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Melatonin induces autophagy in neuroblastoma by alleviating Pak2-mediated endoplasmic reticulum stress

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Abstract. Neuroblastoma (NB), the most common extracranial solid tumor in children, remains challenging to treat due to limited therapeutic efficacy and poor prognosis. Emerging evidence highlights the critical roles of endoplasmic reticulum (ER) stress and autophagy in cancer progression. The present study investigated the therapeutic potential of melatonin in neuroblastoma and its underlying mechanisms. Using Neuro-2a (N2a) cells, it demonstrated that melatonin alleviated ER stress by upregulating ER chaperones glucose-regulated protein (GRP)78 and GRP94 and the pro-apoptotic protein CHOP, while enhancing autophagic activity. Western blotting revealed increased LC3-II/I ratios, elevated autophagy-related protein 5 and Beclin1 levels, and reduced p62 expression, indicating autophagy induction. Immunofluorescence and transmission electron microscopy confirmed the dose-dependent accumulation of autophagosomes. ER stress inhibitor 4-phenylbutyric acid attenuated melatonin-induced autophagy, linking ER stress relief to autophagic activation. Mechanistically, melatonin upregulated p21-activated kinase 2 (Pak2), which suppressed mTOR phosphorylation and activated unc-51-like kinase 1, thereby modulating the AMP-activated protein kinase (AMPK) pathway. Pak2 overexpression amplified melatonin's ER stress-alleviating effects, whereas Pak2 knockdown or AMPK inhibition diminished its efficacy. These findings established that melatonin suppresses neuroblastoma growth by mitigating Pak2-mediated ER stress to induce cytotoxic autophagy. The present study provided novel insights into melatonin as a promising therapeutic agent for neuroblastoma, warranting further exploration in preclinical models and clinical trials.

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Introduction

Neuroblastoma (NB), the most common extracranial solid tumor in infants and young children, accounting for 8-10% of childhood malignancies (1). Childhood NB is difficult to treat and has a poor prognosis with single treatment. Although the 5-year survival rate of neuroblastoma patients has increased from <20-50% in the past few decades, it still accounts for ~15% of all childhood cancer deaths (2). Current treatment approaches aim to improve the effectiveness of treatment by adding immunotherapy and targeted therapy to standard regimens (3).

Autophagy is the main intracellular material transport mechanism, responsible for transporting various intracellular substances to lysosomes for degradation and recycling. Recent studies have focused not only on the function of autophagy in tumor cells themselves, but also on the role of autophagy in the tumor microenvironment and the functions of related immune cells. There is increasing evidence showing how autophagy and its related processes affect the development and progression of cancer, which helps guide the design of anticancer therapeutics based on inhibiting or promoting autophagy (4). In the tumor microenvironment, autophagy of tumor cells can be induced by a combination of intracellular and extracellular stress signals, including metabolic stress, hypoxia, redox stress and immune signals (5). In response to metabolic stress, tumor cells rewire their own metabolic pathways by upregulating nutrient transporters and activating autophagy (6). Mechanistically, 5'-AMP-activated protein kinase (AMPK) and mTOR complex 1 (mTORC1) are two opposing regulatory kinases. After target phosphorylation in the pre-autophagy initiation complex, AMPK is located at the activation site and mTORC1 is located at the inhibition site (7,8). To initiate autophagy, AMPK phosphorylates six different sites of unc-51-like kinase 1 (ULK1) and S91/S94 of Beclin 1 (9). Wang *et al* (10) predicted a risk signature of four autophagy-related genes for neuroblastoma survival that was associated with tumor immunity. Bishayee *et al* (11) demonstrated that the RNA-binding protein HuD promotes autophagy and tumor stress survival by inhibiting mTORC1 activity and increasing ARL6IP1 levels. Ugun-Klusek *et al* (12) found that monoamine oxidase-A promotes protective autophagy

in human SH-SY5Y neuroblastoma cells through Bcl-2 phosphorylation. These results suggest that autophagy can promote apoptosis of neuroblastoma cells.

The endoplasmic reticulum (ER) is involved in a number of cellular functions, including protein synthesis, calcium homeostasis or phospholipid synthesis. Under stressful situations, the ER environment is disrupted and protein maturation is impaired, leading to the accumulation of misfolded proteins and a characteristic stress response called the unfolded protein response. ER stress and the unfolded protein response (UPR) can mediate toxic consequences through various pathogenic mechanisms (including cell death, fibrosis and inflammatory signaling pathways) (13). Activation of PerK can also promote the production of pro-inflammatory IL-6 and IL-8 by increasing p38 and PerK signaling pathways, while CHOP can regulate the transcription of cytokines including IL-6. In certain types of cancer, ER stress leads to the release of proinflammatory cytokines, including IL-6 and TNFα, each of which contains XBP1s binding sites in its promoter. These cytokines can promote pathology by driving inflammation and, in some cases, cancer cell proliferation (14). A number of studies have demonstrated that ER stress can trigger the autophagy process (15). Celesia *et al* (16) demonstrated that reactive oxygen species (ROS)-dependent ER stress and autophagy play an important role in the mechanism of action of TBT-F in colon cancer cells. The antitumor drug ABTL0812 impairs the growth of neuroblastoma cells through ER stress-mediated autophagy and apoptosis (17). Therefore, the relationship between autophagy and altered oxidative stress is evident, following the pathway of ER stress and/or mitochondrial changes.

Melatonin regulates circadian rhythms and is associated with improved sleep, scavenging of ROS, anti-aging effects, and seasonal and circadian rhythms. There is a mutual relationship between melatonin and autophagy. Ge *et al* (18) found that autophagy has a potential role in regulating melatonin synthesis in rat pineal cells. At the same time, the therapeutic potential of melatonin in cancer, neurodegenerative diseases, viral infections and obesity is related to its role as an autophagy regulator, in which melatonin regulates ER stress, autophagy and apoptosis (19,20). Zhang *et al* (21) found that melatonin increased cardiomyocyte autophagy by regulating the VEGF-BGRP78PERK signaling pathway, thereby alleviating diabetic cardiomyopathy. In preclinical studies, fetal hypoxia caused autophagy and mitochondrial damage in ovarian granulosa cells, which was alleviated by melatonin supplementation (22). In terms of anti-inflammatory effects, melatonin alleviates sepsis-induced small intestinal damage by upregulating SIRT3-mediated oxidative stress, inhibiting mitochondrial protection and inducing autophagy (23). However, the therapeutic effect of melatonin on neuroblastoma and its underlying mechanism remain to be elucidated.

The present study investigated whether melatonin reduces ER stress and impairs neuroblastoma tumor growth, enabling cytotoxic autophagy and apoptosis. Specifically, it investigated whether melatonin induces the transcription of glucose-regulated protein (GRP)78 and GRP94 and CHOP in neuroblasts and alleviates ER stress. In addition, it also explored how melatonin regulates p21-activated kinase 2 (Pak2) and its mediated signaling pathway to alleviate ER stress in neuroblastoma. This provided a new treatment option for patients with high-risk neuroblastoma.

