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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin prevents abnormal mitochondrial dynamics resulting from the neurotoxicity of cadmium by blocking calcium-dependent translocation of Drp1 to the mitochondria

Abstract: Cadmium (Cd) is a persistent environmental toxin and occupational pollutant that is considered to be a potential risk factor in the development of neurodegenerative diseases. Abnormal mitochondrial dynamics are increasingly implicated in mitochondrial damage in various neurological pathologies. The aim of this study was to investigate whether the disturbance of mitochondrial dynamics contributed to Cd-induced neurotoxicity and whether melatonin has any neuroprotective properties. After cortical neurons were exposed to 10 μM cadmium chloride ($CdCl_2$) for various periods (0, 3, 6, 12, and 24 hr), the morphology of their mitochondria significantly changed from the normal tubular networks into punctuated structures within 3 hr. Following this pronounced mitochondrial fragmentation, Cd treatment led to signs of mitochondrial dysfunction, including excess reactive oxygen species (ROS) production, decreased ATP content, and mitochondrial membrane potential ($\triangle\Psi m$) loss. However, 1 mM melatonin pretreatment efficiently attenuated the Cd-induced mitochondrial fragmentation, which improved the turnover of mitochondrial function. In the brain tissues of rats that were intraperitoneally given 1 mg/kg $CdCl_2$ for 7 days, melatonin also ameliorated excessive mitochondrial fragmentation and mitochondrial damage in vivo. Melatonin's protective effects were attributed to its roles in preventing cytosolic calcium ($[Ca^{2+}]_i$) overload, which blocked the recruitment of Drp1 from the cytoplasm to the mitochondria. Taken together, our results are the first to demonstrate that abnormal mitochondrial dynamics is involved in cadmium-induced neurotoxicity. Melatonin has significant pharmacological potential in protecting against the neurotoxicity of Cd by blocking the disbalance of mitochondrial fusion and fission.

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Introduction

The dynamic nature of mitochondria has renewed our appreciation of the physiological and pathological roles of mitochondrial dysfunction in human health and diseases [1–3]. Mitochondrial dynamics with constant mitochondrial fission and fusion not only orchestrate mitochondrial morphology and distribution, but also determine the fundamental mitochondrial biological processes and regulate mitochondrial quality control [2, 4, 5]. Moreover, the impairment of mitochondrial dynamics greatly contributes to the development and progression of various neurodegenerative diseases [6, 7]. Accumulating clinical and experimental evidence indicates that continuous mitochondrial fission without the balance of mitochondrial fusion always leads to excessive mitochondrial fragmentation and mitochondrial damage that is critical to induce neuronal death [8]. Among the series of mitochondrial dynamin-related GTPases, the pathological roles of dynamin-related protein 1 (Drp1) in triggering mitochondrial fission have been highlighted [9–11]. It has been reported that neurotoxins, such as beta-amyloid and 6-hydroxydopamine, impaired the balance of mitochondrial dynamics by upregulating Drp1 gene expression, promoting the translocation of Drp1 to the mitochondria, or regulating the post-translational modification of Drp1 [9, 12–14].

Cadmium (Cd) is a common toxic environmental and occupational pollutant with high neurotoxicity. Epidemiological and clinical data have shown that Cd exposure results in a variety of neurological symptoms, including severe headaches, visuomotor dysfunction, peripheral neu-

ropathy, learning disabilities and hyperactivity, olfactory dysfunction [15–18]. Acute Cd poisoning produces parkinsonism symptoms, and occupational exposure had a cause and effect relationship with amyotrophic lateral sclerosis [16, 19]. However, the mechanisms underlying Cd-induced neurotoxicity still remain unclear. Our recent study showed that Cd-induced hepatotoxicity led to mitochondrial fragmentation which may have been Drp1 dependent [20]. Predictably then, the impairment of mitochondrial dynamics may be involved in the neurotoxicity of Cd.

Recent evidence indicates that melatonin exerts efficient protection in reducing metal-induced toxicity [21]. As a broad spectrum antioxidant [22–24], melatonin has pleiotropic effects as well as neuroprotective properties [25–27]. It is capable of rapidly crossing the blood–brain barrier and accumulating at high concentrations in nerve cells [28, 29]. Melatonin's multiple contributions toward the maintenance of mitochondrial homeostasis have made it become an increasingly interesting pharmacological agent against neurodegenerative diseases [30–32]. Interestingly, recent studies reveal that melatonin has the potential to rescue the abnormal mitochondrial dynamics induced by the mtDNA T8993G mutation and methamphetamines [33–35]. However, it still remains obscure how melatonin prevents detrimental mitochondrial fission in pathological conditions, and whether melatonin directly affects mitochondrial dynamics, as the beneficial roles of melatonin in mitochondrial dynamics may not be the cause, but rather the consequence of its protective effects in rescuing mitochondrial damage.

Based on the above analysis, the current study was designed to investigate the following issues: (i) whether the disturbance of mitochondrial dynamics contributes to Cd-induced neurotoxicity, (ii) if so, whether melatonin has direct benefical effects in remodeling the balance of mitochondrial dynamics, which contributes to attenuate mitochondrial damage; and (iii) how melatonin protects against the impairment of mitochondrial dynamics during the neurotoxicity of Cd.

Materials and methods

Cell culture and treatment

Primary cortical neurons were dissected from newborn Sprague Dawley rats, as previously described [36]. All procedures were performed in strict accordance with the guidelines of the Animal Care Committee of the Third Military Medical University. The mature neurons were exposed to 10 μM cadmium chloride ($CdCl_2$) (Sigma, St Louis, MO, USA) for various periods (0, 3, 6, 12, and 24 hr). The $CdCl_2$ was dissolved in distilled and deionized water to produce a 10 mM stock solution, which was then used to produce serial dilutions in the cell culture medium before application. To detect the protective effects of melatonin, the cortical neurons were pretreated with 1 mM melatonin (Sigma) for 2 hr prior to $CdCl_2$ exposure. Melatonin (0.5M) was freshly dissolved in ethanol (ACS regent, ≥99.5%) and stored at 4°C. It was further diluted in the culture medium before application.

Time-lapse confocal imaging and mitochondrial morphology analyses

To observe the changes in the mitochondrial morphology in the cortical neurons, the $CdCl_2$-treated cells were incubated with the MitoTracker Red CMXRos probe (200 nM) (Invitrogen Corp., Carlsbad, CA, USA) for 30 min at 37°C according to the manufacturer's instructions. After being washed twice in cold PBS, the living cells were visualized under a Leica TCS SP2 confocal laser scanning microscope (TCS SP2, Germany) by excitation at 579 nm and by emission at >599 nm.

For time-lapse confocal imaging, the cells were placed in a well-equipped live imaging station with a constant temperature of 37°C, 95% humidity, and 5% CO_2 for the duration of the experiment. Individual mitochondria were tracked in real time, and images were acquired every 10 min for 3 hr, as previously described [13, 37].

For regular image acquisition, a series of single confocal 2D images were captured and quantitatively analyzed using the ImageJ software (NIH), as previously described [38, 39]. The aspect ratio (AR; major axis/minor axis) and form factor (FF; the reciprocal of circularity value) were used to evaluate the mitochondrial fragmentation (particles) of each individual mitochondrion. Lower AR and FF values represent more mitochondrial fragmentation.

