Neurochemical Research (2019) 44:1851–1868
https://doi.org/10.1007/s11064-019-02820-6

ORIGINAL PAPER

Melatonin Improves Memory Deficits in Rats with Cerebral Hypoperfusion, Possibly, Through Decreasing the Expression of Small-Conductance Ca^{2+}-Activated K^+ Channels

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Received: 15 December 2018 / Revised: 2 May 2019 / Accepted: 27 May 2019 / Published online: 11 June 2019

Abstract
This study investigated the expression pattern, regulation of expression, and the role of hippocampal small-conductance Ca^{2+}-activated K^+ (SK) channels in memory deficits after cerebral hypoperfusion (CHP) with or without melatonin treatment, in rats. Adults male Wistar rats (n = 20/group) were divided into (1) a sham (2) a sham + melatonin (3) a two-vessel occlusion (2-VO) model, and (4) a 2-VO + melatonin. Melatonin was administered (i.p.) to all rats at a daily dose of 10 mg kg^{-1} for 7 days starting at the time of 2-VO-induction. In contrast to 2-VO rats, melatonin increased the latency of the passive avoidance learning test and decreased time to find the hidden platform in Water Morris Test in all tested rats. In addition, it concomitantly downregulated SK1, SK2, and SK3 channels, downregulated mRNA levels of TNFα and IL-1β, enhanced BDNF levels and activity of PKA levels, and restored the levels of cholinergic markers in the hippocampi of the treated-rats. Mechanistically, melatonin significantly prevented CHP-induced activation of ERK1/2, JNK, and P38 MAPK at least by inhibiting ROS generation and enhancing the total antioxidant potential. In cultured hypoxic hippocampal neurons, individual blockage of MAPK signaling by the MEK1/2 inhibitor (U0126), but not by the P38 inhibitor (SB203580) or JNK inhibitor (SP600125), completely prevented the upregulation of all three kinds of SK channels. These data clearly confirm that upregulation of SK channels plays a role in CHP-induced memory loss and indicate that melatonin reverses memory deficits after CHP in rats, at least by, downregulation of SK1, SK2, and SK3 channels in their hippocampi.

Keywords Melatonin · Cerebral hypoperfusion · ERK1/2 · SK channels · Memory

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Introduction

Cerebral hypoperfusion (CHP) due to cerebrovascular disease is a common medical event in elderly people and is considered a prominent risk factor of geriatric cognitive and memory dysfunction [1]. In the last decades, numerous advanced studies have been conducted to understand the molecular basis of CHP-induced neurodegeneration and its associated cognitive deficits [2]. In this regard, multiple mechanisms have been suggested including elevation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), overproduction of ROS and proinflammatory cytokines as a result of both glutamate-dependent and glutamate-independent neural toxicity [1, 2].

At present, it is widely accepted that hippocampal neural excitability and synaptic plasticity are regulated by small-conductance Ca^{2+}-activated K^+ (SK or Kca2) channels [3]. Within the nervous system, SK channels usually open in response to any significant rise in $[Ca^{2+]}i]$ [3]. Three subtypes of SK channels have been identified in the mammalian hippocampus, namely, SK1 (Kca2.1), SK1 (Kca2.2), and SK3 (Kca2.3) [4]. Due to their role in the reduction of synaptic plasticity, the bulk of evidence is now illustrating the crucial function of these channels in the regulation of memory and memory encoding [3, 5]. Indeed, lower levels of SK2 and SK3 mRNA are seen in the hippocampus during the early stages of spatial learning [6], and higher expression of SK3 channels with a concomitant reduction in learning abilities in the hippocampus of aged mice was reported [7]. In addition, memory defects are observed in mice overexpressing SK2 [3]. Unfortunately, the involvement of SK channels in cognitive deterioration as well as their regulation, expression, and activities in the hippocampus of animals or humans during or after CHP have never been investigated. Such information may widen our knowledge about the above disorders and maximize our therapeutic options with more effective treatments.

On the other hand, melatonin (N-acetyl-5-methoxytryptamine) is a key endogenous indole-amine secreted and released by the pineal gland and some extra-pineal tissues of mammals [8, 9]. Within the CNS, melatonin performs an essential function in the regulation of circadian rhythms [10]. In addition, melatonin and its metabolites possess well-known anti-oxidant, anti-inflammatory, and anti-apoptotic properties because of its direct or indirect activation of numerous signaling pathways [10, 11]. Of note, melatonin can cross the blood–brain barrier to exert its effect on brain tissues [8, 9]. Given these characteristics, numerous studies have shown the ability of exogenous-melatonin to inhibit apoptosis induced-neurodegeneration and improve learning and memory in various animal models of neurobehavioral disorders including stroke, Down syndrome, dementia, and Alzheimer's disease [8, 9, 12, 13]. To a large extent, these neurobehavioral protective effects of melatonin are related to its antioxidant and antiapoptotic properties and to its participation in protection from mitochondrial dysfunction [10, 11]. Nonetheless, it is largely unknown whether melatonin affords its neurobehavioral protection and reversal of memory loss, especially after CHP, by regulating the expression levels of the above-mentioned SK channels.

On the other hand, the roles of mitogen-activated protein kinases (MAPKs) as important signaling pathways within the mammalian nervous system in health and disease have been reported [14-16]. Members of the MAPK family include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and P38 [14]. Studies have uncovered over-activation of all MAPKs in the hippocampi and/or cortices after CHP or a cerebral ischemic insult, in vivo [15, 17]. Among them, the ERK1/2 cascade can be activated by growth factors, mitogens, ROS, or increased $[Ca^{2+}]i$, thus leading to either cell survival or apoptosis [16-18]. In contrast, JNK and P38 cascades respond to inflammatory cytokines, ROS, or cellular stress to promote inflammation and neural-cell death [19]. Moreover, MAPKs perform crucial functions in the nervous system by enhancing neuronal synaptic plasticity and expression and via activation of various ion channels [15, 16]. Very recently, melatonin was found to significantly downregulate all MAPKs in the brain of rats exposed to ischemia [20]. Given the participation of ROS, $[Ca^{2+}]_i$, and inflammation in CHP and in melatonin's neuroprotective effect, these observations allowed us to hypothesize that MAPKs are deeply involved in the regulation of SK channels during cerebral hypoperfusion with or without melatonin treatment.

Melatonin was shown to protect the brain of rodent from oxidative damage induced by cerebral hypoperfusion and improve memory in these rats mainly by reducing ROS, microglial activation and inflammation [21, 22]. Given the above-mentioned interconnected evidence, this study was carried out on rat's hippocampus to investigate the expression pattern and the role of SK channels in memory deficits after CHP with or without melatonin treatment and to investigate if they are regulated through MAPKs members.

Materials and Methods

Animals and Experimental Design

Adults pathogen-free male Wistar rats (aged 6 weeks and weighing 120–130 g) were obtained from the Animal Research facility of King Khalid University in Abha, KSA. All the animals were kept under a photoperiod of 12 h light and 12 h darkness with automatically regulated temperature (22–23 °C) and free access to water and food. This study was carried out in accordance with the regulations set by our institution, which follow the regulations of laboratory animal care and use published by the US National Institutes of Health (NIH publication No. 85–23, revised in 1996).

The Animal Model of Global CHP (2-VO)

Permanent bilateral occlusion of the common carotid arteries with hypotension (2-VO) was applied to induce CHP as previously described by our group with little modification [23]. In brief, prior to the surgical procedure, the rats were deprived of food and water for 12 h. The rats were anesthetized using a mixture of 2.5% isoflurane (Baxter, Deerfield, IL, USA) in 33% oxygen and 67% nitrous oxide using a face mask as shown by others [21, 24]. After confirmation of deep anaesthesia, a horizontal midline cervical incision was made to carefully separate and expose the common carotid arteries from the sheath and vagus nerve, which were then double-ligated with coated Vicryl (R) plus antibacterial/Polyglactin 910 3/0 absorbable surgical suture (Ethicon, Johnson & Johnson, UK) approximately 1 cm inferior to the origin of the external carotid artery. Then, incisions were closed in layers. The sham-operated animals underwent a similar procedure, except that the common carotid arteries were not occluded. During the surgical operation, hypothermia was prevented by means of a heated table, and body temperature was continuously monitored with a rectal probe. Eye lubricant ointment was applied to both eyes to prevent corneal injury. After the operation, all the rats were placed on a heated table until they recovered from the anesthesia and then moved to clean and ventilated cages and provided with food and water.

Experimental Groups

Melatonin (Cat. No. M5250, Sigma-Aldrich, St. Louis, USA) was dissolved in ethanol and further dissolved in saline solution. The final concentration of ethanol used in this preparation was 1% (the vehicle). Rats were divided into 4 groups (n = 20/each) of (1) sham: administered the vehicle (1% ethanol in saline solution) (2) sham + Melatonin (sham + Mel): were sham rats and administered melatonin (10 mg kg^{-1}) (3) 2-VO: were 2-VO-induced rats and administered the vehicle, and (4) 2-VO + melatonin (2-VO + Mel): were 2-VO-induced rats and administered melatonin (10 mg kg^{-1}). All treatments were given intraperitoneally (i.p.) on a daily basis for 7 days (started directly during induction of cerebral hypoperfusion and given every next day at 8:00 a.m.). The dosage and route of administration of melatonin were adopted from previous studies in rats, mice and Mongolian gerbils [21, 22, 24, 25] where they showed that melatonin at this dose protects the brains against cerebral ischemia. All rats which were didn't survive the surgery or died after the surgery in any group were replaced.