Materials and methods

Cell recovery and passaging. Neuro-2a cells (N2a cells; cat. no. CL0383; Hunan Fenghui Biotechnology Co., Ltd.) were used for cell studies. A tube of N2a cells were placed on -80˚C ice and the cryopreservation tube placed in a 37˚C water bath until the cryopreservation solution melted into liquid. Then, 1 ml of the solution was transferred to a 15 ml centrifuge tube, and 1 ml of 10% DMEM was added, mixed and centrifuged (room temperature, 1,000 x g for 5 min). After the cells were resuspended, they were transferred to a T25 culture flask and 5 ml DMEM was added. The cells were observed under a microscope to be single cells and evenly distributed. The cells were then transferred to a cell culture incubator at 37˚C, and 5% CO_2 for culture. At 24 h after cell recovery, the adhesion and cell density of N2a cells were observed and fresh 5 ml DMEM was replaced. At this time, it was observed that 70-80% of the cells were round, with a small number of spindle-shaped cells, and the cell density accounted for ~60% of the T25 flask. When the cell density in the T25 flask reached ~90%, the cell culture was passaged using the 1-to-2 method to avoid growth inhibition.

Cell proliferation. Since N2a cells proliferate rapidly, neuroblastoma cells were plated at $3x10^5$ cells/well in 6-well plates with 1.5 ml of culture medium added, so that the cell confluence of each well was 60-70% and observed after 24 h. After the cells adhered, new culture medium was replaced and designated drugs were used for intervention treatment.

Reagents and instruments. Melatonin reagent, ER stress inhibitor 4-phenylbutyric acid (4-PBA) and AMPK inhibitor Dorsomorphin (Dorso) were purchased from Beijing Solarbio Science & Technology Co., Ltd.; ER-related protein GRP78 was purchased from Wuhan Servicebio Technology Co., Ltd.; Beclin1 was purchased from BIOSS; GRP94, CHOP, autophagy-related protein P62, autophagy-related protein 5 (ATG5), LC3, β-Actin, mTOR, phosphorylated (p)-mTOR, ULK1, phosphorylated (p)-ULK1 and Pak2 were provided by Wuhan Sanying Biotechnology. The PAK2 overexpression lentivirus packaging and PAK2 interference lentivirus packaging were provided by Beyotime Biotechnology. The experimental instruments and equipment are as follows: fluorescence microscope (Olympus Corporation), transmission electron microscope (Hitachi High-Technologies Corporation), chemiluminescence imager (Tanon4800 Multi; Tanon Science and Technology Co., Ltd.), and constant temperature cell culture incubator (Thermo Scientific Forma Series II; Thermo Fisher Scientific, Inc.).

PAK2 overexpression and knockdown. The PAK2 overexpression lentivirus packaging and PAK2 interference lentivirus packaging were provided by Beyotime Institute of Biotechnology (3rd-generation lentiviral packaging system). PAK2 overexpression lentivirus: The mouse PAK2 interference lentivirusAK2 gene sequence (accession no. NM_008779.3; https://www.ncbi.nlm.nih.gov/nuccore/NM_008779.3/) is available

in the National Center for Biotechnology Information database. The PAK2 overexpression lentivirus was packaged using the pLOV-UbiC-EGFP vector and then 293T cells (cat. no. CRL-3216; American Type Culture Collection) were transfected with the expression vectors. To construct stable PAK2 overexpression and knockdown cell lines, lentiviruses were packaged using a three-plasmid system. In brief, HEK293T cells were co-transfected with 8 μg pLOV-UbiC-EGFP plasmid, 6 μg psPAX2 plasmid and 2 μg pMD2.G plasmid at a mass ratio of 4:3:1. Viral supernatant was collected 48 and 72 h after transfection and concentrated to obtain high titer virus solution. The optimal multiplicity of infection (MOI) was determined to be 20. Screening was performed 48 h after infection, using puromycin at a concentration of 2 μg/ml for 7 days to remove uninfected cells. Subsequently, the concentration of puromycin was reduced to 0.5 μg/ml for maintenance culture, and stable cell lines were finally obtained. The PAK2 overexpression lentivirus was harvested, filtered and concentrated. To establish short hairpin (sh)RNA-mediated PAK2 knockdown, pLKO.1-puro lentiviral plasmids were used containing the validated mouse PAK2 shRNA Seq [TRCN0000023619; mRNA Target: GCU GAUGAAGUUGCUGAGUAU. Constructing the full shRNA Transcript with U bases: 5'-(GCUGAUGAAGUUGCUGAG UAU)-[CUCGAG)-(AUACUCAGCAACUUCAUCAGC)-3']. The PAK2 shRNA lentiviral plasmid was co-transfected with the packaging constructs psPAX2 and pMD2.G by Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) into the human 293T cells. After 24 and 48 h, virus particles were collected, filtered through a 0.45-μm PES filter and then used to infect the N2a cells. The lentivirus without the transgene was used as the negative control (NC) and was produced in the same manner as the inhibitor vector. Transfection efficiency was confirmed by RT-qPCR and western blot analysis.

Melatonin intervention and protein extraction. Different concentrations of melatonin, 1, 5, 10, 20 and 0 μm (control group) were placed into the proliferating N2a cells and cultured in a cell culture incubator at 37˚C and 5% CO_2 for 48 h. Then the old culture medium was discarded, each well washed with 1 ml PBS, 300 ml RIPA lysis buffer + PMSF protease inhibitor (100:1) mixture added and lysed on ice for 5 min. Then, the cells in the well plate were scraped and transferred into a new 1.5 ml tube for further lysis on ice for 30 min with shaking every 10 min. The tube was placed at 4˚C and 10,000 x g for 15 min, and 280 μl of the supernatant was aspirated. After quantification of sample proteins by BCA analysis, each sample was mixed with loading buffer and denatured in a high-temperature water bath. Finally, divide into 3 tubes and store at -20˚C.