Determination of the mitochondrial parameters

As previously described [36, 40], the oxidative stress in the cortical neurons and rat brain tissues was determined using a 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe and a lipid peroxidation MDA (malondialdehyde) assay kit (Beyotime, Shanghai, China), respectively. ATP was measured with an ATP Determination Kit (Invitrogen Corp.). The mitochondrial membrane potential ($\triangle\Psi m$) was detected in the cortical neurons with a mitochondrial membrane potential assay kit with JC-1 (Invitrogen Corp). The cardiolipin content of the cortical neurons was estimated by staining them with nonyl acridine orange (NAO) (Invitrogen Corp), a high-affinity cardiolipin dye. The cells were cultured with 5 μM NAO in growth medium at 37°C for 30 min. Representative images of the cortical neurons were acquired with a confocal laser scanning microscope. The excitation/emission fluorescence of NAO was measured at 490/540 nm. The mtDNA copy number was assayed by quantitative real-time PCR. As previously described [36, 40], the mtDNA amplicon was generated from a cytochrome c oxidase subunit I (COX I) and the nuclear amplicon was generated by amplifying the β-actin segment. All experiments were repeated five times.

Cell viability assay

Cell viability was analyzed using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol as previously described [36].

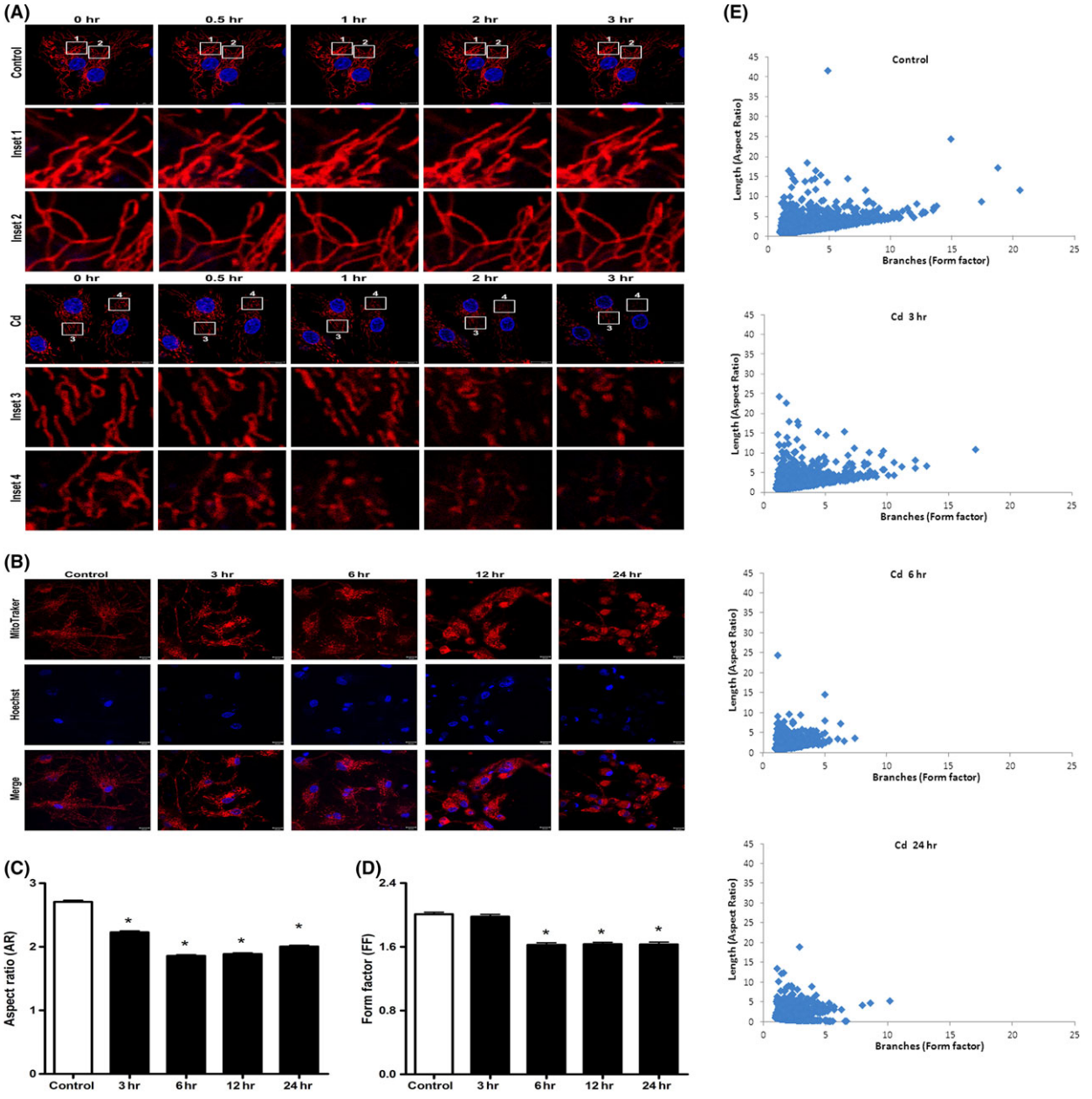

Fig. 1. $CdCl_2$ exposure triggered excessive mitochondrial fragmentation in cortical neurons. MitoTracker Red CMXRos (red) was applied as the specific probe to label the mitochondrial morphology. The neuronal nucleus was labeled with Hoechst 33258 (0.5 μg/mL). (A) Representative individual mitochondria were tracked in real time using time-lapse microscopy immediately after the cortical neurons were exposed to 10 μM $CdCl_2$. (B) The representative changes in the mitochondrial morphology were obtained by confocal microscopy of the cortical neurons treated with 10 μM $CdCl_2$ for various periods (0, 3, 6, 12, and 24 hr) and analyzed by ImageJ software. (C–E) The aspect ratio (AR) and form factor (FF) represent the ratio of the lengths of major and minor axis and the reciprocal of the circularity value, respectively. Smaller AR and FF values indicate increased mitochondrial fragmentation. N = 2415–4243 mitochondria. The values are presented as the means ± S.E.M. (n = 6), $^*P < 0.05$ versus the control group.

