. All data were analyzed by observers blind to the group. The animals' movement was analyzed using a SMART video tracking system, v3.0 (Panlab Harvard Apparatus, Barcelona, Spain).

2.3. Western blotting analysis

Mouse brains were rapidly removed and the hippocampi dissected and stored at −80 °C. The tissue samples, as reported previously [29], were homogenized with RIPA buffer (50 mM Tris-HCL, 150 mM NaCl, 1% Triton X 100, 0.1% sodium dodecylsulphate, 0.5% sodium deoxycholate, phosphatase inhibitor cocktail, and protease inhibitor cocktail) and the protein concentrations of each sample measured according to the Bradford protocol [32]. The antibodies of phosphorylated CaMKII (pCaMKII, Santa Cruz Biotechnology; SCBT, Cat# sc-32289, RRID: AB_626786), CaMKII (SCBT Cat# sc-5306, RRID: AB_626788), propeptide BDNF (proBDNF, SCBT Cat# sc-65514, RRID: AB_1128219), TrkB (Cell Signaling Technology; CST, Cat# 4603, RRID: AB_2155125), phosphorylated GluN2A (pGluN2A, CST Cat# 4206, RRID: AB_2112292), GluN2A (CST Cat# 4205, RRID: AB_2112295), phosphorylated GluN2B (pGluN2B, CST Cat# 14544, RRID: AB_2798506), GluN2B (CST Cat# 5355, RRID: AB_10922589), β-III tubulin (SCBT Cat# sc-51670, RRID: AB_630408), synaptophysin (CST Cat# 36406, RRID: AB_2799098), and actin (Merck Millipore Cat# MAB1501, RRID: AB_2223041) were used to examine each target protein level in various tissue samples. Species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, MA, USA) were applied to recognize the primary antibodies and visualize them through Luminata™ HRP Chemiluminescence detection reagents (Millipore, MA, USA) and exposure to X-ray film (Kodak). Immunoblot bands were quantified using Image J software (National Institutes of Health, Bethesda, MD).

2.4. Immunohistochemical staining

Paraformaldehyde fixed mice brains were coronally sectioned into 25 μm thick tissue slices and the synaptic protein, synaptophysin, was immunostained by mouse/rabbit HRP using the 3, 3′-diaminobenzidine (DAB) technique. DAB staining was performed as described in previous studies [26]. Brain sections were incubated for 30 min with a penetration buffer (0.3% Triton X-100 in PBS) at room temperature, and then rinsed thrice with PBS. The samples were thoroughly covered with hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity after PBS washes. The sections were then incubated in an immunoblocking buffer (1% bovine serum albumin in PBS) for 1 h. The anti-synaptophysin antibodies (CST Cat# 36406, RRID: AB_2799098) were diluted with the immunoblocking buffer and incubated overnight at 4 °C. After brief PBS rinsing, the brain sections were incubated in the immunoblocking buffer diluted HRP-conjugated secondary antibody for 1 h at room temperature. Four PBS washes were performed before a 3-min incubation with freshly prepared DAB at room temperature. After rinsing with distilled water four times, the samples were dried and mounted onto coverslips with Permount®. The sample slides were observed and photographed by an Olympus BX53 fluorescent microscope (Olympus, Tokyo, Japan).

2.5. Statistical analysis

Data were expressed as mean and standard error of the mean (SEM) (mean ± SEM). Significance was assessed by using analysis of variance and subsequently analyzed by Tukey's post-hoc test. A value of $P < 0.05$ was considered statistically significant. The statistical software

employed to analyze the data in this study was Graph Pad Prism 7 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Melatonin reverses methamphetamine-induced learning and memory impairments in mice

To determine whether MEL could attenuate the METH-induced cognitive impairment in mice, the MWM task was used to assess the performance of learning and memory after MEL treatment. The training and testing time points of the water maze are shown in Fig. 1. The visible platform, performed on the first day after habituation session, showed no significant different between group demonstrating intact visual competence and no sensorimotor abnormality (Fig. 2A; $F_{3,38} = 1.136$, $p = 0.347$). The results of hidden platform training trials over five consecutive days showed that METH-treated mice had a significantly longer latency period (Fig. 2B; $F_{3,45} = 3.385$, $p < 0.05$) and swimming path length (Fig. 2C; $F_{3,36} = 25.32$, $p < 0.0001$) than the NSS, MEL and METH+MEL groups, before reaching the hidden platform, without changing swimming speed (Fig. 2D; $F_{3,33} = 0.2867$, $p = 0.835$). The latency time suggested that the administration of METH significantly impairs the learning ability of mice. However, MEL treatment effectively rescued METH-induced impairment of learning and memory. The probe trial was performed for testing the animal memory after the learning of the platform position is finished. In a probe trial, compared with the other three groups of animals, the METH-treated mice clearly had the longest latency period (Fig. 2E; $F_{3,45} = 3.951$, $p < 0.05$) and length of the swimming path to reach the platform (Fig. 2F; $F_{3,44} = 4.568$, $p < 0.01$). Additionally, all other probe trial analyses indicated that METH-treated mice spent the shortest time in the target quadrant (Fig. 2G; $F_{3,45} = 7.066$, $p < 0.001$), were the least in number entering the target quadrant (Fig. 2H; $F_{3,45} = 5.357$, $p < 0.01$), and the least in number swimming across to the platform position (Fig. 2I; $F_{3,45} = 3.912$, $p < 0.05$). Considering the results of all the MWM tests together, METH-treated mice showed the worst performance in spatial learning and memory

