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Modulation of α7nAchR by Melatonin Alleviates Ischemia and Reperfusion-Compromised Integrity of Blood Brain Barrier through Inhibiting HMGB1-Mediated Microglia Activation and CRTC1-Mediated Neuronal Loss

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Abstract

The only food and drug administration (FDA)-approved drug currently available for the treatment of acute ischemic stroke is tissue plasminogen activator (tPA), yet the therapeutic benefits of this drug are partially outweighed by the increased risk of hemorrhagic transformation (HT). Analysis of the NIH trial has shown that cigarette smoking protected tPA-treated patients from HT, however, the underlying mechanism is not clear. Nicotinic acetylcholine receptors (nAChR) has shown anti-inflammatory effect and modulation nAChR could be a strategy to reduce ischemia/reperfusion-induced blood brain barrier (BBB) damage. Since melatonin could regulate the expression of α7nAchR and melatonin's neuroprotective effect against ischemic injury is mediated via α7nAChR modulation, here, we aim to test the hypothesis that melatonin reduces ischemia and reperfusion (I/R)-induced BBB damage through modulation of α7nACh receptor (α7nAChR). Mice were subjected to 1.5 h ischemia and 24 h reperfusion and at the onset of reperfusion, mice received intraperitoneal administration (i.p.) of either drug or saline. Mice were randomly assigned into five groups: Saline; α7nAChR agonist PNU282987; Melatonin; Melatonin + Methyllycaconitine (MLA, α7nAChR antagonist) and MLA group. BBB permeability was assessed by detecting the extravasation of Evan's blue and IgG. Our results showed that I/R significantly increased BBB permeability accompanied by occludin degradation, microglia activation, and high mobility group box 1 (HMGB1) release from the neuron. In addition, I/R significantly induced neuronal loss accompanied by the decrease of CREB regulated transcriptional coactivator 1 (CRTC1) and p-CREB expression. Melatonin treatment significantly inhibited the above changes through modulating α7nAChR. Taken together, these results demonstrate that melatonin provides a protective effect on ischemia/reperfusion-induced BBB damage, at least in part, depending on modulation of α7nAChR.

Introduction

Blood brain barrier (BBB) damage after ischemia significantly influences stroke outcome (Liu et al. 2020). The main effective strategy for saving ischemic brains is the rapid revascularization of arterial territories to restore tissue perfusion (Grossman and Broderick 2013). Tissue plasminogen activator (tPA) is currently the only FDA-approved drug available for the treatment of acute ischemic stroke in the clinic. However, the therapeutic benefits of tPA are outweighed by its narrow treatment time window (3-4.5h) (Tissue plasminogen activator for acute ischemic stroke 1995) with the risk of a more than six-fold increase in hemorrhagic transformation (HT) after thrombolysis (Intracerebral hemorrhage after intravenous t-PA therapy for ischemic stroke. The NINDS t-PA Stroke Study Group 1997) and the high mortality following hemorrhage. These factors severely limited clinical application of tPA (Wardlaw et al. 2012). The damage of the integrity of BBB which starts at the ischemic stage and aggravates at the reperfusion stage (Jin et al. 2014), is the pathological basis of HT after tPA treatment in stroke, especially following delayed tPA treatment (Jickling et al. 2014; Liebner et al. 2018). Exploring the underlying mechanism of the BBB disruption is desirable for understanding the adverse effects of tPA therapy. Although reperfusion-induced BBB injury has been an important research topic for decades and protecting the integrity of BBB is known to effectively alleviate cerebral ischemic damage (Won et al.

2015; Chen et al. 2009), there is no effective treatment that has been approved by the FDA to protect BBB from reperfusion-induced damage so far.

Analysis of a landmark National Institutes of Neurological Disorders and Stroke (NINDS) recombinant tPA (r-tPA) stroke trial indicated that cigarette smoking protected tPA-treated patients from HT (Tissue plasminogen activator for acute ischemic stroke 1995; Intracerebral hemorrhage after intravenous tPA therapy for ischemic stroke. The NINDS tPA Stroke Study Group 1997). The prevalence of HT among tPA-treated smokers was significantly lower than the non-smoking tPA-treated patients, indicating that a smoking-triggered cascade possibly protected the BBB from ischemia/reperfusion (I/R)-induced damage. Nicotine has been shown to increase the production of plasminogen activator inhibitor-1 (PAI-1), a tPA inhibitor, within brain endothelial cells (Zidovetzki et al. 1999). More importantly, nicotine plays a protective role in reducing the toxicity of the tar and nitric oxide (components in cigarettes) to endothelial cells (Naik et al. 2014). However, due to the addiction and other side effects (Semenova et al. 2018), nicotine is not a feasible or ethical clinical treatment for patients. More recently, the cholinergic anti-inflammatory pathway has been found to inhibit the release of cytokines by the modulation of nicotinic acetylcholine receptors (nAChRs) (Pavlov et al. 2009). Hence, modulation nAChRs may be a strategy to reduce I/R-induced BBB damage.

The α7 subtype of nicotinic acetylcholine receptor (α7nAChR) is the second most abundant receptor in the nervous system and possesses multiple biological roles in neuronal survival, neurodegeneration and cognitive processes (Bencherif et al. 2014). Accumulating evidence shows that activation of α7nAChR can alleviate BBB disruption induced by I/R (Han et al. 2014a; Han et al. 2014b; Zou et al. 2017), hemorrhage (Krafft et al. 2012; Krafft et al. 2013), subarachnoid hemorrhage (SAH) (Duris et al. 2011) and experimental traumatic brain injury (Dash et al. 2016) through decreasing neuroinflammation and oxidative stress (Han et al. 2014b) and increasing the expression of claudin-5 and occludin (Kimura et al. 2019). However, in clinical trials of schizophrenia-associated cognitive deficits, multiple α7nAChR agonists including EVP-6124 and GTS-21 failed to be approved for marketing because of the side effect of cardiotoxicity (Beinat et al. 2015).

High mobility group box 1(HMGB1) not only plays a critical role as a nuclear DNA-binding protein, but also as a cytokine-like mediator of systemic inflammation, for example, HMGB1 plays an important role in BBB damage after stroke (Nishibori et al. 2020). In addition, serum HMGB1 levels were significantly elevated in patients with cerebral ischemia (Goldstein et al. 2006) and HMGB1 has been reported to be released from neurons early following ischemic injury, and acts as a mediator linking acute brain damage and subsequent inflammatory processes (Qiu et al. 2008). Of note, α7nAChR is involved in OGD-induced HMGB1 release in primary cultured neurons, and expression of α7nAChR was significantly decreased after reoxygenation (Wang et al. 2012).

Melatonin improves the functional integrity of endothelial cells (Hobson et al. 2018; Lee et al. 2018; Zhao et al. 2018), especially, shown protective effects against BBB disruption that was induced by excitotoxicity in neonatal rats (Moretti et al. 2015), transient focal cerebral ischemia in young mice (Chen

et al. 2006b; Chen et al. 2006a) and lipopolysaccharide challenge in old mice (Wang et al. 2017). It is noteworthy that melatonin attenuates neuroinflammation and α7nAchR mRNA expression in lipopolysaccharide-stimulated rat astrocytoma cells (Niranjan et al. 2012). In addition, melatonin regulates the autophagic flux via upregulation of α7nAchR (Jeong and Park 2015), more important, Parada et al. showed that the melatonin's neuroprotective effect against ischemic injury is mediated via α7nAChR modulation (Parada et al. 2014). However, it is unclear whether the effect of melatonin on I/R-induced BBB damage is through modulation of α7nAChR, and if it is, the underlying mechanism is even not clear.

Here, we aim to investigate the effect of melatonin on I/R-induced BBB disruption in mice and examine the relationship between melatonin and α7nAChR modulation. Our results indicated that melatonin significantly reduced I/R-induced BBB disruption, occludin degradation, microglia activation, increased the loss of neuronal and the release of high mobility group box 1 (HMGB1) from the neuron, and decreased p-CREB and CREB regulated transcriptional coactivator 1 (CRTC1) expression. These effects could be reversed by the α7nAChR antagonist.

Materials And Methods

Animals

C57BL/6J male mice (8-10 weeks, 23-25g), purchased from SLAC Company (Shanghai, China), were housed 5 per cage under the vivarium with constant temperature (23±1°C) and controlled light (12-h light/12-h dark cycle). All animals had free access to food and water. The Soochow University Committee on Animal Care approved the animal procedures which were performed following the NIH Guide for the Care and Use of Laboratory Animals. Details of animal use could be found in Figure legends.