Animal exposure

Twenty-four adult (8 wk old, 180–220 g) male Sprague Dawley (SD) rats were purchased from the Experimental Animal Center at the Third Military Medical University (Chongqing, China). The rats were housed at 25°C under standard conditions with a 12-hr light–dark cycle and a constant temperature. For the cadmium exposure, randomly selected rats were intraperitoneally injected with 1 mg/kg of $CdCl_2$ for 7 days. $CdCl_2$ was dissolved in 0.9% physiological saline. To detect the neuroprotective effects of melatonin against Cd, 10 mg/kg melatonin was intraperitoneally administrated 2 hr before Cd injection for 7 days. Melatonin was freshly prepared every time before injection. An additional six randomly selected rats were intraperitoneally injected with 0.9% physiological saline to serve as controls. The rats were sacrificed by decapitation 24 hr after the last injection and the brain tis-

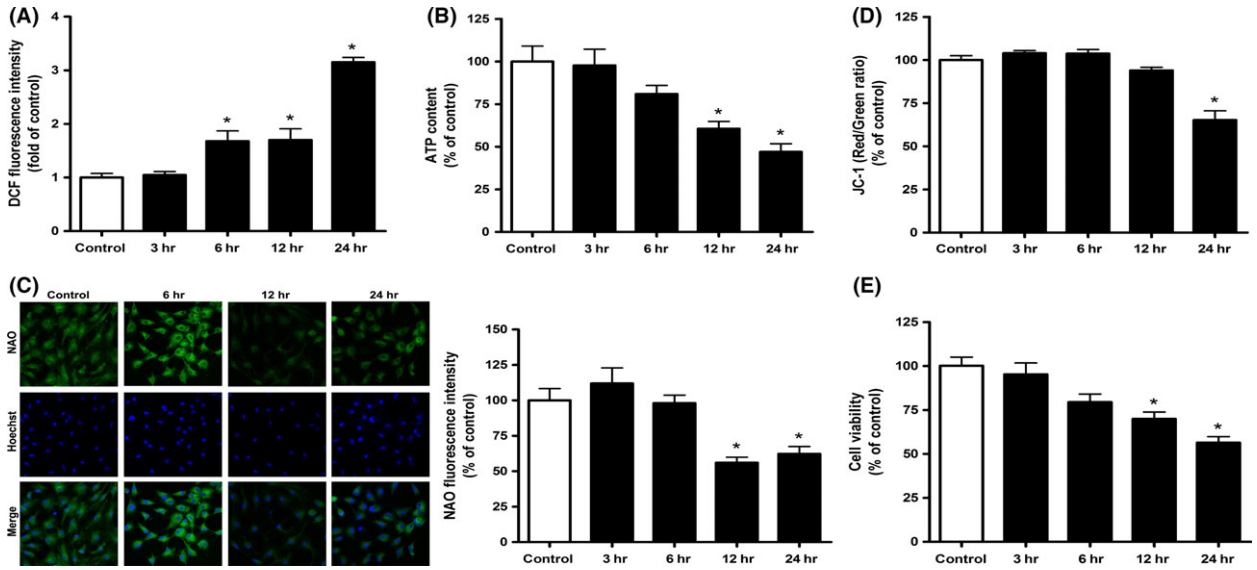

Fig. 2. $CdCl_2$ exposure induced mitochondrial dysfunction in the cortical neurons in a time-dependent manner. (A) The ROS production, (B) ATP levels, (C) cardiolipin content, (D) $\triangle\Psi m$, and (E) cell viability were assayed after the cortical neurons were treated with 10 μM $CdCl_2$ for various periods (0, 3, 6, 12, and 24 hr). The results are expressed as a percentage of the control, which was set to 100%. The values are presented as the means $\pm$ S.E.M. (n = 5), $^*P < 0.05$ versus the control group.

sues were harvested for analysis. All of the procedures were approved by the Animal Care Committee of the Third Military Medical University.

Transmission electron microscopy

The frozen brain tissues were rinsed in PBS and fixed in 10% buffered formaldehyde for 72 hr before being embedded in paraffin. The sections were deparaffinized, rehydrated with a graded ethanol series, and quenched with PBS three times. The brain sections were cut into small pieces (1 mm^3). After they were fixed in 2.5% glutaraldehyde and precooled to 4°C, the brain sections were postfixed with 2% osmium tetroxide in 0.1 M PBS, rinsed, dehydrated, and embedded. Random thin sections (0.05 μm) were cut using glass knives, and the sections were collected on naked copper mesh grids and then stained with uranyl acetate and lead citrate. The sections were viewed using a Hitachi-7500 electron microscope (Hitachi-7500; Hitachi Company, Tokyo, Japan).

Western blot analysis for mitochondrial dynamics-related proteins

The cortical neurons were lysed with cell lysis buffer in the presence of a cocktail of proteinase/phosphatase inhibitors (Cell Signaling Technology, Inc., Danvers, MA, USA) and centrifuged at 12 000 g for 30 min at 4°C. The extracted proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked and incubated with various primary antibodies overnight at 4°C. We used the following primary antibodies for the Western blots: anti-Drp1 (BD Biosciences, Bedford, MA, USA), anti-fission 1 (Fis1) (Abcam, Cambridge, MA, USA), anti-mitofusin 1 (Mfn1) (Abcam), anti-mitofusin 2 (Mfn2) (Abcam), anti-optic atrophy 1 (OPA1) (Abcam), anti-phospho-Drp1 (serine 616) and anti-phospho-Drp1 (serine 637) (Cell Signaling Technology), and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The specific Odyssey secondary antibodies used were IRDye800 donkey anti-rabbit and IRDye680 donkey anti-mouse IgG antibodies. The fluorescent signals were detected and quantified using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). For the mitochondrial Drp1 protein level analysis, mitochondrial extracts were isolated from primary cultured cortical neurons with the Cell Mitochondria Isolation Kit as previously described [20]. Then, the mitochondrial Drp1 protein levels were determined as described above.

Measurement of the intracellular calcium ($[Ca^{2+}]_i$) concentrations

To measure cytosolic calcium levels in intact cortical neurons, the cells were loaded with 2 μM Fluo-4 AM (Invitrogen Corp) for 20 min at room temperature. To measure the acute effect of Cd on $[Ca^{2+}]_i$ change, the culture medium was changed into the Hibernate A solution, and the neurons were stimulated with $CdCl_2$ (10, 50 and 100 μM), and a series of images were captured every 5 s by confocal microscopy using a Zeiss LSM 780 microscope. To measure the chronic effects of Cd on $[Ca^{2+}]_i$, cortical neurons were exposed to 10 μM $CdCl_2$ for 12 hr, briefly washed with HBSS solution without Ca^{2+} and Mg^{2+}, and then loaded with an HBSS solution containing 2 μM Fluo-4 AM at 37°C for 30 min in the dark. Representative images of the cortical neurons were captured with a TCS SP2 confocal laser scanning microscope at excitation/emission wavelengths of 480/525 nm.

