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 the modulation of α7nAChR.

Keywords Melatonin · Blood–brain barrier · α7nAChR · HMGB1 · Stroke

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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 tPA therapy for ischemic stroke. The NINDS tPA 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 protects 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 (Kraft et al. 2012; Kraft 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 N2 and O2 (70% N2: 30% O2), 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, 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 2 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 non-specific 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% non-fat 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 mm² 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 Fig. 1B and C, 1.5 h ischemia and 24 h reperfusion significantly increased EB leakage (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2B). Same as melatonin, PNU282987 significantly alleviated I/R-induced EB leakage (Fig. 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 (Fig. 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 (Fig. 2D, **P<0.01 vs. the non-ischemic (NI) hemisphere), and both PNU282987 and melatonin treatment significantly inhibited the degradation of occludin (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4A). The effect of melatonin was partially reversed by MLA (Fig. 4A). Consistant with immunofluorescent study, the western blot result showed that, PNU282987 and melatonin significantly inhibited HMGB1 decrease in the ischemic hemisphere (Fig. 4B, *P < 0.05 vs. NI hemisphere, **P < 0.05 vs. the Saline group) and MLA could partly inhibit the effect of melatonin (Fig. 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 (Qin et al., 2019), 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 (Fig. 5). Melatonin or PNU282987 inhibited the activation and necrosis of microglia (Fig. 5A). In addition, melatonin decreased microglia activation-mediated IgG leakage in the ischemic hemisphere (Fig. 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 (Fig. 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 (Fig. 6B). These results demonstrate that melatonin exerts beneficial effects on I/R-induced neuronal injury.

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**Fig. 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.
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, Fig. 7A). MLA reversed the effect of melatonin and significantly decreased the level of CRTC1 (##P<0.01, Fig. 7A) when it was treated alone, suggesting that melatonin...
Fig. 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 that accompanied IgG leakage. N=3, scale bar=50 μm.
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 (Fig. 7C). MLA alone reversed the effect of melatonin. These results were further confirmed by western blot (Fig. 7B), suggesting that melatonin inhibited the decrease of CRTC1 and p-CREB via modulation of α7nAChR.

**Fig. 6** Melatonin treatment decreased I/R-induced neuronal loss. (A) Representative confocal micrographs showed the immunostaining 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, while 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.
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 tPA therapy for ischemic stroke. The NINDS tPA 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 cardio-toxicity (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-inflammatory molecule. 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. 2010; Ellwood et al. 2000; Verrijdt et al. 2002), upon I/R, 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, increase the expression of CREB transcriptional coactivator CRTC1, and reduce the degradation of tight junction protein and 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.

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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, ZXHANG, XH, ZXHAO, and YL performed experiments, analyzed the data, made the figures, and drafted the manuscript.

Data Availability

The data are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest

The authors declare that they have no competing interest.