Focal Cerebral Ischemia and Reperfusion model in mice

During surgical procedures, C57BL/6 mice were anesthetized with isoflurane (4% for induction, 1.75% for maintenance) in a mixture of N_2 and O_2 (70% N_2: 30% O_2), with the body temperature kept constant at 37.5±0.5°C by a heating pad. To mimic cerebral ischemic stroke, middle cerebral artery (MCA) occlusion (MCAO) was induced using an intraluminal monofilament as described previously (Gu et al. 2012). To summarize, the right common and internal carotid arteries were isolated and ligated through a midline incision of the neck under a microscope. A 6-0 nylon monofilament thread with silicon-coated tip was inserted into the right internal carotid artery through the common carotid artery, blocking the blood flow to MCA. After 90 min of occlusion, the thread was removed to allow reperfusion for 24 h. After completion of the surgical procedures, the incision was sutured and the mice were placed in a controlled temperature condition (24-25°C) to recover from anesthesia.

Drug administration and experimental groups

After MCAO, mice were randomly assigned into five different treatment groups: 1,“Saline”(containing 2% DMSO) group; 2, PNU282987 (a potent agonist of α7 nAChR,10 mg/kg) group; 3, Melatonin (15 mg/kg) group; 4, Melatonin+methyllycaconitine (MLA, an antagonist of α7 nAChR, 6 mg/kg) group; 5, MLA (6 mg/kg) group. The mice were intraperitoneally administered with either saline or one of the four treatments at the onset of reperfusion.

Assessment of Evan’s blue (EB) dye leakage

Leakage of EB dye from brain is a marker of BBB disruption (Sun et al. 2017). Hence, BBB disruption was determined by assessing the extravasation of EB dye (Sigma). EB (2%) was injected (3 ml/kg) through the tail vein after 22 h of reperfusion. After 2 h of the EB injection, mice were perfused transcardially with ice-cold PBS, then the brain was quickly removed and sliced into 1 mm coronal sections with a brain matrix. EB leakage appeared as blue on brain sections and quantitative assessment was done by detecting the EB contents (Liu et al. 2017). In brief, ischemic and non-ischemic brain hemispheres were weighed and homogenized in 50% wt/vol trichloroacetic acid (Sigma). After centrifugation (14000 g for 15 min) at 4°C, the supernatant was collected, and the OD values of supernatants were measured at 620 nm on a microplate fluorescence reader (Infinite M200 Pro; TECAN, Grodig, Austria). The quantity of EB was calculated according to the gradient concentrations of EB standard curve. The dye content (μg) detected in each sample was quantified as EB leakage and expressed as per gram of brain tissue (μg/g). The EB leakage was analyzed by investigators that were blind to treatment group designation.

Evaluation of BBB permeability by detection of immunoglobulin G leakage

Immunoglobulin G (IgG) leakage is another method to evaluate BBB permeability. As we described previously (Wang et al. 2016), the 20-μm-thick cryosection was fixed with 4 % paraformaldehyde (PFA) for 20 min, then incubated with Cy3-conjugated Affinity Pure Goat anti-Mouse IgG (1:500, Jackson, 112-165-167, USA) for 2 h. Immunostaining was visualized in an LSM 700 microscope (Carl Zeiss) and images were obtained.

Immunostaining for CRTC1, HMGB1, Iba-1, NeuN, occludin, p-CREB

The 20-μm-thick frozen slices were fixed with 4 % PFA for further analysis as we described previously (Zhang et al. 2019). In brief, the sections were blocked with 5 % goat serum for 2 h to inhibit nonspecific binding and incubated with primary antibody against HMGB1 (1:800; Abcam, ab79823, UK), CRTC1 (1:100; Cell Signaling Technology, #2587, USA), occludin (1:100; Invitrogen, 711500, USA), Iba-1 (1:2000; Wako, 019-19741, Japan), NeuN (1:800; Merck Millipore, MAB337, USA), p-CREB (1:100; Cell Signaling Technology, #9198, USA) overnight at 4°C. After being washed with PBS 3 times, sections were incubated with 488- or Cy3-conjugated secondary antibody (anti-rabbit, 1:800, Life Technology, A11008, USA; anti-mouse, 1:800, KPL, 072-01-18-06, USA) for 2 h in dark at room temperature, followed by DAPI staining for 5 min. Color images were snapped by investigators that were blind to treatment group designation using a confocal microscope (Zeiss LSM 700, Carl Zeiss). Neuron number was blindly measured in images captured from non-ischemic and ischemic hemispheres using Image J.

Western blot analysis for HMGB1, CRTC1, p-CREB, and occludin

Tissues in ischemic (I) and non-ischemic (NI) hemispheres were achieved at 24 h after MCAO (Yang et al. 2018). Proteins (30 μg of total protein) were boiled and electrophoresed in 10 % or 12 % SDS-PAGE acrylamide gels. Then the proteins were transferred onto PVDF membrane (Millipore, Billerica, MA, USA), and blocked for 2 h in TBS-T containing 5% nonfat milk. The membranes were incubated with primary antibodies against HMGB1 (1:20000; Abcam, ab79823, UK), CRTC1 (1:1000; Cell Signaling Technology, #2587, USA), p-CREB (1:1000; Cell Signaling Technology, #9198, USA), or occludin (1:300; Invitrogen, 711500, USA) overnight at 4°C, washed in TBS-T, and then followed by incubation with corresponding HRP conjugated anti-rabbit (1:3000; Boster, BA1054, China) or anti-mouse antibodies (1:3000; Boster, BA1050, China) for 2 h at room temperature. The protein bands were developed with an enhanced luminescence reagent (Millipore) and photographed with ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). The intensities of protein band were quantitated via normalization to β-actin as the expression of the ratios of target proteins/β-actin.

TUNEL assay

The TUNEL Apoptosis Detection Kit (Yeasen, 40307ES20, China) was used to detect apoptosis cells according to the instructions from the manufacturer. Briefly, at first, brain sections were re-hydrated and nuclear was stripped with proteinase K. A mixture of 488-labeled nucleotides and terminal deoxynucleotidyl transferase was applied onto brain sections for 60 min at 37°C in a dark humidified incubator and followed by DAPI staining for 5 min. Incubation with labeling solutions without the enzyme served as a negative control (Yang et al. 2011). Microvessels labeled with RECA-1 were counted in images captured from ischemic hemispheres by using National Institutes of Health Image J. Indicators of animal identity on slides were blinded to the investigator. The number of TUNEL cells was calculated as the mean of the numbers per mm^2 obtained from the imaged sections.

Statistical analysis

The data were expressed as mean ± SEM. The theoretical normal distribution of values was calculated. T-test was used for within-group or two-group comparison, and one-way ANOVA with Newman-keuls comparison post hoc test was used to evaluate the difference between groups. All statistical analyses were performed with the SPSS 17.0 software, and plotting was carried out by GraphPad Prism software version 5.0. Differences were considered to be significant when $P<0.05$.

Results

Melatonin alleviated ischemia-reperfusion (I/R)-induced BBB damage

To examine the effect of melatonin treatment on I/R-induced BBB damage in mice, we first evaluated the extent of EB leakage as a measure of the BBB permeability. As shown in Figure. 1B and C, 1.5 h ischemia and 24 h reperfusion significantly increased EB leakage (Figure. 1C, $*P<0.05$ vs. the non-ischemic

hemisphere), and melatonin at a dose of 15 mg/kg (Parada et al. 2014) significantly alleviated EB extravasation (Figure. 1C, $^{\#}P<0.05$ vs. the Saline group in ischemic hemisphere).

The loss of occludin and claudins proteins has been seen by our and other groups in the I/R-compromised BBB (Jin et al. 2013; Liu et al. 2009). Here, our immunostaining results showed that melatonin also decreased I/R-induced degradation of occludin (Figure. 1D) in mice. These results suggest that melatonin effectively alleviated I/R-induced BBB disruption and tight junction protein occludin degradation.