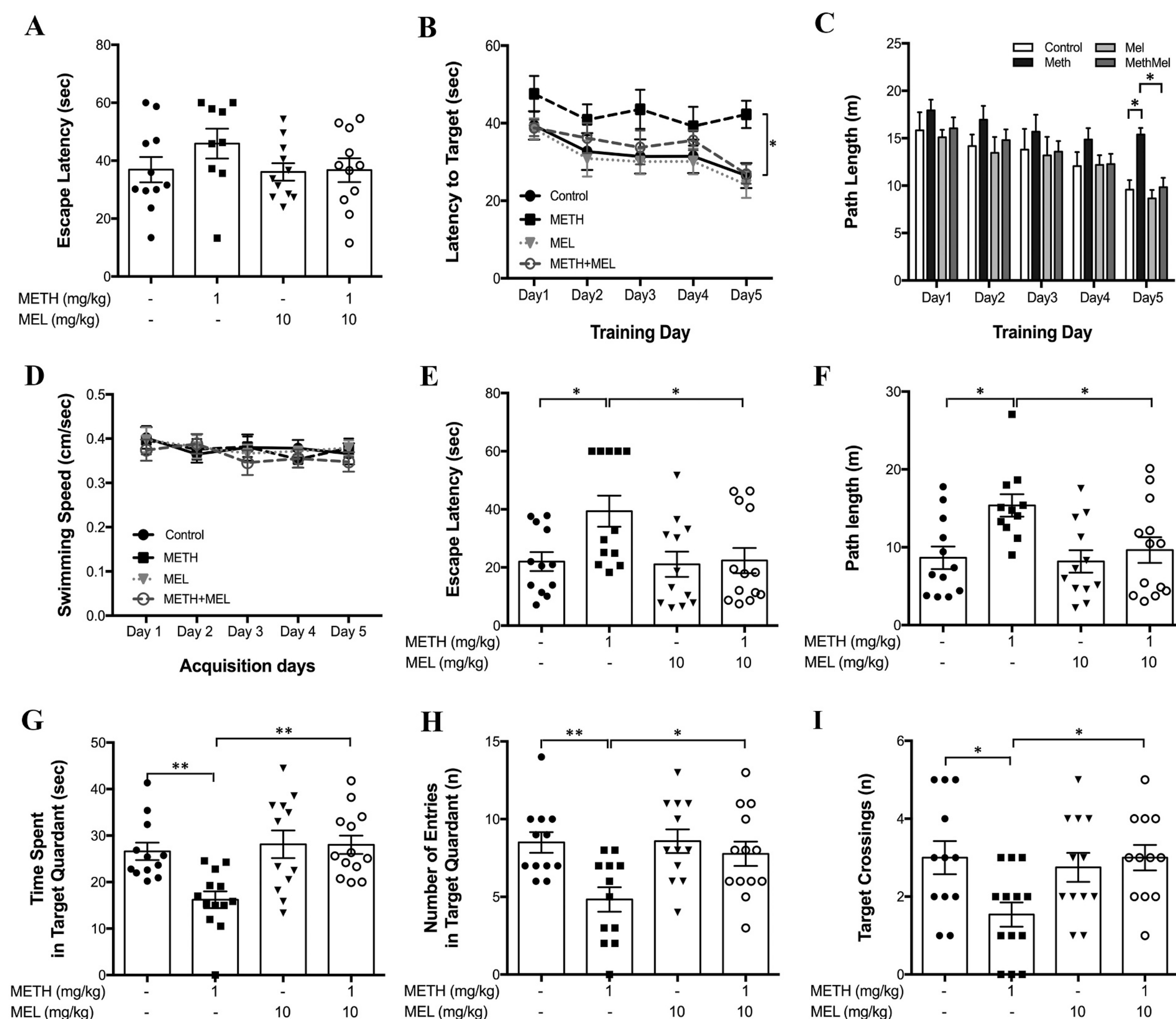

Fig. 2. Morrison water maze task demonstrating that melatonin administration improves learning and memory impairment of methamphetamine-treated mice. The visible platform test, performed on the first day after habituation session, showed no significant difference between groups demonstrating intact visual competence and no sensorimotor abnormality (panel A). The latency time (panel B) and path length (panel C) of reaching the platform showed that the METH treatment robustly compromised learning, and the group administered with METH+MEL significantly improved learning ability on day 5 of the training trials, without changing swimming speed (panel D). Both escape latency in finding the platform location (panel E) and travel distance to the platform (panel F) of the probe test showed similar results: the METH+MEL group had shorter latency time and distance for reaching the platform in the probe test. Other analytical results of the probe test, including the time spent in the platform quadrant (panel G), the number of entries in the platform quadrant (panel H), and the number of crossings to the platform location (panel I), suggested that MEL administration effectively rescued the METH-impaired memory. * and ** denote statistical significance at $P < 0.05$ and $P < 0.01$, respectively. ($n = 12$).

capability, but MEL administration post-METH (METH+MEL treated group) enabled effective recovery from the METH-induced impairment of learning and memory.