References

An C, Shi Y, Li P, Hu X, Gan Y, Stetler RA, Leak RK, Gao Y, Sun BL, Zheng P, Chen J (2014) Molecular dialogs between the ischemic brain and the peripheral immune system: dualistic roles in injury and repair. Prog Neurobiol 115:6–24
Beinat C, Banister SD, Herrera M, Law V, Kassiou M (2015) The therapeutically potential of alpha7 nicotinic acetylcholine receptor (alpha7 nAChR) agonists for the treatment of the cognitive deficits associated with schizophrenia. CNS Drugs 29(7):529–542
Benchef M, Narla ST, Stachowiak MS (2014) Alpha7 neuronal nicotinic receptor: a pluripotent target for diseases of the central nervous system. CNS Neurol Disord Drug Targets 13(5):836–845
Bitner RS, Bunnelle WH, Anderson DJ, Briggs CA, Buccafusco J, Curzon P, Decker MW, Frost JM, Gronien JH, Gubbins E, Li J, Malysz J, Markosyan S, Marsh K, Meyer MD, Nikkel AL, Radek RJ, Robb HM, Timmermann D, Sullivan JP, Gopalakrishnan M (2007) Broad-spectrum efficacy across cognitive domains by alpha7 nicotinic acetylcholine receptor agonism correlates with activation of ERK1/2 and CREB phosphorylation pathways. J Neurosci 27(39):10578–10587
Chen TY, Lee MY, Chen HY, Kuo YL, Lin SC, Wu TS, Lee EJ (2006) Melatonin attenuates the postischemic increase in blood-brain barrier permeability and decreases hemorrhagic transformation of tissue-plasminogen activator therapy following ischemic stroke in mice. J Pineal Res 40(3):242–250
Chen HY, Chen TY, Lee MY, Chen ST, Hsu YS, Kuo YL, Chang GL, Wu TS, Lee EJ (2006) Melatonin decreases neurovascular oxidative/nitrosative damage and protects against early increases in the blood-brain barrier permeability after transient focal cerebral ischemia in mice. J Pineal Res 41(2):175–182
Chen B, Friedman B, Cheng Q, Tsai P, Schim E, Kleinfeld D, Lyden PD (2009) Severe blood-brain barrier disruption and surrounding tissue injury. Stroke 40(12):e666-674
Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M (2003) TORCs: transducers of regulated CREB activity. Mol Cell 12(2):413–423
da Fonseca AC, Matias D, Garcia C, Amaral R, Geraldo LH, Freitas C, Lima FR (2014) The impact of microglial activation on blood-brain barrier in brain diseases. Front Cell Neurosci 8:362

Dash PK, Zhao J, Kobori N, Redell JB, Hylin MJ, Hood KN, Moore AN (2016) Activation of alpha 7 cholinergic nicotinic receptors reduce blood-brain barrier permeability following traumatic brain injury. J Neurosci 36(9):2809–2818

de Wit NM, Vannol J, Kamermans A, Hendriks J, de Vries HE (2016) Inflammation at the blood-brain barrier: The role of liver X receptors. Neurobiol Dis. https://doi.org/10.1016/j.nbd.2016.09.015

Dudvarski Stankovic N, Teodorczyk M, Ploen R, Zipp F, Schmidt MHH (2016) Microglia-blood vessel interactions: a double-edged sword in brain pathologies. Acta Neuropathol 131(3):347–363

Duris K, Manaenko A, Suzuki H, Rolland WB, Krafft PR, Zhang JH (2011) alpha7 nicotinic acetylcholine receptor agonist PNU-282987 attenuates early brain injury in a perforation model of subarachnoid hemorrhage in rats. Stroke 42(4):3530–3536

Ellwood KB, Yen YM, Johnson RC, Carey M (2000) Mechanism for activation of alpha-7 nicotinic acetylcholine receptors. Mol Cell Biol 20(12):4359–4370

Goldstein RS, Gallowitsch-Puerta M, Yang L, Rosas-Ballina M, Huston JM, Czura CJ, Lee DC, Ward MF, Bruchfeld AN, Wang H, Lesser ML, Church AL, Litroff AH, Sama AE, Tracey KJ (2006) Elevated high-mobility group box 1 levels in patients with cerebral and myocardial ischemia. Shock 25(6):571–574

Grossman AW, Broderick JP (2013) Advances and challenges in treatment and prevention of ischemic stroke. Ann Neurol 74(3):363–372

Gu Y, Zheng G, Xu M, Li Y, Chen X, Zhu W, Tong Y, Chung SK, Liu KJ, Shen J (2012) Caveolin-1 regulates nitric oxide-mediated matrix metalloproteinases activity and blood-brain barrier permeability in focal cerebral ischemia and reperfusion injury. J Neurochem 120(1):147–156

Han Z, Shen F, He Y, Degos V, Camus M, Maze M, Young WL, Su H (2014) Activation of alpha-7 nicotinic acetylcholine receptor reduces ischemic stroke injury through reduction of pro-inflammatory macrophages and oxidative stress. PLoS One 9(8):e105711