Western blotting. After collecting cells from each group for protein extraction, the denatured proteins were loaded onto the gel (40 μg per well) for electrophoresis using separation and concentration gels. After electrophoresis, the proteins were transferred to a PVDF membrane of the appropriate size. Each antibody was detected using a constant current of 290 mA, followed by a quick blocking solution (cat. no. G2052-500ML; Wuhan Servicebio Technology Co., Ltd.) for 10 min, washed twice with TBS +0.05% Tween (TBST) at room temperature for 3 min each and incubated with the primary antibody after blocking. Membranes were incubated overnight at 4˚C, then the secondary antibody was applied at room temperature for 1 h and washed three times with TBST for 5 min each. An ECL chemiluminescence substrate kit (ultra-sensitive; cat. no. BL520A; Biosharp Life Science) was used, and the developer was added. Luminol and HRP were used to form luminous images. Developer was to allow chemiluminescence imaging (Tanon4800 Multi; Tanon Science and Technology Co., Ltd). The membranes were washed with antibody eluent (cat. no. G2079-100M; Wuhan Servicebio Technology Co., Ltd) to remove bound primary antibody at room temperature, and then washed twice with TBST, each time for 5 min. The membranes were incubated with the internal control antibody at room temperature in 2 h, using mouse β-actin (1:10,000; cat. no. 66009-1-Ig; Wuhan Sanying Biotechnology). Following which the membranes were incubated with a secondary antibody and developed in room temperature 1 h again. The experiment was repeated twice. The antibodies used in this experiment were as follows: Rabbit resist grp94 (1:8,000; cat. no. 14700-1-AP; Wuhan Sanying Biotechnology), CHOP (1:20,000; cat. no. 15204-1-AP; Wuhan Sanying Biotechnology), LC3 (1:20,000; cat. no. 14600-1-AP; Wuhan Sanying Biotechnology), ATG5 (1:5,000; cat. no. 10181-2-AP; Wuhan Sanying Biotechnology), Beclin1 (1:5,000; cat. no. bs-1353R; BIOSS), P62(1:5,000; cat. no. 18420-1-AP; Wuhan Sanying Biotechnology), PAK2 (1:10,000; cat. no. 21401-1-AP; Wuhan Sanying Biotechnology), ULK1 (1:10,000; cat. no. 68445-1-Ig; Wuhan Sanying Biotechnology), p-ULK1(1:50,000; cat. no. 80218-1-RR; Wuhan Sanying Biotechnology), mouse resist grp78 (1:10,000; cat. no. GB15098; Wuhan Servicebio Technology Co., Ltd), mTOR (1:10,000; cat. no. 66888-1-Ig; Wuhan Sanying Biotechnology) and p-mTOR (1:5,000; cat. no. 67778-1-Ig; Wuhan Sanying Biotechnology). Secondary antibodies were used as follows: HRP-conjugated Goat Anti-Rabbit IgG (H+L) (1:6,000; cat. no. SA00001-2; Wuhan Sanying Biotechnology) and HRP-conjugated Goat Anti-Mouse IgG (H+L) (1:6,000; cat. no. SA00001-1; Wuhan Sanying Biotechnology).

Immunofluorescence. N2a cells were seeded into 24-well plates, with $1x10^5$ cells per well. After 24 h, cells were treated with the indicated treatments and 48 h later, immunofluorescence was performed. First, cells were washed twice with pre-cooled PBS, then fixed with anhydrous ethanol in room temperature for 15 min, washed three times for 5 min each time with PBST and then blocked with 10% blocking solution, 20 μl bovine serum (cat. no. S12012-100g; Shanghai Yuanye Biotechnology Co., Ltd.) + 180 μl 0.25% Triton X-100, at room temperature for 45 min. The blocking solution was discarded and the primary antibody, 200 μl rabbit LC3 (1:200; cat. no. 14600-1-AP; Wuhan Sanying Biotechnology) was added and incubated at 4˚C overnight, followed by secondary antibody CoraLite488-conjugated Goat Anti-Mouse IgG (H+L) (1:200; cat. no. SA00013-2; Wuhan Sanying Biotechnology) at room temperature in the dark for 1 h. The cell nuclei were stained with Hoechst 33342 (5 mg/ml) at room temperature for 10 min, washed and mounted with an anti-fluorescence quencher, and then images captured with a fluorescent microscope (200X).

Transmission electron microscopy observation of autophagosomes. Culture medium was discarded, 1 ml of PBS was added to each well for washing, and then 1 ml of trypsin was added for digestion, followed by the addition of 10% DMEM to terminate the digestion and the cells incubated at 300 x g for 5 min at room temperature. After discarding the supernatant, when the cell pellet was ~5 mm^3, 2.5% glutaraldehyde fixative was used at room temperature in the dark for 30 min and then transferred to 4˚C for storage. The cells were stained with 2% uranyl acetate saturated alcohol solution for 8 min in the dark; 2.6% lead citrate solution was used to avoid carbon dioxide staining for a further 8 min. Following which infiltration embedding was performed as follows: i) Acetone:812 embedding agent (1:1) at 37˚C for 2-4 h; ii) acetone:812 embedding agent (1:2) at 37˚C for overnight infiltration; and iii) pure 812 embedding agent at 37˚C for 5-8 h. The pure 812 embedding agent was poured into the embedding plate, and the samples were inserted into the embedding plate and oven at 37˚C overnight. Subsequently, the embedding plate was transferred to the oven at 60˚C for polymerization for 48 h and the resin block was removed. The fixed precipitates were sectioned (thickness, 60 nm) and images captured using a transmission electron microscope (cat. no. HT7800; Hitachi, Ltd.).

Construction of PAK2 overexpression and inhibition N2a cell model. N2a cells were digested with 0.25% trypsin digestion solution (cat. no. G4004-100 ml; Servicebio) for 1 min, centrifuged at 300 x g for 5 min, and resuspended. The resuspended N2a cells were added to 6-well plates, and $3x10^5$ N2a cells were added to each well. After 24 h, when the cell confluence reached 50-70%, 6 μl of PAK2 overexpression lentivirus stock solution ($1x10^9$ TU/ml) was added. When MOI=20, 6 μl of PAK2 inhibition lentivirus stock solution and polybrene were added to make the final concentration of 5 μg/ml. After 48 h, 1, 5, 10 and 20 μm of melatonin were added to the corresponding groups, and cell protein was extracted after 48 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from homogenized the cell pellets using an RNA extraction buffer (AgBio, Inc.) following the manufacturer's guidelines. Subsequently, mRNA was reverse transcribed into cDNA using a cDNA synthesis kit (Takara Bio, Inc.). A mixture of 1 μl of synthetic cDNA and specific primers was used to amplify inflammatory cytokine target genes by SYBR Premix Ex Taq2 (Takara Bio, Inc.). The primers used in the present study were as follows: Pak2 forward (F), 5'-ACACCAGCACTGAACACCAA-3', and reverse (R), 5'-CAATCTGCGCTTCGTCCATG-3'; and GAPDH F, 5'-AGGTCGGTGTGAACGGATTTG-3' and R, 5'-TGTAGACCATGTAGTTGAGGTCA-3'. The following thermocycling conditions were used for qPCR: initial step at 50˚C for 2 min; 95˚C for 10 min; 45 cycles of 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 15 sec. GAPDH was used as a housekeeping and internal control gene to assess the relative expression of target genes. The comparative Cq method ($2^{-\Delta\Delta Cq}$) was used to calculate the expression change of the target gene relative to the reference gene (24). Data analysis was performed using the software CFX Manager™ (BioRadCFXManager; version 2.2; Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 (Dotmatics). All values are presented as mean ± SD. Multiple comparisons were performed with one-way analysis of variance followed by Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Melatonin reduces ER stress in N2a neuroblastoma cells. Melatonin has been shown to play a role in ER stress in various diseases and can directly or indirectly interfere with ER-related sensors and downstream targets of UPR, but there are currently no studies on ER stress in N2a neuroblastoma cells (25). The present study used the UPR to drive the increased expression of the ER chaperones GRP78 and GRP94 and the Perk downstream protein CHOP to verify that melatonin alleviates ER stress in N2a neuroblastoma cells (26,27). After N2a cells were treated with melatonin at different concentrations for 48 h, western blotting showed that the expression of GRP94, GRP78 and CHOP proteins increased (Fig. 1A). The rapid proliferation of N2a cells caused the accumulation of misfolded and unfolded proteins in the ER lumen, triggering the ER stress response. At the N2a cell level, after melatonin treatment, the expression of CHOP (Fig. 1B), GRP78 (Fig. 1C) and GRP94 (Fig. 1D) proteins increased, which can help accelerate the processing of misfolded and unfolded proteins in the ER, thereby alleviating ER stress and maintaining the homeostasis of the intracellular environment.