Passive Avoidance Test for Retention Memory

The retention memory of all rats was tested using the passive avoidance learning test (PALT) according to Barkur and Bairy on the morning of day 8 [26]. In the test, the retention memory of the rat is measured by its ability to remember a previously exposed electrical foot shock delivered 2 h prior to the evaluation. In the test, the passive avoidance apparatus used consists of a wooden box (50 × 50 × 35 cm) with a larger and brightly illuminated room and a smaller (15 × 15 × 15 cm) dark compartment supplied with a grid floor which is connected to an electrical shock source. In addition, both rooms are separated from each other by a door. The retention memory test was divided into 2 stages of (1) exploration and learning and (2) retention evaluation. During the first exploration phase, while the door was open, each rat was placed in the illuminated large room and allowed to explore it freely for 3 trails, each of 5 min at 30 min intervals. At the end of 3rd test trial, as the rat stepped into the dark compartment, the door was closed and a single foot shock (50 Hz, 1.5 mA, for 1 s) was delivered directly through the grid floor. Here, the rat was kept in dark for an additional 10 s. Then, the rat was returned to its cage and the test was repeated the next day (day 9). The time latency needed for the animals to step into the dark area is recorded. Normal rats usually avoid entering this dark area.

Morris Water Maze (MWM)

The spatial learning and memory function in all groups of rats was also analyzed using Morris water maze (MWM) on 4 consecutive days (end of day 10–13) [27, 28]. In brief, the maze was a circular swimming pool (180 cm in diameter) and was filled with water (60 cm deep, 22 ± 2 °C). The circular platform (diameter is 10 cm) was submerged at 2 cm below the surface of the milky water and all animals were tested to find this hidden platform (90 s/trial). The test was repeated five times for each rat/trail and the average time taken for all trails was presented marker of its cognitive performance. Escape latencies defined as the time needed to find the platform during Morris water maze (MWM).

Hippocampus Collection

Directly after WMT, all rats were killed by cervical dislocation after being anesthetized with sodium pentobarbital (60–70 mg kg^{-1}, i.p.) and their brains were removed and immediately placed in an ice-cold dish. Next, the hippocampus was quickly excised from each brain according to the neuroanatomy atlas under a stereomicroscope, then weighed and frozen in liquid nitrogen and stored at − 80 °C for further use. Six hippocampi per group were homogenized separately in 1 mL of ice-cold buffered saline (pH 7.4) containing a protease inhibitor mixture (Sigma-Aldrich) and the supernatants were stored at − 80 °C and used later for the biochemical analysis. The other 14 hippocampi per group were frozen at − 80 °C and used later to extract RNA for quantitative PCR (qPCR) or western blotting analysis.

Biochemical Assays in Hippocampal Homogenates

Malondialdehyde (MDA) levels, as a lipid peroxidation marker, were measured as levels of thiobarbituric acid reactive substances (TBARS) using a commercially available assay kit (cat. No. NWK-MDA01, NWLSS, USA). Total antioxidant capacity (TAC) was determined calorimetrically via the reaction with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid. In the test, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) served as a standard, and the results were expressed in micromoles of Trolox equivalents per milligram of protein. ROS levels were determined using the reaction with 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The concentration of choline acetyltransferase (AchT) was determined by means of a commercially available ELISA kit (ChAT, cat. No. SEB929Ra, Cloud-Clone Corp., Houston, TX, USA). Colorimetric commercially available kits were used to determine the levels of acetylcholine (Ach) (cat. No. Cell Biolabs, Inc, STA-603, San Deigo, CA, USA) and the activity of acetylcholine esterase (AChE) (cat. No. ab138871, Abcam, UK). All the tests were carried out according to the manufacturers' instructions and were run in triplicate.

Isolation of Hippocampal Neurons and Cell Culture

Primary cultures of hippocampal neurons were prepared from foetal rat brains in accordance with the methods of Chen and Aguilera [29]. In brief, pregnant Wistar rats were killed by decapitation after CO_2 sedation. Foetal rats in their 18 days of age were rapidly and carefully isolated, their heads were separated in ice-cold PBS, and their brains were excised under a dissecting microscope. Hippocampi were digested with 1 mg mL^{-1} collagenase II (Invitrogen, Carlsbad, CA, USA) dissolved in 25 mM HEPES (Invitrogen), pH 7.4, containing 4 mg mL^{-1} BSA, 0.7 mM dibasic sodium phosphate, 137 mM potassium chloride, 5 mM sodium chloride, 0.2 mg mL^{-1} DNase (activity 500 U mg^{-1}), 1.0 mg mL^{-1} glucose, and 100 µg mL^{-1} gentamicin, all of which were purchased from Sigma-Aldrich. After that, hippocampal cells were mechanically dissociated by gentle pipetting and then centrifuged at 2000 rpm for 2 min. The resultant pellet was resuspended and plated at the density of 3×10^5 cells mL^{-1} in six-well plates pre-coated with poly-L-lysine in Dulbecco's modified Eagle's medium (DMEM) with 5% of foetal bovine serum and 5% of horse serum (Sigma-Aldrich) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h before replacement of the medium with the neurobasal medium supplemented with B27 (Invitrogen), 0.5 mM L-glutamine, and a penicillin/streptomycin solution. The medium was refreshed every 2 or 3 days during cultivation for an additional 10 days by the removal half the volume of media and replace with the same volume of Neurobasal medium. To suppress glial-cell proliferation, 5 µM cytosine arabinoside (AraC) (Sigma-Aldrich) was added to the neurobasal medium at day 1 of the culture. The dose of AraC was used at its lowest effective dose (5 µM) on day 1 as suggested by Seibenhener and Wooten [30] to be used once and before day 3.

Hypoxia Induction and Treatments

Cultures were maintained in modular incubator chambers (Cat. No. MIC-101, Billups-Rothenberg) for 12 h at 37 °C in a humidified atmosphere of 95% N_2 and 5% CO_2 to induce hypoxia, whereas normoxic cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2. The JNK inhibitor, SP600125 (10 µM) or its inactive analogue N1-methyl-1,9,pyrazoloanthrone (NM1,9,PA) (100 µM) [28], or the MEK1/2 inhibitor, U0126, or its inactive analogue U0124 (Cell Signaling Technology, Danvers, MA, USA) (both at 10 µM) [31], or the P38 inhibitor, SB203580 or its inactive analogue SB202474 (each at 5 µM), [32], or melatonin (50 µM) were added 1 h before hypoxia. All the inhibitors and their analogs, as well as melatonin, were prepared in DMSO and then diluted to their final concentration in PBS where the final DMSO concentration was 0.1%. DMSO was added to the medium of normoxic and hypoxic cells as vehicle control (0.1%). Nevertheless, the dose of melatonin used in this part of the study was selected based on repetitive dose dependence experiments on hypoxic hippocampal cells incubated with ascending doses of melatonin (10–100 µM), where both 50 and 100 µM yielded ~ 80–90% cell survival (relative to control). Data are not presented here for simplicity.

Determination of Cell Viability

At the end of the incubation periods, cultured neuronal cells were stained with 0.4% trypan blue (Sigma-Aldrich). The stained cells were regarded as dead and total cell numbers were counted in six non-overlapping visual fields of five wells under a light microscope (200 × magnification).

Whole-Cell Lysate Preparation

Proteins from all hippocampus specimens were extracted with 1 mL of RIPA buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0) to which protease inhibitor (cat. No. P8340, Sigma-Aldrich) and phosphatase inhibitor cocktails (PhosSTOP, cat. No. PHOSS-RO, Sigma-Aldrich) were added. For cultured hippocampi, the medium was first removed, and the cells were lysed with the Tissue Protein Extraction Reagent (T-PER, Pierce, Rockford, IL, USA) in a ratio of 1 g to 20 mL of the reagent supplied with the protease and phosphatase inhibitors as described above followed by centrifugation at 10,000×*g* for 5 min to pellet cells and tissue debris.

Western Blotting

Protein concentrations in the protein extracts from cultured hippocampal cells and from rat hippocampi isolated from rat brains were measured by the Bradford assay and then were separated (60 µg of protein per well) by SDS-PAGE in a 10% gel. After the transfer of the proteins to a nitrocellulose membrane and blocking (5% milk in TBST buffer), the membranes were incubated with various monoclonal and polyclonal antibodies: against BDNF (cat. No. ab108319, 15 kDa) purchased from Abcam, Cambridge, UK, SK1 (cat. No. sc17991, 60–62 kDa), SK2 (KCNN2 [K_{Ca}2.2], cat. No. sc101991, 57 kDa), SK3 (KCNN2 [K_{Ca}2.3], cat. No. sc28621, 70 kDa), and β actin (cat. No. sc-1616, 43 kDa) purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against SPAK/JNK (cat. No. 9252, 46 and 54 kDa), P-SAPK/JNK (Thr183/Tyr185, cat. No. 9251, 46 and 54 kDa), p44-24 MAPK (ERK1/2, cat. No. 9101, 42 and 44 kDa), phospho-p44-24 MAPK (p-ERK1/2, Thr202/Tyr204, cat. No. 9101, 42 and 44 kDa), P38 (cat. No. 9211, 43 kDa), p-P38 MAPK (Thr180/Tyr182, cat. No. 9211, 43 kDa PKA-Cα (cat. No. 4782, 42 kDa), and phospho-PKA-C (Thr197, cat. No. 4781, 42 kDa) were purchased from Cell Signaling Technology. Membranes were stripped for detection of various antibodies four times at a maximum of 4 times and detection of phosphorylated forms was performed first. Phosphorylated proteins were always detected first and the reference gene was always detected lastly. Images were scanned on a C-DiGit Blot Scanner (LI-COR, USA) and analyzed in the supplied Image Studio DiGits software. Protein expression levels were presented as relative expression levels towards that of β-actin.