Han Z, Li L, Wang L, Degos V, Maze M, Su H (2014) Alpha-7 nicotinic acetylcholine receptor agonist treatment reduces neuroinflammation, oxidative stress, and brain injury in mice with ischemic stroke and bone fracture. J Neurochem 131(4):498–508

Hardingham GE, Fukunaga Y, Badling H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nature neuroscience 5(5):405–414

Hayakawa K, Qiu J, Lo EH (2010) Biphasic actions of HMGB1 signaling in inflammation and recovery after stroke. Ann N Y Acad Sci 1207:50–57

Hobson SR, Gurusinghe S, Lim R, Alers NO, Miller SL, Kingdom JC, Wallace EM (2018) Melatonin improves endothelial function in vitro and prolongs pregnancy in women with early-onset preeclampsia. J Pineal Res 65(3):e12508

Irving EA, Barone FC, Reith AD, Hadingham SJ, Parsons AA (2000) Differential activation of MAPK/ERK and p38/SAPK in neurones and glia following focal cerebral ischaemia in the rat. Brain Res Mol Brain Res 77(1):65–75

Jaillard A, Corru C, Durieux A, Moulin T, Boutitie F, Lees KR, Hommel M (1999) Hemorrhagic transformation in acute ischemic stroke The MAST-E study. MAST-E Group. Stroke 30(7):1326–1332

Jeong JK, Park SY (2015) Melatonin regulates the autophagic flux via activation of alpha-7 nicotinic acetylcholine receptors. J Pineal Res 59(1):24–37

Jickling GC, Liu D, Stamova B, Ander BP, Zhan X, Lu A, Sharp FR (2014) Hemorrhagic transformation after ischemic stroke in animals and humans. J Cereb Blood Flow Metab 34(2):185–199

Jin X, Liu J, Liu KJ, Rosenberg GA, Yang Y, Liu W (2013) Normobaric hyperoxia combined with minocycline provides greater neuroprotection than either alone in transient focal cerebral ischemia. Exp Neurol 240:9–16

Jin X, Liu J, Liu W (2014) Early ischemic blood brain barrier damage: A potential indicator for hemorrhagic transformation following tissue plasminogen activator (tPA) thrombolysis? Curr Neurovasc Res 11(3):254–262

Khatri R, McKinney AM, Swenson B, Janardhan V (2012) Blood-brain barrier, reperfusion injury, and hemorrhagic transformation in acute ischemic stroke. Neurology 79(13 Suppl 1):S52–S57

Kimura I, Dolgu S, Takata F, Matsumoto J, Kawahara Y, Nishihira M, Sakada S, Saisho T, Yamauchi A, Katoaka Y (2019) Activation of the alpha7 nicotinic acetylcholine receptor upregulates blood-brain barrier function through increased claudin-5 and occludin expression in rat brain endothelial cells. Neurosci Lett 694:9–13

Krafft PR, Altay O, Rolland WB, Duris K, Lekic T, Tang J, Zhang JH (2012) alpha7 nicotinic acetylcholine receptor agonism confers neuroprotection through GSK-3beta inhibition in a mouse model of intracerebral hemorrhage. Stroke 43(3):844–850

Krafft PR, Caner B, Klebe D, Rolland WB, Tang J, Zhang JH (2013) PHA-543613 preserves blood-brain barrier integrity after intracerebral hemorrhage in mice. Stroke 44(6):1743–1747

Lee FY, Sun CK, Sung PH, Chen KH, Chua S, Sheu JJ, Chung SY, Chai HT, Chen YL, Huang TH, Huang CR, Li YC, Luo CW, Yip HK (2018) Daily melatonin protects the endothelial lineage and functional integrity against the aging process, oxidative stress, and toxic environment and restores blood flow in critical limb ischemia area in mice. J Pineal Res 65(2):e12489

