Melatonin inhibits postischemic matrix metalloproteinase-9 (MMP-9) activation via dual modulation of plasminogen/plasmin system and endogenous MMP inhibitor in mice subjected to transient focal cerebral ischemia

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Keywords:	stroke, matrix metalloproteinase, plasminogen/plasmin system, tissue inhibitor of metalloproteinases (TIMP), melatonin

Melatonin inhibits postischemic matrix metalloproteinase-9 (MMP-9) activation via dual modulation of plasminogen/plasmin system and endogenous MMP inhibitor in mice subjected to transient focal cerebral ischemia

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Abstract

We have shown that melatonin attenuated matrix metalloproteinase-9 (MMP-9) activation and decreased the risk of hemorrhagic transformation following cerebral ischemia-reperfusion. We, herein, investigate the possible involvement of the plasminogen/plasmin system and endogenous MMPs inhibitor underlying the melatonin-mediated MMP-9 inhibition. Mice were subjected to 1-hr ischemia and 48-hr reperfusion of the right middle cerebral artery. Melatonin (5 mg/kg) or vehicle was intravenously injected upon reperfusion. Brain infarction and hemorrhagic transformation were measured. Extracellular matrix (ECM) damage was determined by Western immunoblot analysis for laminin protein. The activity and expression of MMP-2 and -9 were determined by gelatin zymography, in situ zymography and Western immunoblot analysis. In addition, the activities of tissue and urokinase plasminogen activators (tPA and uPA) were evaluated by plasminogen-dependent casein zymography. Endogenous plasminogen activator inhibitor (PAI) and tissue inhibitors of MMP (TIMP-1) were investigated by using enzyme linked immunosorbent assay (ELISA) and Western immunoblot analysis, respectively. Cerebral ischemia-reperfusion induced increased MMP-9 activity and expression at 12-48 hr after reperfusion onset. Relative to controls, melatonin-treated animals had significantly decreased MMP-9 activity and expression ($P<0.05$), in addition to reduced brain infarction and hemorrhagic transformation as well as improved laminin protein preservation. This melatonin-mediated MMP-9 inhibition was accompanied by reduced uPA activity ($P<0.05$), as well as increased TIMP-1 expression and PAI activity ($P<0.05$, respectively). These results demonstrate the melatonin's pleuripotent mechanisms for attenuating postischemic MMP-9 activation and neurovascular damage, and further support it as an add-on to thrombolytic therapy for ischemic stroke patients.

Key Words: stroke; matrix metalloproteinase; plasminogen/plasmin system, tissue inhibitor of metalloproteinases (TIMP), melatonin.

Introduction

Recent studies have highlighted the involvement of matrix metalloproteinases (MMPs) in the pathogenesis of cerebral ischemic-reperfusion damage. Following the onset of ischemia, MMP enzymes were excessively activated and expressed [1-4]. The activated and over-expressed MMPs induce endothelial damage, and can further cleave protein components of the extracellular matrix (ECM) such as collagen, proteoglycan and basal laminin, leading to transmigration of inflammatory cells and large, toxic molecules into the brain parenchyma [4-6]. In particular, delayed vascular reperfusion induces disruption of the blood-brain barrier (BBB) by up-regulated MMPs [4, 6, 7]. These consequences can result in deleterious brain edema and hemorrhage, and considerably increase post-stroke morbidity and mortality [2, 7, 8]. MMPs are, therefore, thought to be the terminal enzymes in the ECM remodeling cascade. It has been demonstrated that inhibition of MMP-9 either by genetic or pharmacological approach could be beneficial in animals subjected to cerebral ischemia-reperfusion [2-4]. One treatment modality for ischemic stroke patients, therefore, is to provide an add-on to the current thrombolytic therapy so as to inhibit postischemic MMP activation and to decrease the thrombolysis-induced hemorrhagic transformation and brain edema [6, 9, 10, 11, 12].

The catalytic activities of MMPs are highly regulated at multiple levels, including transcription, secretion, zymogen activation, and inhibition by tissue inhibitors of metalloproteinases (TIMPs) [4, 10, 13]. Notably, the active forms of MMPs are stoichiometrically regulated by the TIMPs. Additionally, the plasminogen system has been implicated in the turnover and maintenance of the basal lamina [5, 10, 14, 15]. This complex system of activators and inhibitors of different enzymes controls a series of the processes through which proenzyme plasminogen is activated to plasmin either by tissue (t-PA) or urokinase plasminogen activator (u-PA). Specially,

the u-PA, which is primarily involved in cell surface proteolysis and ECM degradation, has been reported to increase in the rodent ischemic brain after experimental stroke [10, 14]. Regulation of endogenous plasminogen activator synthesis and release, therefore, could be important mechanism underlying the MMP-9-mediated damage caused by cerebral ischemia.

Melatonin (*N*-aceyl-5-methoxytryptamine) has a variety of pharmacological actions that may be beneficial in the treatment of acute stoke [16-18]. This natural neuroprotectant and its metabolites are well known to be potent free radical scavengers and antioxidants [19, 20]. We have previously shown that exogenous administration with melatonin reduced oxidative damage, protected against both gray and white matter and synapto-dendritic pathology, and improved neurobehavioral and electrophysiological outcomes following transient focal cerebral ischemia in mice and rats, respectively [21-23]. In the context of neurovascular protection after stroke, we have also demonstrated that melatonin improved the preservation of the BBB permeability, attenuated the oxidative/nitrosative damage to the ischemic neurovascular unit, and decreased a risk of hemorrhagic transformation following the recombinant tissue plasminogen activator (t-PA) therapy in experimental ischemic stroke models in mice [9, 11]. It is also known that melatonin consistently modulates MMP-9 activity and expression in various experimental models of gastric ulcer, endometriosis and spinal cord injury [24-29]. Alternatively, we have demonstrated that melatonin effectively attenuated post-ischemic MMP-9 activation, and, thus, inhibited systemic leukocyte transmigration into the ischemic brain and local cerebral microglial activation at the subacute stage of focal cerebral ischemia in rats [12, 30]. While some melatonin-mediated inhibitory effects for postischemic MMP-9 activation have been characterized [12], its molecular basis and the possible involvement of TIMPs remains not clarified in the context of stroke [26, 27]. In attempt to extend our initial work, we

further measured the time-course changes of MMP activation and its inhibitory effect with melatonin therapy at acute and subacute stages of stroke. We also explored the possible mechanisms of action underlying melatonin's inhibitory ability against the MMP-9 activation and increased expression following stroke.