Melatonin can induce autophagy in N2a neuroblastoma cells. To evaluate the effect of melatonin on autophagy in N2a neuroblastoma cells, the present study used western blotting to detect autophagy-related proteins ATG5, P62, BECLIN1, LC3BI/LC3BII and electron microscopy to observe the formation of autophagosomes and immunofluorescence for verification (28). The western blotting results of N2a cells treated with melatonin for 48 h showed that the ratio of protein LC3II/LC3I increased, the expression of ATG5 and Beclin1 proteins increased, and the expression of p62 protein decreased. There was a negative association between p62 and autophagy levels, and a positive association between Beclin1 and autophagy levels (Fig. 2A-E). The results showed that as the concentration of melatonin increased, the expression of autophagy-related proteins also increased, indicating that cellular autophagy was enhanced.

Regarding LC3B-green fluorescence, the fluorescence signal of positive samples should show a bright and uniform morphology, which indicates strong autophagic activity. Conversely, weak or absent fluorescence signal represent that autophagy activity is weak or not occurring. As the concentration of melatonin increased, the LC3B-green fluorescence intensity gradually increased (Fig. 2F and G).

The cell pellets collected after melatonin treatment were examined by transmission electron microscopy. There are three types of autophagy markers: Isolation membrane,

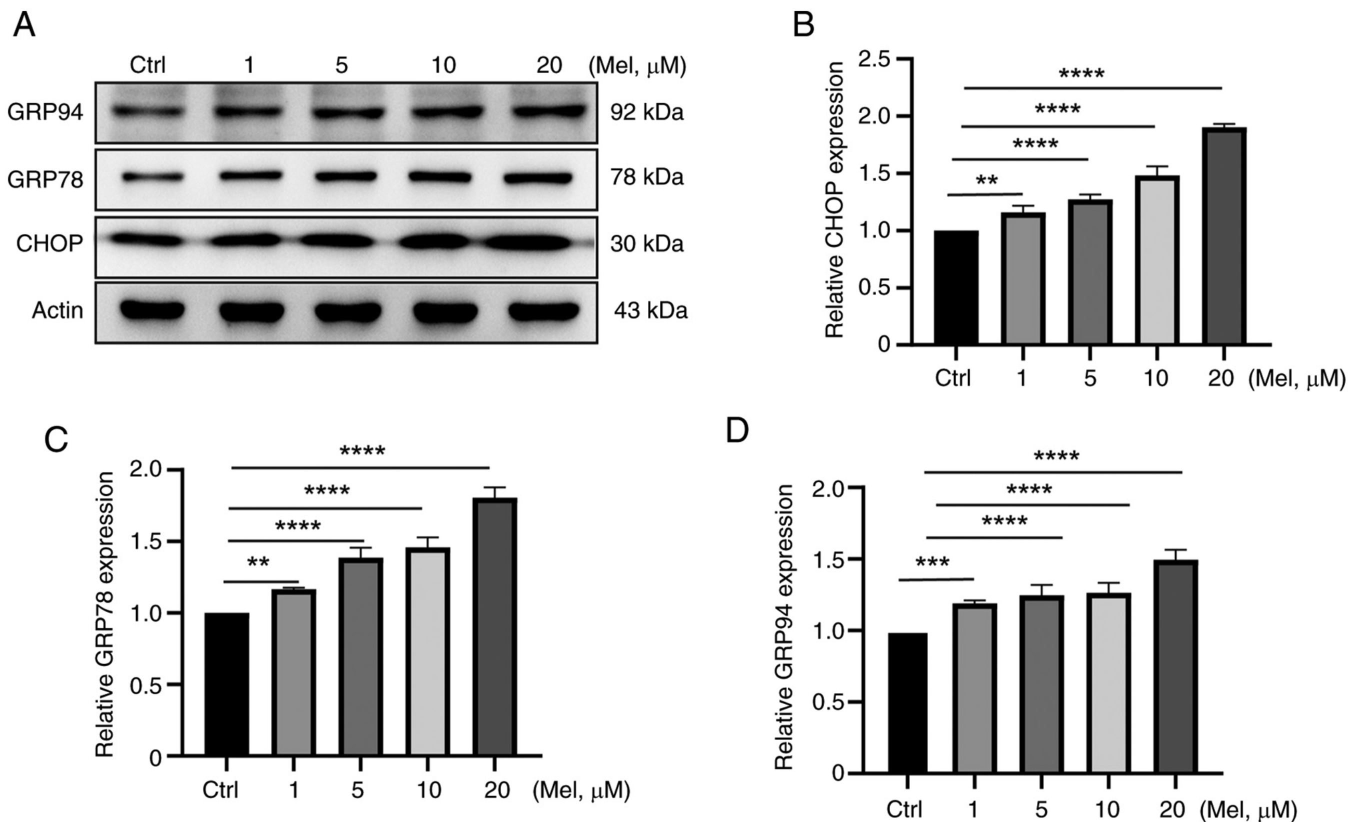

Figure 1. Melatonin reduces ER stress in N2a neuroblastoma cells. N2a cells were treated with melatonin at 1, 5, 10, 20 μM concentrations for 48 h. (A) Western blotting detection showed that the expression of (B) CHOP, (C) GRP78 and (D) GRP94proteins increased. **P<0.01, ***P<0.001, ****P<0.0001, ns, not significant; ER, endoplasmic reticulum; N2a, Neuro-2a; GRP, glucose-regulated protein; Mel, melatonin.

autophagosome and autolysosome. The present study experiment mainly observed the autophagosome. As the concentration of melatonin increased, the number of autophagosomes gradually increased (Fig. 2H).

Therefore, through western blotting detection, transmission electron microscopy and immunofluorescence, it can be concluded that as the concentration of melatonin increased, the ability of N2a neuroblastoma to undergo autophagy increased.

Effects of different concentrations of melatonin on autophagy in N2a neuroblastoma cells via ER stress. In order to further verify the relationship between melatonin and ER stress and autophagy in neuroblastoma cells, the effects of different concentrations of melatonin on autophagy in neuroblastoma cells were detected by western blotting after treatment with the ER stress inhibitor 4-PBA (Fig. 3A). The expression of p62 protein is inversely proportional to the intensity of autophagy, so after the inhibitor treatment, its expression increased, indicating that the intensity of autophagy was weakened (Fig. 3B). Compared with the group treated with the same concentration of melatonin, the expression of autophagy-related proteins (ATG5, BECLIN1, LC3B) in the group treated with 4-PBA was relatively reduced (Fig. 3C-E).

Similarly, immunofluorescence (LC3B) revealed that autophagy was weakened after treatment with 4-PBA, and the green fluorescence was reduced compared with that of the same concentration of melatonin (Fig. 3F and G).