Melatonin alleviated the disruption of BBB integrity partially through the modulation of α7 nAChR

In the central nervous system, there is a melatonin-dependent variation of the function and number of binding sites of nicotine (Markus et al. 2003). Further, α7nAChR activation has been shown to play a critical role in protecting the integrity of BBB (Han et al. 2014a; Han et al. 2014b; Zou et al. 2017). Therefore, we further investigated whether melatonin could prevent the BBB disruption from I/R injury through modulating the α7nAChR. Agonist (PNU282987, 10 mg/kg) (Parada et al. 2013) and antagonist (MLA, 6 mg/kg) (Parada et al. 2014) of α7nAChR were recruited to mimic or block the effect of melatonin against α7nAChR. EB leakage was used to evaluate the BBB integrity (Figure. 2B). Same as melatonin, PNU282987 significantly alleviated I/R-induced EB leakage (Figure. 2C, $^{*}P<0.05$ vs. the Saline group). When combined with MLA, the protective effect of melatonin was partially abolished, and the treatment with MLA alone aggravated the BBB damage (Figure. 2C). These results suggest that melatonin's protective effect is partially mediated via α7nAChR modulation.

Activation of the α7nAChR has been shown to upregulate BBB function through increasing claudin-5 and occludin expression in rat brain endothelial cells (Kimura et al. 2019). Hence, we next investigated the effect of α7nAChR on I/R-induced occludin degradation by modulating α7nAChR. As seen in western blot results, I/R induced a dramatic reduction of occludin expression (Figure. 2D, $^{**}P<0.01$ vs. non-ischemic (NI) hemisphere), and both PNU282987 and melatonin treatment significantly inhibited the degradation of occludin (Figure. 2D, $^{\#}P<0.05$ vs. the Saline group). MLA partly abrogated melatonin's effect, and treatment with MLA alone aggravated the occludin degradation induced by ischemic stroke (Figure. 2D), suggesting that melatonin's protective effect on occludin degradation is partially mediated via α7nAChR modulation.

Melatonin suppressed the release of HMGB1 from neurons through modulation of α7nAChR

HMGB1 is released from neurons after the onset of brain ischemia (Qiu et al. 2008). Early release of HMGB1 can be an important factor in the initial inflammatory response in ischemic penumbra (An et al. 2014) and damage the BBB integrity (Li et al. 2018). Therefore, HMGB1 secretion might play critical roles in mediating BBB disruption in brain I/R injury. For this reason, we examined whether melatonin could reduce HMGB1 release from neurons following ischemic stroke. Co-staining HMGB1 and NeuN showed that the HMGB1 was mainly present in neurons in the NI hemisphere, I/R significantly increased the release of HMGB1 as the positive signal of HMGB1 was decreased and the co-localization of HMGB1 and

NeuN was almost diminished. Our results showed that treatment with melatonin could inhibit the HMGB1 secretion from neurons which is induced by I/R (Figure.3).

We next investigated whether the effect of melatonin on reducing the HMGB1 release was due to the modulation of α7nAChR. Immunostaining results showed that I/R injury increased the release of HMGB1, while PNU282987 and melatonin treatment inhibited the release of HMGB1 (Figure. 4A). The effect of melatonin was partially reversed by MLA (Figure. 4A). Consistant with immunofluorescent study, the western blot result showed that, PNU282987 and melatonin significantly inhibited HMGB1 decrease in the ischemic hemisphere (Figure. 4B, $^{*}P<0.05$ vs. NI hemisphere, $^{\#}P<0.05$ vs. the Saline group) and MLA could partly inhibit the effect of melatonin (Figure. 4B). These data suggest that melatonin suppressed the release of HMGB1 through the modulation of α7nAChR.

Melatonin treatment suppressed I/R induced microglia activation

After stroke, microglia are rapidly activated and transformed into phagocytes, which secrete a variety of inflammatory mediators, leading to the BBB disruption (Dudvarski Stankovic et al. 2016; Su et al. 2008). We detected the morphological and biochemical changes in cortex and striatum because ischemic stroke induced both cognition and motor function impairment and BBB damage and brain injury were found in cortex and striatum (Liu et al. 2017). Immunofluorescence results showed that microglia were activated into amoeboid morphology or even necrosis in ischemic cortex and striatum after I/R injury (Figure. 5). Melatonin or PNU282987 inhibited the activation and necrosis of microglia (Figure.5A). In addition, melatonin decreased microglia activation-mediated IgG leakage in the ischemic hemisphere (Figure.5B), suggesting that I/R-induced microglia activation aggravated BBB damage, and this impact could be attenuated by melatonin via modulation of α7nAChR.

Melatonin treatment decreased I/R-induced neuronal loss

Melatonin at a dose of 15 mg/kg reduced infarct size and improved motor skills in photothrombotic stroke, this effect was partially suppressed by MLA (Parada et al. 2014). Neuronal loss was quantitated by analyzing the number of neurons in ischemic hemisphere relative to the non-ischemic hemisphere. I/R induced a significant number reduction of neurons (Figure. 6A), and melatonin treatment significantly prevented this reduction. The result was further confirmed by TUNEL staining which could work as a quantitative assay to determine the protective effect of melatonin against I/R-induced apoptosis. In TUNEL staining, the positively stained apoptotic nuclei were observed in ischemic hemisphere. There was a significant increase of TUNEL-positive cells in ischemic hemisphere of the Saline group, whereas melatonin reversed this change (Figure. 6B). These results demonstrate that melatonin exerts beneficial effects on I/R-induced neuronal injury.

Melatonin treatment inhibited I/R-induced CRTC1 and p-CREB decrease through modulation of α7nAChR

Neurons played an important role in maintaining the integrity of BBB (Lo et al. 2003). CREB regulated transcriptional coactivator 1 (CRTC1), the most efficient transcriptional coactivator of CREB, is critical for

neuron survival after OGD (Sasaki et al. 2011). Here, we explored the role of CRTC1 and p-CREB in I/R-induced neuronal loss. Our data showed that the expression of CRTC1 was reduced in ischemic tissue, and this degradation was significantly inhibited by PNU282987 or melatonin (*P<0.05 vs. Saline group, Figure.7A). MLA reversed the effect of melatonin and significantly decreased the level of CRTC1 ($^{\#\#}P$<0.01, Figure.7A) when it was treated alone, suggesting that melatonin inhibited the decrease of CRTC1 partly via modulation of α7nAChR.

CREB has been reported to be a key element to protect the brain against ischemic insult (Khatri et al. 2012; Hardingham et al. 2002; Mabuchi et al. 2001) and phosphorylated CREB protein (p-CREB) is surrounding the infarct area after 90-min MCAO (Irving et al. 2000; Tanaka et al. 1999). We next checked the role of p-CREB in I/R-induced neuronal loss. Immunofluorescence results showed that p-CREB protein level was reduced in ischemic brain, and this decrease was inhibited by PNU282987 or melatonin treatment (Figure.7C). MLA alone reversed the effect of melatonin. These results were further confirmed by western blot (Figure.7B), suggesting that melatonin inhibited the decrease of CRTC1 and p-CREB via modulation of α7nAChR.

Discussion

Cerebral edema and hemorrhage transformation (HT) are two consequences of BBB damage following ischemia and reperfusion. The incidence of HT occurs up to 44% after tPA treatment, and due to the high mortality after HT the clinical use of tPA is severely limited (Lees et al. 2010; Jaillard et al. 1999). BBB damage is the pathological basis of tPA-induced HT after stroke. Thus, protecting the integrity of the BBB to reduce the risk of HT is an urgent problem, however, no effective strategy to protect BBB is currently available. In this study, we have investigated the effect of melatonin on I/R-induced BBB disruption. Our important findings include: 1) Cerebral I/R injury destroyed the integrity of BBB by promoting HMGB1 release from neurons, activating microglia, and degrading the TJP occludin; 2) Melatonin inhibited this effect by reducing HMGB1 release and microglia activation via modulating the α7nAChR; 3) Modulation of α7nAChR by melatonin also reduced I/R-induced neuronal loss through increasing the expression of CRTC1 and p-CREB (Fig. 8).