Real-Time PCR

Primers used to study mRNA levels of various types of SK channels and β-actin in the rat hippocampus are shown in Table 1. Primers sequences of tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) were adopted from Hu et al. [33]. In brief, total RNA was extracted from each frozen hippocampus (5 rats/group) using the RNeasy Mini Kit (cat. No. 74,104, Qiagen Inc.). The concentration of isolated RNA was measured on a NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total RNA was reverse-transcribed (Omniscript RT Kit, cat. No. 205,111, Qiagen Inc.) in the presence of primers for 1 h. qPCR analyses of SK1, SK2, and SK3 mRNAs were carried out on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using the Ssofast Evagreen Supermix (Bio-Rad), and mRNA levels were calculated by the $^{\Delta\Delta}$CT method. β-Actin served as a reference gene. Data were obtained in triplicate for each gene, and average readings from five rats were presented as a relative expression towards β-actin mRNA. Each qPCR plate contained no template controls to prevent DNA contamination.

Statistical Analysis

All these analyses were performed in GraphPad Prism statistical software, version 6 (GraphPad Software, La Jolla, CA, USA). All the data were tested for normality of the distribution by the Kolmogorov–Smirnov test, followed by the Levene test for variance equivalence. The time latencies measured in MWM within each group on different days of testing were analyzed by one-way ANOVA with repeated measures followed by Tukey's post hoc test for multiple comparisons. Latencies in both PALT and MWM from each day of testing and all other measured biochemical parameters were compared between groups by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. *P* values less than 0.05 were assumed to reflect a significant difference. Data are presented as means ± SD.

Results

Melatonin Improves Hippocampal-Dependent Retention and Memory Function in Control and 2-VO-Induced Rats

As shown in Fig. 1a, b, 2-VO-induced rats had significantly lower time latency to enter the dark area during the PALT and had significantly longer escape times to find the hidden platform overall the 4 testing days during the WMT, as compared to sham-operated rats. These data indicate that 2-VO-induced rats have impaired retention and spatial memory, respectively. However, sham + Mel-treated rats showed significantly longer time to enter the dark area during PALT and had shorter escape latencies, only at day 3, of the WMT, as compared to sham-operated rats (Fig. 1a, b). Similarly, 2-VO + Mel-treated rats had longer time latency to enter the dark area and shorter escape latency overall the testing

Table 1 Primers used in qPCR

bp	Primer sequence	Target
241	F: 5′-CTGTGGGAAGGGCGTGTGTCTG-3′ R: 5′-CCGAACCCGGCTTTGGTCTGG-3′	SK1 (kCNN1/KCa2.1)
218	F: 5′-GTGCTCTTGGTTTTTAGTATCTCG-3′ R: 5′-CAACCTGCACCCATTATTCC-3′	SK2 (KCNN2 KCa2.2)
400	F: 5′-GCCAACAAGCGGAAAAACCAAAAC-3′ R: 5′-CCAGGCGTGCCGTCCAGAAGAAC-3′	SK3 (KCa2.3)
110	F: 5′-CGTTGACATCCGTAAAGAC-3′ R: 5′-TAGGAGCCAGGGCAGTA-3′	β-Actin

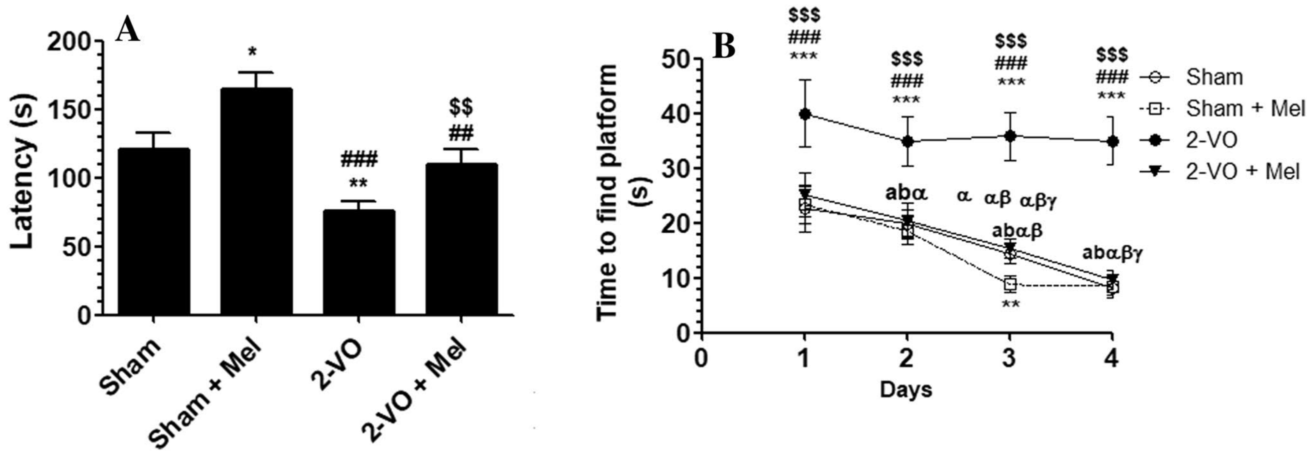


Fig. 1 Time latencies during *the passive avoidance learning test (PALT and Morris water maze (MWM).* **a** The latency is identified as the time needed for the rat to enter the dark area. **b** The escape latency is identified as the time needed for the rat to find the hidden platform. Values are expressed as mean ± SD for 20 rats/group. *,**,***Sham-operated rats at $p < 0.05$, $p < 0.01$; and $p < 0.001$, respectively. $^{\#,\#\#,\#\#\#}$Sham + melatonin (Mel) at $p < 0.05$, $p < 0.01$; and $p < 0.001$, respectively. $^{\$,\$\$,\$\$\$}$2-VO-induced rats + melatonin (Mel) at $p < 0.05$, $p < 0.01$; and $p < 0.001$, respectively

days of WMT as compared to 2-Vo-induced rats (Fig. 1a, b). These data suggest that melatonin improves both retention and spatial memory of both healthy and 2-VO-induced rats. Of note, there was no significant difference in time latency to enter the dark area or escape latency measured at days 1–4 when a comparison was made between 2-VO + Mel and sham-operated rats.

Melatonin Prevents the Generation of Oxidative Stress in the Hippocampi of Rats with Induced CHP and Enhances the Antioxidant Potential In Both Control and Rats with Induced CHP

It should be mentioned here that we could not find any significant difference between the average weights of hippocampi among all the groups (Fig. 2a). Cerebral hypoperfusion (CHP) induced by the 2-VO operation significantly increased levels of ROS and MDA and concomitantly decreased TAC in the hippocampi of rats, as compared to sham-operated rats (Fig. 2b–d). Nonetheless, as compared to 2-VO rats, melatonin post-administration to 2-VO rats significantly attenuated the increases in ROS and MDA levels and raised TAC in the hippocampi of the treated rats, as compared to 2-VO-induced rats and the levels of all these biochemical ends were not significantly different as compared to sham-operated rats (Fig. 2b–d). However, while levels of ROS remained unchanged, levels of MDA were significantly lower and levels of TAC were significantly higher in the hippocampi of sham + Mel as compared to their corresponding basal levels measured in the hippocampi of the sham-operated rats (Fig. 2b–d).

Melatonin Prevents the Upregulation of mRNA and Protein Levels of SK1, SK2, and SK3 Channels in the Hippocampi of Control and Rats with Induced CHP

All SK channels were expressed in the hippocampus of control rats, where both mRNA and protein levels of SK1 and SK 2 were high while those of SK3 was barely detectable (Fig. 3a, b). Nonetheless, 2-VO-induced rats had significantly increased hippocampal mRNA and protein levels of SK1, SK2, and SK3 channels, as compared to sham-operated rats (Fig. 3a, b). On the other hand, the hippocampi of control + Mel or 2-VO + Mel-treated rats showed significantly decreased mRNA and protein levels of all these SK, as compared to sham or 2-VO-induced rats, respectively. Of note, the mRNA and protein levels of SK1, SK2, and SK3 channels in 2-VO + Mel-treated rats were not significantly different as compared to their levels detected in the hippocampi of sham-operated rats (Fig. 3a, b).

