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ORIGINAL ARTICLE

Melatonin Rescue Oxidative Stress-Mediated Neuroinflammation/Neurodegeneration and Memory Impairment in Scopolamine-Induced Amnesia Mice Model

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Abstract

Cognitive decline and memory impairment induced by oxidative brain damage are the critical pathological hallmarks of Alzheimer's disease (AD). Based on the potential neuroprotective effects of melatonin, we here explored the possible underlying mechanisms of the protective effect of melatonin against scopolamine-induced oxidative stress-mediated c-Jun N-terminal kinase (JNK) activation, which ultimately results in synaptic dysfunction, neuroinflammation, and neurodegeneration. According to our findings, scopolamine administration resulted in LPO and ROS generation and decreased the protein levels of antioxidant proteins such as Nrf2 and HO-1; however, melatonin co-treatment mitigated the generation of oxidant factors while improving antioxidant protein levels. Similarly, melatonin ameliorated oxidative stress-mediated JNK activation, enhanced Akt/ERK/CREB signaling, promoted cell survival and proliferation, and promoted memory processes. Immunofluorescence and western blot analysis indicated that melatonin reduced activated gliosis via attenuation of Iba-1 and GFAP. We also found that scopolamine promoted neuronal loss by inducing Bax, Pro-Caspase-3, and Caspase-3 and reducing the levels of the antiapoptotic protein Bcl-2. In contrast, melatonin significantly decreased the levels of apoptotic markers and increased neuronal survival. We further found that scopolamine disrupted synaptic integrity and, conversely, that melatonin enhanced synaptic integrity as indicated by Syntaxin, PSD-95, and SNAP-23 expression levels. Furthermore, melatonin ameliorated scopolamine-induced impairments in spatial learning behavior and memory formation. On the whole, our findings revealed that melatonin attenuated scopolamine-induced synaptic dysfunction and memory impairments by ameliorating oxidative brain damage, stress kinase expression, neuroinflammation, and neurodegeneration.

Keywords Amnesia · Reactive oxygen species (ROS) · Brain-derived neurotrophic factor (BDNF) · Cyclic AMP response element-binding protein (CREB) · Scopolamine · Melatonin

Introduction

Alzheimer's disease (AD) is a highly prevalent neurodegenerative disorder, and the number of patients gradually increases with the passage of time. The main etiology of AD has yet to be elucidated definitively. The pathological hallmarks of AD include the accumulation of senile plaques, neurofibrillary tangles and cholinergic neuronal degeneration (Embury et al. 2017; Schliebs and Arendt 2011; Spires-Jones and Hyman 2014). AD pathology also includes oxidative stress, neuroinflammation and neuronal death, which consequently leads to memory impairment (Kanninen et al. 2011; Lee et al. 2012). Cholinergic system dysfunction causes a decline in acetylcholine (Ach) levels and plays a critical role in the pathogenesis of dementia (Goverdhan et al. 2012; Shao et al. 2014). These cognitive impairments may be caused by changes in morphology, such as structural changes in neurons, synapses and nerve fibers; changes in neurotransmitter levels; or oxidative brain damage (Haider et al. 2016). Currently, therapeutic approaches have focused on activating cholinergic neurotransmitters by increasing acetylcholine levels or decreasing acetylcholine degradation by acetylcholinesterase (Farlow et al. 2011; Shintani and Uchida 1997).

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Scopolamine, a nonselective antimuscarinic agent, leads to progressive impairment of learning and memory principally by blocking central cholinergic signaling (Konar et al. 2011; Pariyar et al. 2017; Park et al. 2012). It is a well-known phenomenon that scopolamine generates reactive oxygen species (ROS) and results in oxidative stress, a critical factor that results in AD-like dementia (Budzynska et al. 2015; Venkatesan et al. 2016). Furthermore, studies have shown that ROS-induced activation of c-Jun N-terminal kinase (JNK), a stress kinase (Kadowaki et al. 2005), reduces the phosphorylation of protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) (Pariyar et al. 2017). Akt activation is required for memory and learning as well as cell survival, while ERK activation induces the expression of cyclic AMP response element-binding protein (CREB), which is involved in antiapoptotic and memory proteins expression (Duarte et al. 2008; Pariyar et al. 2017). Additionally, downregulation of a neurotrophic factor, brain-derived neurotrophic factor (BDNF), has been linked to AD-like pathology (Scott Bitner 2012; Song et al. 2015). Previous studies have reported that scopolamine significantly reduces the levels of these proteins (Lee et al. 2018).

Melatonin (N-acetyl-5-methoxytryptamine) is a multifunctional mammalian neurohormone of the pineal gland that is excreted into the blood and cerebrospinal fluid (CSF) and plays an important role in the regulation of seasonal and circadian rhythms (Rodriguez et al. 2004). Its production substantially decreases with age, and this decrease is related to age-related neurodegeneration (Ali and Kim 2015; Karasek and Reiter 2002). CSF melatonin levels are significantly reduced to levels one-half the levels of young individuals in aged populations with early AD-like neuropathological changes in the brain (Liu et al. 1999; Zhou et al. 2003). In fact, it has been confirmed that removing the pineal gland results in hippocampal deformities that are reversed by oral administration of melatonin (Ling et al. 2009). Common indications of melatonin reduction in AD patients are sleep disruptions, nightly restlessness, and sundowning, all of which are more frequently observed in the elderly population than in other populations and particularly in patients with AD (Ferguson et al. 2010). Melatonin has strong antioxidant and free radical-scavenging properties, exerts anti-inflammatory effects, decreases cholinesterase activity and prevents mitochondrial damage and apoptosis; it also maintains the integrity and functionality of cellular membranes (Hoppe et al. 2010; Wen et al. 2016).

In the present study, we investigated the neuroprotective effect of melatonin in scopolamine-induced oxidative stress-mediated JNK activation, Akt/ERK/CREB signaling disruption, synaptic dysfunction, memory impairment, neuroinflammation and neurodegeneration. This protective effect of melatonin may be direct via its antioxidant/antiapoptotic/anti-inflammatory effects or indirect via its positive regulation of p-Akt/p-ERK/p-CREB and other synaptic proteins.

Materials and Methods

Chemicals

Scopolamine, melatonin, and 20,70-dichlorodihydrofluorescein diacetate (DCFHDA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male wild-type C57BL/6 N mice (8 weeks old, weighing 25–30 g, $n = 40$) were obtained from Samtako Bio, Osan, Korea. The animals were acclimatized for 7 days; they were maintained under controlled temperature (25 °C) and lighting (12/12 dark/light cycle) and were allowed to have free access to water and food. Efforts were made to use the smallest possible number of animals and to minimize their suffering. All the experimental techniques were performed in accordance with the guidelines of the animal ethics committee of the Division of Applied Life Sciences, Biology Department, Gyeongsang National University, South Korea.

Mice were randomly grouped into three groups: 1) a control vehicle-treated group (control, $n = 12$), 2) a scopolamine-treated group (S, $n = 14$), 3) a scopolamine+melatonin treated group (S + M, n = 14), 4) and a melatonin alone group (Melat). Treatments were given I.P. for 14 days. Scopolamine (1 mg/kg) was dissolved in saline, while melatonin (10 mg/kg) was dissolved in 0.1% dimethyl sulfoxide (DMSO); the volumes were made up for the administration.

Behavioral Study

The behavior of the mice was assessed using the Morris Water Maze (MWM) and Y-maze tasks. Twenty to thirty minutes after drug (scopolamine or scopolamine plus melatonin) administration according to the treatment plan, the behavioral tasks were performed. The MWM is a well-known test to assess memory function, and the MWM was performed as our group reported previously (Lu et al. 2010) with minor changes. Each mouse received training for two consecutive days. Latency (sec) to escape from the water (to reach the hidden platform) was measured for each trial (for 5 consecutive days). The probe test was executed on day 6 for the assessment of memory consolidation. The platform was removed during the test, and each mouse was allowed to freely swim for 1 min. The time spent by the mice in the target quadrant and the number of crossings over the previously hidden platform location were calculated. Memory consolidation was represented by the time spent in the target quadrant. The behavioral data were recorded using SMART, a video-tracking software package by the Panlab Harvard Apparatus bioscience company (Holliston, MA, USA).

For the Y-maze task, each mouse was placed in the middle of the device and was allowed to move freely through the maze for 3 8-min sessions. The series of arm entries was digitally recorded. The successive entry of the mice into the three arms in overlapping triplet sets was defined as spontaneous alternation. The percentage (%) of alternation behavior was calculated as the successive entries into three different arms consecutively/total number of arm entries −2 × 100. A greater percentage of spontaneous alternation behavior reflected improved cognitive function.