In the present study, we, therefore, examined whether intravenous administration with melatonin (5 mg/kg) at reperfusion onset could reduce the MMP-2 and MMP-9 activation and increased expression at acute and subacute stages of ischemic stroke and, therefore, improve the preservation of the ECM integrity in the ischemic brain. We also explored possible involvement of the plasminogen/plasmin system and TIMPs underlying the melatonin-mediated MMP-9 inhibition following transient focal cerebral ischemia in mice.

Materials and Methods

All procedures performed were approved by the Subcommittee on Research Animal Care of the University Medical Center, whose standards meet the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals).

Animal preparation, anesthesia, and monitoring

Adult male C57 black (C57BL/B6) mice, weighing 20-26 g, were supplied by the University Laboratory Animal Center, and were allowed free access to food and water before and after surgery. Animals were anesthetized with 1-2 % halothane in 70 % N_2O/30 % O_2. During surgery, body temperature was maintained at 37.0±0.5 °C, which corresponds to brain temperature of 37.2±0.5 °C [31, 32], using a thermostatically controlled heating blanket and rectal probe (Harvard Apparatus, South Natick, MA, USA).

Experimental model, drug administration, and grouping of animal

Focal cerebral ischemia was employed by intra-arterial suture occlusion of the proximal right middle cerebral artery (MCA) [12, 21, 23]. Briefly, the bifurcation of the right common carotid artery was exposed under an operating microscope. A 6-0 nylon suture, with its tip rounded by heating over a flame and subsequently coated with silicone (Merck KGaA, Darmstadt, Germany), was advanced 7.5-8.5 mm from the external into the internal carotid artery until the tip occluded the origin of the MCA. After closure of the operative sites, the animals were allowed to awaken from the anesthesia and temporarily transferred to a cage with a heating lamp (ambient temperature ≈26±1 ºC). During another brief period of anesthesia, the suture was gently removed at 60 min of MCA occlusion. Reperfusion was ensured by an improvement in the ipsilateral local cortical blood perfusion (LCBF) at a defined area of the ischemic core cortex to about 50% of baseline following an initial

decrease to about 20% of baseline caused by MCA occlusion as determined by Laser-Doppler flowmetry (LDF, Laserflo BMP^2, Vasamedics, St. Paul, MN, USA). After the surgical procedures, the animals were kept in a cage with a heating lamp and monitored for 2 h and then transferred into the home cage (ambient temperature ≈24±1 ºC).

Melatonin (Sigma-Aldrich Co., St Louis, MO, USA) was dissolved in a mixture of PEG 400 (Sigma-Aldrich) and 0.9% normal saline (3:7, vol/vol). Fresh drug solution was prepared in a dark hood shortly before the administration. Animals were given intravenously either with melatonin (5 mg/kg, n=25) or vehicle (PEG-saline, n=26) at the initiation time of reperfusion (i.e., 60 min after the onset of ischemia). Samples of the ischemic and non-ischemic brain tissues obtained from the 2 groups of animals (n=6, for both groups at each time interval) were used for zymographic examinations at 4, 12, 24 or 48 hr after the onset of reperfusion. A secondary set of animals, received melatonin (5 mg/kg, n=33) or vehicle (PEG-saline, n=33) upon reperfusion, was used to evaluate the brain infarction (n=8 for both groups), *in situ* zymographic examinations (n=8 for both groups), plasminogen-dependent casein zymography (n=8 for both groups) as well as Western immunoblot (n=8 for both groups) at 24 hr of reperfusion. In an additional subset of animals receiving melatonin (5 mg/kg, n=6) or vehicle (PEG-distilled water, n=6), the right femoral artery was cannulated by a PE 10 catheter for measuring arterial blood gases, glucose and hematocrit prior to and during ischemia and at 20 min after reperfusion. Blood sampling was also employed at 24 hr of reperfusion for measuring endogenous plasminogen activator inhibitor (PAI) activity. The dose of melatonin was based on the pharmacokinetic study of exogenous melatonin in animal models [33] and the neuroprotective dose-responsive studies of melatonin in rodent models of transient focal cerebral ischemia [9, 11, 16, 21-23, 34].

LCBF monitoring

Laser-Doppler flowmetry was used for LCBF measurements [12, 21, 23]. The scalp was incised along the midline, and two 1.0-mm diameter areas in bilateral parietal bones were thinned 0.5 mm posterior and 4 mm lateral to the Bregma for placement of the LDF probes (model P436, Vasamedics, St. Paul, MN, USA). The region is close to the core of the infarction caused by MCA occlusion on the operated side. Another 1.0-mm diameter area in right parietal bone was thinned 1.0 mm posterior and 1.5 mm lateral to the Bregma for additional LCBF measurements [35]. LCBF was serially measured prior to and during the MCA occlusion, upon a brief period (10 min) of reperfusion and at 30 min after the onset of reperfusion. The LCBF data were expressed as a percentage of the baseline values.

Animal sacrifice and quantification of ischemic brain damage and hemorrhage

Following 24 h of survival, sacrifice was performed under anesthesia by transcardiac perfusion accomplished with 150 ml of 3.7% formaldehyde in 0.1 M phosphate-buffered saline (PBS). After postfixation overnight, the brains were embedded in Optimal Cutting Temperature compound (OCT, Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. The brains were sectioned 40 μm thick on a cryostat (HM-500O, Microm International GmbH, Walldorf, Germany). Serial sections of 40 μm at eight preselected coronal levels, with 1-mm intervals from the stereotaxic coordinates of the Bregma AP +2.22 to –4.78 mm [35], were mounted on poly-L-lysine-coated (Sigma Chemical Co.) slides and dried at 37℃ overnight.