Melatonin Downregulates All Types of MAPKs in the Hippocampi of Control and Rats with Induced CHP

Protein levels of total ERK1/2, JNK1/2, and P38 did not significantly change whereas the amounts of p-ERK1/2, p-JNK1/2, and p-P38 significantly increased in the hippocampi of 2-VO-induced rats, as compared to sham-operated rats (Fig. 4a–c). On the other hand, melatonin post-administration to control or to 2-VO-induced rats significantly lowered the phosphorylation rate of all these MAPKs members, as compared to sham or 2-VO-induced rats, respectively (Fig. 4a–c). While protein levels of p-ERK1/2

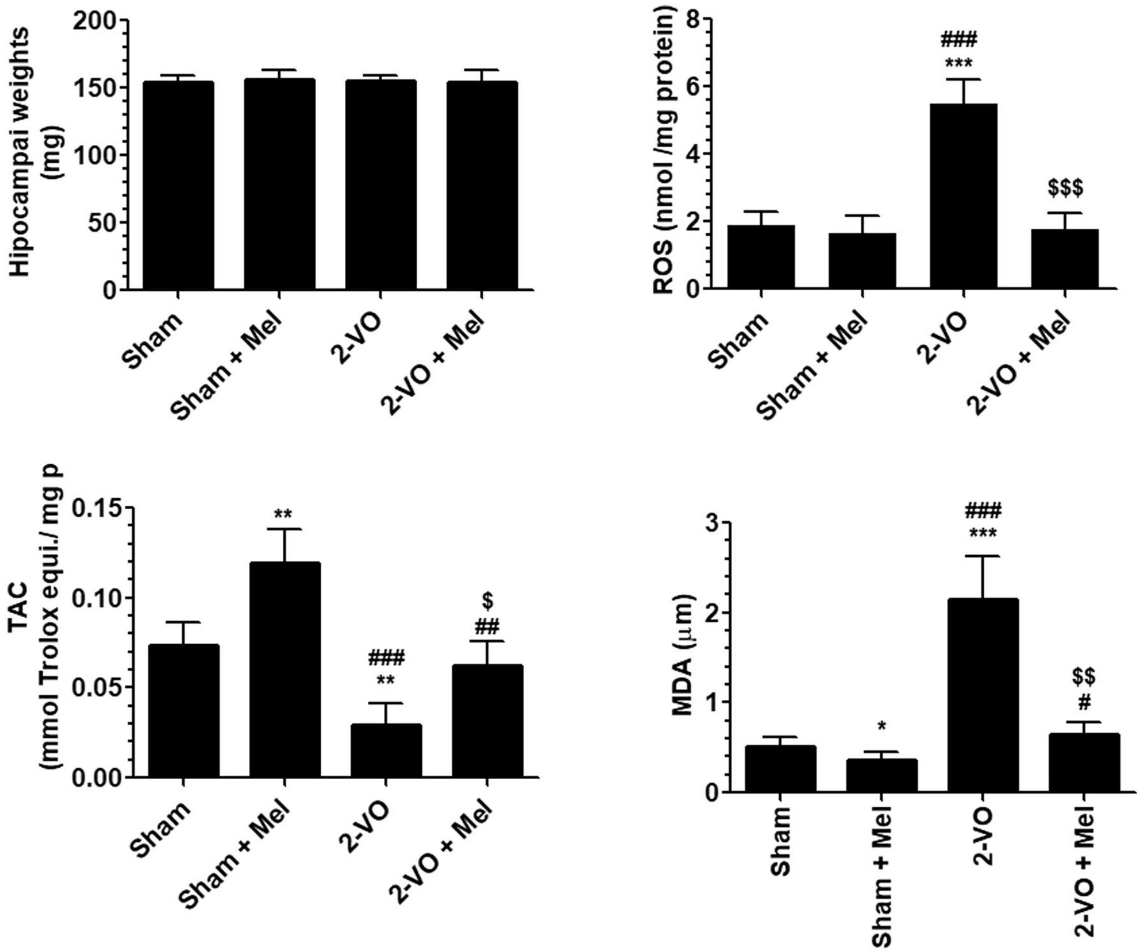


Fig. 2 Average hippocampal weights and hippocampal levels of reactive oxygen species, total antioxidant capacity (TAC), and Malondialdehyde MDA in all groups of rats. Values are expressed as mean ± SD for 6 rats/group. *,**,***Sham-operated rats at $p<0.05$, $p<0.01$; and $p<0.001$, respectively. #,##,###Sham + melatonin (Mel) at $p<0.05$, $p<0.01$; and $p<0.001$, respectively. $$,$$$2-VO-induced rats + melatonin (Mel) at $p<0.01$ and $p<0.001$, respectively

remained slightly but significantly increased, protein levels of p-P38 and p-JNK1/2 were not significantly different as compared to their levels measured in sham-operated rats (Fig. 4a–c).

Melatonin Enhanced Cholinergic Function and Upregulated BDNF in the Hippocampi of Control and Rats with Induced CHP

Significantly lowered levels of ACh, protein levels of BDNF, and AChT activity with a concomitant increase in the activity of AChE were seen in the hippocampi of 2-VO rats, as compared to sham-operated rats (Fig. 5a–d). On the contrary, sham + Mel and 2-VO + Mel-treated rats had significantly increased levels of ACh, protein levels of BDNF, and activity of AChT and had a significant decrease in the activity of AChE, as compared to sham or 2-VO-treated rats respectively (Fig. 5a–d). Among all, the activity of AChE remained slightly and significantly higher than its levels measured in the hippocampi of sham-operated rats (Fig. 5a–d).

Melatonin Downregulated mRNA Levels of TNF-α and IL-1β in the Hippocampi of Control and Rats with Induced CHP

Total levels of PKA were not significantly altered in all groups of rats. However, there was a significant increase in mRNA levels of TNF-α and IL-1β with a concomitant decrease in p-PKA in the hippocampi of 2-VO-induced rats (Fig. 6a–c). The mRNA levels of TNF-α and IL-1β, as well as, protein levels of p-PKA were significantly decreased whereas the levels of p-Akt were significantly increased in the hippocampi of both control + Mel and 2-VO + Mel treated rats (Fig. 6a–c).

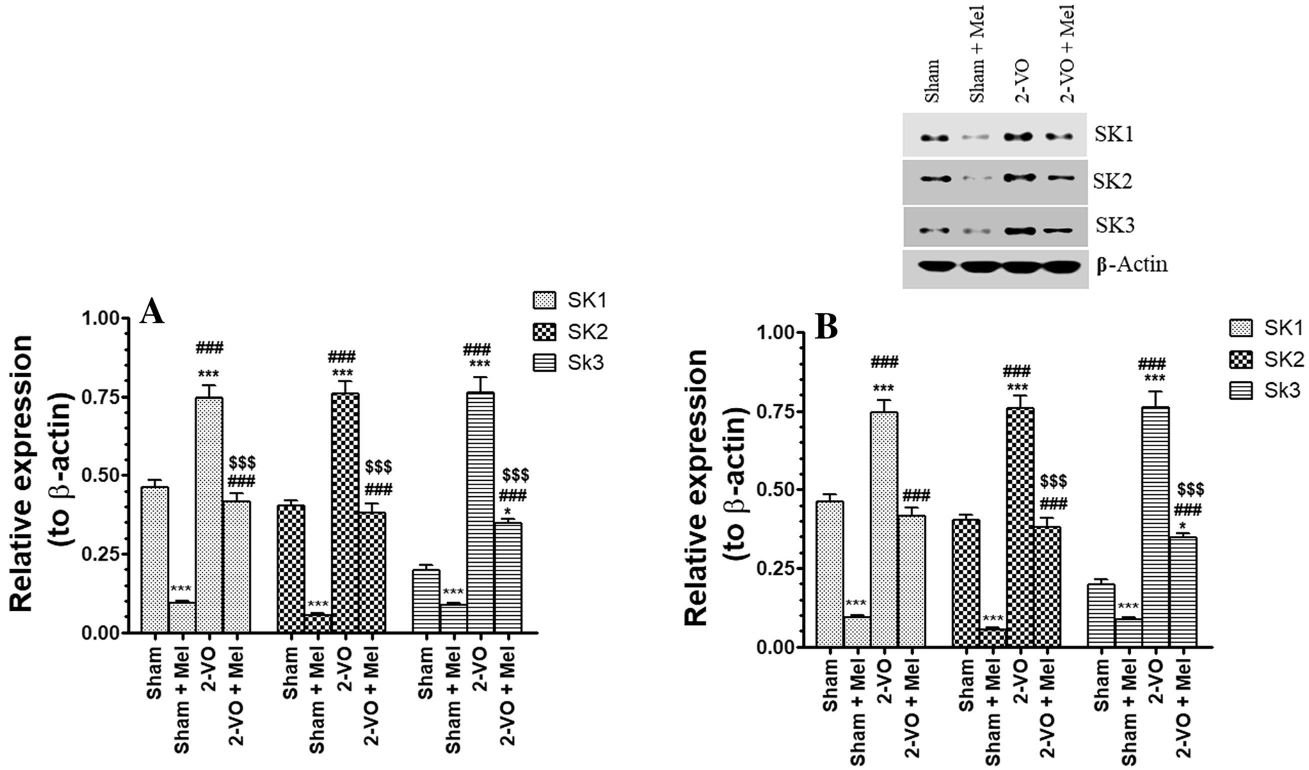


Fig. 3 mRNA and protein levels of SK1, SK2, and SK3 in the hippocampi of all groups of rats. Values are expressed as mean ± SD for 6 rats/group. *,***Sham-operated rats at $p < 0.01$ and $p < 0.001$, respectively. ###Sham + melatonin (Mel) at $p < 0.001$. $$$2-VO-induced rats + melatonin (Mel) at $p < 0.001$

Melatonin Downregulates all MAPKs and SK Channels in Cultured Hippocampal Cells

As in the in vivo experiments, all SK channels were expressed in the cultured hippocampal cells isolated from rats. Exposure of cultured hippocampal cells to hypoxia, with or without DMSO, significantly increased the protein levels of p-ERK1/2, p-JNK, p-P38, SK1, SK2, and SK3 and significantly reduced cell survival as compared to the normoxic cells (Figs. 7a–c, 8a–d). The addition of melatonin to the hypoxic cultures significantly lowered the proteins levels of p-ERK, p-JNK, p-P38, SK1, SK2, and SK3 and significantly increased cell survival, as compared to their corresponding levels in hypoxic cells (Figs. 7a–c, 8a–d).