Cell Culturing and Drug Treatment

The HT22 (mouse hippocampal) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10%) and penicillin/streptomycin (1%) in a 5% CO_2 incubator at 37 °C. When confluency reached 70%, the cells were pretreated for 1 h with scopolamine (3 mM) followed by melatonin (100 μM) or SP600125 (20 μM), a JNK inhibitor, for 24 h.

Cell Viability Assay

To assess cell viability, cells were cultured at a density of 1×10^4 cells per well containing 100 μl of DMEM in 96-well plates. After the attachment of the cells, the medium was replaced with fresh medium containing scopolamine (3 mM) and one of two concentrations of melatonin (100 or 200 μM). The cells were incubated for another 24 h followed by incubation with MTT solution for 4 h. Then, 100 μl of DMSO per well was added, and the plate was shaken for 10–20 min on a shaker. The absorbance was measured via a microplate reader at 570 nm. The experiments were repeated three times.

Protein Extraction

As described previously (Khan et al. 2017), after the completion of the behavioral studies, mice were euthanized, and the brains (cortex and hippocampus) were collected, frozen on dry ice and then stored at −80 °C. The tissues were homogenized in protein extraction solution (PRO-PREP) according to the manufacturer's instructions (iNtRON Biotechnology, Inc., Sungnam, South Korea). After that, the samples were centrifuged (13,000 rpm at 4 °C for 25 min), and the supernatants were collected and stored at −80 °C. Similarly, HT22 cells were collected in PBS after treatment and centrifuged, and the supernatant was removed. The remaining pellets were dissolved in PRO-PREPTM. The brain homogenates and cell lysates were quantified with a Bio-Rad protein assay solution.

Western Blot Analysis

Immunoblotting was conducted as previously described with some modifications (Sarubbo et al. 2018). We separated solubilized proteins by SDS-PAGE and transferred them to a nitrocellulose membrane. We incubated the membrane with primary antibodies. We then exposed the membrane for 1 h to a horseradish peroxidase-conjugated secondary antibody (1:3000; Promega) and visualized the proteins by using a chemiluminescence-based detection kit (ECL kit; Amersham, Japan).

Antibodies

The antibodies used in this study were anti-Nrf2 (sc-722), anti-HO1 (sc-136,961), anti-p-Akt (sc-514,032), anti-Akt (sc-5298), anti-BDNF (sc-546), anti-PSD-95 (sc-71,933), anti-Syntaxin (sc-12,736), anti-synaptosomal-associated protein 23 (SNAP-23) (sc-374,215), anti-p-JNK (sc-6254), anti-JNK (sc-7345), anti-Caspase 3 (sc-7272), anti-Bax (sc-7480), anti-Bcl2 (sc-7382), anti-TNF-α (sc-52,746), anti-IL-1β (sc-32,294), anti-p-NF-κB (sc-136,548), anti-NF-κB (sc-8008), anti-Iba-1 (sc-32,725), anti-GFAP (sc-33,673) and anti-β-actin (sc-47,778) from Santa Cruz Biotechnology (Dallas, TX, USA). In addition, anti-p-CREB (#87G3), anti-CREB (#48H2), anti-p-IKKα/β (#2697), anti-p-ERK (#9101), anti-ERK (#9102) and anti-Cleaved Caspase-3 (#9664) antibodies were used and were obtained from Cell Signaling Technology (Massachusetts, United States).

Tissue Sample Preparation for Morphological Analysis

After the completion of drug treatments, mice were perfused transcardially with PBS followed by 4% ice-cold paraformaldehyde. The brains were then postfixed for approximately 48 to 72 h in 4% paraformaldehyde and then shifted to 20% sucrose for an additional 72 h. The brains were frozen in O.C.T. compound (A.O., USA), and then 14-μm coronal sections of the cortex and hippocampus were cut using a CM 3050C cryostat (Leica, Germany).

Immunofluorescence

Immunofluorescence staining was carried out as reported previously (Badshah et al. 2016; Rangasamy et al. 2018) with some changes. Briefly, slides containing the 14-μm brain sections were washed two times for 10 min each with PBS (0.01 M) and kept for 1 h in blocking solution (2% normal bovine or donkey serum, depending on the primary antibody used, and 0.3% Triton X-100 in PBS). Next, the slides were kept overnight at 4 °C and incubated with primary antibodies (1:100 dilution) in the blocking solution. After that, the slides were again washed with PBS (0.01 M) and then incubated

with fluorescein isothiocyanate (FITC)-labeled (green) or TRITC-labeled (red) secondary antibodies (1:100) for 1.5–2 h. Finally, the slides were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5–10 min, and glass coverslips were mounted with mounting medium. The staining patterns were examined using a confocal laser-scanning microscope (FV1000MPE) and were evaluated.

Cresyl Violet (Nissl) Staining

Cresyl violet (Nissl) staining was performed for histopathological analysis to assess the degree of neuronal cell death (apoptosis). Slides containing brain sections were washed 2 times for 15 min each with PBS (0.01 M) followed by staining with a cresyl violet (0.5%) solution (containing a few drops of glacial acetic acid) for 10 to 15 min. After that, the slides were washed with distilled water and dehydrated in a graded ethanol series (70%, 95%, and 100%), followed by immersion in xylene. Finally, the slides were covered with glass coverslips using mounting medium. The slides were then examined with a fluorescence microscope. The results were assessed, and the staining density was measured with the ImageJ program.

Reactive Oxygen Species (ROS) and Lipid Peroxidation (LPO) Assays

The ROS assay was conducted as previously described with minor changes (Amin et al. 2017; Qian et al. 2008). The assay is mainly based on the formation of 2′7′ dichlorofluorescein (DCF) from the oxidation of 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The cortical and hippocampal homogenates were diluted 1:20 in ice-cold Lock's buffer to yield 2.5 mg tissue/500 μL as the final concentration. Then, 1 ml of the Lock's buffer mixture (pH ± 7.4), 0.2 mL of homogenates, and 10 mL of DCFH-DA (5 mM) were incubated for 15 min at room temperature to form fluorescent DCF from DCFH-DA. The formation of DCF from DCFH-DA was evaluated via a microplate reader at 484 nm (excitation) and 530 nm (emission). In the absence of homogenate, to enable calculation of DCF formation (background fluorescence), we measured parallel blanks. The ROS levels are expressed as DCF formed (pmol)/amount of protein (mg). The evaluation of LPO is important for the assessment of oxidative stress. The LPO marker free malondialdehyde (MDA) was assessed in the cortical and hippocampal protein lysates using an MDA colorimetric/fluorometric assay kit (BioVision, USA, Cat# K739–100) in accordance with the manufacturer's guidelines.

ROS and LPO Assays (In Vitro)

Cells were cultured in 200 μl of DMEM medium per well in 96-well plates. The cells were incubated for 24 h at 37 °C in a humidified incubator with 5% CO2. After 24 h, scopolamine (3 mM) and/or melatonin (100 or 200 μM) diluted in fresh medium was used to replace the previous medium. ROS quantification was performed as discussed for the in vivo experiment. Likewise, the LPO assay was carried out using a commercially available kit (BioVision, Cat# K739–100).

Cell Viability Assay (MTT Assay)

Cell viability in HT22 cells was examined via MTT assay as described previously (Khan et al. 2018).

Statistical Analysis

The data are expressed as the group mean ± SEM. Prism 6 (GraphPad Software, San Diego, CA) was used for one-way ANOVA with Tukey's post hoc test. Differences between groups were considered significant at $p < 0.05$. The data are presented as the mean ± SEM of 6–7 mice per group and are representative of three independent experiments. Significance: * = $P \leq 0.05$, ** = $P \leq 0.01$, # = $P \leq 0.001$, ## = $P \leq 0.0001$.