Analysis of a landmark of National Institutes of Neurological Disorders and Stroke (NINDS) recombinant tPA (r-tPA) stroke trial showed that the prevalence of HT among smokers was significantly lower than the non-smoking tPA-treated patients (Tissue plasminogen activator for acute ischemic stroke 1995; Intracerebral hemorrhage after intravenous t-PA therapy for ischemic stroke. The NINDS t-PA Stroke Study Group 1997), indicating that activating the nicotine receptor may be a promising strategy to protect I/R-induced BBB damage and reduce the risk of HT. Activation of α7nAChR by currently available agonists has been shown to alleviate BBB damage induced by I/R (Han et al. 2014a; Han et al. 2014b; Zou et al. 2017), hemorrhagic stroke (Krafft et al. 2012; Krafft et al. 2013), SAH (Duris et al. 2011) and experimental traumatic brain injury (Dash et al. 2016). However, in clinical trials, the α7nAChR agonists failed to be approved because of the side effects of cardiotoxicity (Beinat et al. 2015). A safe drug that could protect

BBB and activate α7nAChR would have great translational potential for the clinical treatment of acute ischemic stroke.

Our results showed that melatonin mimicked the effect of α7nAChR agonist PNU282987 to reduce I/R-induced BBB damage, while α7nAChR antagonist blocked this effect, indicating that melatonin exerts its effect through the modulation of α7nAChR. This is consistent with previous studies showing that melatonin provided the neuroprotection effect (Parada et al. 2014) or regulated the autophagic flux (Jeong and Park 2015) via activating α7nAChR. In addition, inactivation of α7nAChR by α-Bgt (a selective α7nAChR antagonist) or MLA inhibited melatonin-mediated protective effects (Jeong and Park 2015; Parada et al. 2014). Therefore, melatonin may be a promising strategy to reduce tPA-induced HT after ischemic stroke.

Our results showed that 15 mg/kg of melatonin significantly inhibited I/R-induced BBB damage and neuronal loss, and this effect could be reduced by selective α7nAChR antagonist MLA. This is consistent with a previous study showing that MLA treatment partially inhibited melatonin's (15 mg/kg) improvement in infarction size and motor skills in a photothrombotic stroke model (Parada et al. 2014). Although melatonin at a dose of 5 mg/kg reduced I/R-induced BBB damage and tPA-induced HT when MCAO duration is shorter (60 min vs 90 min in our study), however, the mechanism was not investigated and melatonin at this dose did not affect neuronal loss (Chen et al. 2006b). Therefore, a higher dose of melatonin is needed to reduce BBB damage and neuronal loss when the duration of MCAO is relatively long.

Our study showed that modulation of α7nAChR by melatonin significantly reduced I/R-induced HMGB1 secretion, which had been shown to disrupt the integrity of BBB (Shichita et al. 2012; Zhang et al. 2011). This is consistent with previous studies showing that cholinergic agonists inhibited HMGB1 release and improve survival in experimental sepsis (Wang et al. 2004), and electroacupuncture pretreatment attenuated cerebral ischemic injury through α7nAChR-mediated inhibition of HMGB1 release in rats (Wang et al. 2012). In addition, in renal ischemic infarction, melatonin can reduce kidney damage by inhibiting the translocation of HMGB1 from nucleoplasm (Zhu et al. 2017). Our finding suggested that HMGB1 secreted from neuron is consistent with previous study showing that HMGB1 is mainly present in neurons or astrocytes nuclei (Hayakawa et al. 2010; Ellwood et al. 2000; Verrijdt et al. 2002), upon I/R, neurons are extremely intolerant to hypoxia, and are easily damaged and secrete HMGB1 outside of the cell.

Our results showed that modulation of α7nAChR by melatonin can attenuate I/R-induced microglia activation, and accompanied by the decrease of IgG leakage. This is consistent with the idea that melatonin acted as an anti-inflammation molecular. Inflammation plays a very important role in BBB damage after stroke (de Wit et al. 2016), and one of the most important pro-inflammatory alarms in ischemic stroke is HMGB1 (Singh et al. 2016) which could activate microglia to transform into phagocytic cells and secrete various factors, such as TNF-α, IL-1, IL-6, and nitric oxide (Singh et al. 2016), causing BBB destruction and HT (da Fonseca et al. 2014).

Our results showed that the α7nAChR agonists PNU282987 or melatonin could significantly inhibit the decrease of p-CREB which plays a very important role in the neuroprotective effects of hypoxic-ischemic brain damage (Khatri et al. 2012) and reduce the down-regulation of CRTC1, the most efficient transcriptional coactivator of CREB (Conkright et al. 2003), exerts a critical effect on neurons survival after OGD (Sasaki et al. 2011). These results are consistent with previous study reporting that α7nAChR agonists can increase cognitive function by increasing CREB phosphorylation (Bitner et al. 2007).

We demonstrated that I/R induced a significant decrease of α7nAChR and melatonin treatment could inhibit such effect, therefore modulation of nicotinic acetylcholine receptors by melatonin may be relevant for therapy with cholinergic drugs (Markus et al. 2010).

Conclusions

In summary, our results demonstrate that modulation of α7nAChR by melatonin can prevent the I/R-induced HMGB1 release, reduce the activation of microglia, and increase the expression of CREB transcriptional coactivator CRTC1, reduce the degradation of tight junction protein, reduce BBB damage, supporting that melatonin, as a safe and effective BBB protective drug, has great translational potential for reducing tPA associated HT after acute ischemic stroke.

Declarations

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Competing interests

The authors declare that they have no competing interest.

Authors' contributions

GQZ and XJ are the principal investigators at the two collaborating institutions and are responsible for project design, supervision of technical personnel, interpretation of results, and preparation of manuscript drafts. HL, JZ and WLL provided advice on experimental design and interpretation, and comments on the manuscript. SC,YS, FL, XZHANG, XH, XZHAO and YL performed experiments, analyzed the data, made the figures and drafted the manuscript.