3.2. Melatonin ameliorates methamphetamine-induced hippocampal-neuronal death

Neuron-specific class III beta-tubulin (β-III tubulin, also known Tuj 1) is a structural protein expressed in central and peripheral neurons, which contributes to microtubule stability in neuronal bodies and neurites, as well as in axonal transportation. β-III tubulin is found in newly generated and postmitotically differentiated neurons [33] and is commonly used as a pan neuron marker. In this study, β-III tubulin was applied in Western blotting to evaluate the alteration of hippocampal neurons. METH-treated mice showed a significant reduction in β-III tubulin levels in the hippocampus, compared with the control group. However, MEL-administered mice recovered from the METH-induced β-III tubulin loss (Fig. 3). Treatment with MEL after METH significantly increased the β-III tubulin level ($F_{3,16} = 6.417$, $P < 0.005$). These results indicated that METH-induced hippocampal neuronal loss could be restored by MEL administration.

3.3. Melatonin recovers methamphetamine-induced reduction of hippocampal synaptic proteins

Synaptophysin is one of the major components of the presynaptic vesicle protein, and was thus employed to determine the synaptic density and relative amount of synapses in this study. Western blot analysis showed a significant reduction in the synaptophysin level in the hippocampi of METH-treated mice compared to control mice (Fig. 4A; $F_{3,12} = 14.65$, $P < 0.0005$). The post-METH MEL treatment significantly restored the level of synaptophysin in the hippocampus, compared to METH only-treated mice. Furthermore, immunohistochemistry (IHC) staining against synaptophysin was employed to determine synaptic distribution and density in the mouse hippocampus. IHC images of whole hippocampi (Fig. 4B) demonstrated that the METH-treated hippocampus had less synaptophysin staining (lighter brown, Fig. 4Bb) than controls (Fig. 4Ba), MEL-treated (Fig. 4Bc), and METH+MEL-treated (Fig. 4Bd) hippocampi. The enlarged images of CA3 regions illustrated the density of synaptophysin (brown network-like formation) in the stratum lucidum and stratum radiatum of the controls (Fig. 4Ca). MEL-treated (Fig. 4Cc) and METH+MEL-treated (Fig. 4Cd) hippocampi had a higher density than the METH-treated hippocampus (Fig. 4Cb). Similar results were also observed in the polymorphic layer of the dentate gyrus region. The polymorphic layer of METH-treated mice showed the lowest density of synaptophysin in the dentate gyrus (Fig. 4Db) compared with same area in control (Fig. 4Da), MEL-treated (Fig. 4Dc), and METH+MEL-treated (Fig. 4Dd) mice. Together, IHC results suggested that METH administration remarkably reduced the amount of synaptophysin, whereas treatment with MEL was capable of recovering the synaptophysin level in the CA3 and dentate gyrus of the hippocampus.

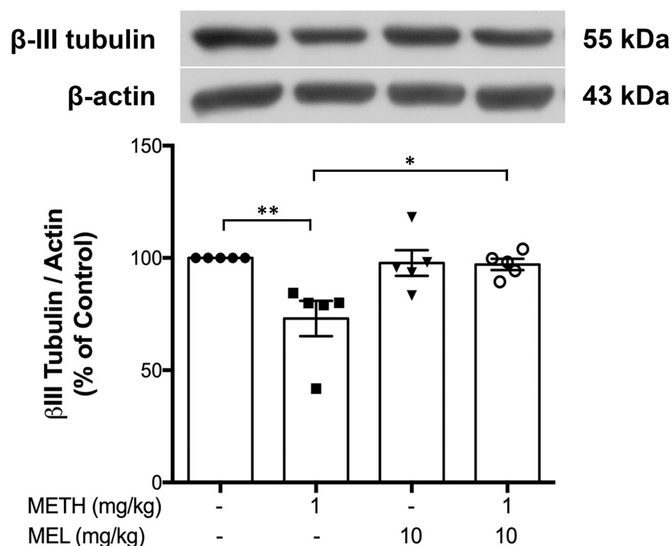

Fig. 3. Melatonin reduces hippocampal neuronal loss in methamphetamine-treated mice. Class III β-tubulin (β-III tubulin) is a specific neuron biomarker. Western blotting was employed to measure changes in β-III tubulin levels in the hippocampi of each mice group. The quantitative Western blots results showed that hippocampal β-III tubulin of METH-treated mice was significantly reduced in comparison with control animals; however, MEL administration restored the β-III tubulin level in the hippocampi. The results implied that MEL restored METH-induced neuron loss and neuronal formation. ** denotes statistical significance at $P < 0.01$ ($n = 5$).