Lees Kennedy R, Bluhmki Eirich, von Kummer Rüdiger, Brott Thomas G, Toni Danilo, Grotta James C, Albers Gregory W, Kaste Markku, Marler John R, Hamilton Scott A, Tilley Barbara C, Davis Stephen M, Donnan Geoffrey A, Hacke Werner (2010) Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. Lancet 375(9727):1695–1703

Li M, Chen S, Shi X, Lyy C, Zhang Y, Tan M, Wang C, Zang N, Liu X, Hu Y, Shen J, Zhou L, Gu Y (2018) Cell permeable HMGB1-binding heptamer peptide ameliorates neurovascular complications associated with thrombolytic therapy in rats with transient ischemic stroke. J Neuroinflammation 15(1):237

Liebner S, Dijkstraen RM, Reiss Y, Plate KH, Agalliu D, Constantin G (2018) Functional morphology of the blood–brain barrier in health and disease. Acta Neuropathol 135(3):311–336

Liu W, Hendren J, Qin XJ, Shen J, Liu KJ (2009) Normobaric hyperoxia attenuates early blood-brain barrier dysfunction by inhibiting MMP-9-mediated occludin degradation in focal cerebral ischemia. J Neurochem 108(3):811–820

Liu Y, Liu WC, Sun Y, Shen X, Wang X, Shu H, Pan R, Liu CF, Liu W, Liu KJ, Jin X (2017) Normobaric hyperoxia extends neuro- and vaso-protection of N-Acetylcysteine in transient focal ischemia. Mol Neurobiol 54(5):3418–3427

Liu C, Xie J, Sun S, Li H, Li T, Jiang C, Chen X, Wang J, Le A, Wang J, Li Z, Wang J, Wang W (2020) Hemorrhagic Transformation After Tissue Plasminogen Activator Treatment in Acute Ischemic Stroke. Cell Mol Neurobiol. https://doi.org/10.1007/s10571-020-00985-1

Lo EH, Dalkara T, Moskovitz MA (2003) Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 4(5):399–415

Mabuchi T, Kitagawa K, Kuwabara K, Takasawa K, Ohtsuki T, Xia Z, Storm D, Yanagihara T, Hori M, Matsumoto M (2001) Phosphorylation of cAMP response element-binding protein in hippocampal neurons as a protective response after exposure to glutamate in vitro and ischemia in vivo. J Neurosci 21(23):9204–9213

Markus RP, Santos JM, Zago W, Reno LA (2003) Melatonin nocturnal surge modulates nicotinic receptors and nicotine-induced
Semenova S, Jin X, McClure-Begley TD, Tadman MP, Marks MJ, Qin C, Zhou LQ, Ma XT, Hu ZW, Yang S, Chen M, Bosco DB, Wu Pavlov VA, Parrish WR, Rosas-Ballina M, Ochani M, Puerta M, Parada E, Buendia I, Leon R, Negredo P, Romero A, Cuadrado A, Nishibori M, Wang D, Ousaka D, Wake H (2020) High Mobility Group Box-1 and Blood-Brain Barrier Disruption. Cells. https://doi.org/10.3390/cells9122510

Moretti R, Zanin A, Pansiot J, Spiri D, Manganuzzi L, Kratzer LJ, Tian DS (2019) Dual Functions of Microglia in Ischemic Stroke. Neurosci Bull 35(5):921–933

Parada E, Egea J, Buendia I, Negredo P, Cunha AC, Cardoso S, Soares MP, Lopez MG (2013) The microbial alph7-acetylcholine nicotinic receptor is a key element in promoting neuroprotection by inducing heme oxygenase-1 via nuclear factor erythroid-2-related factor 2. Antioxid Redox Signal 19(11):1135–1148

Parada E, Buendia I, Leon R, Negredo P, Romero A, Cuadrado A, Lopez MG, Egea J (2014) Neuroprotective effect of melatonin against ischemia is partially mediated by alph7 nicotinic receptor modulation and HO-1 overexpression. J Pineal Res 56(2):204–212