Data Availability Statement

The data are available from the corresponding author upon reasonable request

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Figures

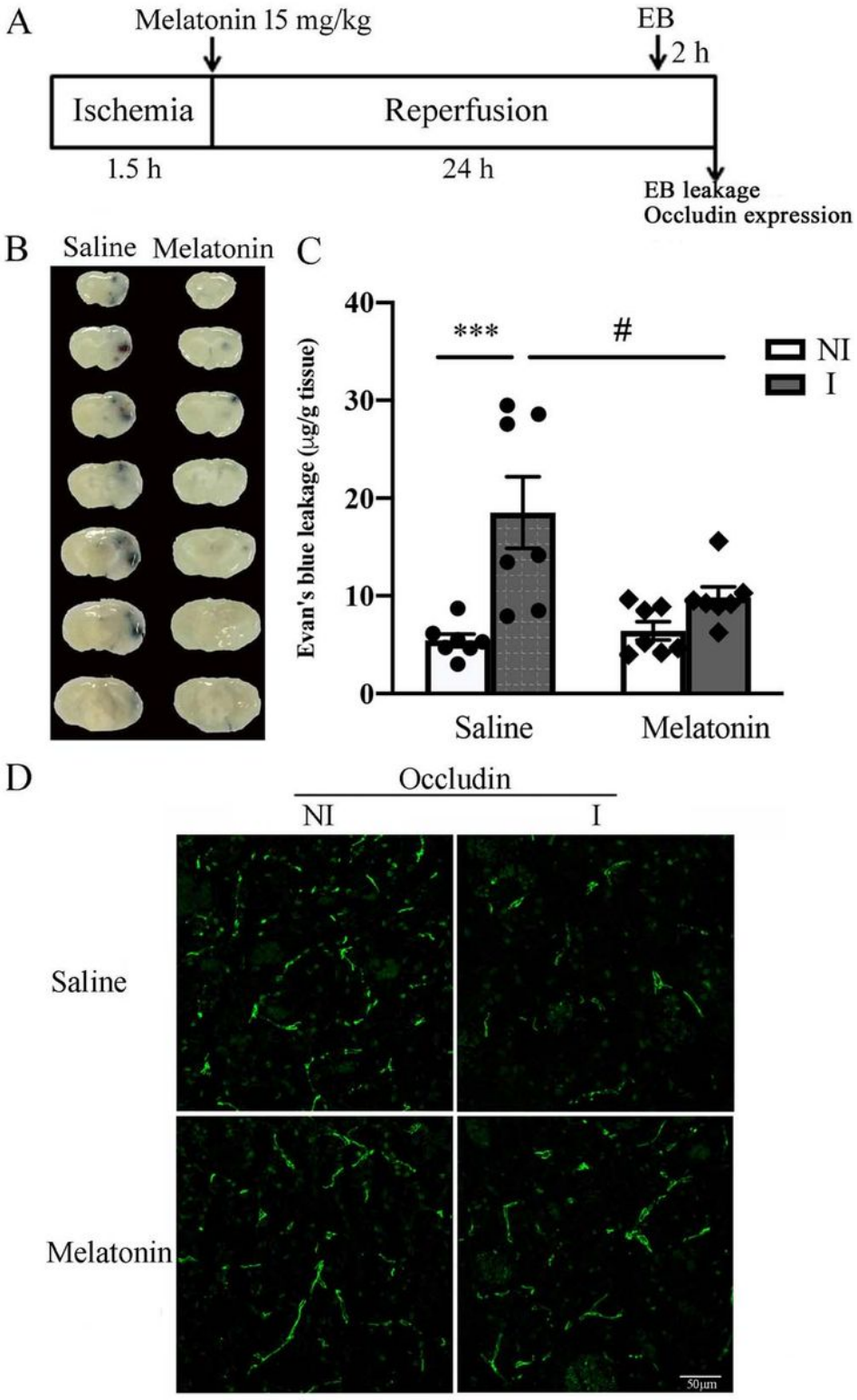

Figure 1

Melatonin treatment alleviated I/R-induced BBB damage in mice. (A) Outline of experimental design. Mice were subjected to 1.5 h MCAO with 24 h reperfusion. (B) Seven consecutive sections showed EB leakage from the saline or melatonin-treated group. (C) EB leakage in the brain tissue was quantitated. Melatonin treatment significantly alleviated the BBB disruption which is induced by I/R injury (*P<0.05 vs. the non-ischemic hemisphere, #P<0.05 vs. the saline group in the ischemic hemisphere, n=7 for each group). (D) Representative confocal micrographs showed that I/R decreased occludin expression, and melatonin treatment ameliorated the degradation. N=3, scale bar =50 μm. All values are subject to normal distribution and T-test was used to evaluate the difference between groups as well as the difference between NI and I hemisphere in the same group.

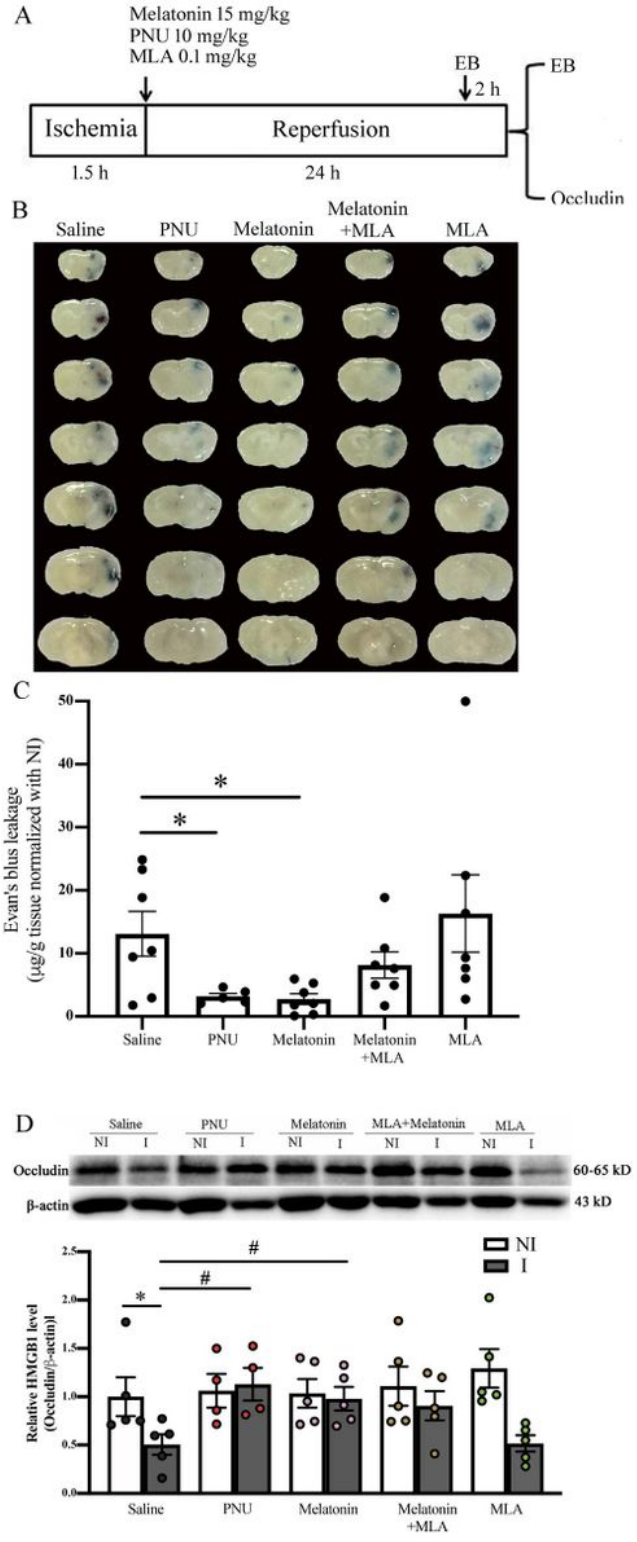

Figure 2

Melatonin treatment reduced I/R-induced BBB damage through modulating α7nAChR. (A) Outline of experimental design. Mice were subjected to 1.5 h MCAO with 24 h reperfusion. (B) Seven consecutive sections showed EB leakage from Saline, PNU282987, Melatonin, Melatonin plus MLA, or MLA group. (C) EB leakage in the brain tissue was quantitated. I/R induced significant BBB damage, and PNU282987 or melatonin treatment alleviated the disruption (*P<0.05 vs. the saline group, n=7 for each group). (D)

Representative immunoblot image demonstrated occludin expression in NI and I hemisphere of each group (upper panel). The band intensity of occludin was quantitated (lower panel). I/R significantly decreased the expression of occludin, whereas both PNU and melatonin treatment ameliorated this change (**P<0.01 vs. the non-ischemic hemisphere, #P<0.05 vs. the saline group in the ischemic hemisphere, n=6 for each group). All values are subject to normal distribution. T-test was used to evaluate the difference between NI and I hemisphere in the same group, and one-way ANOVA with Newman-keuls comparison post hoc test was used to evaluate the difference between groups in ischemic hemisphere.