Sections were stained with 0.5% cresyl violet. Under light microscopy, the areas of neuronal perikarya displaying typical morphological features of ischemic damage

were delineated using a computerized image analyzer (MCID Elite; Imaging Research Inc., St. Catharines, Ontario, Canada). Brain infarction was expressed as a percentage of the contralateral hemisphere volume by using an "indirect method" (area of intact contralateral [left] hemisphere minus area of intact region of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere [11, 22, 32, 36-38]. Hemorrhagic transformation within infarct was also quantified. Accordingly, 3 categories were scored: 0, no observable hemorrhage; 1, microscopic hemorrhage, unable or difficult to detect under macroscopic views; 2, obvious and macroscopic hemorrhage.

Cell Counting for surviving neurons and gelatinase-active cells

Coronal sections obtained between the Bregma AP -0.22 and -0.78 mm was chosen [12, 23, 30]. Six random and nonoverlapping regions (500x400 μm^2) were sampled for the ischemic core (the unstained area), the inner (right side to the margin between the stained and unstained area), and the outer (left side to the margin between the stained and unstained area) boundary zones of the infarct areas at the parietal cortex. Cell counts were expressed as the mean number of viable neurons per mm^2.

Gelatin Zymography

Brain samples were homogenized and centrifuged. Gelatinase activity in the supernatants was extracted and purified with gelatin-Sepharose 4B (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) by the method described previously [1-3, 5, 12]. Samples normalized for protein concentration were mixed with sample buffer and loaded onto 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 1.25 mg/mL gelatin. After electrophoresis, the gel was washed with 2.5% Triton X-100

buffer for three times. The gel was incubated in 100 mL of 50 mM Tris-HCl (pH 7.4) containing 5 mM CaCl2 , 200mM NaCl and 0.2% Brij 35 at 37℃ overnight. Gel was stained with 0.25% Coomassie blue R-250 (Sigma) and destained appropriately.

In situ zymography

After transcardial perfusion with ice-cold PBS, pH 7.4, brains were quickly removed without fixation and frozen in 2-methylbutane with liquid nitrogen [12]. Sections (10 μm) were cut on a cryostat and incubated at 37℃ overnight in 0.05 M PBS, containing 2 μg of fluorescein isothiocyanate (FITC)-labeled gelatin (Molecular Probes, Eugene, OR, USA). The gelatin with a fluorescent tag remains caged (no fluorescence) until the gelatin is cleaved by gelatinase activity. This method detects regionally specific gelatinolytic activity but does not distinguish between MMP-9 and MMP-2 [12]. The section was washed in PBS for 5 min three times. The brain sections were then incubated with 2.5×10^{-3} mg/ml Hoechst 33258 (Molecular Probes) in PBS for 15 s in a dark chamber and then they were rinsed in distilled H_2O and mounted with Aquamount (Shandon, Pittsburgh, PA, USA).

We used a Zeiss Axioskop 2 Mot microscope equipped with a digital CoolSnap-Pro cf camera (Media Cybernetics, Inc., Carlsbad, CA, USA) and a semiautomated image analysis system (MCID Elite) for the measurements. The density of the gelatinase-active cells, relative to the total number of the Hoechst-staining cells, on the ischemic and contralateral sides were observed and compared between the melatonin- and the vehicle-treated animals.

Plasminogen-dependent casein zymography

Polyacrylamide gels were copolymerized with casein (5 mg/ml; Sigma) and plasminogen (0.01 U/ml, i.e., 0.07 μg/ml; Sigma), as described previously [1, 5, 14]. The sample buffer did not contain β-mercaptoethanol. After renaturing the enzymes in

the gel by removing the SDS with 2.5% Triton X-100 for 1 hr at room temperature, the enzymatic digestion in the gel proceeded in an incubation buffer containing 5 mM CaCl2, 50 mM Tris/HCl, pH 7.4, 200 mM NaCl and 0.2% Brij 35 at 37℃ for 24 hr. To visualize the enzymatic digestion, the gels were stained with Coomassie blue R-250 (Sigma). The lysis zones representing the enzymatic digestion appeared as clear zones in the gel. Transparent zones of lysis correspond to t-PA and uPA, respectively.

Western blot analysis

Samples of the ischemic and the contralateral, intact brain tissues were quickly dissected on dry ice after animals' sacrifice. Sample was homogenized in lysis buffer, containing 1% Triton X-100, 20 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.5% deoxycholate, 1 mM ethylenediamenetetraacetic acid, 0.1% SDS, and was centrifuged at 18,000 g for 60 min at 4℃ [12, 23]. Protein concentrations of each sample were determined with a BCA protein assay kit (Pierce, Rockford, IL).

Cell lysate of 30 μg protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE), and was transferred onto polyvinylidene difluoride microporous membranes (IPVH00010, Millipore; Billerica, MA, USA). Membranes were blocked with 5% milk, then probed with primary antibodies against MMP-9 (monoclonal rabbit anti-MMP-9, dilution 1:500; Chemicon International, Temecula, CA, USA), laminin (polyclonal rabbit anti-laminin, dilution 1:1000; Abcam, Cambridge, UK), tissue inhibitor of metalloproteinase-1 (polyclonal rabbit anti-TIMP-1, dilution 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), extracellular-regulated kinase (monoclonal rabbit anti-ERK, dilution 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) and diphospho-ERK-1/2 (monoclonal mouse anti-diphospho-ERK-1/2, dilution 1:1000; Sigma), and then incubated with horseradish peroxidase–conjugated immunoglobulin (1:5,000; Chemicon International)

at room temperature for 1 hr. Bound antibody was visualized with the Amersham ECL system (GE Healthcare Bio-Sciences Corp.). Membranes were then probed for β-actin (1:10,000; Chemicon International). Optical densities were measured by a Luminescent Image Analyzer (Fujifilm LAS-3000; Fuji Photo Film Co., Tokyo, Japan).