Results

Melatonin Mitigates Oxidative Stress Induced by Scopolamine Administration

To study the potential protective effect of melatonin against scopolamine-induced oxidative brain damage in mice, a ROS assay was carried out. ROS can lead to DNA damage, and as a result, neurodegeneration occurs. The results showed that scopolamine induced oxidative stress and significantly elevated reactive oxygen species (ROS) production compared to the vehicle. On the other hand, the group that received scopolamine along with melatonin showed significantly reduced ROS generation levels compared to the group treated with scopolamine alone (Fig. 1b). Similarly, melatonin markedly reduced LPO levels in the brain (cortex and hippocampus) homogenates of the scopolamine+melatonin-administered group compared to the scopolamine-only group (Fig. 1c). Similarly, the ROS and LPO results were confirmed in vitro in mouse hippocampal HT22 cells (Fig. 1e, f). The ROS and LPO results showed that oxidative stress was induced by scopolamine administration (Skalicka-Wozniak et al. 2018). To measure cell viability, we performed an in vitro MTT assay using HT22 cells. The MTT results showed that scopolamine (3 mM) reduced cell viability, while the two concentrations of melatonin (100 and 200 μM) protected neuronal cells (Fig. 1d). Previous studies have demonstrated that nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling is one of the most important antioxidant responses and that Nrf2 binds

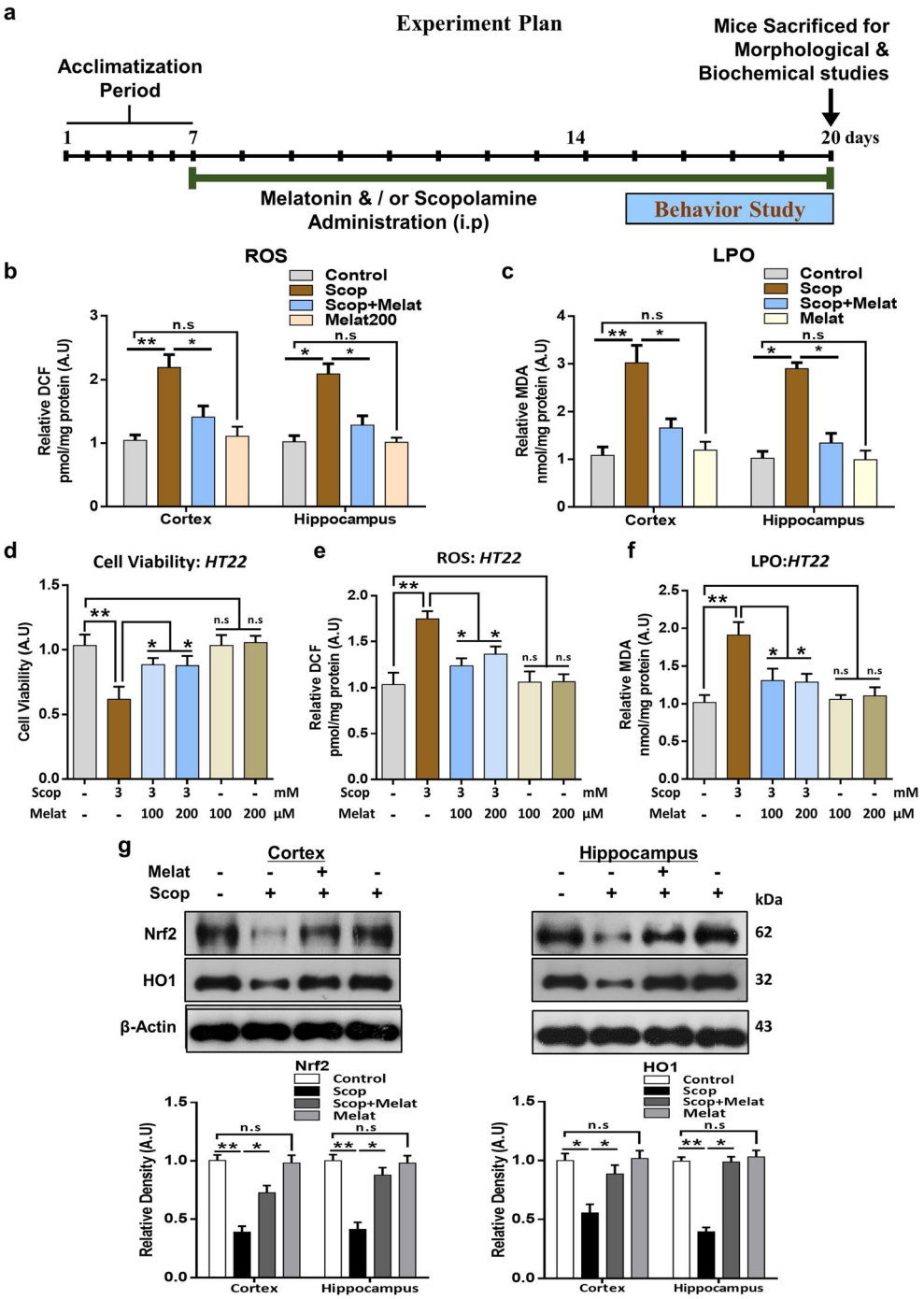

Fig. 1 Melatonin regulates the levels of endogenous antioxidant defense molecules by regulating the expression of Nrf2 and HO-1, protecting against scopolamine-induced oxidative stress. **a** The overall schedule of the experiments conducted in the current study is shown. **b**, **c** Representative quantitative results of the ROS and LPO assays, respectively. **d** Bar graph of the MTT (cell viability) assay results. Scopolamine (3 mM) reduced cell viability. Treatment with melatonin at two concentrations (100 and 200 μM) increased the cell viability of HT22 cells after 24 h. **e**, **f** Representative quantitative results of the ROS and LPO assays, respectively, in HT22 cells. Treatment with melatonin at both concentrations (100 and 200 μM) significantly decreased scopolamine-induced (3 mM) elevations in ROS levels. g Immunoblot results showing the relative expression of proteins in the different experimental groups and a graphical representation showing the differences among the experimental groups. The data are presented as the mean ± SEM of 6–8 mice per group and are representative of three independent experiments. Significance: * = $P \leq 0.05$, ** = $P \leq 0.01$

to the antioxidant responsive element (ARE), which is vital for the transcription and expression of numerous key antioxidative enzymes, including heme-oxygenase 1 (HO-1) (Itoh et al. 2004). Thus, we measured the effects of melatonin on the activation of Nrf2/HO-1 signaling in the brain (cortex & hippocampus) of scopolamine-treated mice. The immunoblot results revealed that the expression of the Nrf2 and HO-1 proteins was decreased in scopolamine-treated mice, while melatonin administration considerably augmented the protein levels of Nrf2 and HO-1 (Fig. 1g).

Effect of Melatonin on JNK/Akt/ERK Signaling against Scopolamine Treatment in Mouse Brain and In Vitro in HT22 Cells

To assess the effects of scopolamine-induced oxidative stress-mediated activation of stress kinases, the expression level of p-JNK protein was evaluated via western blot. Interestingly, the protein level of JNK was significantly elevated in the scopolamine group, whereas melatonin reduced the elevated expression of p-JNK in vivo. Similarly, to determine whether

melatonin reversed the downregulation of p-Akt/p-ERK signaling caused by scopolamine treatment, we performed western blot analysis of cortex and hippocampus protein lysates. The results showed that the protein levels of p-Akt, p-Akt and p-ERK were significantly lower in scopolamine-treated mice than in control vehicle-treated mice in both the cortical and hippocampal regions. However, melatonin administration significantly increased the levels of these proteins in both areas, as shown in Fig. 2a. To confirm these in vivo beneficial effects of melatonin, we examined the protein levels of activated p-JNK, p- Akt and p-ERK in HT22 cells in vitro. Again, the western blot data showed that scopolamine significantly increased the protein levels of p-JNK protein while reducing the protein expression levels of p-Akt and p-ERK. However, melatonin alleviated the activation of JNK and recovered the p-Akt and p-ERK protein levels in a similar way to that of SP600125 (a pharmacological inhibitor of JNK), suggesting that JNK is the major contributor to this signaling pathway (Fig. 2b). Furthermore, in vitro immunofluorescence analysis showed that p-JNK immunoreactivity was significantly higher in scopolamine-treated HT22 cells than in control cells. However, melatonin and SP600125 prevented scopolamine-induced JNK activation (Fig. 2c).

Melatonin Treatment Reversed the Scopolamine-Induced Decreases in the P-CREB and BDNF Proteins

Previous studies have reported that learning-related protein expression is induced via the CREB transcription factor in LTP experiments activating the CREB signaling cascade. Similarly, scopolamine-induced amnesia is reversed by estrogen receptor agonists, which activate the CREB signaling pathway (Pariyar et al. 2017). Thus, we determined the protein expression levels of CREB and BDNF, which play important roles in memory and learning. Western blot analysis showed that scopolamine reduced CREB levels in both the cortex and hippocampus, consistent with the results for Akt and ERK signaling. However, melatonin significantly recovered the expression levels of these proteins. In the same way, we also examined the protein level of BDNF, showing that scopolamine treatment alone considerably decreased the BDNF protein level compared to the control treatment. Remarkably, melatonin significantly augmented BDNF expression to levels higher than those after scopolamine administration alone (Fig. 3a). Furthermore, we determined the CREB protein level in HT22 cells in vitro. Scopolamine significantly reduced CREB levels; however, melatonin, like SP600125, substantially enhanced CREB levels (Fig. 3b). Immunofluorescence analysis also supported the p-CREB result obtained with western blot, as melatonin treatment significantly improved the p-CREB immunofluorescence reactivity in the CA1 and DG regions of the hippocampus in the scopolamine + melatonin-administered group (Fig. 3c).