3.4. Melatonin restores methamphetamine-induced decrease in the phosphorylation of NMDA receptors and CaMKII

NMDA receptors are known to be associated with synaptic plasticity, long-term potentiation, and long-term depression, which are the processes involved in memory formation [7,34]. The activated NMDA receptors not only allow the Ca^{2+} and Na^{+} influx leading to membrane depolarization, but Ca^{2+} also acts as a secondary messenger to activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), following a downstream signaling necessary for LTP induction [35]. Western blot analysis showed that the phosphorylation levels of GluN2A (pGluN2A) and GluN2B (pGluN2B) subunits were significantly decreased in the hippocampi of METH treated mice (Fig. 5A; $F_{3,16} = 7.81$, $P < 0.005$, and Fig. 5B; $F_{3,16} = 4.265$, $P < 0.05$). Moreover, the phosphorylation/activation of the downstream signaling molecule, CaMKII (pCaMKII), was also remarkably suppressed compared with control and METH+MEL-treated mice (Fig. 5C; $F_{3,16} = 16.03$, $P < 0.0001$). The suppressed phosphorylation of GluN2A, GluN2B, and CaMKII suggested that METH administration could lower the function of NMDA receptors.

3.5. Melatonin reverts the methamphetamine-induced alteration of brain-derived neurotrophic factor and TrkB

Western blotting results showed that the hippocampal pro-BDNF peptide was significantly lower in METH-treated mice than in controls and METH+MEL-treated mice (Fig. 6A; $F_{3,16} = 7.798$, $P < 0.005$). Furthermore, the hippocampi of METH-treated mice expressed higher levels of TrkB protein than those in the control and METH+MEL treated groups (Fig. 6B; $F_{3,16} = 8.742$, $P < 0.005$). The low BDNF expression in the METH-treated mouse hippocampus suggested that METH administration might not only impair learning and memory ability (Fig. 2), but also had negative effects on neuronal survival, neurogenesis, neuronal growth and differentiation.

4. Discussion

Methamphetamine (METH) is a widely abused psychostimulant drug with complicated CNS neurotoxicity. The neurotoxic effects of METH include disrupting neuronal structures and its molecular components, activating astroglial and microglial inflammation, and causing neuronal death. The mechanisms of METH-induced neurotoxicity mostly correlate with oxidative stress, excitotoxicity, and neuroinflammation [36]. Eventually, METH-induced neurotoxicity causes structural and physiological alterations in several brain regions of both humans [37,38] and rodents [28,39,40], and leads to neuronal disorders. The main topic of our study was to evaluate the post-treatment effect of MEL on METH-induced behavioral and molecular deficits. The anti-amnesic effect of