Electron microscopy revealed that the number of vacuolar autophagosomes after treatment with 4-PBA was also reduced compared with that after treatment with the same concentration of melatonin (Fig. 3H).

Melatonin regulates Pak2 expression and detects the activation of the downstream AMPK signaling pathway of Pak2. Pak2 was tested by western blotting to detect whether melatonin could regulate Pak2 expression, and the activation of the AMPK signaling pathway downstream of Pak2 was detected by western blotting. With the increase of melatonin concentration, the expression of PAK2 protein gradually increased, indicating that melatonin can regulate Pak2 expression. For all shRNA and overexpression vector transfections, it was confirmed that the transfection was successful (Fig. S1). In the detection of AMPK signaling pathway: the total protein of MTOR and ULK1 remained basically unchanged, but the expression of phosphorylated protein of MTOR decreased, and the expression of phosphorylated protein of ULK1 increased. This indicated that after melatonin treatment, the MTOR activity in the AMPK signaling pathway was inhibited, resulting in a decrease in the MTOR phosphorylation level. It promoted the activity of ULK1, indicating that the expression of Pak2 can affect the activation of the downstream AMPK signaling pathway, thereby participating in regulating the initiation and progress of cellular autophagy (Fig. 4A-D).

RNA was isolated from N2a cells treated with different concentrations of melatonin and the ability to express Pak2

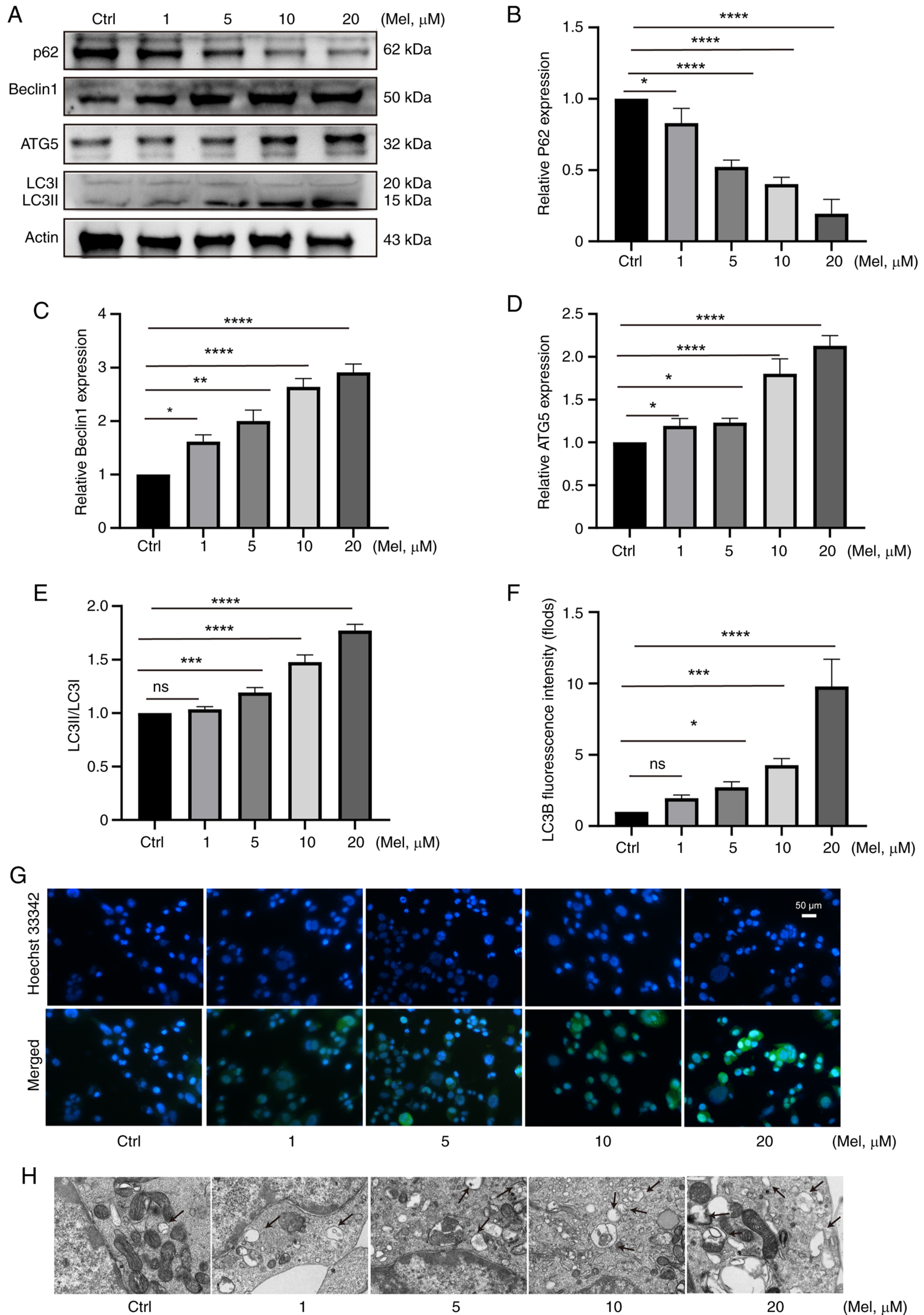

Figure 2. Melatonin can induce autophagy in N2a neuroblastoma cells. N2a cells were treated with melatonin at 1, 5, 10, 20 μM concentrations for 48 h. (A) western blotting detection showed (B) the expression of p62 protein decreased, (C) the expression of Beclin1 proteins and (D) ATG5 increased and (E) the ratio of protein LC3II/LC3I increased. (F and G) As the concentration of melatonin increased, the intensity of LC3 staining (green) gradually increased. (H) As the concentration of melatonin increased, the number of autophagosomes gradually increased. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; ns, not significant; N2a, Neuro-2a; ATG5, autophagy-related protein 5; Mel, melatonin.