Pavlov VA, Parrish WR, Rosas-Ballina M, Ochani M, Puerta M, Ochani K, Chavan S, Al-Abed Y, Tracey KJ (2009) Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. Brain Behav Immun 23(1):41–45

Qin C, Zhou LQ, Ma XT, Hu ZW, Yang S, Chen M, Bosco DB, Wu LJ, Tian DS (2019) Dual Functions of Microglia in Ischemic Stroke. Neurosci Bull 35(5):921–933

Qiu J, Nishimura M, Wang Y, Sims JR, Qiu S, Savitz SI, Salomone S, Moskowitz MA (2008) Early release of HMGB1 from neurons after the onset of brain ischemia. J Cereb Blood Flow Metab 28(5):927–938

Sasaki T, Takemori H, Yagit a Y, Terasaki Y, Uebi T, Horike N, Takagi H, Susumu T, Teraoka H, Kusano K, Matano O, Oyama N, Sugiyama Y, Sakoda S, Kitagawa K (2011) SIK2 is a key regulator for neuronal survival after ischemia via TORC1-CREB. Neuron 69(1):106–119

Semenova S, Jin X, McClure-Begley TD, Tadman MP, Marks MJ, Markou A (2018) Differential effects of withdrawal from intermittent and continuous nicotine exposure on reward deficit and somatic aspects of nicotine withdrawal and expression of alpha4beta2 nAChRs in Wistar male rats. Pharmacol Biochem Behav 171:54–65

Shichita T, Agot T, Kamouchi M, Kitazono T, Yoshimura A, Ooboshi H (2012) Novel therapeutic strategies targeting innate immune responses and early inflammation after stroke. J Neurochem 123(Suppl 2):29–38

Sinha V, Roth S, Veltkamp R, Liesz A (2016) HMGB1 as a Key Mediator of Immune Mechanisms in Ischemic Stroke. Antioxid Redox Signal 24(12):635–651

Su EJ, Fredriksson L, Geyer M, Folestad E, Cale J, Andrae J, Gao Y, Peters K, Mann K, Yepes M, Strickland DK, Betsholtz C, Erikson U, Lawrence DA (2008) Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. Nat Med 14(7):731–737

Sun Y, Chen X, Zhang X, Shen X, Wang M, Wang X, Liu WC, Liu CF, Liu J, Liu W, Jin X (2017) β2-Adrenergic receptor-mediated HIF-1alpha upregulation mediates blood brain barrier damage in acute cerebral ischemia. Front Mol Neurosci 10:257

Tanaka K, Nagata E, Suzuki S, Dembo T, Nogawa S, Fukuzumi Y (1999) Immunohistochemical analysis of cyclic AMP response element binding protein phosphorylation in focal cerebral ischemia in rats. Brain Res 818(2):520–526

The NINDS t-PA Stroke Study Group (1997) Intracerebral hemorrhage after intravenous t-PA therapy for ischemic stroke. Stroke 28(11):2109–2118

Verrijdt G, Haelens A, Schoenmakers E, Rombauts W, Claessens F (2002) Comparative analysis of the influence of the high-mobility group box 1 protein on DNA binding and transcriptional activation by the androgen, glucocorticoid, progesteron and mineralocorticoid receptors. Biochem J 361(Pt 1):97–103

Wang H, Liao H, Ochani M, Justman I, Lin X, Yang L, Al-Abed Y, Metz C, Miller EJ, Tracey KJ, Ulloa I (2004) Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med 10(11):1216–1221

Wang Q, Wang F, Li X, Yang Q, Xu N, Huang Y, Zhang Q, Gou X, Chen S, Xiong L (2012) Electroacupuncture pretreatment attenuates cerebral ischemic injury through alph7 nicotinic acetylcholine receptor-mediated inhibition of high-mobility group box 1 release in rats. J Neuroinflammation 9:24