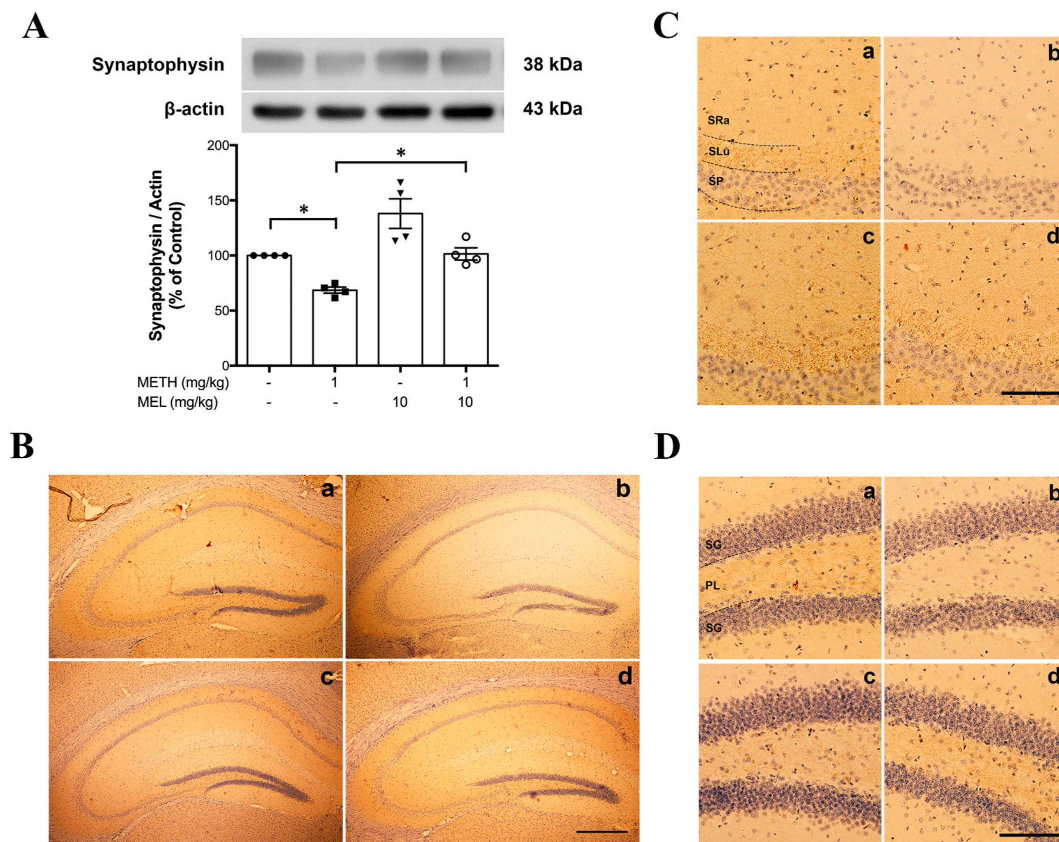

Fig. 4. Melatonin restored the methamphetamine-induced hippocampal reduction of synaptic proteins. Western blot demonstrated that the synaptophysin level was significantly lower in METH-treated mice than in control and METH+MEL groups (panel A). Immunohistochemically stained hippocampal images with anti-synaptophysin (light brown to brown) and hematoxylin (blue) show that the hippocampus of METH-treated animal (panel Bb) had a lighter staining of both synaptophysin and nuclei comparing with control, MEL-treated, and METH+MEL- treated groups (panels Ba, Bc, and Bd; scale bar = 500 μm). The enlarged images of the CA3 region demonstrated that the lowest level of synaptophysin (brown network-like formation) was detected in the stratum lucidum and the stratum radiatum of METH-treated mice (panel Cb) compared with control, MEL-treated, and METH+MEL-treated animals (panels Ca, Cc, and Cd; scale bar = 100 μm). Similar results were observed in the dentate gyrus of the hippocampus. The lowest synaptophysin level was measured in the polymorphic layer of METH-treated mice (panel Db) compared to control, MEL-treated, and METH+MEL-treated animals (panels Da, Dc, and Dd; scale bar = 100 μm). Together with the results of Western blot and immunohistochemistry, MEL administration could recover METH-induced loss of synaptic formation. Abbreviation: SG: stratum granulosum; PL: polymorphic layer; SP: stratum pyramidales; SLu: stratum lucidum; SRa: stratum radiatum. * denote statistical significance at $P < 0.05$ ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MEL has been well documented in human studies on patients with cognitive decline [38,41], and in AD and aging mouse models [25,30,42,43]. However, the mechanism by which MEL administration post-METH counteracts METH-induced neurotoxicity is still unclear.

Since spatial learning and memory has been strongly associated with hippocampal synaptic plasticity, long-term potentiation (LTP), NMDA receptor function, and BDNF [40,44], we postulated that the NMDA receptors, CaMKII (downstream signaling molecule of NMDA receptors), synapses, BNDF, and TrkB (BDNF receptor) should correlate with MEL treatment. The METH-treated mouse model was employed in this study to understand the physiological changes in neuron number, GluN2A and GluN2B (subunits of NMDA receptors), CaMKII signaling molecule, synapse formation, BDNF, and TrkB in the hippocampus. The MWM behavioral study demonstrated that mice after a 7-day METH-administration robustly showed disrupted spatial learning and memory. These behavioral results are consistent with the findings of previous studies where repeated 1 mg/kg METH administration, once a day for 7 days, induced impairment of recognition and spatial memory in mice [28,45,46]. Similar to our previous study, post-METH administration of MEL also effectively improved METH-induced learning and memory impairment [29].

Beta-III–tubulin is one of seven β–tubulin isotypes found in neurons and involved in cell proliferation and neurogenesis [47]. In this study, we measured the neuronal specific marker, β-III tubulin, to indirectly determine the neuron number and volume (outgrow-neurites) in the hippocampus. The immunoblotting results demonstrated that the hippocampal β-III tubulin level was significantly reduced in METH-treated mice compared with controls, while post-METH MEL administration elevated the β-III tubulin back to baseline level (Fig. 3). Previous studies have reported that MEL protects neurons against amphetamine-induced hippocampal dopaminergic fiber degeneration [48], enhances hippocampal progenitor cell proliferation [49], and attenuates the methamphetamine-induced inhibition of neurogenesis [50,51]. Our β-III tubulin results also suggested that post-METH MEL-administration could enhance either –or both– neurite-outgrowth or neurogenesis processes in the hippocampus. The quantity and distribution of hippocampal synapses were examined to understand their correlation with the aforementioned β-III tubulin results. Immunoblotting analysis and immunohistochemistry images (Fig. 4) showed that MEL-administration restored the METH-induced synaptophysin reduction in the hippocampus. Our finding is in accordance with previous studies that reported the suppression of synaptophysin expression and synaptic transmission in the hippocampus by amphetamine administration [48,52,53].