Effect of Melatonin on Glial Activation and NF-κB Mediated Neuroinflammation in Scopolamine-Administered Mice

Glial cells (astrocytes and microglia) play an important role in inflammation as well as inflammatory neurodegeneration because they are the sources of several cytokines (Block et al. 2007; Combs 2009). Thus, we studied the protective effect of melatonin against glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba-1), the main indicators of reactive astrocytes and microglia, respectively. The results revealed that significantly elevated protein levels of GFAP and Iba-1 were present in the scopolamine-injected mice, while cotreatment with melatonin reduced the levels of these proteins (Fig. 4a). Similarly, the immunofluorescence results also supported the western blot results for GFAP, as the number of GFAP-reactive cells was substantially reduced in the scopolamine-treated mice compared to the vehicle-treated control mice. However, melatonin treatment together with scopolamine significantly reduced the number of active GFAP cells in the cortical and hippocampal regions compared to scopolamine treatment alone (Fig. 4b). Furthermore, the expression of NF-κB has been found to be elevated during the aging process (Calabrese et al. 2011), and postmortem analyses of the brains of AD patients have revealed the presence of NF-κB in neurons and neurofibrillary tangles (Terai et al. 1996). Melatonin cotreatment significantly attenuated the elevated expression of p-NF-κB. Moreover, NF-κB overexpression may lead to the activation of several proinflammatory markers that are associated with neurodegeneration. The levels of activated inflammatory markers, such as TNF-α and IL-1β, were examined in cortex and hippocampus samples from scopolamine-treated mice via western blot analysis. The results showed that melatonin significantly decreased scopolamine-induced neuroinflammation by reducing the TNF-α and IL-1β expression levels (Fig. 5a). These findings were further confirmed via confocal microscopy. The results showed that TNF-α immunoreactivity was significantly upregulated by scopolamine administration and that melatonin significantly downregulated the immunoreactivity (Fig. 5b).

Melatonin Reduced Neuronal Apoptosis and Neurodegeneration in Scopolamine-Treated Mice

The administration of scopolamine induced neuronal apoptosis in the mice. Thus, the expression of various proapoptotic and antiapoptotic markers was examined by western blotting. The results suggested that after scopolamine administration, the levels of the proapoptotic protein

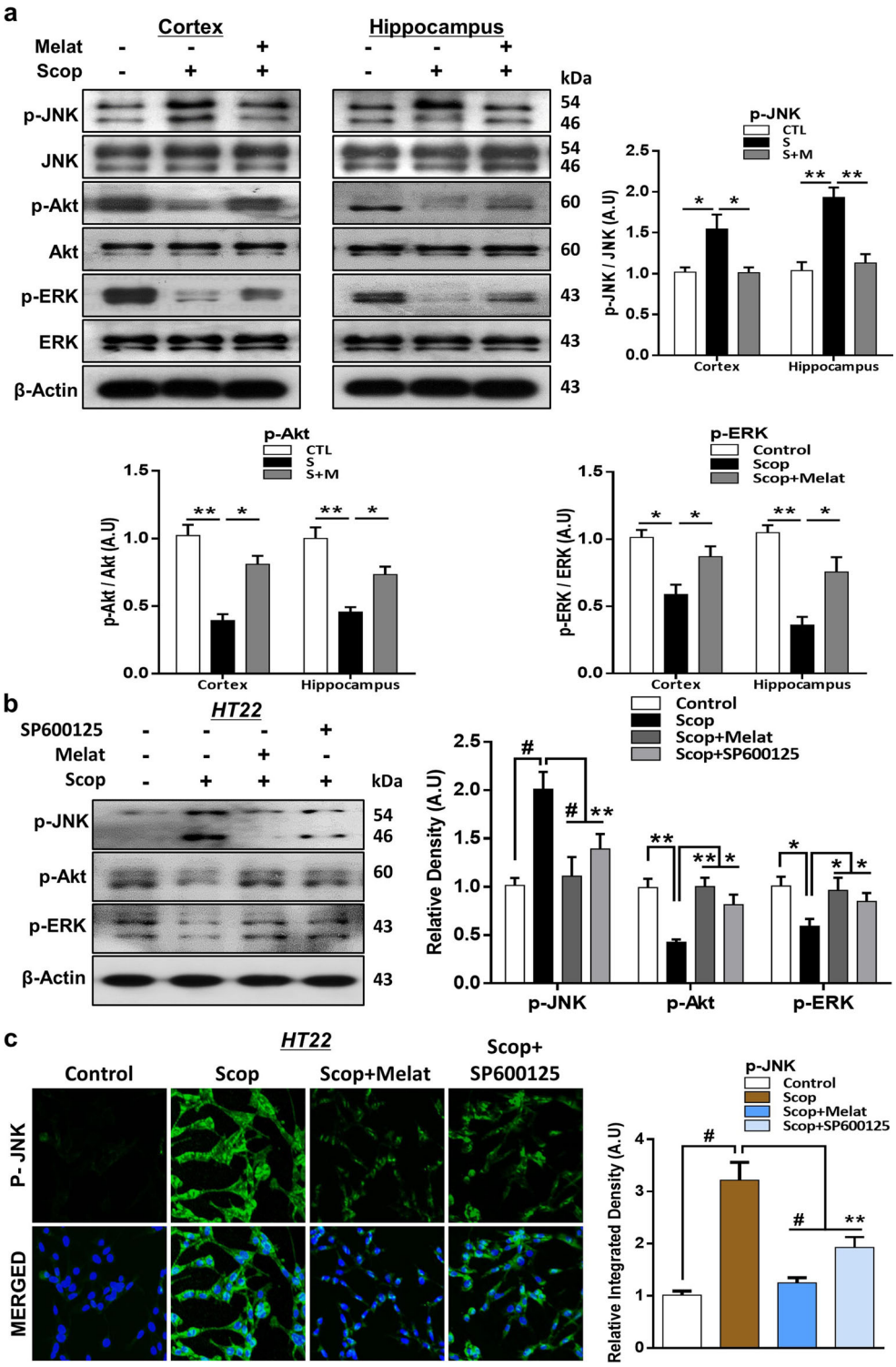

Fig. 2 Effect of Melatonin on p-Akt/p-ERK/p-CREB signaling under scopolamine treatment. **a** The western blot results for p-JNK/JNK, p- Akt/Akt, and p-ERK/ERK in the cortical and hippocampal areas of the different experimental groups are shown. Representative bar graphs showing the relative changes in protein expression in the different groups. **b** Western blot analysis of p-JNK, p- Akt, and p-ERK in HT22 cells treated with scopolamine, scopolamine + melatonin, and scopolamine + SP600125. **c** Representative confocal microscopy images of the immunoreactivity of p-JNK (green, FITC; blue, DAPI) in HT22 cells exposed to scopolamine, scopolamine + melatonin, and scopolamine + SP600125. The data are presented as the mean ± SEM of 6–8 mice per group and are representative of three independent experiments. Significance: * = $P \leq 0.05$, ** = $P \leq 0.01$, # = $P \leq 0.001$

B cell lymphoma 2 (Bcl-2)-associated X protein (Bax) were significantly higher than those after vehicle administration in the control mice group. Similarly, scopolamine also significantly downregulated the levels of the antiapoptotic B cell lymphoma 2 (Bcl-2) protein, overall increasing the ratio of Bax/Bcl-2 in the mouse brain (hippocampus and cortex). As shown in the results, cotreatment with melatonin and scopolamine reduced the amount of Bax and improved the Bcl-2 protein levels. Scopolamine treatment prompted the activation of Caspase-3. Conversely, cotreatment with melatonin caused a significant decrease in the expression of activated Caspase-3 in the