Assay for endogenous plasminogen activator inhibitor (PAI)

According to the manufacturer's instructions, plasma samples of 100 μl were added to 96-well coated microtiter strip plate for the assay of active PAI-1 activity by using a PAI-1 activity assay kit (Innovative Research, Southfield, MI, USA). The lower limit of detection for the PAI assay was 0.02 ng/ml. The absorbance was measured at 450 nm using a plate reader (Stat Fax 2100, Awareness Technology, Inc., FL, USA).

Neurobehavioral testing and body weight measurements

Body weight measurement was employed daily. A battery of sensory-motor tests was conducted prior to and after the ischemia-reperfusion insult and on a daily basis up to 48 hr after reperfusion by two observers unaware of treatment protocol. Briefly, two neurologic grading systems were used: (1) a sensorimotor grading scale modified from previously published methods [12, 21, 23, 36] with five categories (0 to 4) for forward and sideways visual placing tests of the affected forelimb and five categories (0 to 4) for motor outcome and (2) a grading scale of 0 to 28 developed by Clark et al [39].

Statistical analysis

All data were expressed as the mean±standard error of the mean (SEM). Paired Students' *t* test was used to evaluate the response to a change in conditions, and

unpaired Students' *t* test was used to evaluate differences between groups. Neurobehavioral and hemorrhagic transformation scores were analyzed by Mann-Whitney *U* test. $P < 0.05$ was selected for statistical significance.

Results

Throughout the course of experiments, five animals (3.9 %) died spontaneously prior to completing the recovery protocol and were excluded: 3 were in the vehicle-injected group and 2 were in the melatonin-treated group. The postischemic core temperature and LCBF were not statistically different at each sampling time interval between controls and study animals (data not shown). The other physiological parameters of the animals were kept within normal physiologic limits during the course of experiments, and did not differ significantly between melatonin-treated animals and vehicle-injected controls (data not shown).

Following the onset of ischemia, both the 2 groups of animals did not have altered levels in the MMP-2 and MMP-9 enzyme activity at the contralateral, intact brain (Figs. 1A, B). Postischemic activation of MMP-9 enzyme did not occur at 4 hr of reperfusion, but was observed in ischemic brain at 12, 24 and 48 hr after reperfusion onset. As compared with vehicle-injected stroke group, melatonin-treated animals had significantly decreased levels in MMP-9 enzyme activity by 57.6% and 53.9% at 24 and 48 hr after reperfusion onset (Figs. 1A, B; $P<0.05$, respectively). This melatonin-mediated decrease in MMP-9 enzyme activity was accompanied with reduced levels of *in situ* gelatinolytic activity by 63.7% (Fig. 1C; $P<0.05$) and MMP-9 protein expression by 27.9% as well (Fig. 1D; $P<0.05$). Melatonin, however, did not modulate the activity of MMP-2 in the ischemic brain at 4-48 hr after reperfusion onset.

The endogenous MMP inhibitor, TIMP-1, was assessed to determine the influence of melatonin on the MMPs. To ascertain a direct causal relationship between the mitogen-activated protein kinases and MMP-9 activity, samples of brain tissues were subjected to Western blot analysis for expression of ERK-1/2. It was noted that melatonin-mediated decreases in the MMP-9 activity and expression were accompanied

by increased expression of TIMP-1 by 130.0% (Fig. 2A; $P<0.05$). Melatonin also effectively modulates the expression of p-ERK1/2 at 24 hr of reperfusion (Fig. 2B; $P < 0.05$).

The effects of melatonin on plasminogen activator inhibitor (PAI-1) and activators were then tested. It was interesting to note that melatonin-treated animals had increased plasma PAI-1 concentrations by 113.3%, compared to the data in controls (Fig. 3A; $P < 0.001$). As described previously, endogenous t-PA levels in the brain were not significantly increased in both the ischemic and the contralateral, non-ischemic brain at 24 hr of reperfusion (Fig. 3B). The protease u-PA was the only plasminogen activator affected by cerebral ischemia-reperfusion (Fig. 3B). We have observed that melatonin treatment did not affect the levels of the t-PA activity (Fig. 3C; $P > 0.05$). Melatonin treatment also did not change the amount of u-PA activity in the non-ischemic brain, but significantly attenuated the postischemic increase in the u-PA activity by 45.8% in the ischemic brain, compared to the values in controls (Fig. 3D; $P < 0.05$).

Transient MCA occlusion resulted in large ipsilateral cortical and striatal infarcts that were reproducible but variable in size. Animals received an intravenous injection of melatonin (n=8) showed a significant reduction in brain infarct volumes by 39.1% (Figs. 4A, B; 45.3%±3.5 vs. 27.6%±4.3, $P < 0.01$), compared with vehicle-injected controls (n=8). This reflected a melatonin-induced decrease in infarct size by 39.1% in cortex and by 49.5% in caudoputamen (Fig. 4C; $P < 0.05$, respectively), and an increase in the surviving neurons by 302.4% in cortex and by 71.3% in caudoputamen (Fig. 4D; $P < 0.01$, respectively). Besides, melatonin significantly decreased the amount of hemorrhagic transformation within infarct by 77.1% (Figs. 4A; median score (95% confidence interval): controls=2.0 (0.9-2.0) vs. melatonin-treated animals=0.0 (0.5-0.7), $P < 0.01$), and significantly improved the preservation of the ECM protein laminin by 35.4% (Fig. 4E; $P < 0.05$), compared to the data in controls. Moreover, melatonin-treated animals showed

significantly improved sensory, motor and the 28-point neurologic scores taken 48 h after the onset of reperfusion than did the vehicle-injected controls (Table 1; $P < 0.05$).