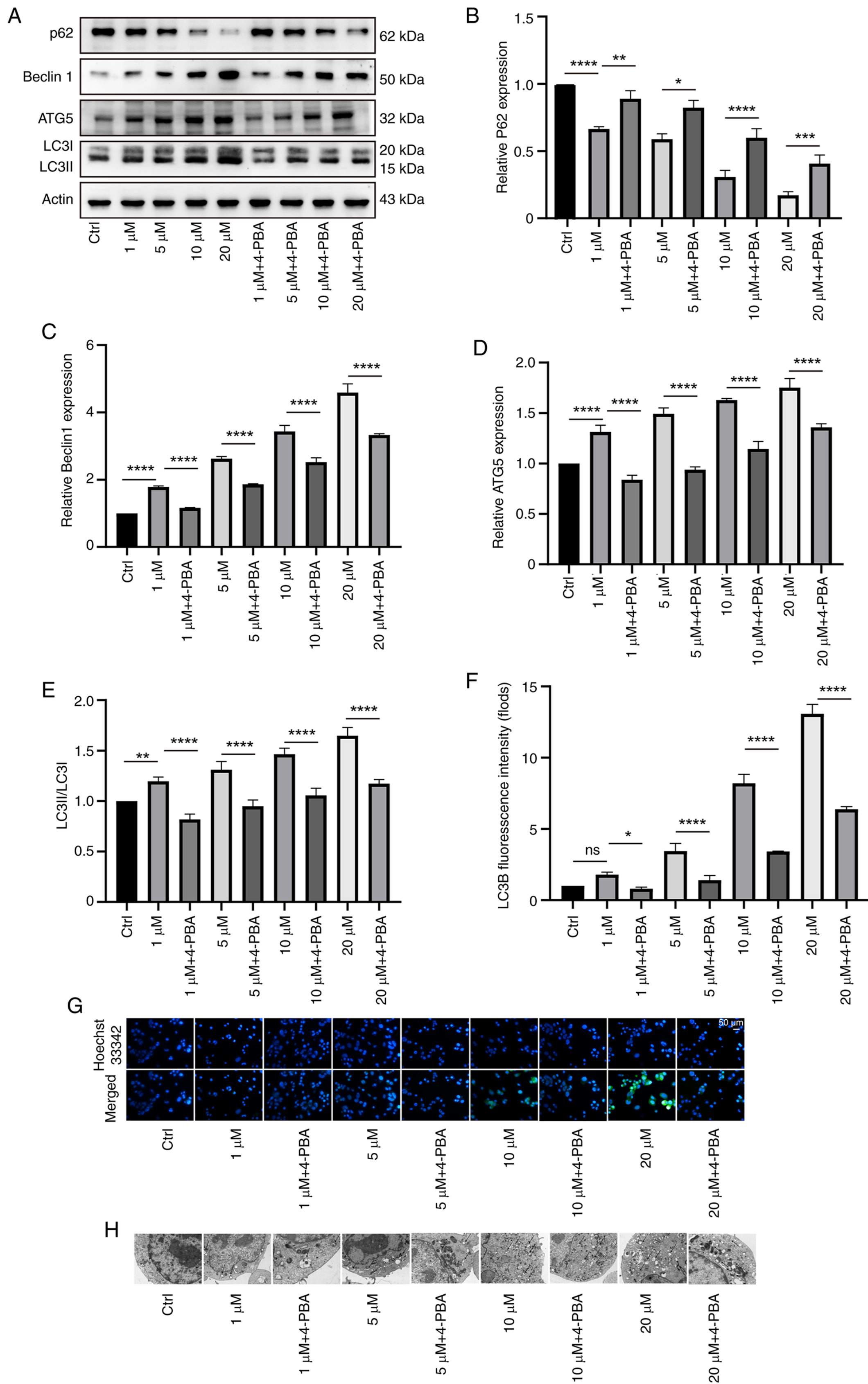

Figure 3. Effects of different concentrations of melatonin on autophagy in N2a neuroblastoma cells via ER stress. (A) N2a cells were treated with melatonin at 1, 5, 10 and 20 μM concentration and with the ER stress inhibitor 4-PBA. (B) The expression of p62 protein was inversely proportional to the intensity of autophagy, its expression increases. Compared with the group treated with the same concentration of melatonin, the expression of autophagy-related proteins (C) ATG5, (D) Beclin1, (E) LC3B in the group treated with 4-PBA was relatively reduced. (F and G) Immunofluorescence of LC3B (green) revealed that autophagy was weakened after treatment with 4-PBA, and the green fluorescence was reduced compared with that of the same concentration of melatonin. (H) Electron microscopy revealed that the number of vacuolar autophagosomes after treatment with 4-PBA was reduced compared with that after treatment with the same concentration of melatonin. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. N2a, Neuro-2a; ER, endoplasmic reticulum; 4-PBA, 4-phenylbutyric acid; ATG5, autophagy-related protein 5; Mel, melatonin.

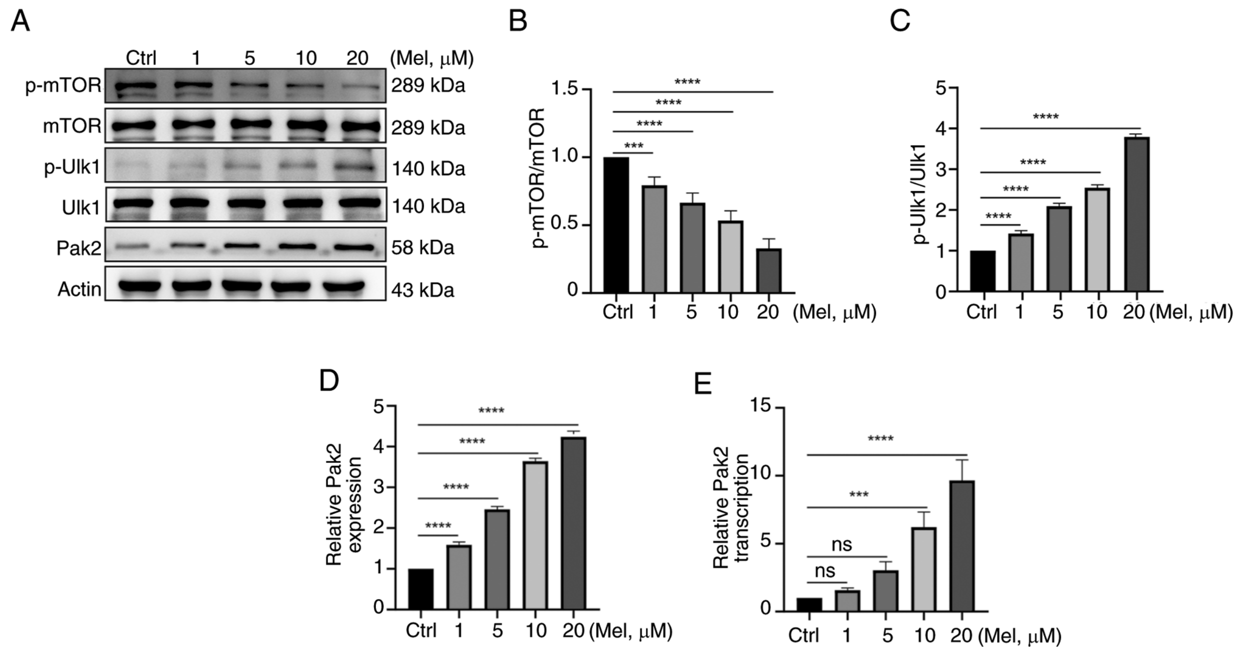

Figure 4. Melatonin regulates Pak2 expression and detects the activation of the downstream AMPK signaling pathway of Pak2. (A) Pak2 and AMPK signaling pathway was tested by western blotting. The total protein of mTOR and ULK1 remained unchanged; (B) however, the expression of phosphorylated protein of mTOR decreased and (C) the expression of phosphorylated protein of ULK1 increased. This indicated that after melatonin treatment, the mTOR activity in the AMPK signaling pathway is inhibited. (D) With the increase of melatonin concentration (1, 5, 10 and 20 μM), the expression of Pak2 protein gradually increased. (E) Quantitative PCR was used to detect the ability to regulate Pak2 expression increases with the enhancement of melatonin concentration. $^{***}P<0.001$, $^{****}P<0.0001$. ns, not significant; Pak2, p21-activated kinase 2; AMPK, 5'-AMP-activated protein kinase; ULK1, unc-51-like kinase 1; Mel, melatonin.

was measured by qRT-PCR analysis. As the dose of melatonin increased, especially when the melatonin concentration was ≥10 μm, the ability of relative Pak2 transcription was significantly increased (Fig. 4E).