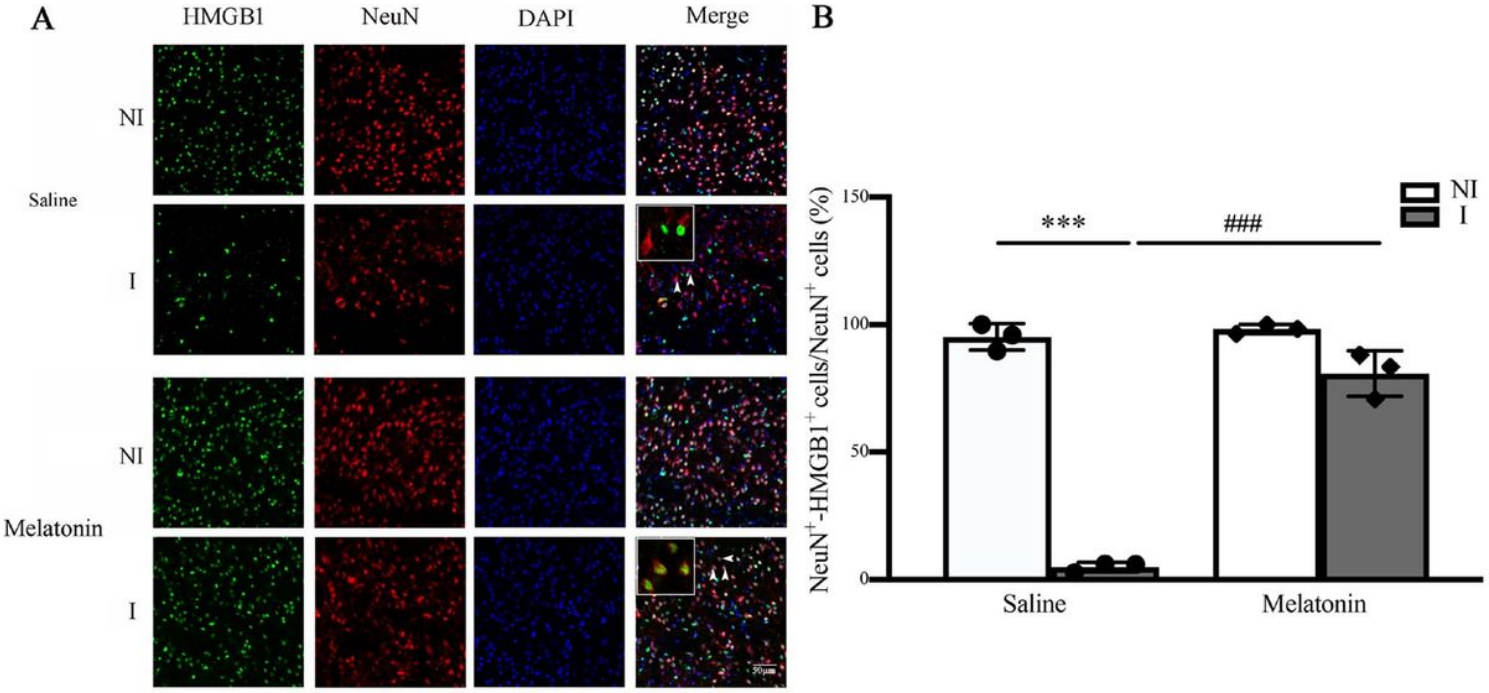

Figure 3

Melatonin treatment reduced I/R-induced HMGB1 release from neurons. Representative confocal micrographs showed co-staining of HMGB1 (green) and NeuN (red) in the brain. (A) compared with non-ischemic hemisphere, I/R decreased the number of NeuN/HMGB1-positive cells in cortex of ischemic hemisphere, and melatonin treatment ameliorated this change. (B) The number of NeuN/HMGB1-positive cells in ischemic hemisphere was quantitated after normalization to the NeuN-positive cells. The results indicated that melatonin suppressed the HMGB1 release from neuron after ischemic stroke (###P<0.01 vs. the saline group in the ischemic hemisphere). N=3, scale bar=50 μm. Data were expressed as mean±SEM. T-test was used to evaluate the difference between groups in the ischemic hemisphere.

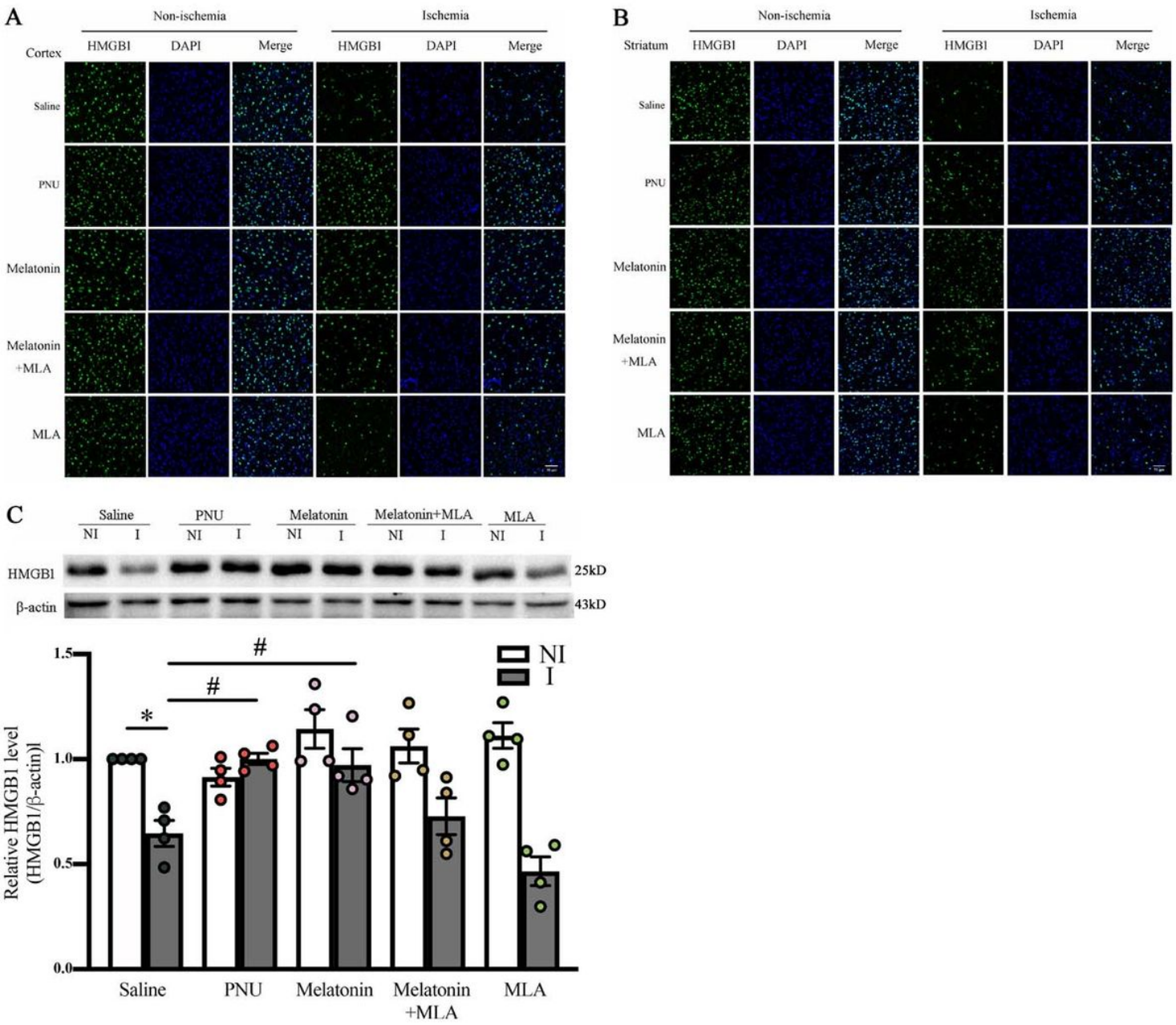

Figure 4

Melatonin treatment inhibited I/R-induced HMGB1 release through modulating α7nAChR. (A) Representative confocal micrographs showed HMGB1 (green) expression in non-ischemic (NI) and ischemic (I) hemisphere of mice from Saline, PNU282987, Melatonin, Melatonin plus MLA, or MLA group. I/R decreased the staining number of HMGB1, and treatment with PNU282987 or melatonin ameliorated this change. When in combination with melatonin, MLA blocked the protective effect of melatonin. N=3, scale bar=50 μm. (B) Representative immunoblot showed the bands of HMGB1 (upper panel). The band intensity of HMGB1 was quantitated (lower panel). I/R induced a significant decrease of HMGB1 expression in the brain (*P<0.05, **P<0.01, ***P<0.001 vs. the non-ischemic hemisphere). Treatment with PNU282987 or melatonin prevented the I/R-induced HMGB1 reduction (#P<0.05 or ##P<0.01 vs. the saline group in the ischemic hemisphere). N=5 for each group. Data were expressed as mean±SEM. All

values are subject to normal distribution. T-test was used to evaluate the difference between NI and I hemisphere in the same group, and one-way ANOVA with Newman-keuls comparison post hoc test was used to evaluate the difference between groups in ischemic hemisphere