Discussion

The present study demonstrates that administration with melatonin upon reperfusion resulted in persistent attenuation in postischemic increases in MMP-9 enzyme activity and protein expression at the subacute stage of 24-48 hrs after transient focal cerebral ischemia in mice. These melatonin-induced decreases in MMP-9 enzyme activity and protein expression were correlated with reduced u-PA activity as well as increased TIMP-1 expression and PAI-1 activity. Consequently, melatonin improved the preservation of the ECM integrity and reduced brain infarct volumes and hemorrhagic transformation as well. This neuroprotection cannot be accounted for by changes in hemodilution (as measured by blood hematocrit), arterial blood pressure, heart rate or differences in core temperature, because these were not significantly different when compared between vehicle-injected and melatonin-treated animals.

Several mechanisms might have actually mediated the melatonin-induced MMP-9 inhibition observed here and as described previously [12, 22-27]. First, reactive oxygen species may play an important role in the regulation of distinct signaling cascades [13, 40-44], many of which act upon the production of matrix metalloproteinases (MMP). Redox-sensitive MMP protein expression requires activation of both ERK1/2 and JNK pathways [44, 45]. Thus, the finding with an inhibitory action of melatonin on p-ERK1/2 suggested its possible responsible for transcriptional inhibition of MMP-9 by melatonin. Alternatively, melatonin has also been shown to block the activated protein factor-1, a transcription-stimulating factor binding to the MMP promotor [1, 46, 47]. Moreover, melatonin has been demonstrated to exhibit an inhibitory action for the protein kinase C, which is part of intracellular signal pathway leading to the activating protein factor-1 complex [48, 49]. It was also possible that melatonin might affect the post-translational level by coordination with the catalytic side, thus leading to conformational changes to block active site, and this, however, needs further evaluation.

In the study, we observed that melatonin could reduce MMP-9 activity by increasing the biosynthesis of endogenous MMP inhibitor, TIMP-1. The finding agrees with previous observations that the regulation of TIMP played an inhibition of metalloproteolytic activities by melatonin [26, 27]. Other possible mechanism might have included the melatonin-mediated attenuation in the in vivo activation of pro-MMP zymogens [12]. In the present study, we have noted that the B6 mice, as opposed to the Sprague-Dawley rats, did not expressed observable amount of active forms of MMP-2 and MMP-9 in the ischemic brain. Our previous work has, however, demonstrated that active form of MMP-9 was significantly reduced with melatonin treatment [12].

Another important mechanism underlying the melatonin-mediated MMP inhibition is its ability to modulate the plasminogen/plasmin system [10, 14, 15]. Plasmin, the conversion product of plasminogen by plasminogen activators, is closely involved in the activation of MMPs. A considerable upregulation of u-PA in ischemic brain further suggests the involvement of this protease in the pathogenesis of ischemic brain damage [10, 14]. Thus, the melatonin-induced decrease of u-PA levels and the increase of the biosynthesis of their specific inhibitor PAI-1 would lead to reduced formation of plasmin, which in turn lowered the amount of active MMPs. Modulation of plasminogen activator activity and their inhibitor PAI-1 are, therefore, melatonin's additional targets accounting for its ability for attenuating postischemic MMP-9 activation and, consequently, limiting the maturation of brain damage. Accordingly, melatonin treatment not only effectively reduced the in vivo activation of pro-MMP-9 and the increased level of u-PA activity, but also significantly increased the levels of the endogenous MMP inhibitor, TIMP-1, and plasminogen activator inhibitor, PAI-1, following cerebral ischemia-reperfusion. However, we could not completely exclude the possibility that the melatonin-mediated MMP-9 inhibition observed here might simply reflect its potent neuroprotective action against ischemic stroke, since the results were interpreted on the basis of whole animal data in

the study. Further in vitro studies are needed to clarify this limitation.

Degradation of the ECM by the activated MMPs and the plasminogen/plasmin system resulted in the destabilization of the neurovascular unit, consequently, leading to brain edema and extravasation of blood [6, 10, 12, 15, 50]. Our finding that melatonin effectively attenuated postischemic activation of MMPs via dual modulation of the endogenous MMP inhibitor and the plasminogen/plasmin system further justified its ability to preserve the ECM integrity and decrease hemorrhagic transformation within infarct, as described here and reported previously [9, 11, 12, 30]. These results further highlighted melatonin's protective ability against ischemic neurovascular damage [11], and strongly recommended that it is a useful agent suited as an add-on of thrombolysis in the treatment of ischemic stroke.

Conclusion

Our results demonstrated that melatonin effectively inhibit postischemic MMP-9 activation and increased expression via dual modulations of the endogenous MMP inhibitor and the plasminogen/plasmin system. These results further clarified the melatonin's pleuripotent mechanisms for attenuating postischemic ECM damage and hemorrhagic transformation, and further support it suited as an add-on to thrombolytic therapy for ischemic stroke patients.

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Table 1. Melatonin improves sensorimotor behavioral scores after cerebral ischemia-reperfusion.

	Weight Loss(g)	Neurologic Behavioral Score		
		Sensory	Motor	28-point clinical scale
Vehicle- treated (n = 8)	4.8±0.3	3.0 (2.9-3.1)	2.0 (1.9-2.1)	15.0 (14.6-15.4)
Melatonin-treated (n = 8)	4.2±0.2	1.0 (0.9-1.1)*	1.0 (0.9-1.1)*	9.0 (8.8-9.2)*

Weight data and neurologic behavioral scores are expressed by mean± SEM and by median (95%CI), respectively. Intravenous injection of melatonin (5 mg/kg) 60 min after the onset of the right middle cerebral artery (MCA) occlusion (i.e., upon reperfusion) significantly improved sensorimotor neurologic scores compared to vehicle-injected control values. *P<0.05 vs vehicle data, respectively.