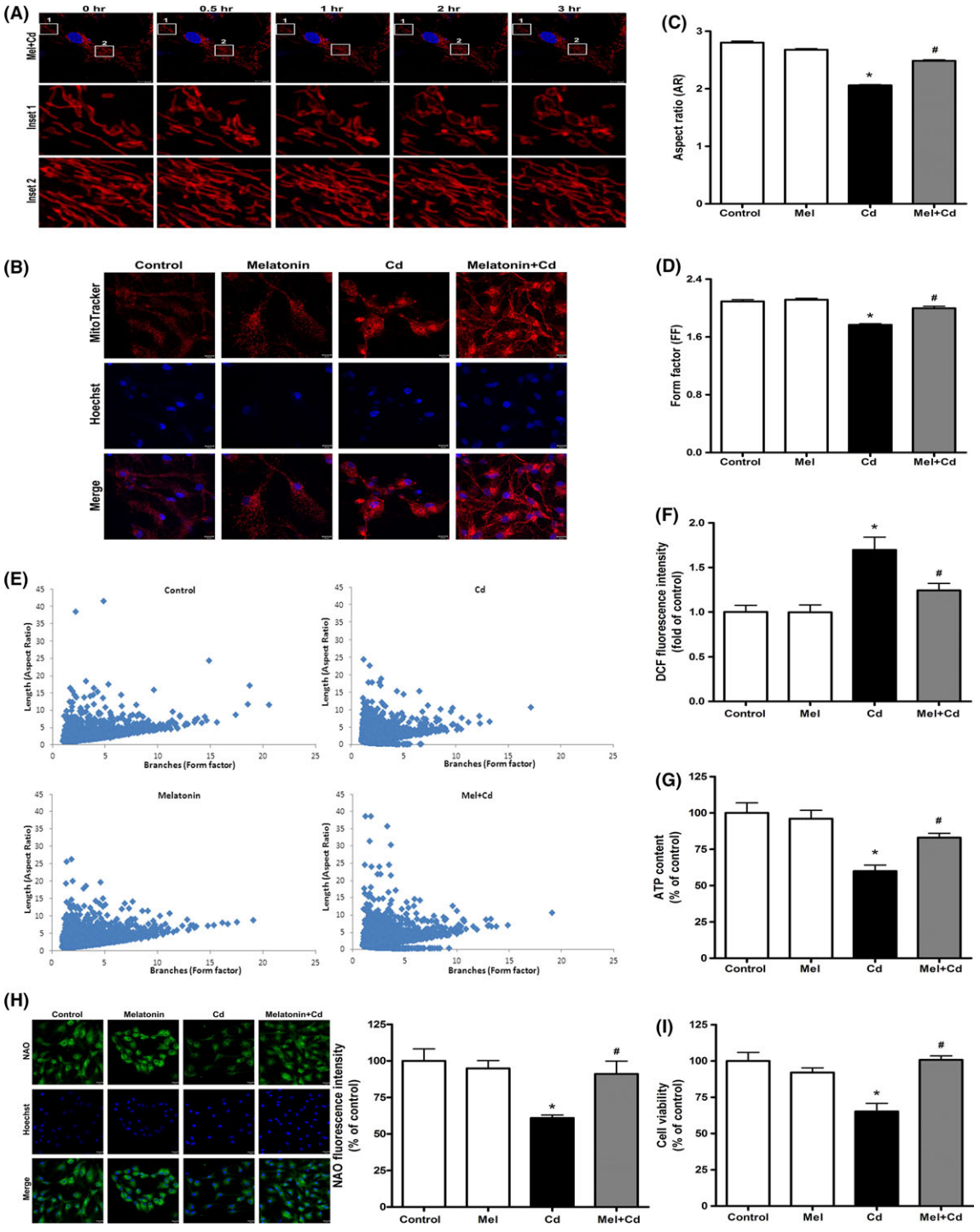

Fig. 3. Melatonin attenuated the $CdCl_2$-induced mitochondrial fragmentation and mitochondrial dysfunction in cortical neurons. The cortical neurons were pretreated with 1 mM melatonin for 2 hr before being exposed to 10 μM $CdCl_2$ for 12 hr. The beneficial effects of melatonin on the mitochondrial morphology were tracked in real time using time-lapse microscopy (A) and detected by regular confocal microscopy (B). (C–E) Melatonin pretreatment improved the AR and FF values in the Cd-treated cortical neurons. N = 4929–7612 mitochondria. Melatonin prevented the ROS overproduction (F), ATP decline (G), cardiolipin reduction (H), and the cell viability decrease (I). The results are expressed as a percentage of the control, which was set to 100%. The values in (C, D, F-I) are presented as the means ± S.E.M. (n = 5), $^{*}P < 0.05$ versus the control group, and $^{\#}P < 0.05$ versus the $CdCl_2$ (10 μM) group.

Statistical analysis

All of the experimental data are expressed as the means ± S.E.M., and each experiment was performed at least three times. The data comparisons among the groups were performed using a one-way ANOVA (Bonferroni's multiple comparison Test), and $P < 0.05$ was considered statistically significant.

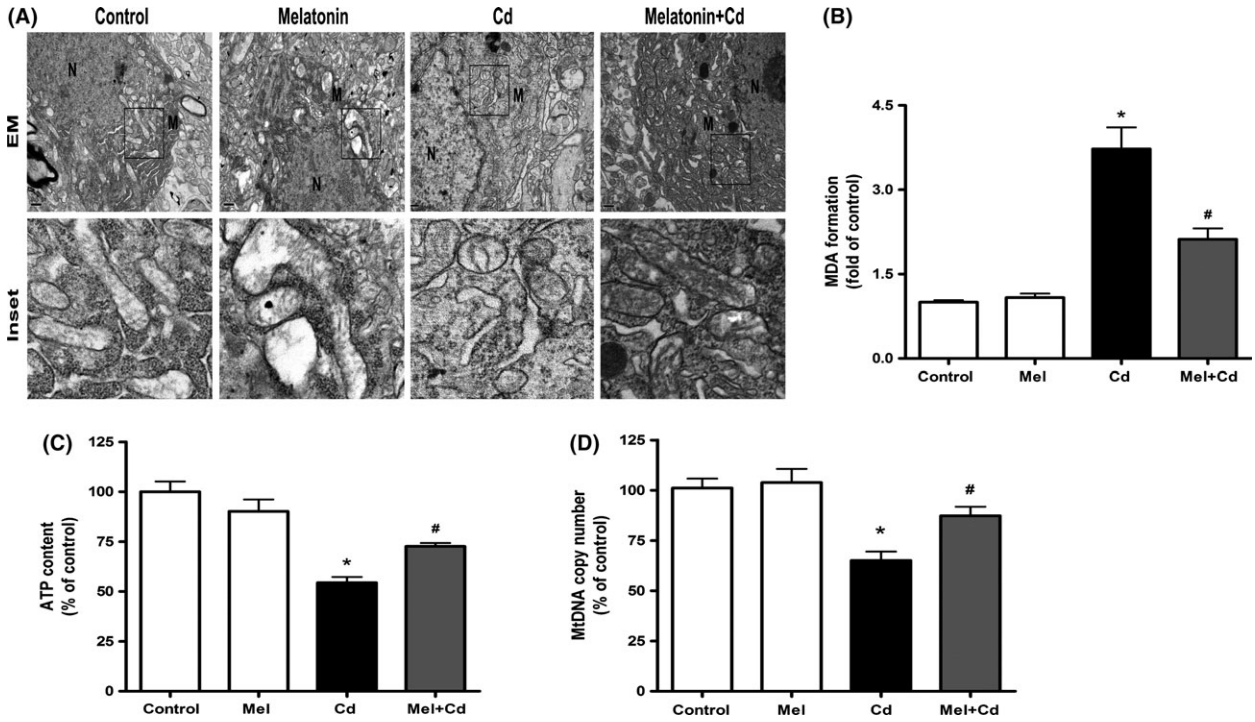

Fig. 4. Melatonin protected against mitochondrial fragmentation and mitochondrial dysfunction in the brain tissues of rats exposed to $CdCl_2$. (A). Representative mitochondrial morphology in the rat brain tissues detected by electron microscopy (EM). N = Nucleus, M = mitochondria. (B) The MDA levels and (C) ATP contents in the rat brain tissues were measured using a commercial kit. (D) The mtDNA copy number was detected by quantitative real-time PCR analysis. The amount of mtDNA was normalized to the internal control, β-actin. The results are expressed as a percentage of the control, which was set to 100%. The values are presented as the means $\pm$ S.E.M. (n = 5), $^{*}P < 0.05$ versus the control group, and $^{\#}P < 0.05$ versus the $CdCl_2$ (10 μM) group.