MEL-administration restored the METH-induced loss of neurons and synapses. We also investigated whether MEL regulates the signal transmission of NDMA receptors in memory/LTP formation. Memory

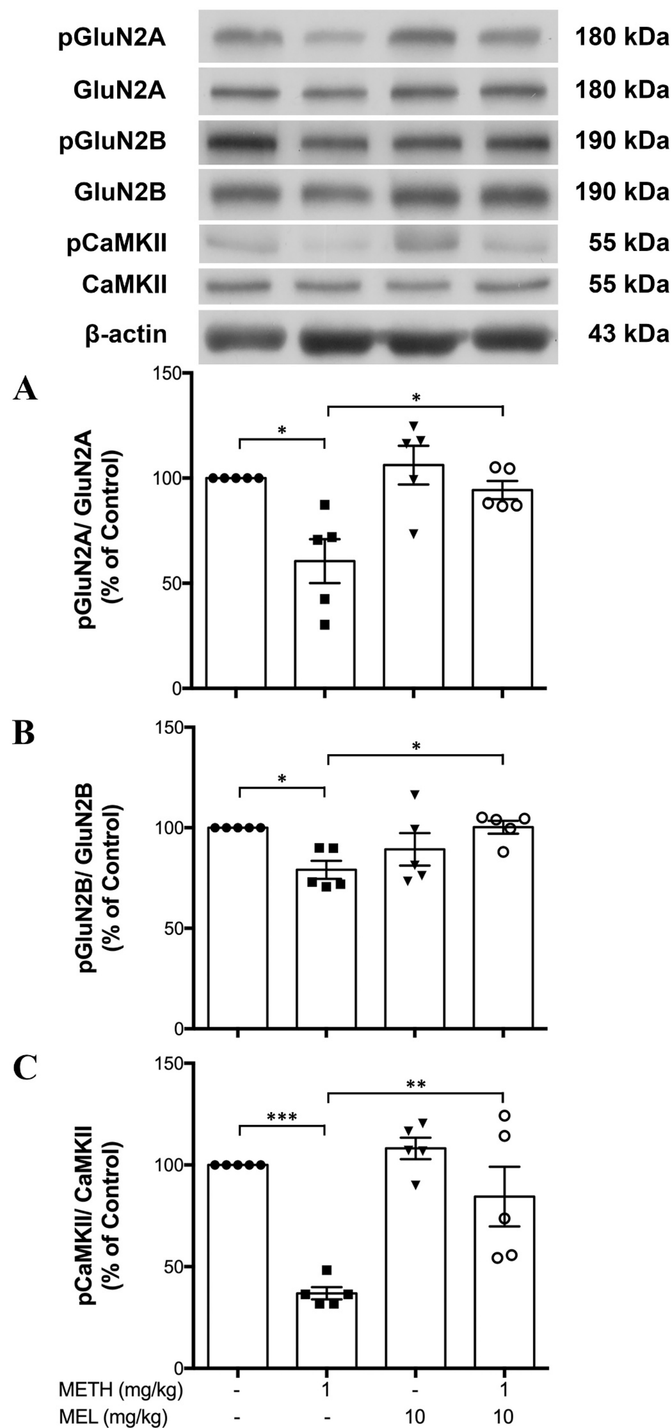

Fig. 5. Melatonin restores METH-decreased phosphorylation of glutamate receptor GluN2A and GluN2B subunits and CaMKII. Western blot showing that the phosphorylation level of GluN2A and GluN2B was significantly lower in the hippocampi of METH-treated rats than that in controls and METH+MEL-treated animals (panel A and B). The downstream signaling, phosphorylation of CaMKII and of glutamate receptors was also robustly decreased in the hippocampi of METH-treated animals compared with those of control and METH+MEL-treated animals (panel C). These results suggested that METH treatment significantly inhibited the activity of glutamate receptors in hippocampal neurons. * and *** denote statistical significance at $P < 0.05$ and $P < 0.001$, respectively ($n = 5$).