Effects of different concentrations of melatonin on ER stress and autophagy in N2a neuroblastoma cells. A neuroblastoma cell model of Pak2 overexpression and expression inhibition was constructed and western blotting was used to detect the effects of different concentrations of melatonin on ER stress in neuroblastoma cells. After constructing the Pak2 overexpression model, different concentrations of melatonin were added and it was found that the expression of GRP94, GRP78 and CHOP proteins increased compared with the Pak2 overexpression and Pak2 inhibition groups with the same concentration of melatonin. This indicated that overexpression of Pak2 increased the level of melatonin in alleviating ER stress in neuroblastoma; conversely, inhibition of Pak2 expression reduces the level of melatonin in alleviating ER stress in neuroblastoma (Fig. 5A-D).

To verify the accuracy, AMPK signaling pathway inhibitors were added into neuroblastoma cells and western blotting used to detect the effects of different concentrations of melatonin on ER stress in neuroblastoma cells. The expression of GRP94, GRP78 and CHOP was compared by different concentrations of melatonin in Dorso and DMSO. This indicates that inhibition of the AMPK signaling pathway reduces the level of melatonin in alleviating ER stress in neuroblastoma (Fig. 5E-H).

Discussion

Melatonin is an important immunomodulatory molecule and exhibits inhibitory effects on cancer growth. Its anti-tumor mechanism is complex and extensive, mainly including the regulation of the estrogen action pathway, affecting the cell cycle, regulating growth factors, interfering with calmodulin and tubulin functions, increasing intercellular gap junctions, affecting cell metabolism and antioxidant and immune-enhancing effects (29). The present study explored whether melatonin could reduce ER stress in N2a neuroblastoma cells and induce autophagy in N2a cells. It studied the relationship between ER stress and autophagy in N2a cells under the action of melatonin, observed the regulation of Pak2 expression by melatonin, detected the activation of AMPK signaling pathway downstream of Pak2 and constructed N2a cell models of Pak2 overexpression and expression inhibition. In contrast to Xing *et al* (30), who applied the hypoxia-reoxygenation (HR) model, proven that melatonin alleviates ER stress in HR injury through the AMPK-Pak2 pathway, the present study applied the tumor microenvironment stress model; the end point of Xing *et al* (30) was cell survival/apoptosis inhibition, whereas the present study focused on autophagy activation. Xing *et al* (30) focused their analysis on apoptosis inhibition, whereas the present study revealed autophagy initiation. Hence, the present study aimed to demonstrate that melatonin induces neuroblastoma autophagy by alleviating Pak2-mediated ER stress, thereby inhibiting the growth of N2a neuroblastoma cells.

Early studies have found that physiological concentrations of melatonin can inhibit human neuroblastoma cells, suggesting that melatonin has anti-proliferation and pro-differentiation effects (31,32). These studies support the present findings that low concentrations of melatonin have the function of promoting differentiation and inducing autophagy. High concentrations of melatonin could induce