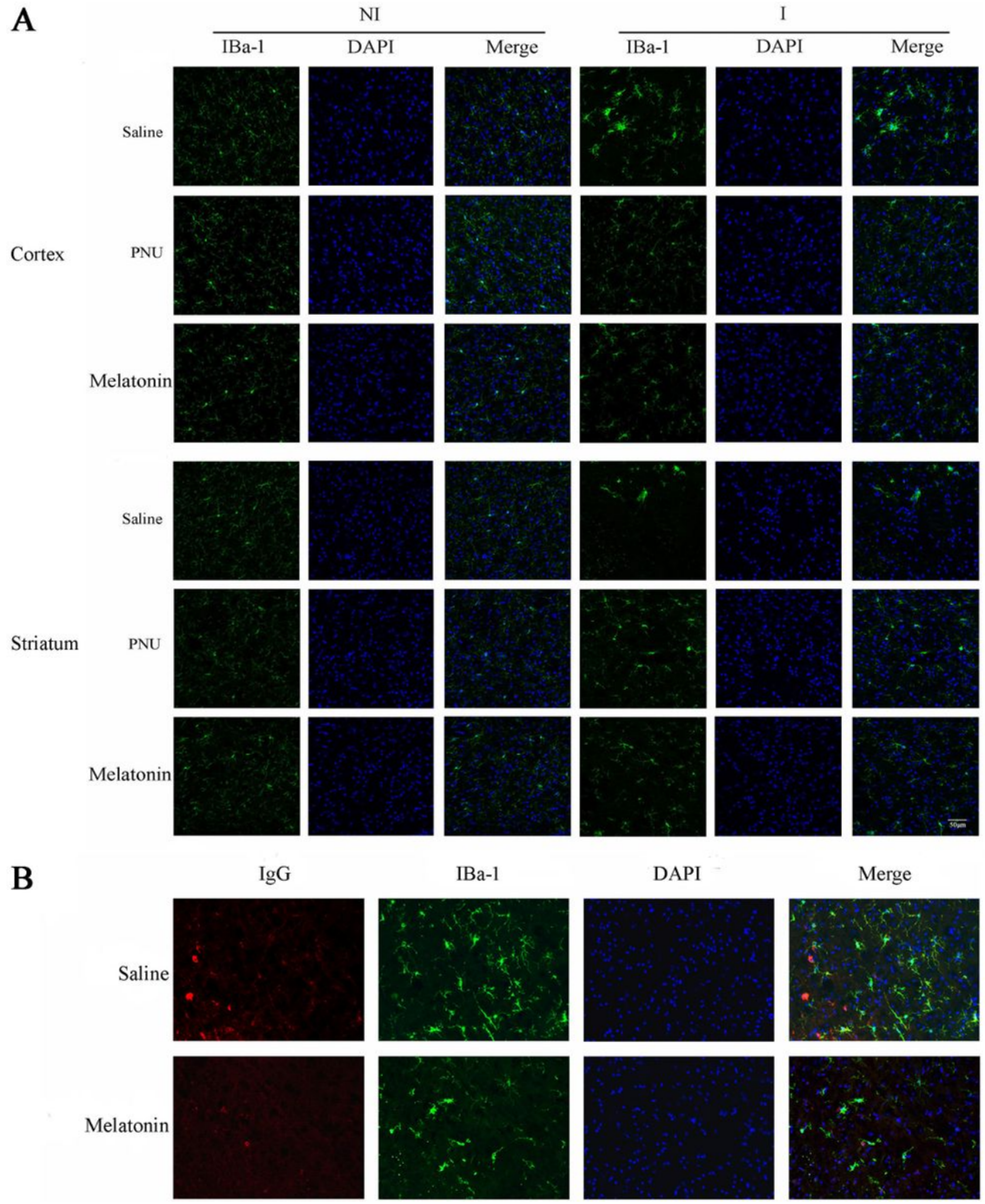

Figure 5

Melatonin treatment reduced I/R-induced microglia activation and IgG leakage. (A) Representative confocal micrographs showed activated microglia (green) with amoeboid morphology in ischemic

hemisphere (I) after I/R injury, and treatment with PNU282987 or melatonin ameliorated this activation in both cortex and striatum. (B) Double immunostaining of leaked IgG (red) and Iba-1 (green) showed that melatonin inhibited the I/R-induced microglia activation accompanied IgG leakage. N=3, scale bar=50 μm.

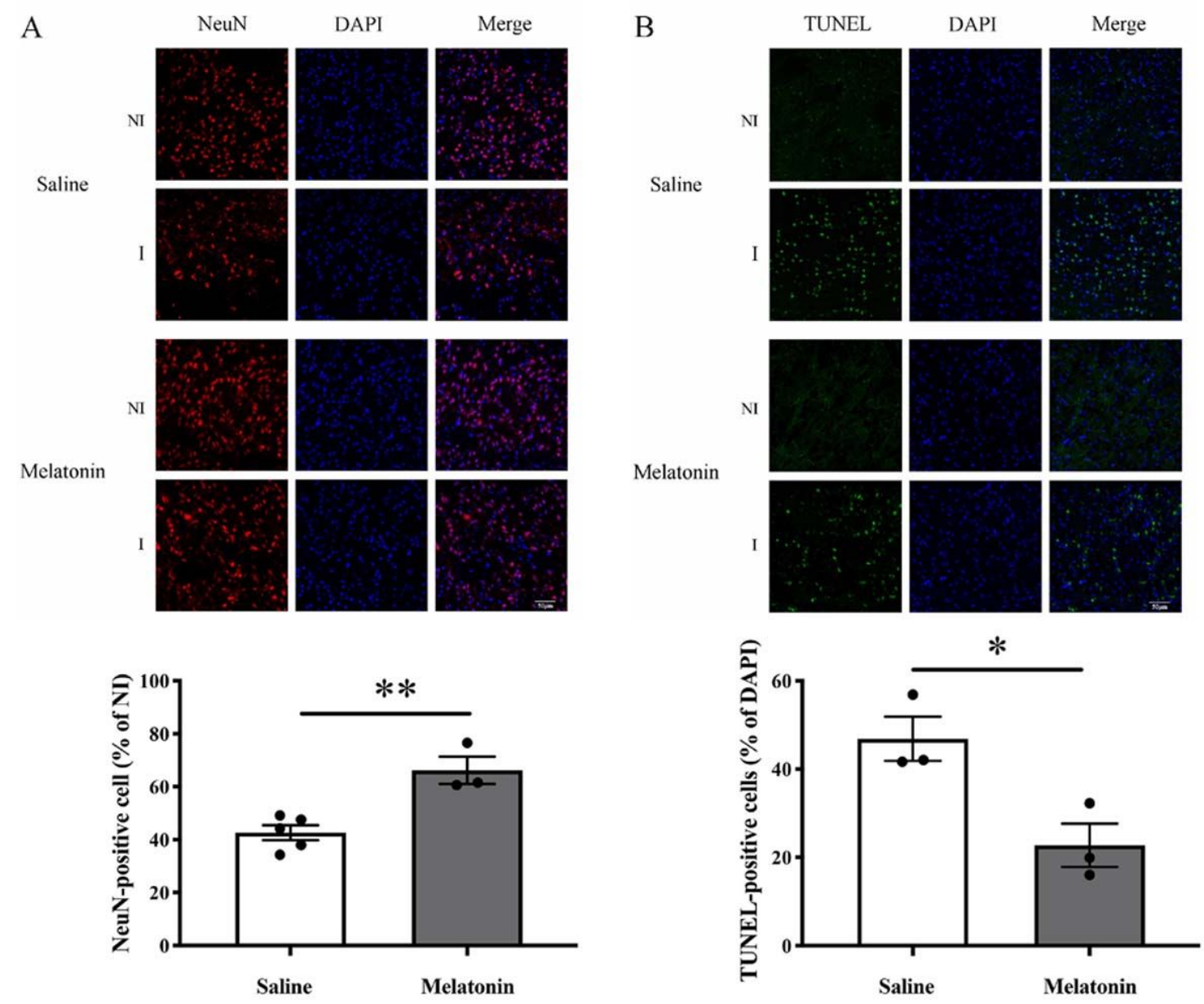

Figure 6

Melatonin treatment decreased I/R-induced neuronal loss. (A) Representative confocal micrographs showed the immunostaining for of neurons (red) in upper panel. The number of neurons lost in ischemic hemisphere was quantitated after normalization to the NeuN-positive cells in NI hemisphere (lower panel). ***P<0.001 vs. the saline group. Data were expressed as mean±SEM, T-test was used to evaluate the difference between groups. N=3 for each group, scale bar=50 μm. (B) Representative confocal micrographs showed TUNEL staining which was used to detect cell apoptosis in ischemic hemisphere (I) after 1.5 h MCAO and 24 h reperfusion (upper panel). TUNEL-positive cell counts were expressed as a percentage of the corresponding DAPI-stained nuclei in NI hemisphere (lower panel). I/R injury

significantly induced cell apoptosis, melatonin treatment remarkably inhibited the apoptosis. **P<0.001 vs. the saline group. Data were expressed as mean±SEM, T-test was used to evaluate the difference between groups. N=3 for each group, scale bar=50 μm.