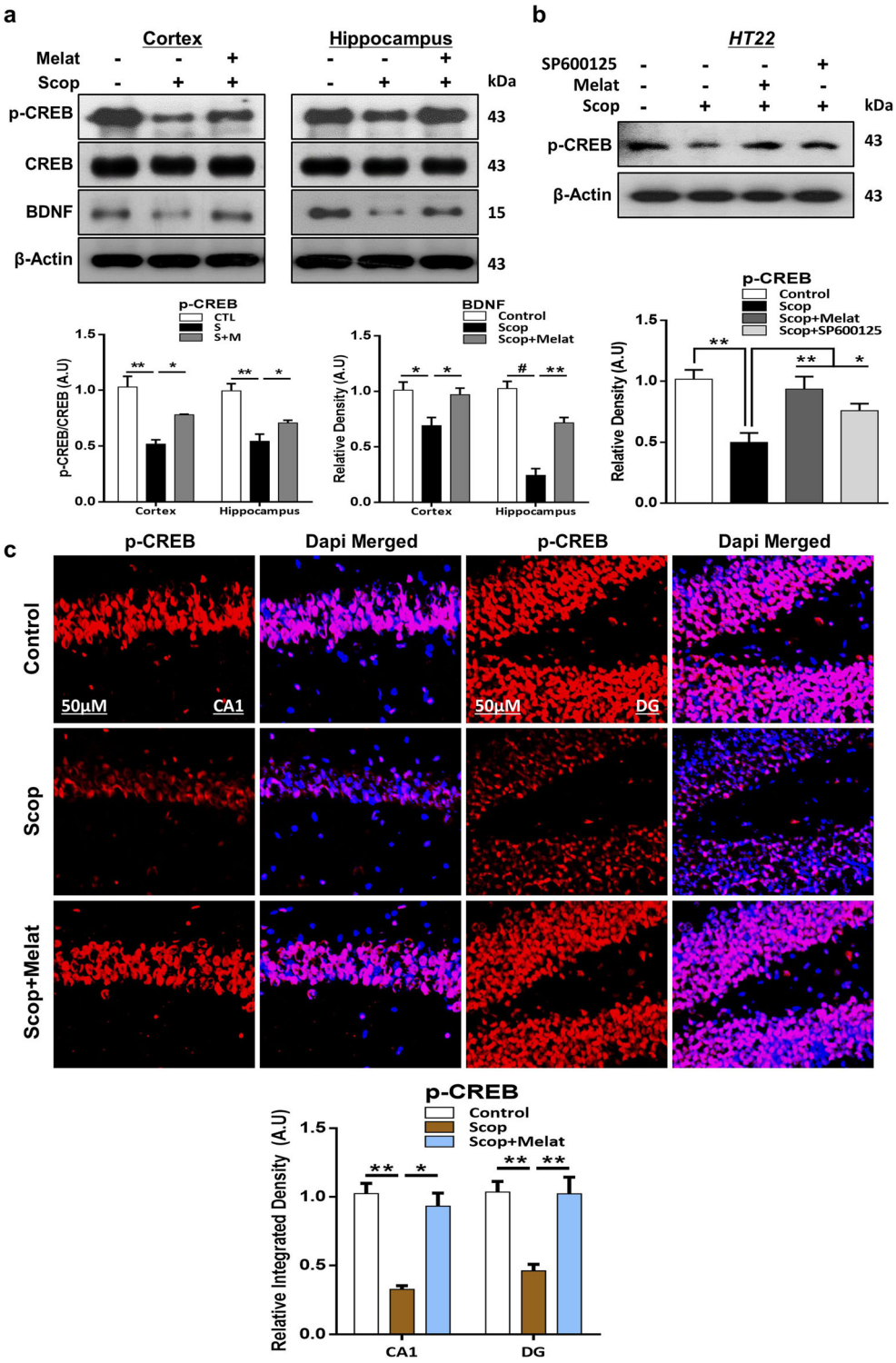

Fig. 3 Melatonin regulated the expression of synaptic proteins in scopolamine-treated mouse brains. **a** Western blot analysis of p-CREB and BDNF protein expression in the mouse cortex and hippocampus following scopolamine and melatonin administration. The bands were quantified using ImageJ software, and the changes are shown with a bar graph. **b** Western blot analysis of p-CREB in HT22 cells treated with scopolamine, scopolamine + melatonin, and scopolamine + SP600125. **c** Confocal microscopy images and quantitative analysis of the expression of p-CREB in the cortex and hippocampus of the experimental groups. The data are expressed as the mean ± SEM of 6–8 mice per group and are representative of three independent experiments. Significance: * = P ≤ 0.05, ** = P ≤ 0.01, # = P ≤ 0.001

hippocampus and cortex of mice (Fig. 6a). Moreover, the degree of neuronal loss was assessed morphologically using cresyl violet stain, which has been reported to be a neuronal antiapoptotic marker (Li et al. 2014). Scopolamine administration alone significantly decreased the amount of surviving neuronal cells. The results clearly display that scopolamine promotes neuronal loss in the hippocampal CA1, CA3, and DG regions as well as the cortical regions. However, melatonin cotreatment preserved neuron survival and prevented scopolamine-induced neuronal loss in the cortical and hippocampal regions of adult mouse brains (Fig. 6b).

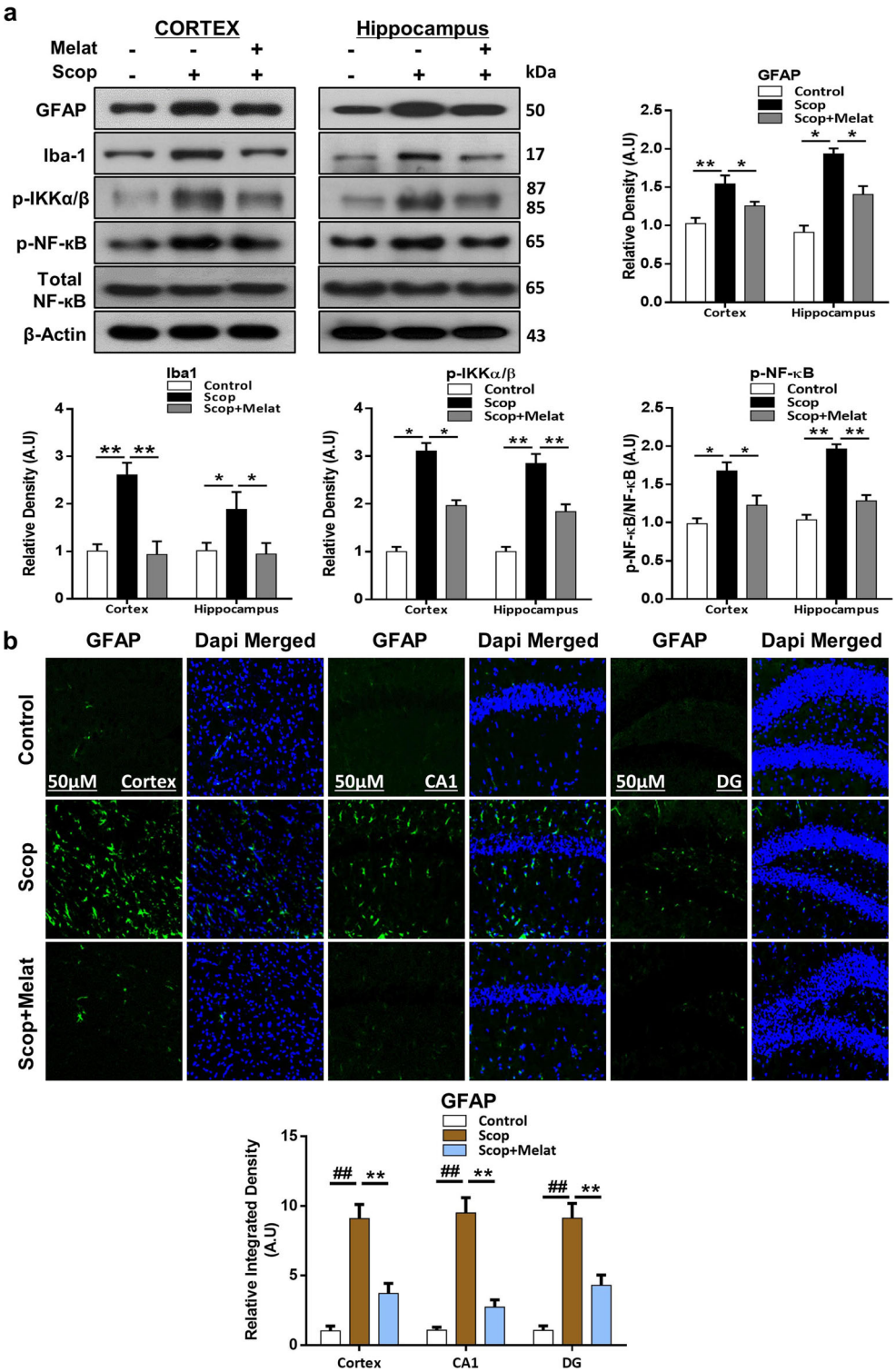

Fig. 4 Melatonin regulated the activation of astrocytes and microglia and the protein expression of p-NF-κB. **a** Western blot results and corresponding bar graphs showing the relative expression of GFAP, Iba-1, p-IKKα/β and p-NF-κB/NF-κB in brain homogenates of the cortical and hippocampal regions of the different experimental groups. The expression was quantified by densitometric analysis. **b** Bar graphs of the relative expression of immunoreactive GFAP in the cortex and hippocampus of the experimental groups. The data are expressed as the mean ± SEM of five mice per group and are representative of three independent experiments. Significance: * = P ≤ 0.05, ** = P ≤ 0.01, # = P ≤ 0.001, ## = *P* ≤ 0.0001