Results

To explore the potential effects of Cd on mitochondrial dynamics, time-lapse microscopy was applied to track the changes in the mitochondrial morphology in real time. The live imaging data showed that the addition of 10 μM $CdCl_2$ gradually altered the dynamics balance of the mitochondrial morphology. Importantly, as early as 3 hr after Cd exposure, the morphology of the majority of the mitochondria shifted from their normally elongated tubular structures into punctuated structures (Fig. 1A). This dramatic mitochondrial fragmentation lasted up to 24 hr and was confirmed by time course analysis through confocal imaging (Fig. 1B). Both the aspect ratio (AR) and form factor (FF) values, which were used to quantify the mitochondrial fission/fusion in living cells, were markedly decreased by 31% and 20% at 12 hr after Cd treatment, respectively (Fig. 1C–E). These results indicated that $CdCl_2$ exposure damaged the balance of mitochondrial fusion and fission, and induced pronounced mitochondrial fragmentation in cortical neurons.

Along with the mitochondrial morphology experiment, time course studies were carried out to examine the adverse effects of Cd on mitochondrial function. As shown in Fig. 2, $CdCl_2$ elevated ROS production to 1.6-fold of that of the controls at 6 hr after exposure (Fig. 2A). Next, the ATP levels and cardiolipin content were clearly decreased by 40% and 44%, respectively, at 12 hr after $CdCl_2$ exposure compared to those of controls (Fig. 2B, C). Subsequently, the $\triangle\Psi$m dissipated to 65% of that of control at 24 hr after $CdCl_2$ treatment (Fig. 2D). In combination with this mitochondrial dysfunction, the cellular viability of the $CdCl_2$-treated cortical neurons showed a time-dependent reduction and was eventually only 56% of that of control (Fig. 2E). These results suggested that mitochondrial dysfunction occurred later than the abnormal mitochondrial dynamics, which probably preceded the mitochondrial damage in the neurotoxicity of Cd in vitro.

To investigate the potential protective effects of melatonin against Cd-induced mitochondrial fragmentation and damage, the cortical neurons were pretreated with 1 mM melatonin 2 hr prior to $CdCl_2$ exposure. Time-lapse analysis and confocal imaging indicated that the melatonin pretreatment efficiently prevented the increase in punctuated mitochondria in the cortical neurons exposed to 10 μM $CdCl_2$ for 12 hr (Fig. 3A,B). Accordingly, melatonin improved the decrease in the AR and FF values (Fig. 3C–E). Following the normalization of mitochondrial dynamics in $CdCl_2$-treated neurons, melatonin efficiently attenuated mitochondrial and cellular damage, including a reduction in ROS overproduction, increasing ATP, cardiolipin contents, and improving cellular viability (Fig. 3F–I).

To further identify the beneficial roles of melatonin in mitochondrial dynamics and function in vivo, the in vitro findings were tested in an animal model by intraperitoneally injecting rats with 1 mg/kg $CdCl_2$ for 7 days. Relative to mitochondrial dynamics in the brain tissue as

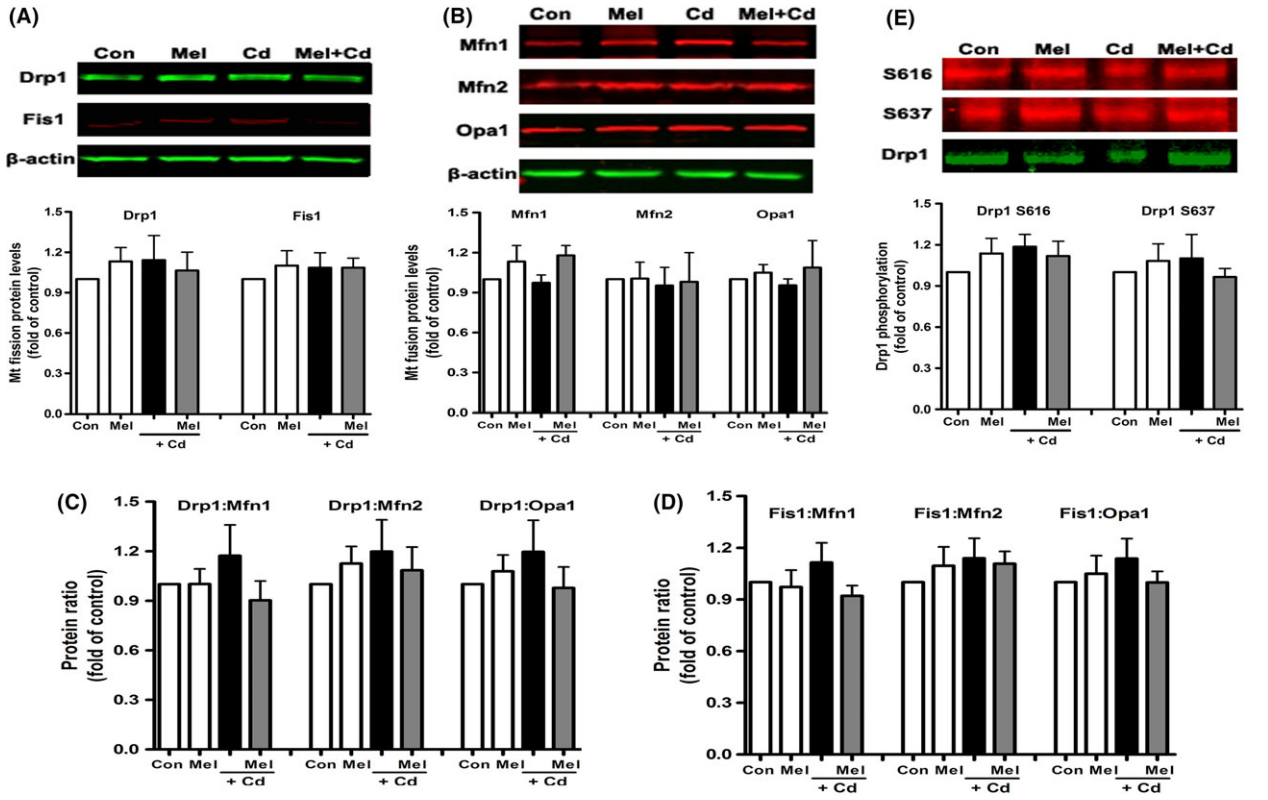

Fig. 5. Cadmium and melatonin did not alter the expression of mitochondrial dynamin-related GTPases or the phosphorylation of Drp1. Western blot analysis was used to detect the expression levels of the mitochondrial fission proteins (Drp1 and Fis1) (A) and mitochondrial fusion proteins (Mfn1, Mfn2, and Opa1) (B) in the Cd-treated neurons. The ratios of the mitochondrial fission proteins (Drp1 and Fis1) to the mitochondrial fusion proteins were quantified as shown in (C) and (D), respectively. Two well-known phosphorylation sites of Drp1 (Ser 616 and Ser 637) were detected in the Cd-treated neurons. Melatonin treatment alone also had no effects on the expression of the mitochondrial fission and fusion proteins or the phosphorylation of Drp1 at Ser616 and Ser637 (A–E). β-actin (42 kDa) was chosen as an internal standard for the amount of protein loaded for each sample. The data are representative of six independent experiments. The results are expressed as the fold of the control, which was set to one. The values are expressed as the means±S.E.M.