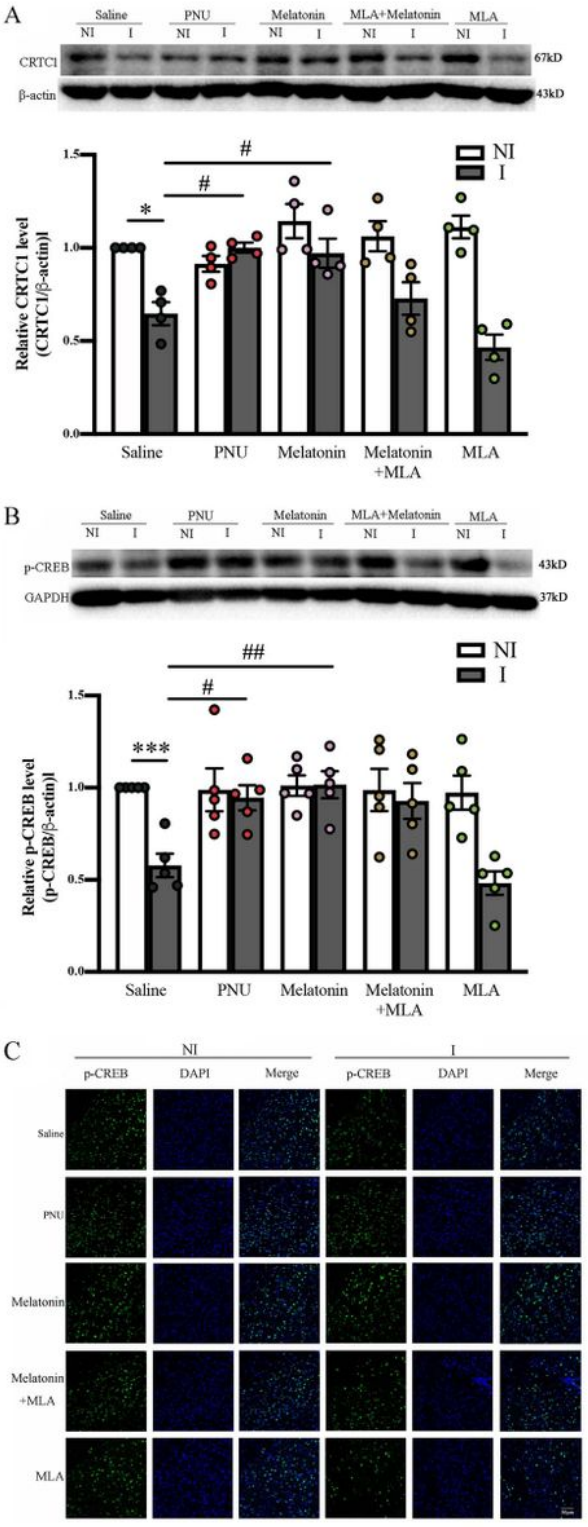

Figure 7

Melatonin treatment inhibited I/R-induced CRTC1 and p-CREB decrease through modulation of α7nAChR. (A) Representative immunoblot showed the bands of CRCT1 (upper panel). The band intensity of CRCT1

was quantitated (lower panel). I/R induced a significant reduction in the expression of CRCT1 (*P<0.05 or ***P<0.001 vs. non-ischemic hemisphere in the same group). Treatment with PNU282987 or melatonin prevented the decrease of CRCT1 protein (#P<0.05 or ##P<0.01 vs. the saline group in ischemic hemisphere) after I/R. N=6 for each group. Data were expressed as mean±SEM. (B) Representative Immunoblot showed the bands of p-CREB (upper panel). The band intensity of p-CREB was quantitated. I/R induced a significant reduction in the levels of p-CREB (*P<0.05 or **P<0.01 vs. the NI hemisphere). Treatment with PNU282987 or melatonin significantly prevented the reduction (#P<0.05 vs. the Saline group). N=5 for each group. Data were expressed as mean±SEM. (C) Representative confocal micrographs showed decreased p-CREB in ischemic hemisphere (I) after I/R. N=3, scale bar=50 μm. All values are subject to normal distribution. Unpaired T-test was used to evaluate the difference between NI and I hemisphere in the same group, and one-way ANOVA with Newman-keuls comparison post hoc test was used to evaluate the difference between groups in ischemic hemisphere.

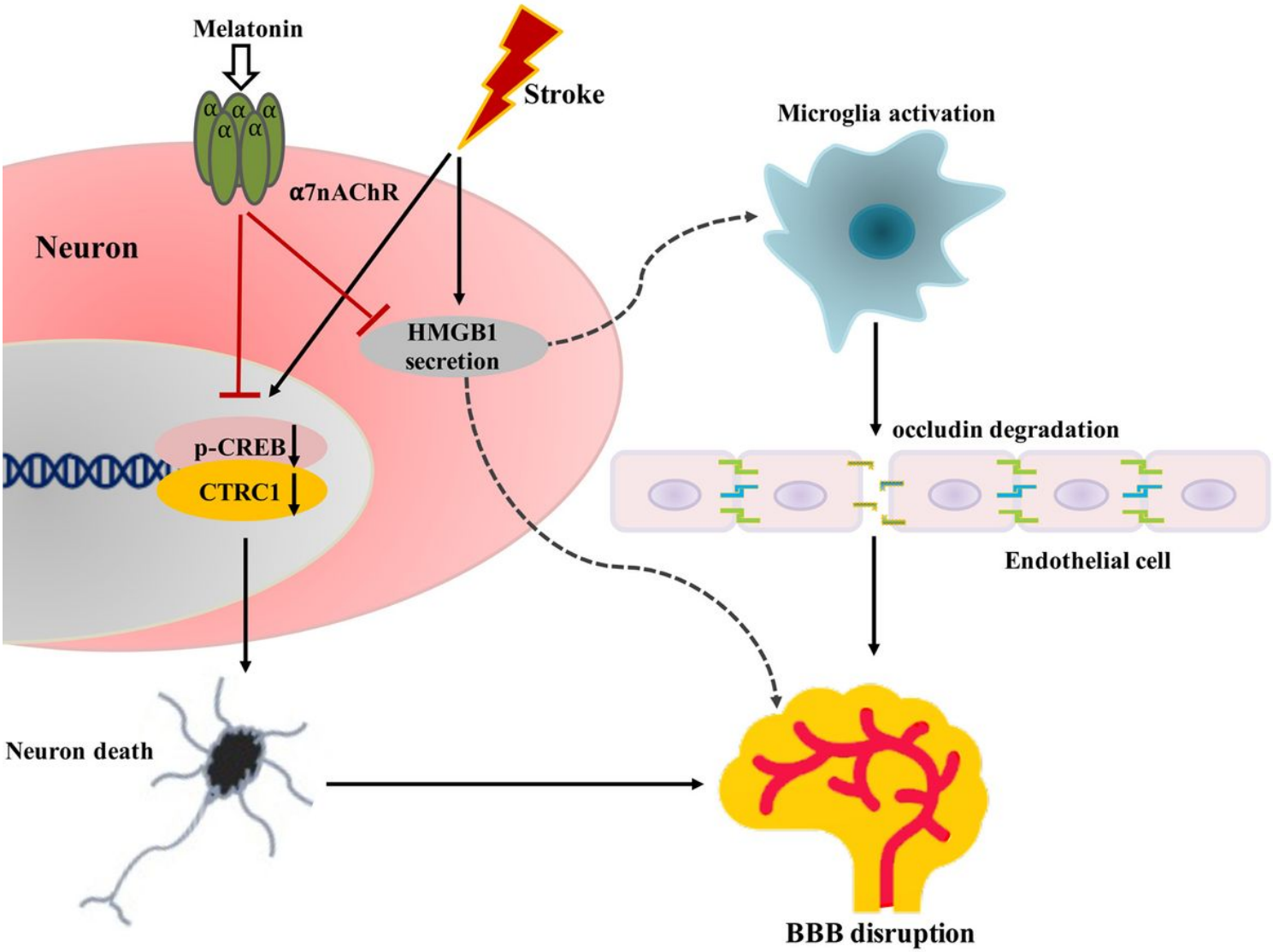

Figure 8

A schema for proposed mechanism underlying melatonin's protective effect on I/R-induced BBB damage in mice. I/R induced BBB disruption accompanied by HMGB1 secretion from neuron, microglia activation,

tight junction protein occludin degradation. In addition, I/R-induced neuronal loss accompanied by CRTC1 and p-CREB decrease. Melatonin treatment could inhibit these effects through modulating α7nAChR.

Supplementary Files

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