Melatonin Improved Synaptic Dysfunction Caused by Scopolamine Treatment

To examine the beneficial effects of melatonin after scopolamine-induced reductions in synaptic proteins, synaptic protein expression was examined in the cortical and hippocampal regions via western blot analysis and confocal microscopy. Our findings showed that scopolamine significantly downregulated Syntaxin, PSD95 (postsynaptic density protein 95) and SNAP-23 protein expression compared to the vehicle. However, melatonin+scopolamine cotreatment significantly improved the expression levels

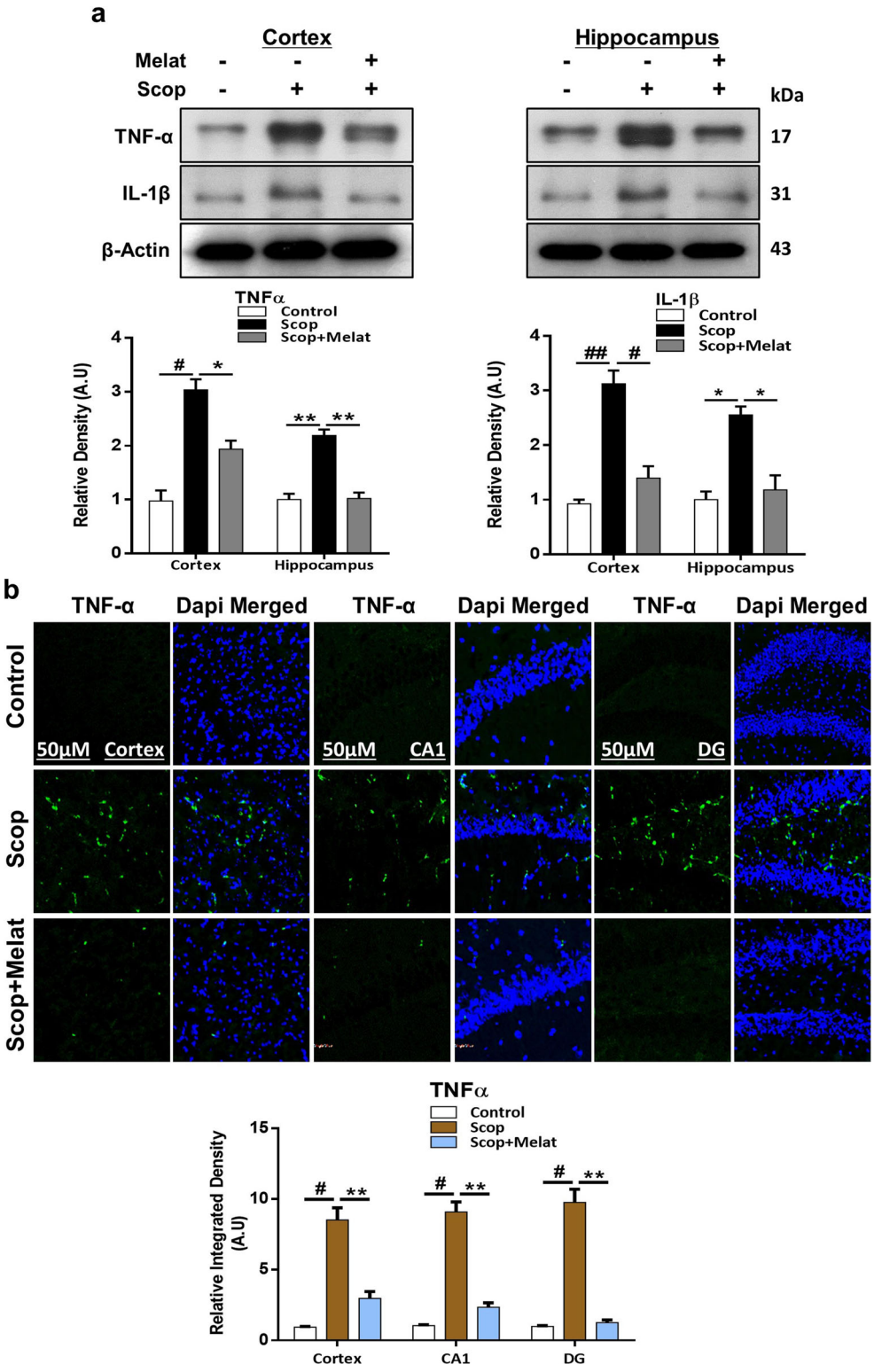

Fig. 5 Melatonin mitigated the neuroinflammation induced by scopolamine. **a** Western blots probed with TNF-α and IL-1β antibodies and bar graphs showing the changes in the protein levels of TNF-α and IL-1β. **b** Bar graph of the relative expression of immunoreactive TNF-α in the cortical and hippocampal areas of the mouse brain. The data are presented as the mean ± SEM of 6–8 mice per group and are representative of three independent experiments. Significance: * = $P \leq 0.05$, ** = $P \leq 0.01$, # = $P \leq 0.001$, ## = $P \leq 0.0001$

of these proteins (Fig. 7a). Furthermore, we confirmed the western blot results with an immunofluorescence assay for synaptophysin (Syp), a presynaptic protein. The results revealed that synaptophysin levels were significantly reduced in the scopolamine-treated group compared with the control group. However, melatonin treatment in combination with scopolamine significantly improved the levels of synaptophysin (Fig. 7b).

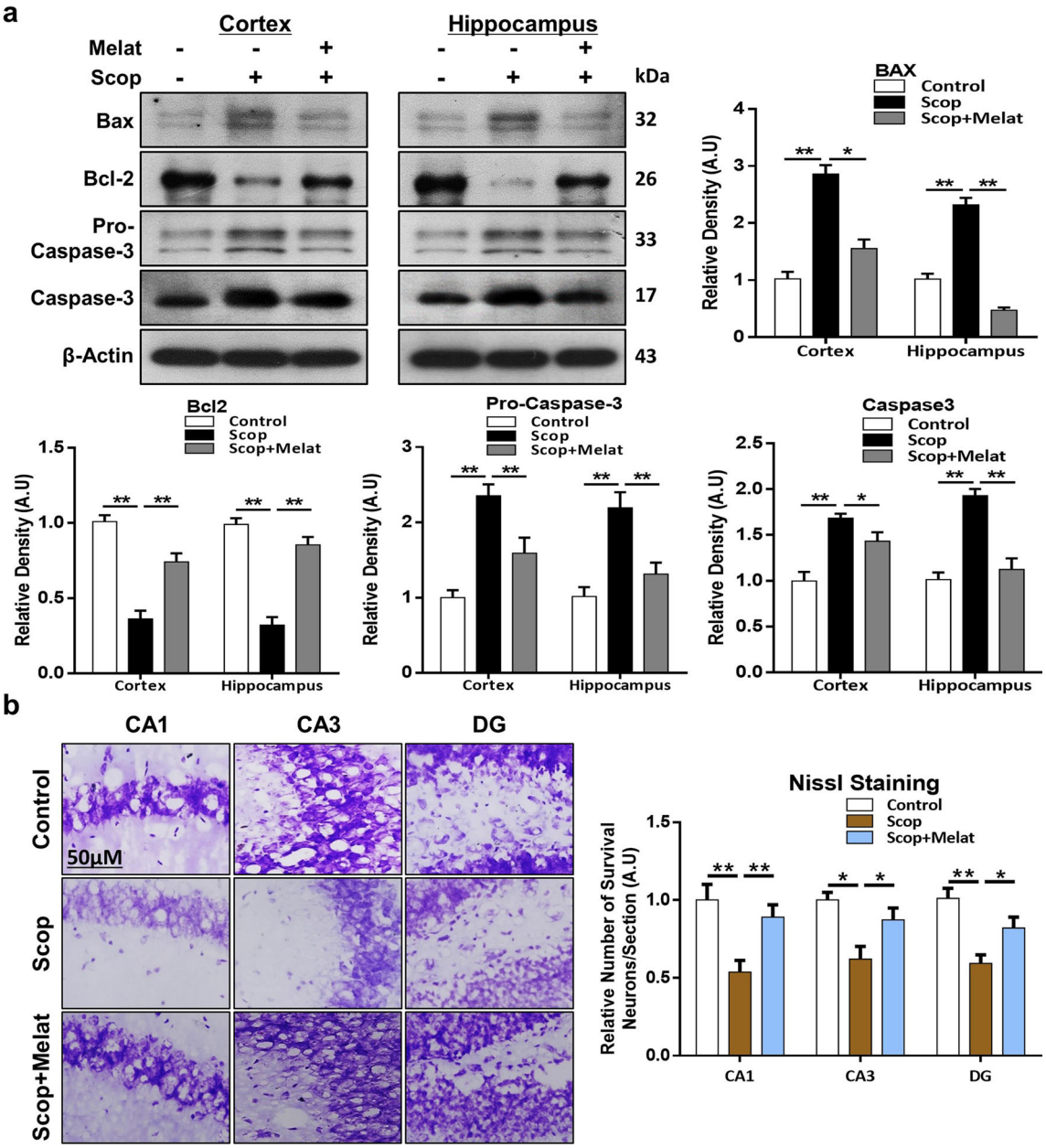

Fig. 6 Melatonin attenuated scopolamine-induced apoptotic cell death by regulating the expression of Bax, Bcl-2, Pro-Caspase-3 and Caspase-3 in the mouse brain. **a** Representative western blots probed with Bax, Bcl-2, Pro-Caspase-3 and Caspase-3 antibodies in the cortex and hippocampus of the experimental groups and bar graphs showing the quantitative results. **b** Images and quantitative analysis of the Nissl-stained hippocampal neurons in the different experimental groups. The targeted areas are the CA1, CA3 and DG regions. The data are expressed as the mean ± SEM ($n = 6$–8 mice/group). Significance: * = $P \leq 0.05$, ** = $P \leq 0.01$