reflected by mitochondrial morphology, electron microscopy indicated that $CdCl_2$ exposure increased the number of smaller mitochondria with fragmented and spotted structures compared to the tubular mitochondrial networks in control group (Fig. 4A). In accordance with the changes in mitochondrial morphology in $CdCl_2$-treated group, Cd disturbed mitochondrial function, as reflected by the rise in MDA levels, and the reduction in ATP levels and mtDNA copy number compared to the controls (Fig. 4B–D). However, melatonin pretreatment (10 mg/kg, 7 days) successfully attenuated the Cd-induced mitochondrial fragmentation, reduced the MDA levels, increased the ATP levels, and maintained the mtDNA copy number (Fig. 4A–D). These findings indicated that melatonin also had protective roles against mitochondrial fragmentation and damage resulting from Cd neurotoxicity in vivo.

To explore the mechanism by which the mitochondrial dynamics were impaired during Cd-induced neurotoxicity, we determined whether melatonin affects the expression pattern of the mitochondrial fission proteins (Drp1 and Fis1) and fusion proteins (Mfn1, Mfn2, and Opa1) in Cd-treated neurons. Western blot analysis indicated that neither Cd exposure nor melatonin treatment altered the expression pattern of these five dynamin-related GTPases and the phosphorylation status of Drp1 (Fig. 5A–E). As Drp1 is the key regulator that mediates mitochondrial fission after translocating to the mitochondria and forming spirals [12, 41], we further investigated the recruitment of Drp1 to the mitochondria by detecting the protein levels of Drp1 in mitochondria isolated from Cd-treated neurons. The Western blot analysis demonstrated that the amount of Drp1 recruited to the mitochondria increased to 1.98-fold in Cd-treated neurons, while melatonin pretreatment efficiently prevented this translocation (Fig. 6A). Furthermore, overexpressing the dominant-negative Drp1 mutant (Drp1 K38A) significantly blocked the excessive mitochondrial fragmentation (Fig. 6B–E), which was similar to the effects of melatonin. This result indicates that melatonin exerted its protective effects on the mitochondrial dynamics through the Drp1 pathway.

Additionally, the inhibitory action of melatonin on the recruitment of Drp1 to the mitochondria was related to blocking the elevation of $[Ca^{2+}]_i$. As soon as $CdCl_2$ was added into the culture medium, there was a significant rise in $[Ca^{2+}]_i$ levels that reached a peak roughly 10 s after Cd treatment (Fig. 7A–C). Moreover, Cd-induced increase in $[Ca^{2+}]_i$ was also detected at 12 hr after Cd treatment (Fig. 7D). These results indicate that the increase in the

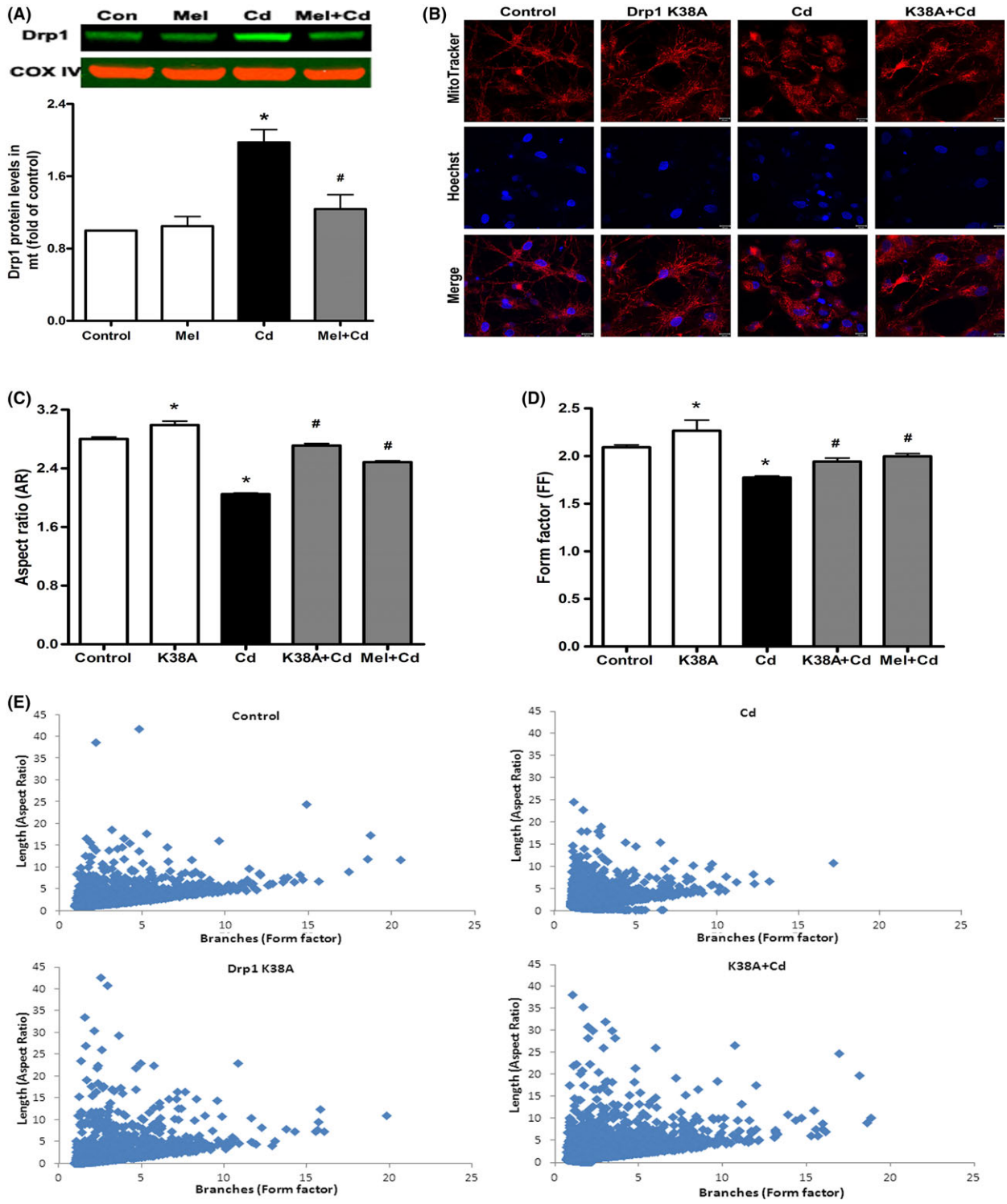

Fig. 6. Melatonin prevented the recruitment of Drp1 to the mitochondria in the Cd-treated cortical neurons. (A) Representative immunoblot and the quantification analysis of the protein levels of Drp1 (84 kDa) in mitochondria isolated from cortical neurons. COX IV (18 kDa) was chosen as an internal standard for the amount of protein loaded for each sample. (B–E) Overexpressing Drp1 K38A attenuated the excessive mitochondrial fragmentation in the Cd-treated cortical neurons. The values in (A, C, D) are presented as the means ± S.E.M. (n = 3–5), $^{*}P < 0.05$ versus the control group, and $^{\#}P < 0.05$ versus the $CdCl_2$ (10 μM) group.