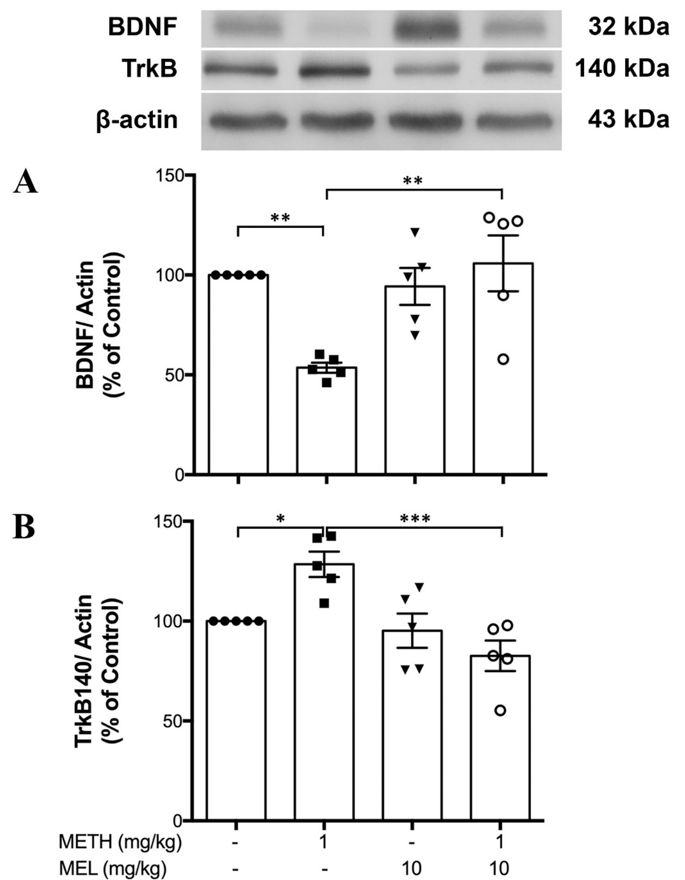

Fig. 6. Melatonin recovers METH-induced alterations of hippocampal BDNF and TrkB protein levels. Western blots indicating that the BDNF level of the METH-treated group was approximately 50% lower than that in control and METH+MEL-treated groups (panel A). Conversely, the protein level of the BDNF receptor, TrkB, was approximately 30% to 40% higher than in control and METH+MEL-treated groups (panel B). These results showed that METH administration altered the protein levels of BDNF and TrkB in hippocampal neurons. MEL treatment could correct the METH-altered expression in both BDNF and TrkB back to normal condition. * and ** denote statistical significance at $P < 0.05$ and $P < 0.01$, respectively ($n = 5$).

formation originates from synaptic plasticity, which is essential for learning and memory, and starts with the activation of NMDA or AMPA receptors [5,54]. Although GluN2A and GluN2B subunits are commonly assembled in hippocampal NMDA receptors, the GluN2A subunit is crucial for promoting neuronal regeneration [55], increasing BDNF expression, and improving learning and memory impairment in an ischemic stroke model [56]. Besides modulating the process of learning and memory [57,58], the GluN2B subunit has functions in synaptogenesis and excitotoxic neuronal death [59]. Steullet reported that NMDA receptor hypofunction is involved in the impairment of synaptic plasticity, learning and memory disability, and other negative CNS repercussions [60]. Hence, we examined the phosphorylation status of two crucial subunits of NMDA receptors, GluN2A and GluN2B. Immunoblotting analysis showed that the phosphorylation of both GluN2A and GluN2B was impaired in the METH-treated hippocampus. Furthermore, the phosphorylation of the downstream signaling molecule of NMDA receptors, CaMKII, was also suppressed by METH-administration. Both Delibas's and Sutcu's studies indicated that MEL modulates LTP and regulates NMDA receptor expression in the brain, by a still unclear mechanism [61,62]. The crucial role of MT_1 and MT_2 melatonin receptors is to sequentially mediate the activation of the PKA and PLCγ signaling cascades that increase the intracellular Ca^{2+} level, leading to