Legends

Fig. 1. (A) Gelatin-dependent zymography of protein extracts from the ischemic and the contralateral, intact brain in the melatonin-treated (T) and the controls (C) animals at 4, 12, 24 and 48 hr after reperfusion. The lytic zones of MMP-9 and MMP-2 were digitized and expressed as optic density (OD) values. (B) Neither the ischemic insult nor the melatonin treatment significantly affected the MMP-2 and MMP-9 activity in contralateral, intact brain. Melatonin-treated animals, however, had significantly reduced MMP-9, but not MMP-2, activity at 24 and 48 hr of reperfusion, compared to controls. (C) Relative to controls, melatonin-treated animals had significantly suppressed *in situ* gelatinolytic activity, as assessed by gelatinase-active cell density. Scale bars=100 μm. (D) The photographs shows typical pattern of changes in MMP-9 protein expression in the ischemic (R) and non-ischemic (L) brain at 24 hr after reperfusion. Densitometric analysis showed that melatonin significantly decreased MMP-9 expression in the ischemic brain. Data are expressed by mean±SEM.

*$P<0.05$ vs. control. $n=6$ animals per group at each time interval.

Fig. 2. Western immunoblot analysis for the tissue inhibitors of MMP (TIMP-1) and the dual-phosphorylated form of extracellular signal regulated kinase (pERK-1/2) and total ERK-1/2 in the ischemic brain. (A) The photographs show that melatonin-treated animals had increased TIMP-1 expression in the ischemic brain, compared to the values in controls. (B) Besides, melatonin attenuated the increased expressions of the p-EKR1/2 in the ischemic brain. *$P<0.05$ vs. control. $n=6$ (each column).

Fig. 3. Assays for endogenous plasminogen activator inhibitor (PAI) and plasminogen-dependent casein zymography. (A) Melatonin-treated animals had significantly increased plasma PAI-1 concentrations, compared to the data in controls (B) Plasminogen-dependent zymography of protein extracts from contralateral, intact and the ischemic brain tissues in the controls and the melatonin-treated animals at 24 hr after reperfusion. The activities of tissue and urokinase plasminogen activators (tPA and uPA) are shown. Melatonin-treated animals had significantly reduced u-PA (C), but not t-PA (D), activity, compared to controls. Data are expressed by mean±SEM. These values were normalized. n=6 animals per group. * P<0.05 vs. control and *** P<0.001 vs. control.

Fig. 4. Melatonin reduced brain damage and hemorrhagic transformation and improved the preservation of extracellular matrix protein in the ischemic brain. A) The cresyl violet-stained coronal sections were from representative animals which received an intravenous injection of vehicle (PE-saline) or melatonin (5 mg/kg) at 60 min after the ischemic onset. Six random and nonoverlapping (500x400 μm^2) regions in the borders of the ischemic parietal cortex and caudoputamen were selected for counting the surviving neurons. Scale bar=5 mm. The inset in the melatonin-treated group showed much attenuated hemorrhagic transformation within infarct, compared to the control group. Scale bar in inset= 100 µm. Additionally, melatonin-treated animals (n=8) had significantly reduced the infarction volume (B) and individual cortical and striatal lesion sizes (C), and significantly increased numbers of surviving neurons (D), compared to controls (n=8). Moreover, improved preservation of laminin protein expression (E) was observed in

melatonin-treated animals, compared to controls. n=6 (each column). * P<0.05 vs. control; ** P<0.01 vs. control, and *** P<0.001 vs. control.