Upregulation of All Three SK Channels Requires Activation of ERK1/2 but not JNK or P38

Given that all MAPKs were upregulated after CHP in vivo and after hypoxia episodes in vitro and because activation of MAPKs can modulate channel expression levels, we aimed to determine which MAPK pathway is responsible for the observed upregulation of SK channels. For this purpose, we pre-incubated the cultured hippocampal neurons with specific blockers (or their inactive analogs as controls) of ERK1/2, JNK, and P38 before induction of hypoxia. Of note, pre-incubation of the hippocampal cells with U0126 (a MEK1/2 inhibitor) but not with its inactive analog, U0124, before hypoxia exposure significantly downregulated and prevented the upregulation of SK1, SK2, and SK3 in the hypoxic cells and significantly enhanced cell survival (Fig. 7b, d). In contrast, pre-incubation of the hypoxic cells with SB203580 (a P38 inhibitor), SP600125 (a JNK inhibitor), or with their inactive analogs did not affect the expression levels of SK1, SK2, or SK3 and their levels remained significantly higher relative to those measured in hypoxic cells (Fig. 8b, d). Among these cells, JNK inhibition increased cell survival and P38 inhibition did not improve cell survival (Fig. 7a), suggesting that inhibition of ERK1/2 and JNK after hypoxia are major mechanisms responsible for the increased hippocampal cell survival and could be the key signaling pathways targeted by melatonin to enhance short- and long-term memory.

Discussion

The exclusive salient findings of this study indicate that (1) CHP induced by the 2-VO procedure impaired memory function and upregulated all three SK channels (SK1, SK2, and SK3) in the hippocampi of rats, in association with increased ROS generation, activation of all MAPK members (ERK1/2, JNK1/2, and P38), and downregulation of BDNF. (2) Besides its ability to restore normal function of the cholinergic system, daily i.p dose of melatonin at 10 mg/kg enhanced memory function in rats with induced CHP by attenuating the increased hippocampal protein levels of all the members of the SK channel family, an effect that is mediated by amelioration of oxidative stress, downregulation of all the members of the MAPK family, and increasing protein levels of BDNF and activity of PKA. (3) In vitro inhibition of MAPKs in cultured hippocampal cells confirmed that activation of ERK1/2 is the signal transduction pathway primarily responsible for the upregulation of all kinds of SK channels and highlights their pathological role in memory deficits after CHP.

Brain CHP is associated with rapid depletion of energy stores and both receptor-dependent and receptor-independent excitotoxicity [1, 2]. Although the massive toxic increase in $[Ca^{2+}]_i$ that is mediated by the direct activation of NMDA receptors and indirectly through L-type Ca^{2+} channels is the hallmark of glutamate receptor excitotoxicity, rapid depletion of glutathione (GSH) stores characterizes glutamate-independent receptor excitotoxicity [1, 2]. Taken together, the increases in ROS and nitrogen reactive species of mitochondrial origin, as well as, increased $[Ca^{2+}]_i$ are considered the main events leading to the death of hippocampal pyramidal neurons and the associated cognitive deficits in rodents, primates, and humans after CHP [1, 34]. Increased ROS generation, cholinergic system dysfunction, and impairment in memory have been described in a rat model of CHP [21, 35–37].

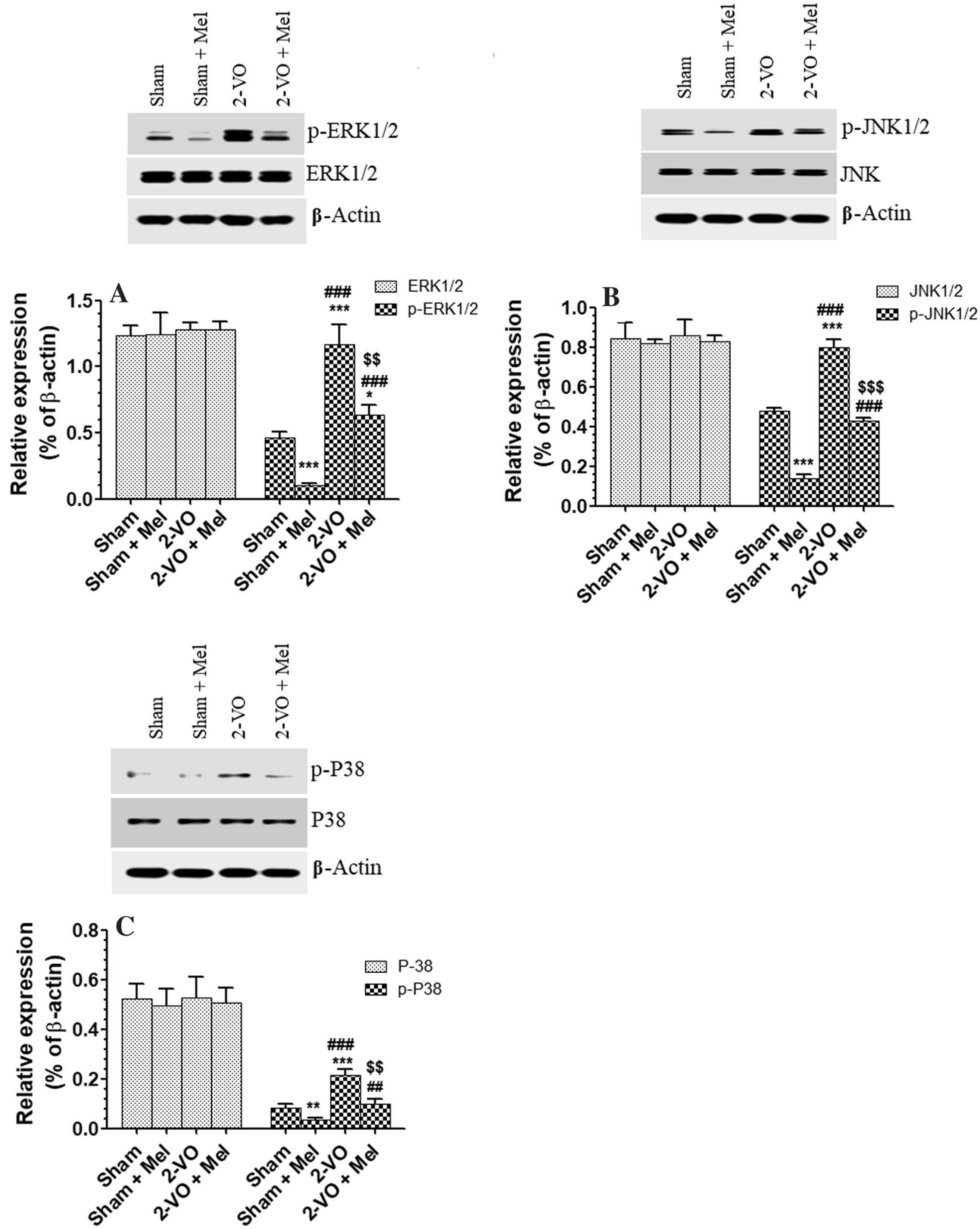
In this study, we primarily aimed to validate our model in relation to ROS generation and its associated memory deficits. In the same line to the above-mentioned study, 2-VO-induced rats showed a decline in retention and spatial memory function as measured by PALT and WMS, respectively. Their hippocampi also these contained significantly lower levels of TAC with concomitant increases in ROS and MDA concentrations, thus supporting numerous evidence in the literature that ROS production is a hallmark of CHP-induced memory loss [1, 2].

Nonetheless, CHP-treated rats had also significantly decreased levels of ACh and AChT activity and had higher activity of AChE, thus demonstrating dysfunction of hippocampal cholinergic function and/or damage of cholinergic neurons possibly due to ROS/Ca^{2+} induced apoptosis. In support of these data, a positive correlation between the levels of ROS and cholinergic disturbances has been established in chronic CHP rat's model and were a major cause of associated cognitive deficits [1, 36–39]. Indeed, ROS are able to induce cholinergic cell death leading to decrease in brain levels of ACh and AChT [1]. Furthermore, chelation of intracellular Ca^{2+} inhibits AChE expression [38]. In addition, it has been shown that CHP can inhibit the PI3K-Akt signal transduction pathway leading to decreased expression of BDNF [40].