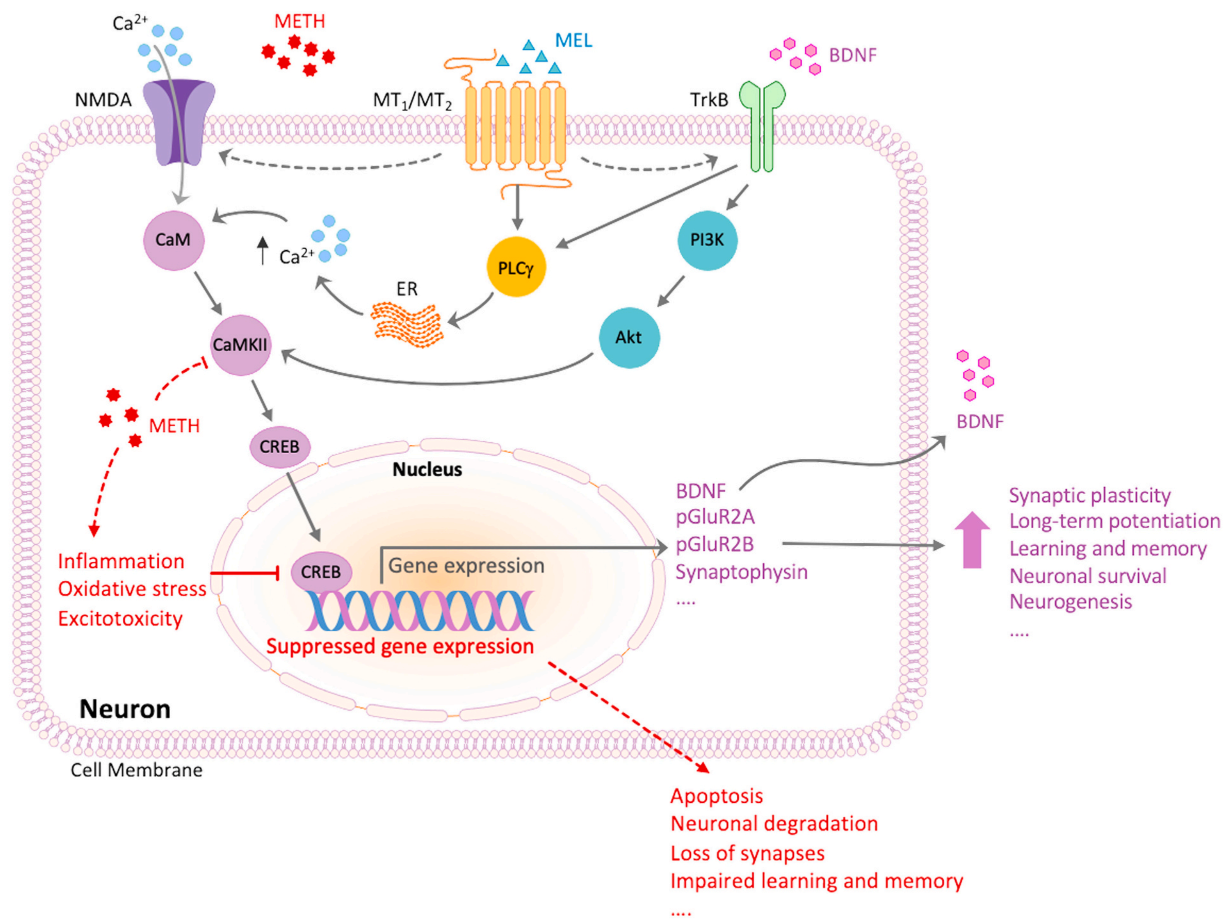

Fig. 7. Cartoon diagram illustrating the possible mechanisms of methamphetamine (METH) neuronal disorder induction as well as melatonin (MEL) restoration of methamphetamine-induced loss of function. METH-administration induces an impairment of learning and memory through the reduction of NMDA receptors and their downstream CaMKII signaling, thereby subsequent loss of BDNF, β–III tubulin, pGluN2A, pGluN2B and synaptophysin. In this study, we have shown that MEL provides the protective and recovering effects against the METH–induced loss in the level of BDNF, synaptophysin, β–III tubulin, pGluN2A, pGluN2B, and CaMKII by activating its receptor MT_1 and MT_2, then triggering downstream PLCγ-Ca^{2+}-CaMKII and TrkB signaling cascade. Together, all results of our study suggested that MEL restores METH-induced impairment on NMDA receptors, synaptic plasticity, neuronal survival, and learning and memory.

CaMKII activation. The activated CaMKII results in the synthesis of targeted proteins involved in many signaling cascades, such as BDNF, GluN2A, and GluN2B subunits, and also acts as a mediator of learning and memory. Thus, our study determined that by involving the CaMKII signaling axes, MEL may have roles in LTP modulation and the improvement of learning and memory formation, as summarized in Fig. 7.

MEL primarily exerts its action through the high-affinity G-protein couple receptors MT_1 and MT_2. However, several protein targets and various enzyme activities have been suggested to interact with MEL [63,64]. MEL receptor-induced signaling cascades are involved in the regulation of BDNF expression. Additionally, the active TrkB activates PLCγ and increases the intracellular Ca^{2+} concentration, which sequentially causes the signaling cascades to modulate activity-dependent plasticity in the hippocampus [65]. The PLCγ-Ca^{2+}-CaMKII signaling cascade is likely a hub for MEL and BDNF. MEL promotes BDNF expression through a PLC-mediated mechanism previously reported [66]. BDNF and TrkB were thus determined by their correlation with MEL treatment. Western blot results indicated that the hippocampal BDNF level was reduced while TrkB level was increased during the withdrawal period of METH treatment (Fig. 6). The decreased BDNF levels in our study are similar to the findings of many previous studies that showed a decrease in serum BDNF levels in METH abusers or in the hippocampi of METH-treated mice during the early withdrawal stage [67–69]. A decrease in the BDNF level was also observed in the dorsal striatum [70] and prefrontal cortex [68] after long-term METH abstinence. It was noted that hippocampal TrkB expression was upregulated by METH treatment, as opposed to the BDNF action; however, we did not find any studies reporting similar results. Therefore, we postulated that the upregulated TrkB was a response to the METH-induced BDNF deficit. However, post-METH MEL administration restored both BDNF and TrkB to the original levels. MEL was recently shown to attenuate scopolamine-induced cognitive impairment via protecting against demyelination through BDNF/TrkB signaling in the ICR mouse dentate gyrus [71]. *N*-acetyl serotonin (NAS), a melatonin precursor, suppressed scopolamine-induced memory impairment and cell death in CA1 and CA3 regions in ICR mice by activating TrkB/CREB/BDNF pathway [72]. Moreover, MEL treatments attenuated the NMDAR antagonist-induced immobility in the forced swim test, promoting accumulation of hippocampal BDNF by events involving MEL receptors [73]. In this study, MEL post-treatment attenuated METH-induced BDNF/TrkB changes in the mouse hippocampus probably via a MEL receptor-dependent mechanism.

Our findings suggest that repeated METH-induced learning and memory impairments may be accompanied by a reduction in the activated-NMDA and BDNF levels, as well as the suppression of their downstream signaling cascades. Nevertheless, MEL administration was capable of restoring METH-induced learning and memory impairment and ameliorating the negative neuronal effects.

5. Conclusion

The present study supports the therapeutic effects of melatonin on learning and memory impairment. Melatonin has protective and recovering effects on glutamate receptors, synapses, and neurotrophic factors which play a role in synaptic plasticity and on learning and memory. However, the signaling cascades directly and indirectly involving melatonin, which may play a role in neural plasticity and memory processes, need to be further investigated.

Declaration of competing interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118844.

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