$[Ca^{2+}]_i$ was the initial step and lasted long enough to induce abnormal mitochondrial dynamics in Cd-treated neurons. To further confirm this, the specific $[Ca^{2+}]_i$ chelator, BAPTA-AM, was applied to block the increase in $[Ca^{2+}]_i$ (Fig. 7B–D), and it efficiently prevented the translocation of Drp1 from the cytosolic to the mitochon-

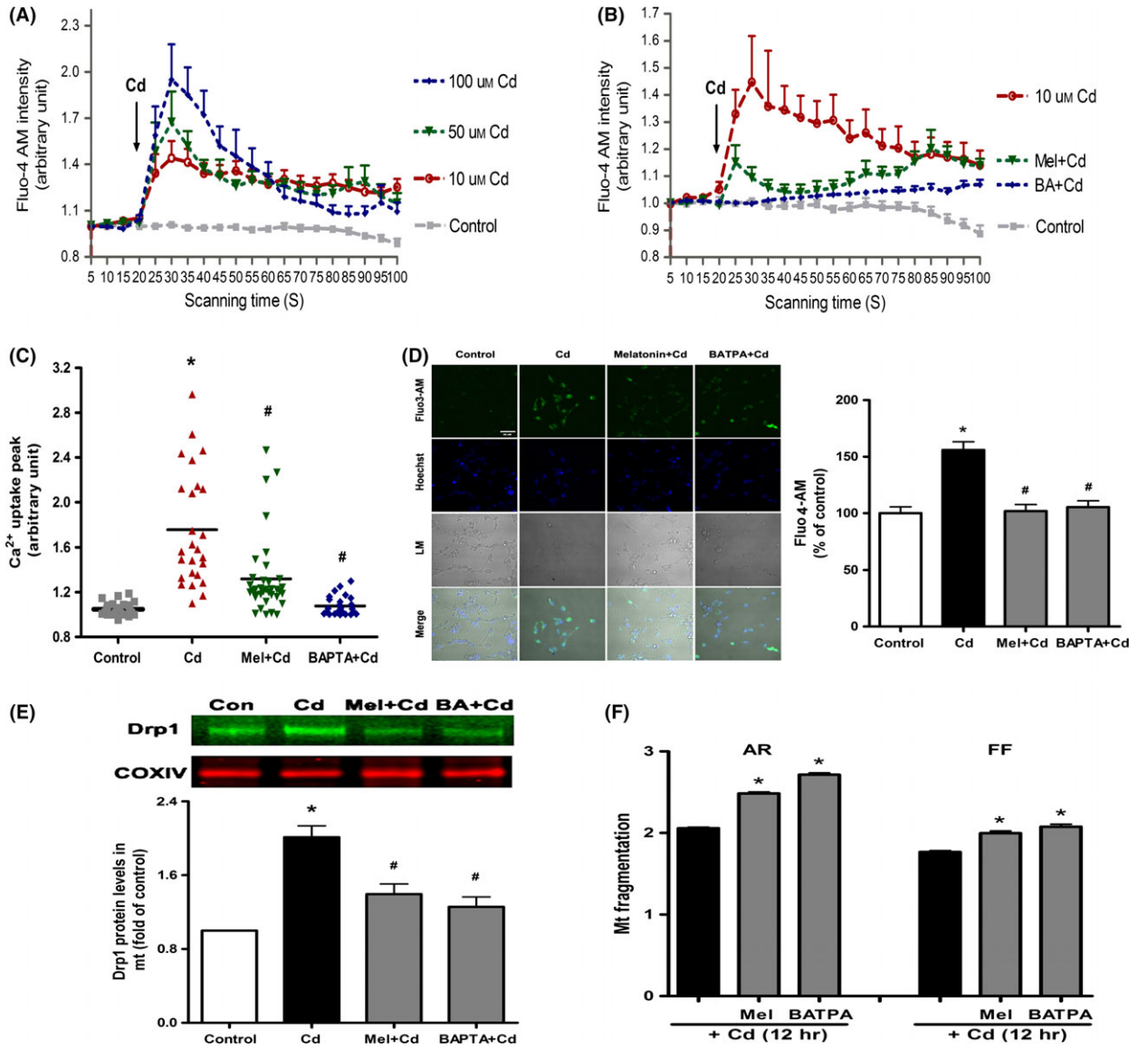

Fig. 7. The beneficial effects of melatonin on Drp1 were related to the increase in $[Ca^{2+}]_i$ levels in cortical neuron. (A) The representative trace shows the acute effect of Cd (10, 50, and 100 μM) in stimulating the rise in $[Ca^{2+}]_i$. Similar to the $[Ca^{2+}]_i$ chelator, BAPTA-AM, melatonin efficiently reduced the increased $[Ca^{2+}]_i$ in the neurons after acute treatment of Cd, reflect by an increased $[Ca^{2+}]_i$ trace (B) and greater $[Ca^{2+}]_i$ peak (C). (D) In cortical neurons with 12 hr treatment with Cd, representative changes of $[Ca^{2+}]_i$ showing melatonin efficiently reduced the increase in $[Ca^{2+}]_i$. Similar to BAPTA-AM, melatonin blocked the recruitment of Drp1 to mitochondria (E) and the excessive mitochondrial fragmentation (F) in Cd-treated cortical neurons. The values in (C-E) are presented as the means ± S.E.M. (n = 3–6), $^*P < 0.05$ versus the Control group, #p $<$ 0.05 versus the $CdCl_2$ (10 μM) group. In (F), $^*P < 0.05$ versus the $CdCl_2$ (10 μM) group.

dria (Fig. 7E), thereby reversing abnormal mitochondrial dynamics (Fig. 7F). Notably, the melatonin pretreatment had essentially the same effects in blocking the increase in $[Ca^{2+}]_i$ in response to either acute (Fig. 7B,C) or chronic Cd exposure (Fig. 7D). Moreover, melatonin exerted BAPTA-AM-like effects in preventing Drp1 translocation and attenuating excessive mitochondrial fragmentation (Fig. 7E,F).