In contrast, a low dose of melatonin significantly improved the retention and spatial memory function in 2-VO-induced rats and successfully decreased oxidative stress, and restored normal function of the cholinergic system and normal levels of BDNF. These results are not surprising given the long history of antioxidant and free-radical–scavenging abilities of melatonin and its metabolites, especially N1-acetyl-N2-formyl-5-methoxykynuramine, which protect from ischemia-induced neural damage [13]. In the same line, melatonin attenuated chronic CHP-induced cognitive impairment and hippocampal neuronal cell damage (after 28 days of CHP) by decreasing oxidative stress, microglial activation and the production of pro-inflammatory cytokines in the ischemic hippocampus of rats [21, 22]. In addition, melatonin can restore normal levels of ACh and AChT and inhibit AChE in the ischemic rat brain and has been found to be useful as an AChE and butyrylcholinesterase inhibitor, especially its cyclic 3-hydroxymelatonin analogs [14, 41, 42].

However, all of the above-mentioned evidence is just confirmatory to the previously published studies that showed possible mechanisms by which CHP induces neural damage and memory deficit and possible actions of melatonin. However, in this study, we aimed to go further and study the expression pattern and possible role of SK channels in memory function during CHP with or without melatonin treatment. This is being based on scientific evidence that showed the presses of all three types of SK channels (SK1, SK2, and SK3) in the hippocampus of rodents and humans [4], and their crucial role they play in regulating cell plasticity, as well as, learning and memory function in both animals and humans [5, 6, 43, 44].

Indeed, blocking or inhibiting these channels increases long-term potentiation (LTP) and improves learning in rodents, whereas stimulation of these channels decreases LTP and learning abilities [43]. Besides, reduced mRNA levels of SK2 and SK3 at the early stages of a spatial learning task were reported [6]. Moreover, elevated expression of SK3 with concomitant reductions in the performance on learning tasks, in synaptic plasticity, and in LTP are seen in the hippocampus of aged mice [7]. Furthermore, in vivo, pharmacological inhibition of SK1 and SK3 with apamin accelerates hippocampus-dependent spatial and


Sham
Sham + Mel
2-VO
2-VO + Mel
p-ERK1/2
ERK1/2
β-Actin
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JNK
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◂**Fig. 4** Protein levels of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and P38 in the hippocampi of all groups of rats. Values are expressed as mean ± SD for 6 rats/group. **,***Sham-operated rats at $p < 0.01$ and $p < 0.001$, respectively. ##,###Sham + Melatonin (Mel) at $p < 0.01$ and $p < 0.001$, respectively. $$,$$$2-VO-induced rats + Melatonin (Mel) at $p < 0.01$ and $p < 0.001$, respectively

non–spatial-memory encoding and improves performance on the novel object recognition task [42, 43]. Besides, memory deficits are seen in mice overexpressing SK2 [3].

Supporting our initial hypothesis, our data for the first time showed increased mRNA and protein levels of SK1, SK2, and SK3 in the hippocampi of 2-VO-induced rats or hypoxic hippocampal cells, all of which were restored by melatonin administration, thus pointing to a possible new pathway of melatonin-induced memory protection via inhibition of SK1, SK2, and SK3 expression. Of big interest is the reduced expression of all these channels in the brains of sham-operated rats administered melatonin. However, the regulation of SK channels in health and disease is poorly described. Nevertheless, in this study, we targeted the role of MAPKs in the regulation of SK channels in the hippocampi of rats multiple reasons. First of all, they are involved in neural-cell survival and death [16, 19], and second, they mediate neuronal synaptic plasticity as well as expression and activation of various ion channels [15]. In addition, melatonin is known to exert its neuroprotective action in animal models of ROS-induced neural injury by modulating MAPKs [20].

After ischemia, ROS and increased [Ca^{2+}] are the best activators of all kinds of MAPKs within various brain regions including the hippocampus [15, 17]. In the ischemic brain or hypoxic cultured neurons, all members of MAPK pathways are activated and the use of specific inhibitors against various MAPKs has confirmed their participation in the cell survival and/or apoptosis as well as memory function [15, 16]. Similarly, in the present study, protein levels of ERK1/2, JNK1/2, and P38 were significantly upregulated in the hippocampus of 2-VO-induced rats and in cultured hypoxic neurons. By contrast, incubating the hippocampal neurons with U0126 and SP600125, specific inhibitors of ERK1/2 and JNK1/2, respectively, enhanced cell survival, whereas pharmacological inhibition of P38 did not rescue cells after hypoxia induction. These data are suggestive of the importance of ERK1/2 and JNK1/2 activation for CHP-induced cell death. Moreover, melatonin significantly inhibited the activity of all MAPKs in the hippocampi of sham-operated rats and 2-VO-induced rats, as well as, in cultured neurons exposed to hypoxia. In support of these findings, melatonin protects the animal brain and restores normal behavior by downregulating all MAPKs after exposure to ischemia [20].

The novel finding of this study is that pharmacological inhibition of JNK and P38 phosphorylation did not affect the expression of SK channels. Nevertheless, the inhibition of ERK1 signaling alone (by U0126) abrogated the increase in the expression of SK1, SK2, and SK3 channels, thereby pointing to the role of this pathway in the memory disturbance after ischemia or CHP. Hence, in addition to its neuroprotective and anti-apoptotic known effects, it can be concluded that inhibition of ERK1/2 mediates melatonin neuroprotection and may participate in the inhibition of SK channels, and all these changes lead to improvement of memory function in healthy as well as after CHP.

Overall, these data clearly identify a possible cross-talk between memory function and expression of SK channels including SK1-3 during CHP and melatonin therapy which are mediated mainly by ERK1/2. However, in spite of these findings, the precise mechanisms by which CHP and melatonin exert these effects remain elusive. However, an important observation in this study remains the significant decrease in BDNF protein levels in the hippocampi of 2-VO-induced rats and its higher levels in the hippocampi of control and 2-V-induced rats administered melatonin which correlates with the reduction/increase in activities of MAPKs and all SK channels. Recent evidence has shown the ability of BDNF to inhibit SK2-mediated AHP and their surface receptor expression possibly by activating serine/threonine protein kinases and most likely protein kinase A (PKA) [45, 46]. Indeed, activation of PKA in COS7 cells repressed the surface expression SK2 while the inhibition of PKA increases it [47]. Based on these results, those authors have suggested that BDNF might enhance the induction of LTP by inhibiting SK-mediated currents through the reduction of SK2 surface expression in CA1 pyramidal neurons, through PKA-dependent mechanism. Of interest, the activity of PKA was significantly reduced in the hippocampi of PKA 2-VO-induced rats and was significantly increased in the hippocampi of both sham-operated and 2-VO-induced rats which administered melatonin. These findings are of much important and could suggest that CHP and melatonin could modulate the expression of all SK channels through BDNF/PKA signaling. However, our data still can't definitely confirm this and further studies at the molecular targeting levels to inhibit BDNF is required to confirm it which will be an interesting area to study.

On the other hand, this study still has some limitations. Most importantly and even their expression was modulated in the hippocampi of rats, in vivo and in vitro, the effect of CHP and melatonin treatment could also affect the expression of SK1-3 in other brain cell types, thus leading to neuroprotection. In this regards, the expression of SK1-3 channels was shown to occur (with various degrees of expression) in neural cells including the limbic system, neocortex, cerebellum, dopaminergic neurons, etc. as well as in non-neural cells including microglial cells and cerebral vasculature [48, 49].