Melatonin Enhanced Learning Memory and Spontaneous Alteration Behavior in Scopolamine-Induced Memory Impairment

The effect of melatonin on learning and spatial memory in mice with scopolamine-induced behavior impairment was analyzed using the MWM. As the results show (Fig. 8a–c), the mean latency to discover the hidden platform decreased steadily throughout training. However, the scopolamine-treated group displayed greater latency to discover the platform, illustrating compromised spatial learning and memory capabilities compared to those of the control group. Our results revealed that melatonin administration significantly attenuated the increased latency to discover the platform during training days caused by scopolamine treatment alone. Next, to assess memory formation, we performed probe trials on day 6 by removing the platform. The number of crossings and the time spent in the target quadrant for the scopolamine-

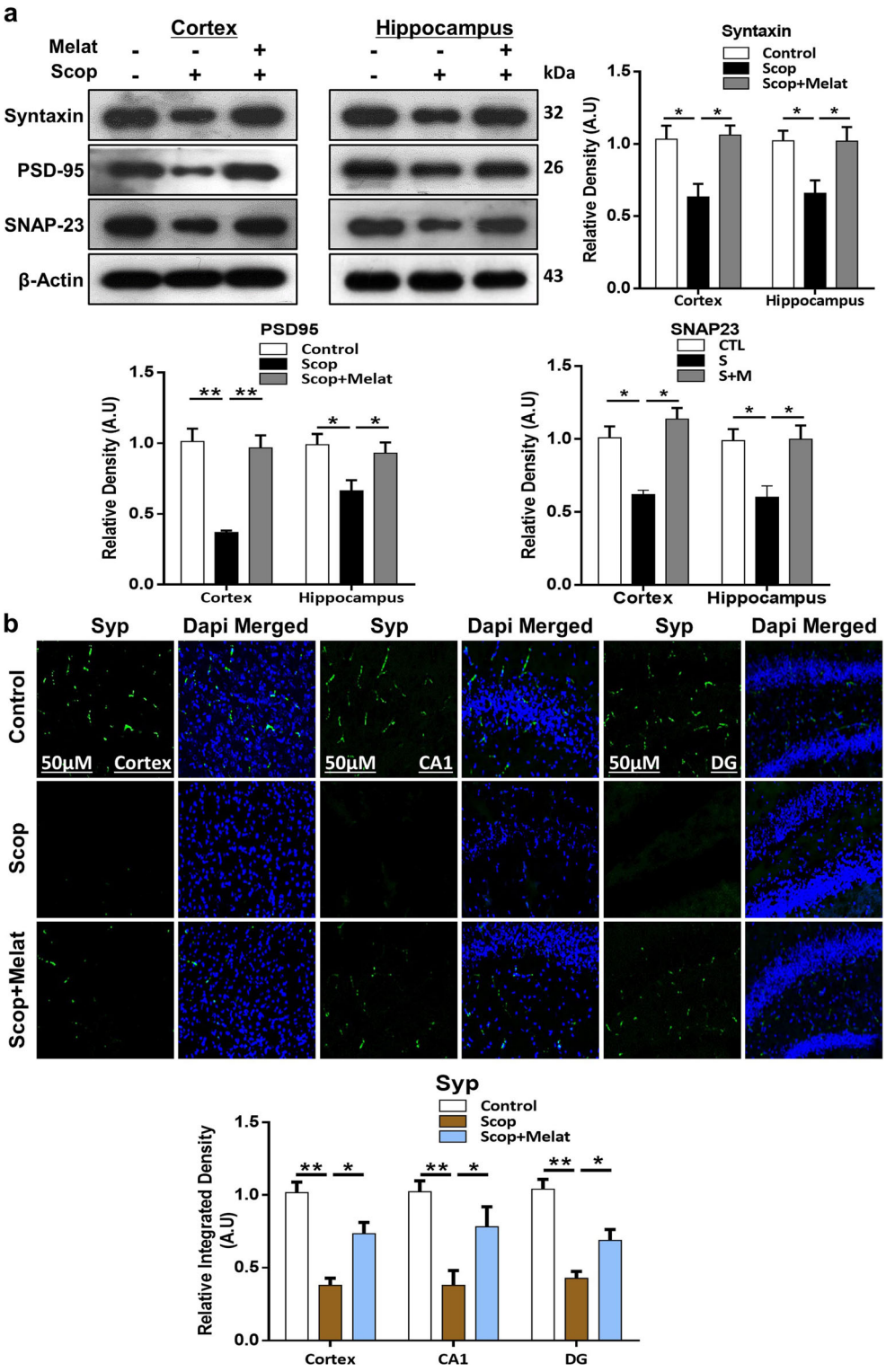

Fig. 7 Melatonin regulated the expression of synaptic proteins in scopolamine-treated mouse brains. **a** Scanned X-ray films of immunoblots and quantitative results showing the expression levels of the proteins (Syntaxin, PSD-95, and SNAPS-23) in the cortex and hippocampus in the experimental groups. **b** Representative immunofluorescence images showing Syp levels in the experimental groups and quantitative results showing the differences. The data are presented as the mean ± SEM of 6–8 mice per group and are representative of three independent experiments. Significance: * = P ≤ 0.05, ** = P ≤ 0.01

administered mice were significantly lower than those for the control mice. However, in the melatonin-cotreated group, the number of platform crossings and the time spent in the target quadrant were significantly increased (Fig. 8d, e). Next, we analyzed exploratory behavior and spatial learning by determining the percentage (%) of spontaneous alternation behavior. An increase in the percentage of spontaneous alternation behavior was assumed to indicate enhanced memory function. Analysis of the spontaneous alternation rate from the center of the Y-maze, which is the percentage of the total number of arm entries that can be counted as successive nonrepetitive triplets, showed that the scopolamine-treated