Discussion

Considering that abnormal mitochondrial dynamics have been highlighted in various pathological conditions of the nervous system, studies that explore potential neuroprotective agents to modulate the balance of mitochondrial fusion and fission have garnered much interest [1, 6, 8, 10, 11]. Herein, our study provides in vivo and in vitro evidence that melatonin efficiently prevented the detrimental shift from mitochondrial fusion to excessive fission in Cd-treated cortical neurons, which preceded mitochondrial dysfunction and contributed to Cd-induced neurotoxicity. Mechanically, the beneficial effects of melatonin in maintaining the balance of mitochondrial dynamics largely relied on its actions in blocking $[Ca^{2+}]_i$-dependent translocation of Drp1 from the cytosol to the mitochondria (Fig. 8). To the best of our

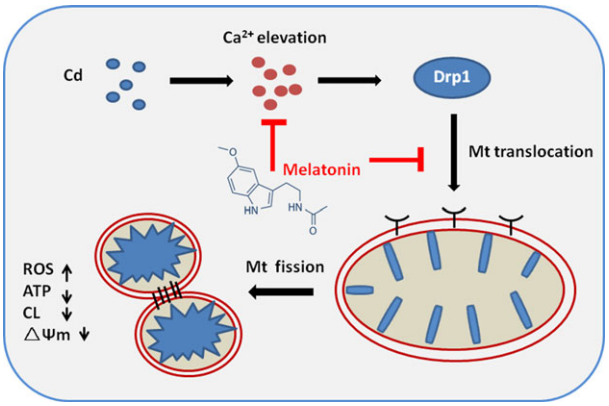

Fig. 8. Schematic model of the protective effects of melatonin against detrimental mitochondrial fission by blocking $[Ca^{2+}]_i$-dependent recruitment of Drp1 to the mitochondria. The events occurred in a specific sequence: first, Cd exposure stimulates an increase in $[Ca^{2+}]_i$ that are responsible for the translocation of Drp1 to the mitochondria. After translocation to the mitochondria, Drp1 triggers excessive mitochondrial fragmentation and subsequently leads to mitochondrial dysfunction and cell death. Melatonin pretreatment prevented abnormal mitochondrial dynamics by blocking the translocation of Drp1 to the mitochondria, which was relied to the beneficial effects of melatonin in reducing the increased $[Ca^{2+}]_i$. As a result, melatonin efficiently protected mitochondrial function and cell viability against Cd-induced neurotoxicity. Cd, cadmium; $[Ca^{2+}]_i$, cytosolic calcium; CL, cardiolipin.

knowledge, our study is the first to investigate the neuroprotective effects of melatonin against Cd-induced neurotoxicity, with a focus on reversing the abnormal mitochondrial dynamics.

Cd induces significant neurotoxicity that is considered to be a possible etiological factor in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [16, 19, 42]. However, its underlying mechanism of toxicity still remains poorly understood. Recent evidence indicates that abnormal mitochondrial dynamics, particularly excessive mitochondrial fission, are increasingly believed to be involved in the pathogenesis of neurodegenerative diseases [8, 10, 13, 14, 37]. Additionally, our recent studies indicated that Cd-induced excessive mitochondrial fragmentation and induced overactivated mitophagy are components of its hepatotoxicity [20, 43]. Therefore, it is worth exploring whether Cd induces its neurotoxicity effects by impairing mitochondrial dynamics. Any disturbances in the mitochondrial dynamics could be promptly reflected by changes in mitochondrial morphology. In the present study, we found that Cd exposure significantly altered the mitochondrial morphology from their normally elongated tubular and filamentous structures to small and spherical particles both in vitro and in vivo. These results indicated that Cd exposure led to abnormal mitochondrial dynamics during Cd-mediated neurotoxicity.

The mitochondrial dynamics control mitochondrial physiology by influencing the exchange of the mitochondrial content between individual mitochondria, through mitochondrial recruitment to critical subcellular compartments, mitochondrial communication with the cytosol, and mitochondrial quality control [1, 5]. Accumulating evidence indicates that disbalance of mitochondrial fusion and fission provokes subsequent mitochondrial dysfunction, which leads to pathological outcomes including cell death in the nervous system [6, 13, 14, 37]. In our present study, mitochondrial fragmentation occurred at 3 hr after Cd exposure, which is much earlier than mitochondrial dysfunction, as reflected by the overproduction of ROS at 6 hr, the decline of ATP levels and cardiolipin content at 12 hr, and the △Ψm disruption at 24 hr. This strongly supported the conclusion that abnormal mitochondrial dynamics precedes mitochondrial damage during Cd-induced neurotoxicity; these results are consistent with previous studies that pointed to excessive mitochondrial fission as the leading cause of neurological pathologies [12, 37, 44].

Importantly, melatonin possesses the ability to prevent the abnormal mitochondrial dynamics, thereby rescuing the Cd-induced mitochondrial damage and neurotoxicity. The dynamic balance of mitochondrial fusion and fission is delicately regulated by two opposing groups of large dynamin-related GTPases [1, 5]. Previous studies indicated that neurotoxins lead to excessive mitochondrial fission by altering the expression patterns of mitochondrial dynamics-related proteins [45, 46]. However, neither Cd treatment nor melatonin administration changed the levels of the mitochondrial fusion and fission proteins in cortical neurons in our study. In contrast, melatonin prevented the translocation of Drp1 from the cytosol to punctuate spots on the mitochondrial surface, which proved to be an essential step for Drp1 to mediate mitochondrial fission [41, 47]. It suggests that in Cd-treated neurons, the fission machinery is present and can be activated through the recruitment of Drp1 to the mitochondria, independent of *de novo* protein synthesis. Furthermore, the inhibitory effects of melatonin on Drp1 translocation largely depended on melatonin's roles in attenuating the increase in $[Ca^{2+}]_i$, similar to Ca^{2+} chelator BAPTA-AM. Although another study showed an elevation of $[Ca^{2+}]_I$ recruited Drp1 to the mitochondria by activating the calcineurin-dependent dephosphorylation of Drp1 Ser637 [41], we did not observe any changes at this phosphorylation site or at Drp Ser616 in the Cd-treated neurons. An unknown post-translational modification mechanism might exist, such as an unidentified phosphorylation site of Drp1, which is involved in the melatonin-mediated regulation of Drp1 translocation to the mitochondria in the Cd-treated neurons.

Taken together, our study supported the hypothesis that melatonin has beneficial roles in preventing the abnormal mitochondrial dynamics by blocking $[Ca^{2+}]_i$-dependent recruitment of Drp1 to the mitochondria. Although mitochondrial dynamics determine multiple mitochondrial functions, as a feedback or in some pathological context, rescuing mitochondrial damage is also helpful for promoting mitochondrial fusion and inhibiting mitochondrial fission [48, 49]. In the neurotoxicity of Cd, time course analysis demonstrated that melatonin directly modulated abnormal mitochondrial dynamics

before its protective effects on mitochondrial function became manifested. With the growing understanding that excessive mitochondrial fission provides insight into the novel mechanisms underlying the pathological conditions in the nervous system [5, 6, 45], the unique characteristics of melatonin in maintaining the balance of mitochondrial dynamics open a new path to reveal its broad neuroprotective actions.

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Author contributions

Shangcheng Xu and Zhou Zhou designed and refined the research; Shangcheng Xu, Huifeng Pi, Ju Tang, Yuming Li, and Huijuan Li carried out the experiment of time-lapse confocal microscopy. Shangcheng Xu, Huiliang Zhang, and Wang Wang analyzed the mitochondrial fission parameters (AR and FF) by ImageJ software. Nixian Zhang and Huifeng Pi performed the measurement of mitochondrial function; Lei Zhang, Pan Guo, Li Tian, Jia Xie, and Min Feng, Ping Deng performed Western blots and analyzed the data; Zhengping Yu, Wang Wang, Russel J. Reiter, Min Zhong, Yanwen Zhang, Mindi He, and Yonghui Lu interpreted the results; Shangcheng Xu prepared figures and drafted the manuscript; Zhengping Yu, Zhou Zhou, and Russel J. Reiter approved final version of the manuscript.

Conflict of interest

No conflict of interests are declared by the authors.

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