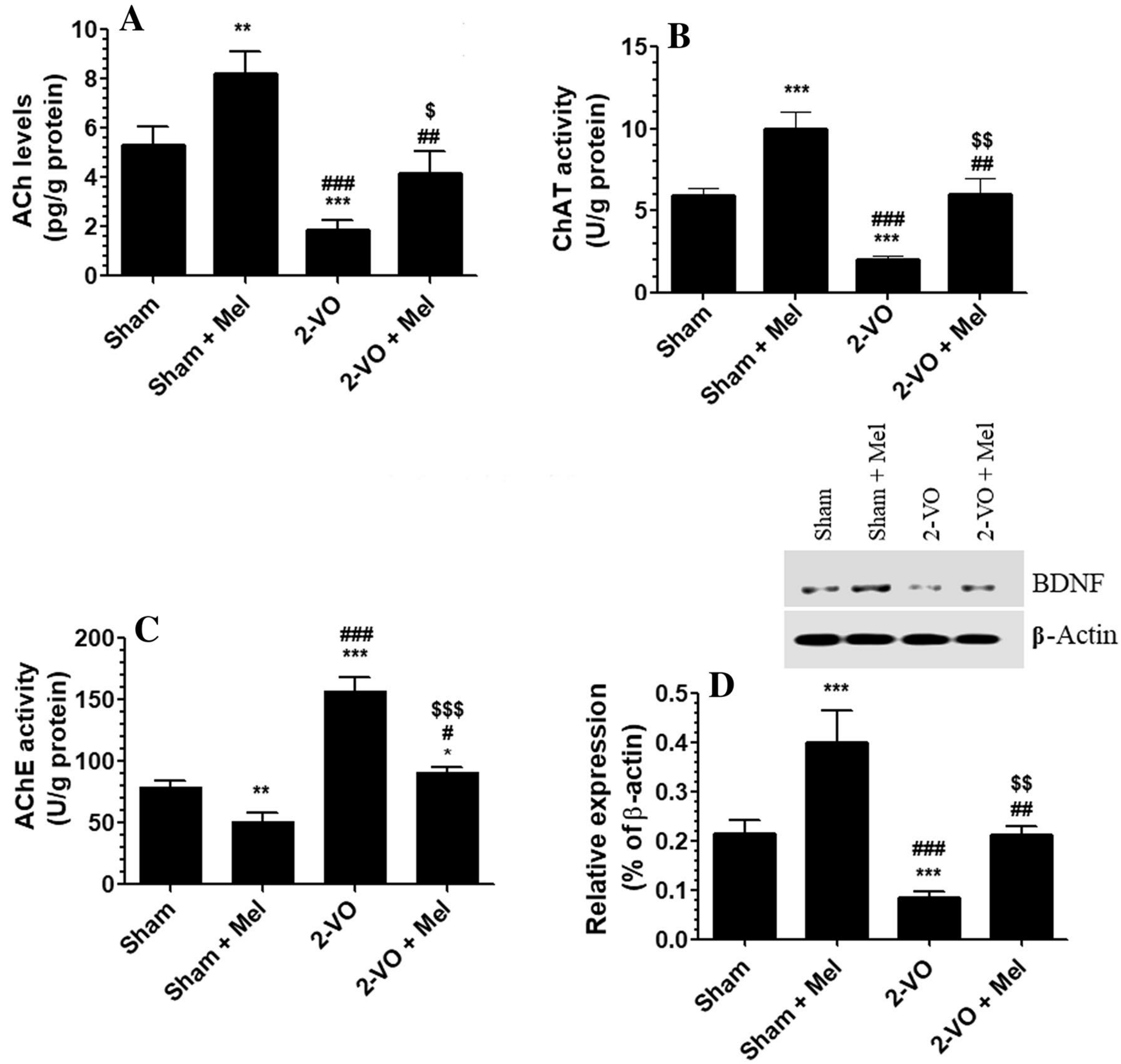

Fig. 5 Levels of acetylcholine (Ach) and activities of choline acetyltransferase (AChT) and AChE acetylcholine esterase (AChE) and protein levels brain-derived neurotrophic factor (BDNF) in the hippocampi of all groups of rats. *,**,***Sham-operated rats at $p<0.05$, $p<0.01$; and $p<0.001$, respectively. #,##,###Sham + melatonin (Mel) at $p<0.05$, $p<0.01$; and $p<0.001$, respectively. $,$$,$$$2-VO-induced rats + melatonin (Mel) at $p<0.05$, $p<0.01$, and $p<0.001$, respectively

Microglial cells are macrophage-like cells of the CNS which are activated by certain insults such ischemia, trauma and microbial invasion, which rapidly response by changing their shapes, upregulating a large number of receptor types, and producing a variety of potentially cytotoxic molecules such as pro-inflammatory cytokines, reactive oxygen intermediates, complement proteins and proteinases, which potentially results in a chronic state of neuroinflammation and neurodegeneration [50-52]. Therefore, it has been suggested that the suppression of microglial activation is a gold strategy to inhibit neurodegeneration after stroke [51, 52]. However, microglial activation and subsequent neuroinflammatory response are hallmarks of experimentally-induced CHP in rats and mice and are a major cause of neural cell death and memory loss [53-55]. Of interest, all SK channels types (1–3) have been detected in microglial cells [48, 49, 56, 57] where SK3 channels were involved in the process of their activation through p38 MAP kinase but not through NF-κB [53]. Indeed, Blocking SK3 channels in microglia reduced their ability to induce apoptosis, caspase 3 activation and tyrosine nitration in neurons [53].

Additionally, it was shown that intronic promoter region of SK2/K_{Ca}2.2 channels in the neurons contains two responsive elements of NF-κB [58]. During microglial activation, the increase TNF-α can facilitate the activation of NF-κB signaling in the neurons and upregulated SK2 channels expression [48, 59]. In this study, even we didn't directly measure the activation of microglial cells, neuroinflammation in the

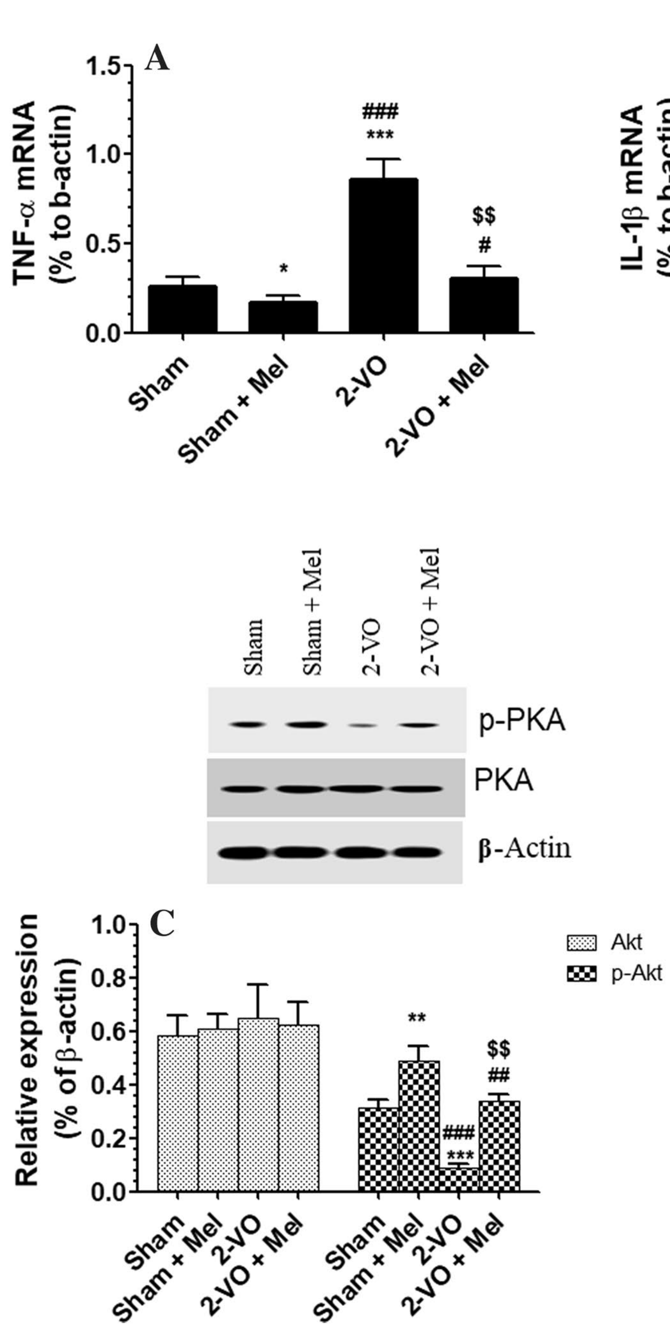

Fig. 6 mRNA of tumor necrosis factor-α and interleukin 1β and protein levels of Akt in the hippocampi of all groups of rats. *,**,***Sham-operated rats at $p<0.05$, $p<0.01$, and $p<0.001$, respectively. #,##,###Sham + melatonin (Mel) at $p<0.05$, $p<0.01$, and $p<0.001$, respectively. $,$$2-VO-induced rats + melatonin (Mel) at $p<0.05$ and $p<0.01$, respectively

hippocampi of 2-VO-induced rats was evident by the significant increase in mRNA levels of IL-1β and TNF-α, both of which were significantly inhibited in the hippocampi of melatonin-treated control or 2-VO-induced rats. Hence, it could be possible that the increase in all SK channels post CHP in rats' hippocampi are mediated by activation on microglial cells and the effect of melatonin is triggered basically by inhibition of neural inflammation and microglia activation. Indeed, melatonin is able to inhibit microglial activation and inhibit neural inflammation, in vivo and in vitro in various models including CHP-induced rats [60-62]. Unfortunately, we didn't measure markers of neuroinflammation and never tested the role of microglial cells in the regulation of SK channels in the hippocampi of rats after CHP with or without melatonin. However, even suggested here as a possible cause, this still remains unlikely given the similar pattern of expression of all