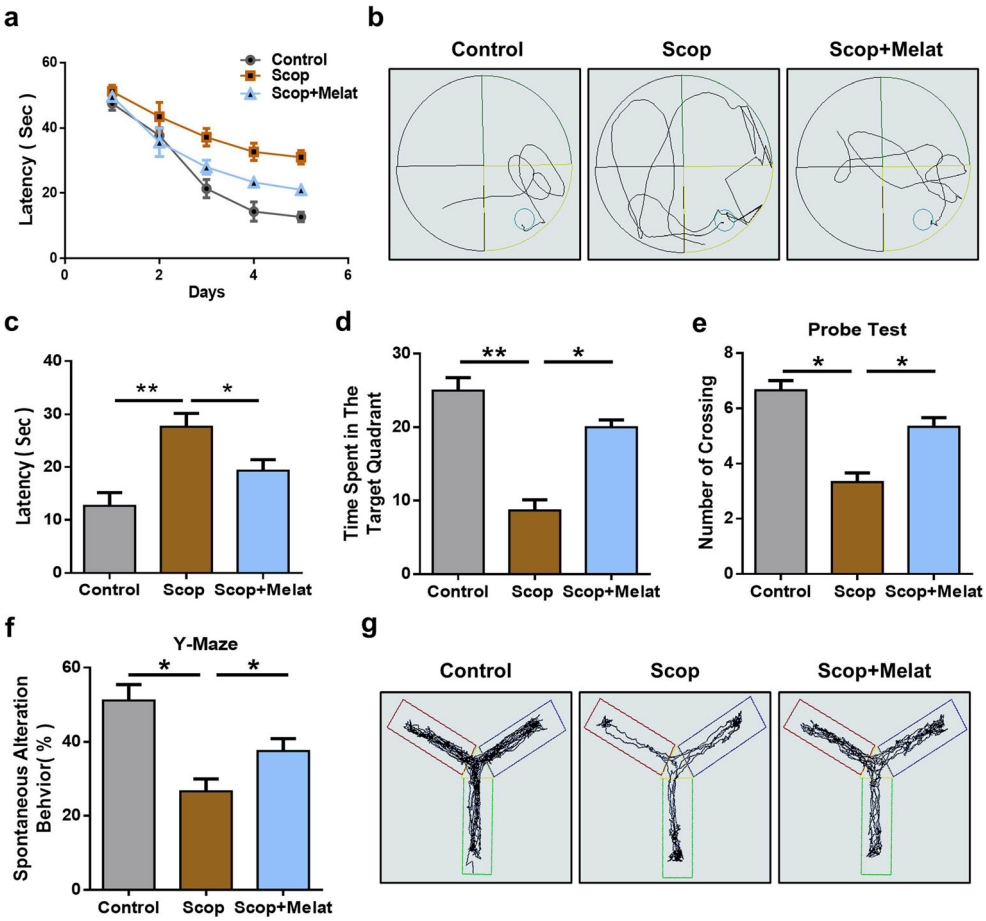

Fig. 8 Effects of melatonin on scopolamine-induced memory dysfunction and behavioral variations in the experimental groups. To evaluate spatial working memory, the MWM and Y-maze tasks were used. **a**, **b** Bar graph showing the latency to reach the hidden platform during the training sessions. The trajectories indicate the path lengths covered by the mice during the probe test. c Escape latency on the 5th (last) day of the training session. **d** Number of crossings over the previous platform location in the probe test. **e** Bar graph showing the time spent in the target quadrant during the probe test session. **f**, **g** Spontaneous alternation behaviors in the Y-maze test and trajectories showing the movement of the mice in the Y-maze trial. The behavioral results are presented as the mean ± SEM of 12–14 mice/group and are representative of 3 independent experiments. Significance: * = P ≤ 0.05, ** = P ≤ 0.01

mice had a lower percentage of alternation than the control mice, thus presenting reduced working memory (Fig. 8f, g). Melatonin treatment in the scopolamine + melatonin-administered group significantly augmented the spontaneous alternation behavior (%) compared to scopolamine treatment alone, indicating that melatonin mitigated memory deficit in the scopolamine-administered mouse model.

Discussion

Synaptic deficit is the early pathological hallmark of AD and is closely related to reduced cognitive function and memory loss (Ohno et al. 2007; Yu et al. 2016). It is well known that scopolamine antagonizes muscarinic receptors and is capable of producing memory deficits in the processes of learning acquisition and consolidation (Du et al. 2015). Similarly, elevated oxidative stress triggers a vicious cycle of synaptic dysfunction, memory deficit, neuroinflammation and apoptosis (Ohno et al. 2007).

Here, we investigated the role of melatonin in protecting against scopolamine-induced oxidative stress in the brain accompanied by a cascade of signaling that results in brain damage. It has been reported that scopolamine-induced memory impairment is characterized by elevated Ca^{2+} influx, oxidative stress, and neuronal cell death (Balaban et al. 2017). Consistent with the fact that melatonin exhibits free radical-scavenging, antioxidant and anti-inflammatory properties, we found that the scopolamine-induced oxidative stress was considerably mitigated by melatonin cotreatment. Melatonin decreased LPO and ROS levels in vivo and in vitro, demonstrating antioxidant activity that played a beneficial role in scopolamine+melatonin-treated mice. Previous studies have suggested that Nrf2 activation provides neuroprotection in AD (Khodagholi et al. 2010). We found that Nrf2 protein levels were reduced in the brains of scopolamine-administered mice and that melatonin administration significantly reversed the decreased expression of the Nrf2 protein. HO-1 upregulation has a potential antioxidant function in the AD brain (Hui et al. 2011; Kamalvand et al. 2003). Our results showed that scopolamine treatment downregulated HO-1 expression; however, cotreatment with melatonin upregulated HO-1 expression.

It has been reported that the persistent production of ROS in inflamed tissues causes prolonged JNK phosphorylation. In addition, JNK activation dominates Akt signaling and affects its downstream pathways (Chaanine et al. 2012). Similarly, many studies have reported that crosstalk between oxidative stress and Akt/ERK signaling plays a critical role in cell

survival and cell death (Kim et al. 2017; Yan et al. 2016). Our findings revealed that the expression levels of p-Akt and p-ERK were significantly decreased by scopolamine; however, melatonin improved p-Akt/p-ERK expression. In addition, the mechanisms of long-term memory formation, neuroplasticity and cell survival are mediated by CREB, while BDNF is important for the modulation of memory processes and learning (Goel et al. 2018). Furthermore, several studies have shown that CREB/BDNF signaling protects against scopolamine-induced synaptic dysfunction, promotes neuronal survival and protects hippocampal neurons from damage (Jana et al. 2013; Koh et al. 2017). Our results showed that p-CREB and BDNF expression levels were significantly reduced by scopolamine, while melatonin ameliorated these decreases in CREB/BDNF protein expression.

The results demonstrated that scopolamine increased the expression of Iba-1 and GFAP, while melatonin cotreatment reduced their expression. Microglia and astrocytes are not only antigen-presenting cells but also effector cells that release several proinflammatory cytokines that promote neurodegeneration. Previous studies have reported that NF-κB, a transcription factor, is overexpressed in activated glial cells, which further induces neuroinflammation by increasing the release of several inflammatory mediators (Maqbool et al. 2013). Melatonin treatment ameliorated the scopolamine-induced overactivation of astrocytes and microglial cells. In addition, the expression of inflammatory markers, such as TNF-α and IL-1β, was also increased in the scopolamine-administered mice, which may be associated with the induction of neuroinflammation-induced memory impairment (Fernandez et al. 1998; Kim et al. 2014). Melatonin reduced the overexpression of TNF-α and IL-1β, preventing neuroinflammation and memory impairment in the scopolamine-administered adult mice. It has been recently reported that long-term scopolamine administration does not potentially induces neuronal loss, but interferes with the proliferation, differentiation and migration of neurons in the DG region of mouse hippocampus (Yan et al. 2014). However, our findings are in line with several other studies which showed that chronic as well as acute scopolamine administration potentially induced apoptosis and consequently leads to the death of hippocampal neurons (Balaban et al. 2017; Jahanshahi et al. 2013; Ray et al. 2014; Sattayasai et al. 2013). It has also been reported that ROS, the main contributors to oxidative stress, induce Bax, a proapoptotic protein, and that Bax overexpression promotes neuronal death. Bcl-2, an antiapoptotic protein, has a function opposite that of Bax (Hou et al. 2014). The ratio of Bcl-2 to Bax regulates cell vulnerability to apoptosis. Bax upregulation and Bcl-2 downregulation are followed by Caspase-3 overexpression, a critical mediator of apoptosis in programmed cell death (Amin et al. 2017; Qian et al. 2008). Our results revealed that scopolamine activates Bax and Caspases while inhibiting Bcl-2 protein expression. Melatonin decreased the overexpression of Bax and the activation of Caspases and promoted Bcl-2 overexpression in scopolamine-treated mice, indicating that melatonin had an antiapoptotic effect in scopolamine-treated mice.

Synaptic dysfunction is associated with decreased level of synaptic proteins including presynaptic synaptophysin, syntaxin, and postsynaptic PSD-95, and SNAP-23 has been studied in AD patients and in animal models of AD (Ali et al. 2015; Amin et al. 2016). Our results showed reduced levels of synaptic proteins in scopolamine-treated mice. Melatonin recovered the reduced synaptic protein levels, significantly improving the expression levels. Melatonin has been found to exhibit a nootropic effect via central excitation and improvement of spatial working memory (Blasko and Grubeck-Loebenstein 2003). Our findings revealed that scopolamine significantly reduced memory function as assessed by the MWM and Y-Maze tests. Cotreatment with melatonin significantly ameliorated the reduced memory function, as indicated by decreases in the escape latency, increases in the total time spent in the target quadrant, and increases in the number of platform crossings in the probe test. With the help of the Y-maze, we detected a decrease in the spontaneous alternation behavior in scopolamine-treated mice that was associated mainly with the hippocampal region (Broadbent et al. 2004).

In summary, our results revealed that melatonin reversed the oxidative stress induced by scopolamine in adult mice. Melatonin reversed oxidative stress-mediated synaptic dysfunction and learning and memory process impairments. Thus, melatonin has substantial potential as a therapeutic intervention for the alleviation of scopolamine-induced deleterious effects. This neuroprotective effect of melatonin needs to be considered in the treatment of memory and synaptic impairments related to neurodegeneration.

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Compliance with Ethical Standards

Conflict of Interest The authors declared no competing financial interests.

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