1

1,2

1

1,3

1

1

1

1

1

1,2,4

1

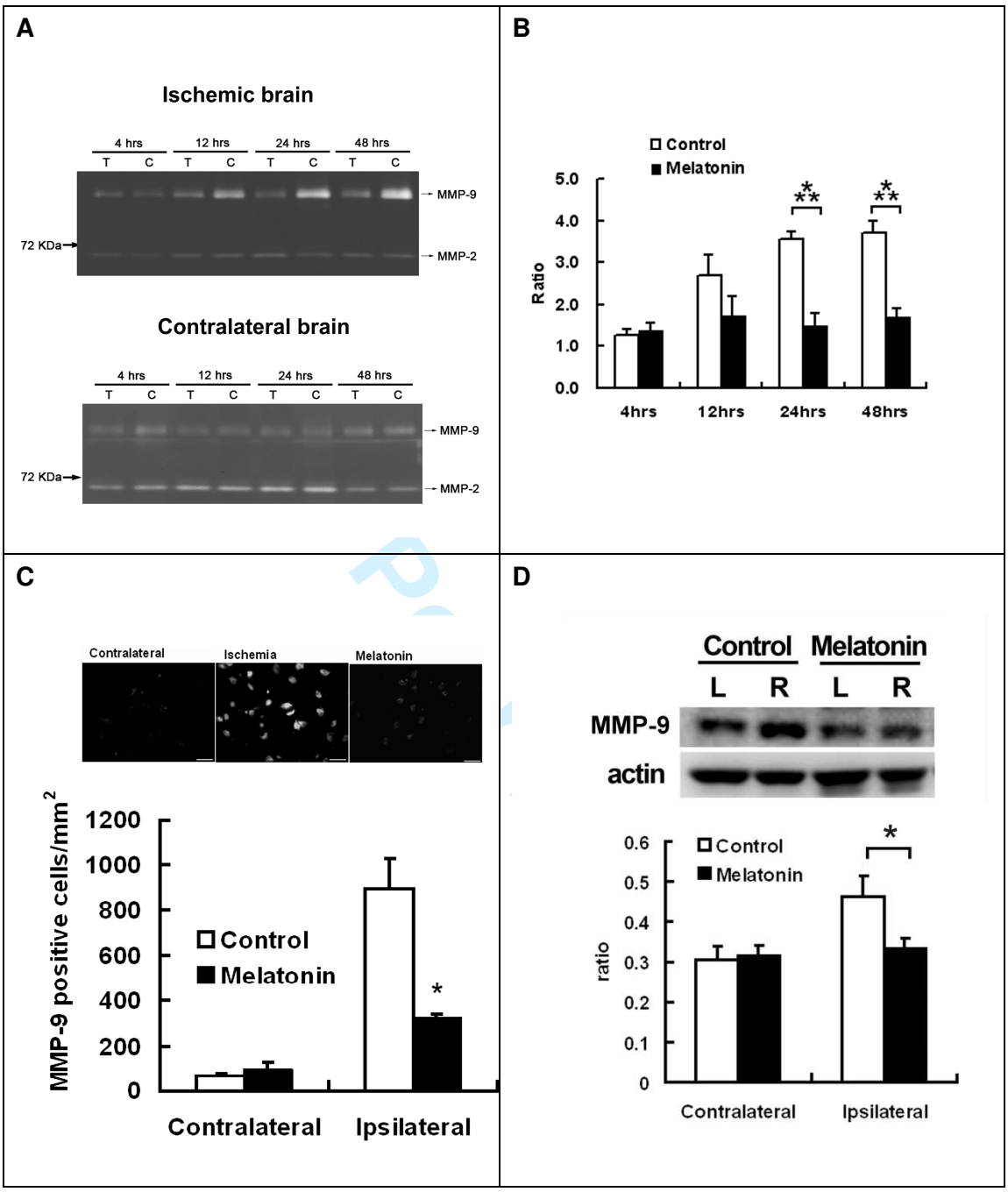
2

3

4

2

Figure 1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12 hrs
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MMP-9
72 KDa
MMP-2
Contralateral brain
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12 hrs
24 hrs
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MMP-9
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B
Control
Melatonin
Ratio
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Figure 2

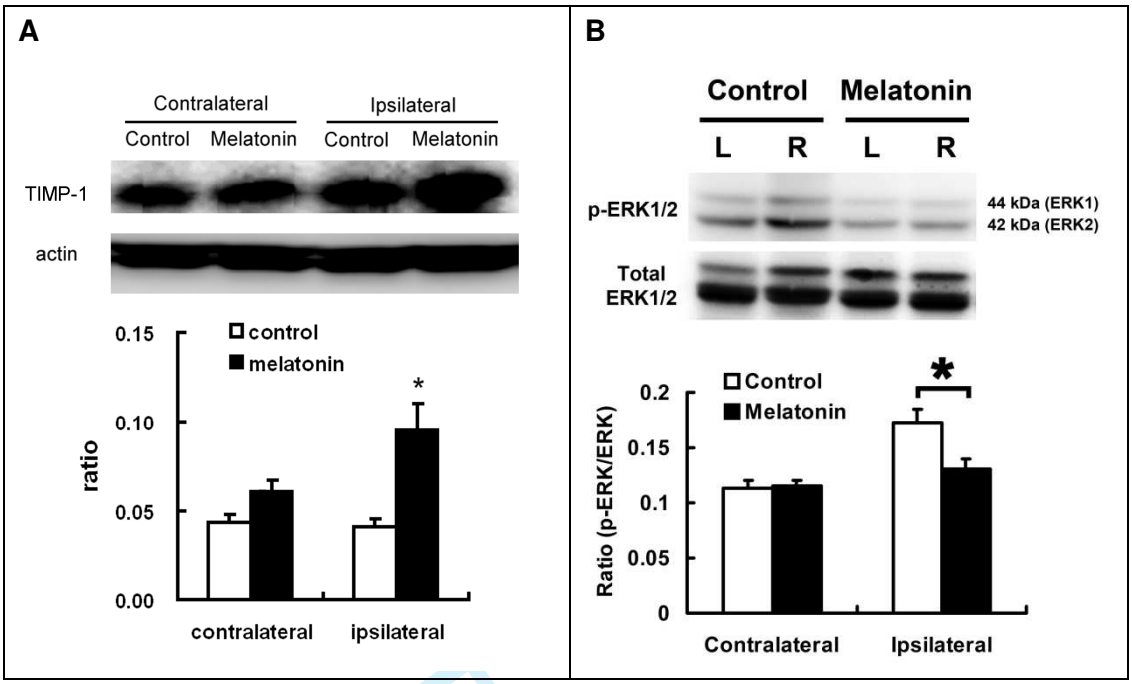
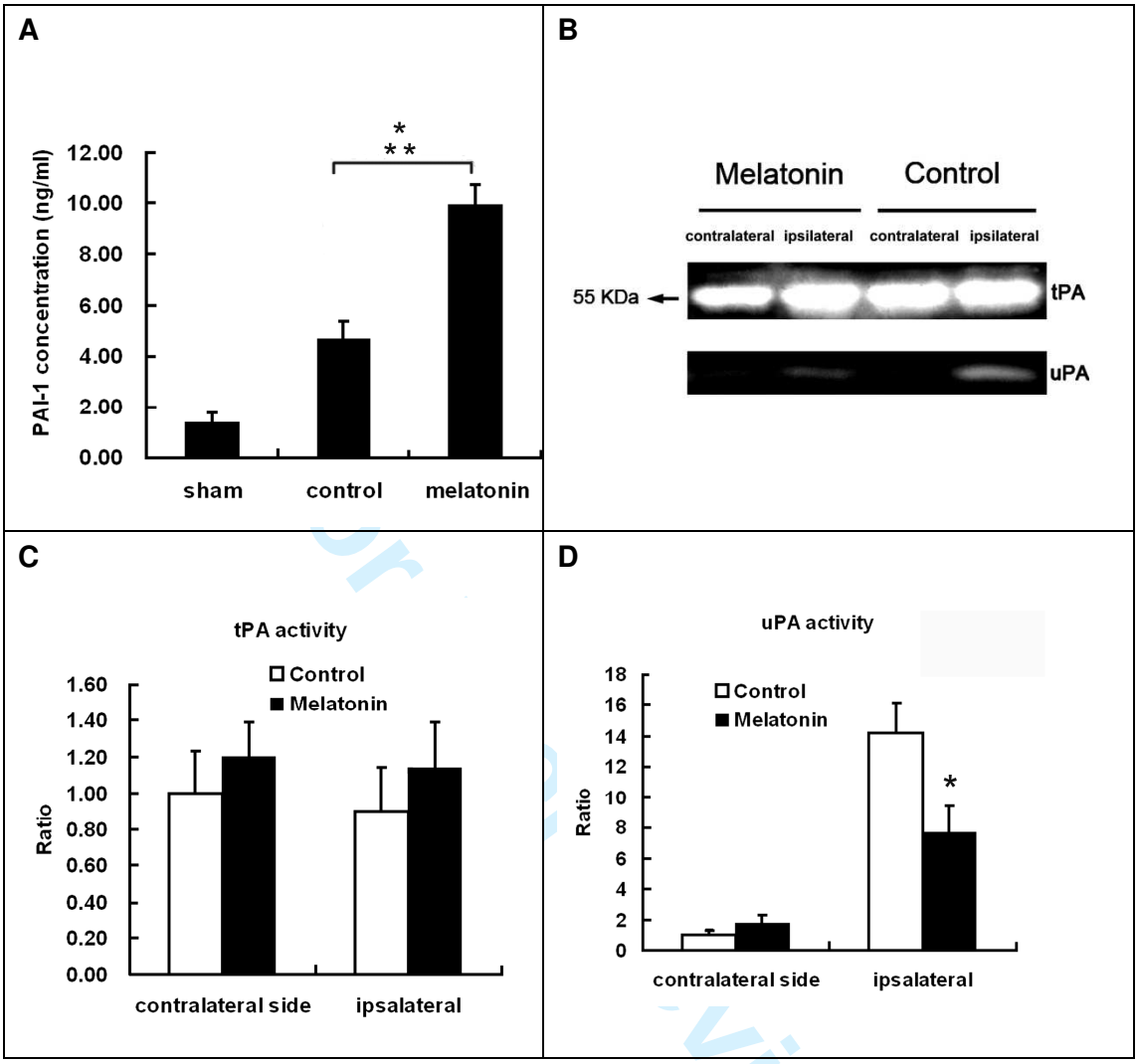