Wang X, Liu Y, Sun Y, Liu W, Jin X (2016) Blood brain barrier breakdown was found in non-infarcted area after 2-h MCAO. J Neurol Sci 363:63–68

Wang X, Xue GX, Liu WC, Shu H, Wang M, Sun Y, Liu X, Sun YE, Liu CF, Liu J, Liu W, Jin X (2017) Melatonin alleviates lipopolysaccharide-compromised integrity of blood-brain barrier through activating AMP-activated protein kinase in old mice. Aging Cell 16(2):414–421

Wardlaw JM, Murray V, Berge E, del Zoppo G, Sandercop P, Lindley RL, Cohen G (2012) Recombinant tissue plasminogen activator for acute ischaemic stroke: an updated systematic review and meta-analysis. Lancet 379(9834):2364–2372

Won S, Sayeed I, Peterson BL, Wali B, Kahn JS, Stein DG (2015) Vitamin D prevents hypoxia/eoxygenation-induced blood-brain barrier disruption via vitamin D receptor-mediated NF-kB signaling pathways. PLoS One 10(3):e0122821

Yang Y, Jalal FY, Thompson JF, Walker EJ, Candelario-Jalil EJ, Li L, Reichard RR, Ben C, Sang QX, Cunningham LA, Rosenberg GA (2011) Tissue inhibitor of metalloproteinases-3 mediates the death of immature oligodendrocytes via TNF-alpha/TACE in focal cerebral ischemia in mice. J Neuroinflammation 8:108

Yang Y, Guan D, Lei L, Liu J, Liu QJ, Yang G, Yan C, Zhai R, Tian J, Bi Y, Fu F, Wang H (2018) H6, a novel hederagenin derivative, reverses multidrug resistance in vitro and in vivo. Toxicol Appl Pharmacol 341:98–105

Zhang J, Takahashi HK, Liu K, Wake H, Liu R, Maruo T, Date I, Ohtsuka A, Mori S, Nishibori M (2011) Anti-high mobility group box-1 monoclonal antibody protects the blood-brain barrier from ischemia-induced disruption in rats. Stroke 42(5):1420–1428

Zhang X, Shen X, Dong J, Liu WC, Song M, Sun Y, Shu H, Towse CL, Liu W, Liu CF, Jin X (2019) Inhibition of Reactive Astrocytes with Fluoroceiclate Ameliorates Learning and Memory Impairment...
Through Upregulating CRTC1 and Synaptophysin in Ischemic Stroke Rats. Cell Mol Neurobiol 39(8):1151–1163
Zhao Z, Lu C, Li T, Wang W, Ye W, Zeng R, Ni L, Lai Z, Wang X, Liu C (2018) The protective effect of melatonin on brain ischemia and reperfusion in rats and humans: In vivo assessment and a randomized controlled trial. J Pineal Res 65(4):e12521
Zhu F, Chong Lee Shin OL, Xu H, Zhao Z, Pei G, Hu Z, Yang J, Guo Y, Mou J, Sun J, Zhu H, Wang Y, Wang M, Yang Q, Liao W, Xu G, Zeng R, Yao Y (2017) Melatonin promoted renal regeneration in folic acid-induced acute kidney injury via inhibiting nucleo-cytoplasmic translocation of HMGB1 in tubular epithelial cells. American journal of translational research 9(4):1694–1707
Zidovetzki R, Chen P, Fisher M, Hofman FM, Faraci FM (1999) Nicotine increases plasminogen activator inhibitor-1 production by human brain endothelial cells via protein kinase C-associated pathway. Stroke 30(3):651–655
Zou D, Luo M, Han Z, Zhan L, Zhu W, Kang S, Bao C, Li Z, Nelson J, Zhang R, Su H (2017) Activation of Alpha-7 Nicotinic Acetylcholine Receptor Reduces Brain Edema in Mice with Ischemic Stroke and Bone Fracture. Mol Neurobiol 54(10):8278–8286

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