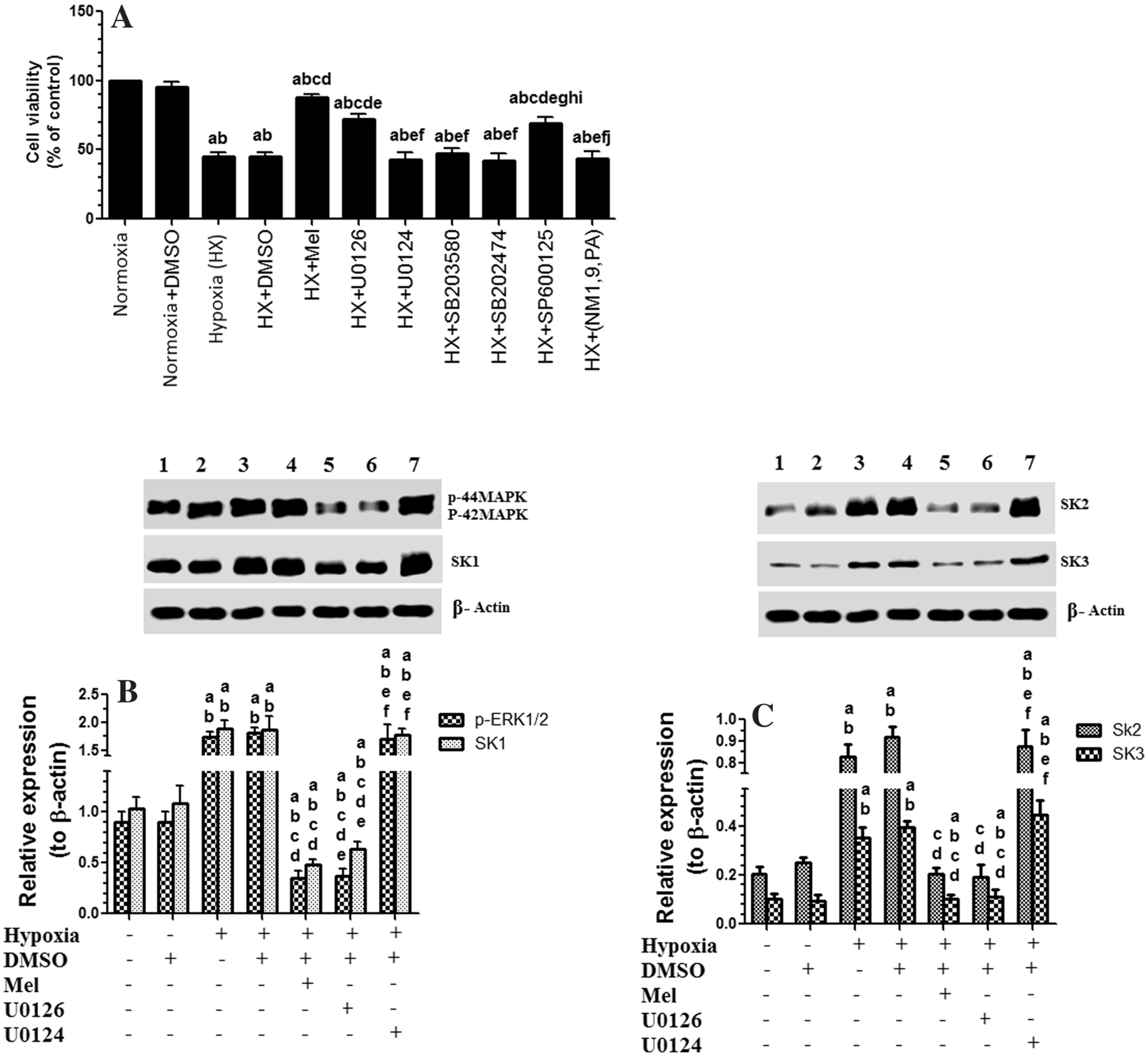

Fig. 7 Cell viability evaluation and protein levels of extracellular signal-regulated kinase 1/2 (ERK1/2) and small-conductance Ca^{2+}-activated K^+ channels (SK1, SK2, and SK3) in the hippocampal neural culture of all treatment groups. Values are expressed as mean ± SD for 5 samples/group. Values were considered significantly different at $P < 0.05$. [a]Significantly different when compared to the normoxia group. [b]Significantly different when compared to group NX + DMSO. [c]Significantly different when compared to group hypoxia (HX). [d]Significantly different when compared to HX + DMSO. [e]Significantly different when compared to HX + Mel. [f]Significantly different when compared to HX + U0126. [g]Significantly different when compared to HX + U0124. [h]Significantly different when compared to HX + SB203580. [i]Significantly different when compared to HX + SB202474. [j]Significantly different when compared to HX + SP600125. *Mel* melatonin

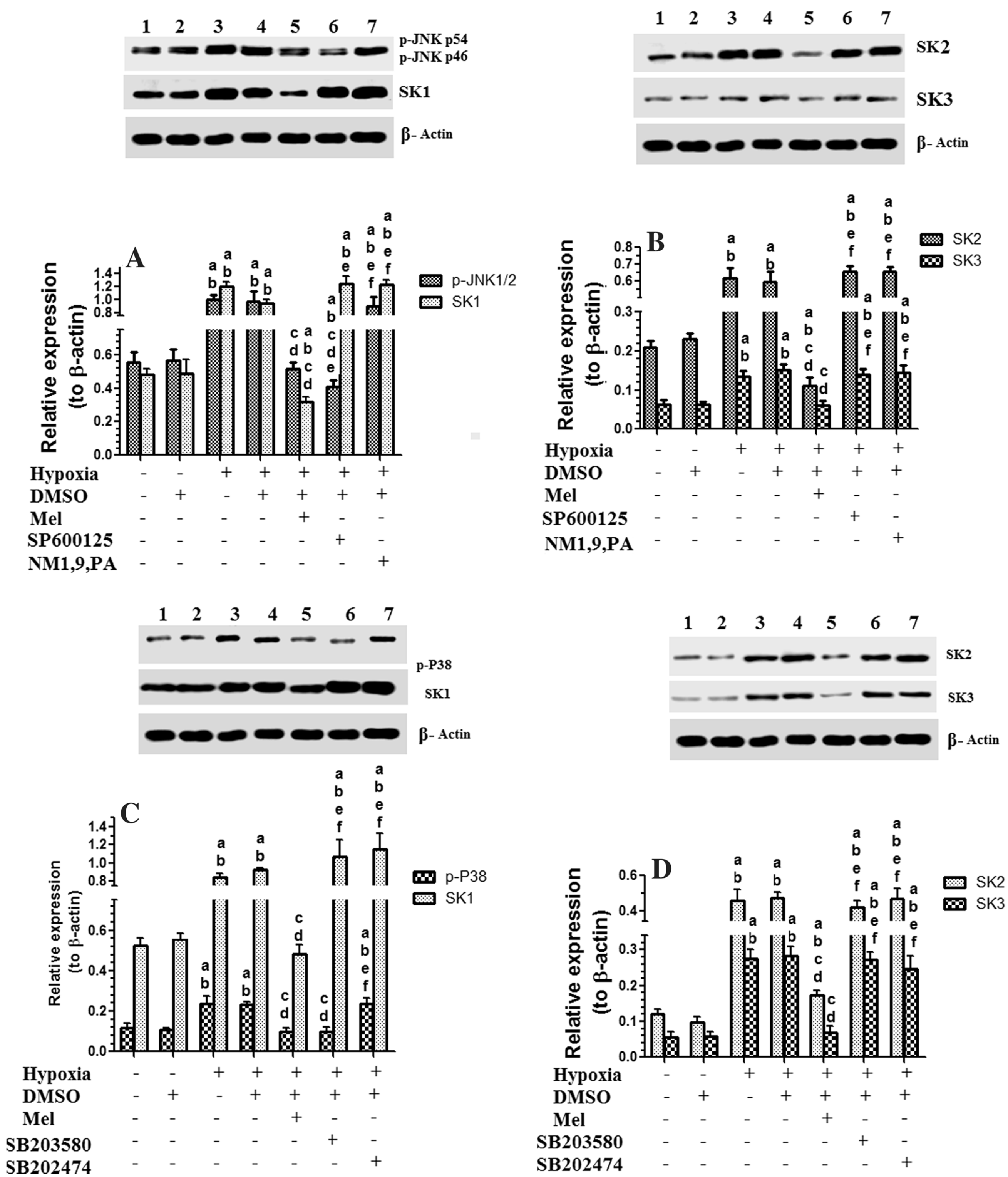

Fig. 8 Protein levels of Jun N-terminal kinase (JNK), P38, and small-conductance Ca^{2+}-activated K^+ channels (SK1, SK2, and SK3) in the hippocampal neural culture of all treatment groups. Values are expressed as mean ± SD for 5 samples/group. Values were considered significantly different at $P < 0.05$. [a]Significantly different when compared to group normoxia. [b]Significantly different when compared to the normoxia + DMSO group. [c]Significantly different when compared to group hypoxia. [d]Significantly different when compared to the hypoxia + DMSO group. [e]Significantly different when compared to group hypoxia + Mel. [f]Significantly different when compared to HX + SP600125 (**a, b**) or to HX + SB203580 (**c, d**). *Mel* melatonin

these channels in a cultured hypoxic hippocampal cell with or without melatonin treatments in absence of microglial cells.

Conclusion

Our study is showing that neurodegeneration and memory deficits that develop in the rat model of CHP are associated with ERK1/2-induced overexpression of all types of SK channels. Through its antioxidant properties and its ability to inhibit MAPKs in the rat hippocampus, melatonin protects from CHP-induced memory loss at least via inhibition of ERK1/2-induced upregulation of SK1, SK2, and SK3 channels, a mechanism that is could be related to upregulation of BDNF-induced activation of PKA or possibly due to inhibition of CHP-induced neuroinflammation.

Acknowledgements The authors would like to thank the technical staff of the animal facility at the Medical College of King Khalid University for their help in the experimental procedure and care of animals this study. The authors also would like to express their gratitude to the deanship of scientific research at King Khalid University for their financial support of the current study via their grant to HFS.

Author Contributions HAD, SME, MAK, and HFS conceived and designed the experimental procedures. HAD, HFS, AMH, and MAA performed the experimental procedure and wrote the material and methods. IH, MA, SME, SA and MAA, MD performed the analysis and revised the final version of the manuscript. HAD, MD, and MAA, MAK, and SA were responsible for the interpretation of the results and wrote the manuscript.

Funding This research is fully funded by the deanship of scientific research at King Khalid University (Grant number KKU_S099_33, 2014).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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