Figure 3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B
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tPA activity
Control
Melatonin
Ratio
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1.40
1.20
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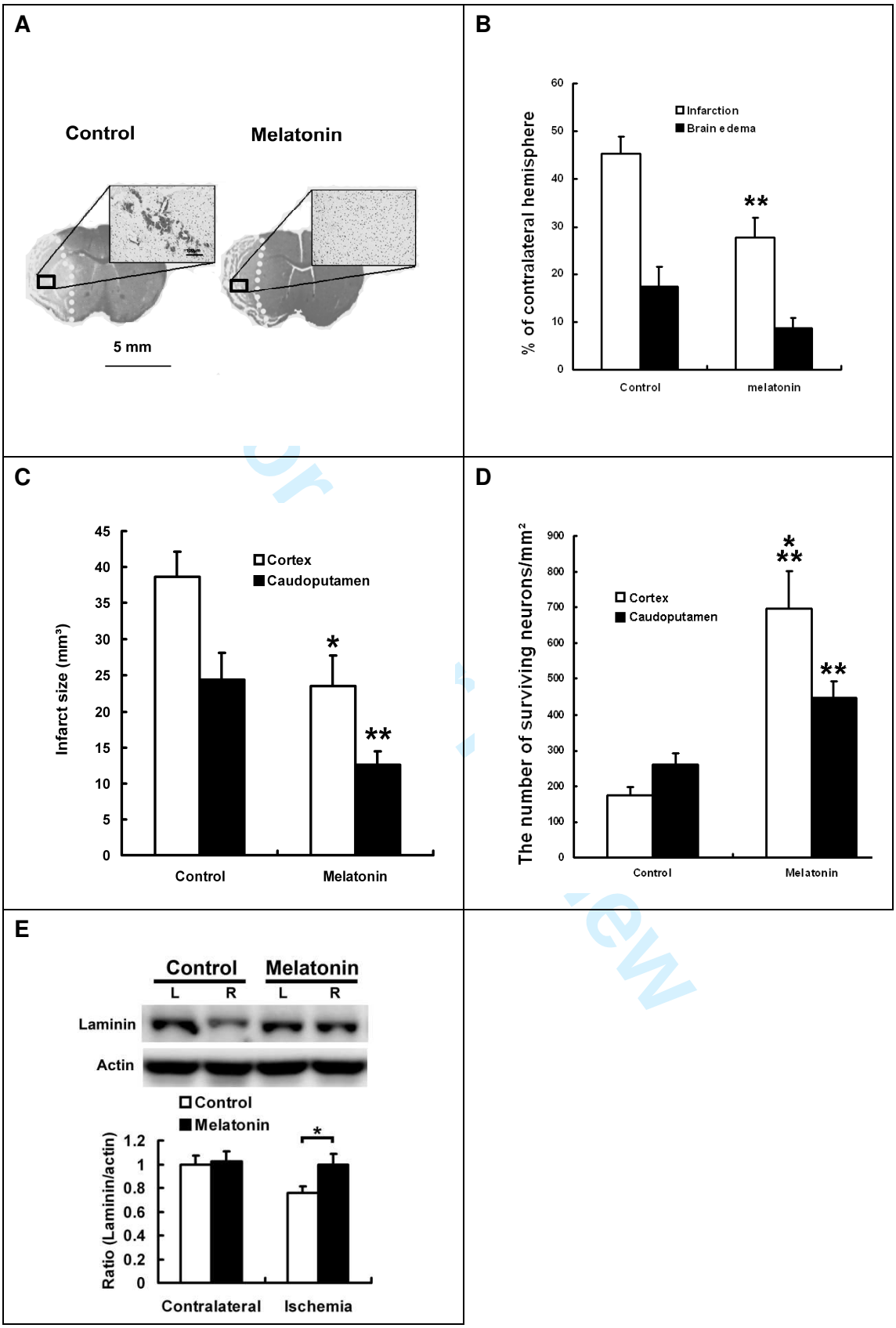
Figure 4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Melatonin
5 mm
B
Infarction
Brain edema
% of contralateral hemisphere
Control
melatonin
C
Cortex
Caudoputamen
Infarct size (mm³)
Control
Melatonin
D
Cortex
Caudoputamen
The number of surviving neurons/mm²
Control
Melatonin
E
Control
Melatonin
L R L R
Laminin
Actin
Control
Melatonin
Ratio (Laminin/actin)
Contralateral
Ischemia