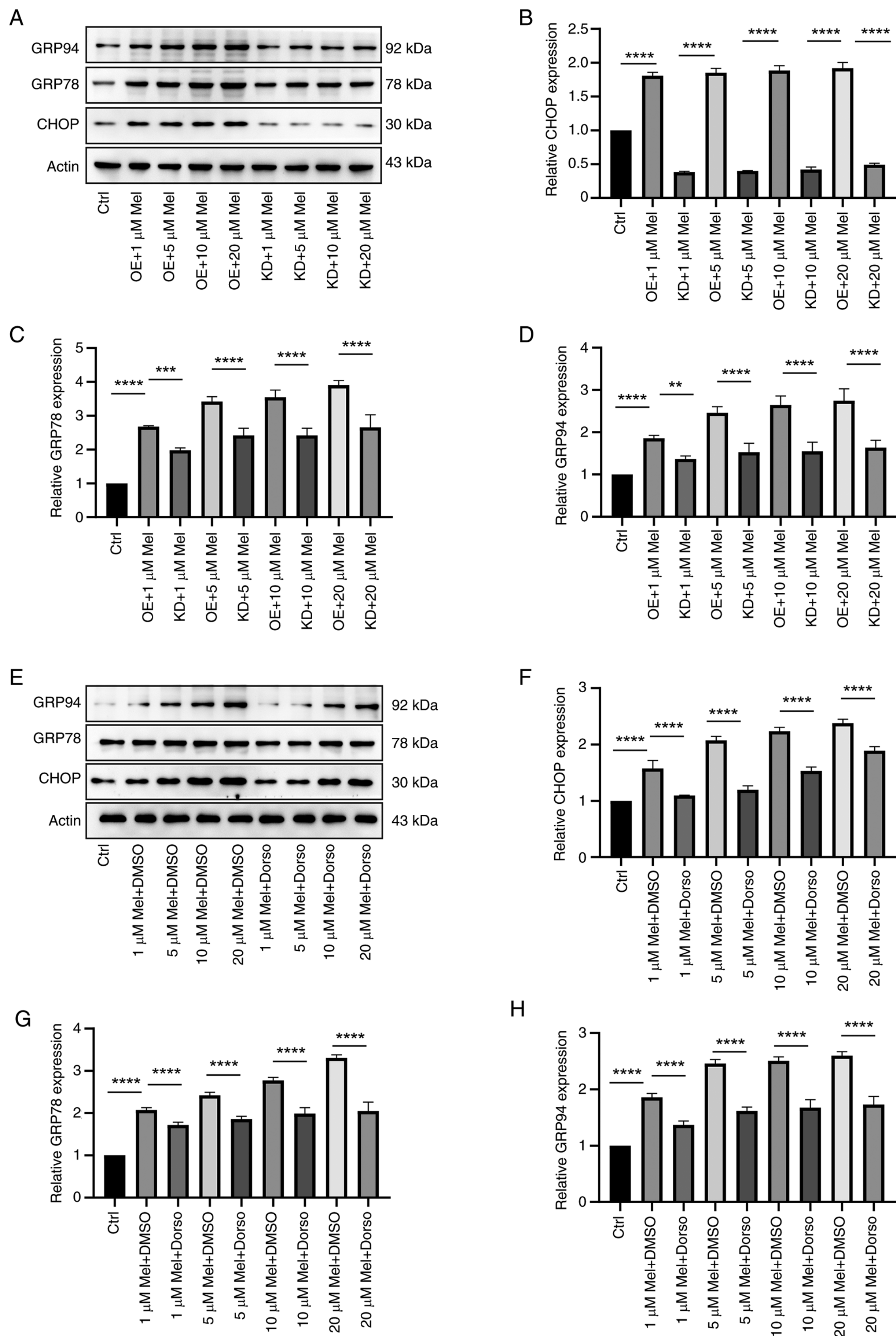

Figure 5. Effects of different concentrations of melatonin on ER stress and autophagy in N2a neuroblastoma cells. (A) The model of Pak2 overexpression and expression inhibition was constructed, and western blotting was used to detect the effects of different concentrations of melatonin on ER stress in neuroblastoma cells. (B-D) The expression of GRP94, GRP78 and CHOP proteins increased compared with the Pak2 overexpression and Pak2 inhibition groups with the same concentration of melatonin. (E) For the groups of AMPK signaling pathway inhibitors and DMSO solvent at the same melatonin concentration, the protein levels of GRP94, GRP78 and CHOP were detected via Western blot analysis. Compared with the AMPK signaling pathway inhibitor and DMSO solvent groups with the same concentration of melatonin, the expression of (F) CHOP, (G) GRP78 and (H) GRP94 proteins was weakened (F-H). **P<0.01, ***P<0.001, ****P<0.0001. ER, endoplasmic reticulum; N2a, Neuro-2a; Pak2, p21-activated kinase 2; GRP, glucose-regulated protein; AMPK, 5'-AMP-activated protein kinase; Mel, melatonin; OE, Pak2 overexpression; KD, Pak2 knockdown; Dorso, dorsomorphin; DMSO, solvent control group.

cell cycle arrest at G_2/M phase, activate caspase-3 and lead to 75% cell apoptosis (33). In a study of Alzheimer's disease, Singrang *et al* (34) found that melatonin was able to inhibit hypoxia-induced amyloid-producing pathway, thereby alleviating Alzheimer's disease-related pathological changes in SH-SY5Y cells. This evidence confirms that melatonin triggers downstream autophagy or apoptosis by alleviating ER stress and that Pak2 is a key regulator. Melatonin upregulates Pak2 through AMPK, which in turn inhibits mTOR and activates ULK1 to determine cell fate (autophagy or apoptosis). In particular, Lee *et al* (32) found that melatonin promoted differentiation through HAS3-mitophagy, while the present study found that melatonin inhibited tumor growth through Pak2-ER stress-autophagy, both pointing to autophagy as a bridge between differentiation and tumor suppression.

In the tumor microenvironment, multiple stressors are enriched to dynamically perturb the protein folding capacity of the ER of malignant tumor cells and stromal cells. In addition to the adverse environmental conditions created by tumors, genetic alterations in cancer cells can exacerbate ER stress and promote sustained activation of the UPR pathway. Co-ordination of the ER stress response is a highly dynamic process that can result in both pro-survival and pro-apoptotic outputs. The intensity and duration of UPR have decisive effects on the fate of cells and a previous study has revealed the role of the ER stress response pathway in the occurrence and development of cancer (35). GRP94 and GRP78 are UPR-driven ER molecular chaperones whose purpose is to clear unfolded proteins and restore ER homeostasis. A previous study has demonstrated that melatonin reduces ER stress by activating ER-associated protein degradation (36). Melatonin inhibits the expression of GRP78 and GRP94 through receptor-mediated mechanisms, blocks the excessive activation of the ER stress signaling pathway and ultimately alleviates BPA-induced testicular cell apoptosis and ER homeostasis imbalance (37). Under conditions of myocardial ischemia-reperfusion injury or HR stress, the protein and mRNA levels of GRP78 are markedly upregulated. Melatonin can markedly reduce the expression of GRP78 by activating Pak2 (38). Based on previous studies, the present study used different concentrations of melatonin to act on N2a cells and found that the expression of GRP94, GRP78 and CHOP proteins increased, proving for the first time to the best of the authors' knowledge that melatonin is also effective in controlling ER stress response in N2a neuroblasts.

Autophagy is a key part of the way tumor cells metabolize. Liu *et al* (39) observed that autophagic degradation of CDK4 was responsible for G0G1 cell cycle arrest in NVP-BEZ235-treated neuroblastoma cells, and Liu *et al* (40) found that Aβ(1-42) ginsenosides Rg1 and Rg2 activated autophagy and alleviated oxidative stress in neuroblastoma cells overexpressing Aβ(1-42). Melatonin also induces autophagy in neuroblastoma cells. Lee *et al* (32) found that melatonin promotes neuroblastoma cell differentiation by activating hyaluronan synthase 3-induced mitochondrial autophagy. The present study detected autophagy-related proteins ATG5, P62, BECLIN1 and LC3BI/LC3BII by western blotting and observed the formation of autophagosomes and immunofluorescence by electron microscopy and found that autophagic activity increased with the increase of melatonin

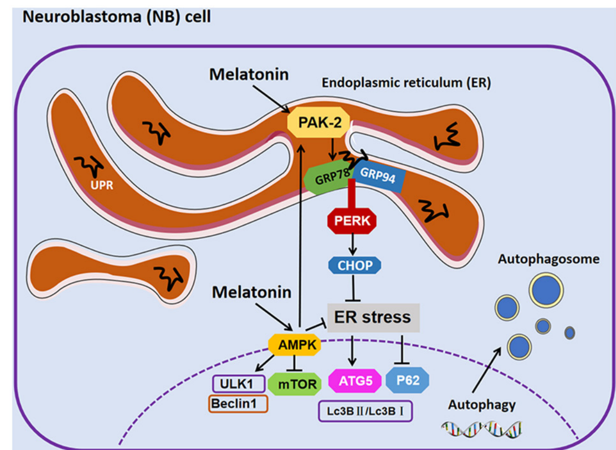

Figure 6. Overview diagram of the present study. Melatonin induces autophagy in neuroblastoma by alleviating Pak2-mediated ER stress. Pak2, p21-activated kinase 2; ER, endoplasmic reticulum; GRP, glucose-regulated protein; AMPK, 5'-AMP-activated protein kinase.

concentration. At the same time, it used western blotting to detect the effect of different concentrations of melatonin on autophagy of neuroblastoma cells after treatment with the ER stress inhibitor 4-PBA, and found that melatonin induces autophagy of neuroblastoma cells by alleviating ER stress.

Pak2 is a novel stress response kinase localized to the ER membrane. A study found that Pak2 is a new therapeutic target for ER stress response (41). Xing *et al* (30) found that melatonin regulates the expression of Pak2 through the AMPK pathway and that inhibition of the AMPK pathway can inhibit melatonin-mediated Pak2 upregulation and promote N2a cell death. The present study observed that with the increase of melatonin concentration, the expression of PAK2 protein gradually increased and after melatonin treatment, although the total protein of MTOR and ULK1 remained basically unchanged, the expression of MTOR phosphorylated protein decreased and the expression of ULK1 phosphorylated protein increased. The aforementioned confirms that in neuroblastoma cells, the expression of Pak2 following melatonin treatment can affect the activation of the downstream AMPK signaling pathway, thereby participating in regulating the initiation and progress of cellular autophagy. The present study simultaneously constructed neuroblastoma cell models with Pak2 overexpression and expression inhibition and added AMPK signaling pathway inhibitors to neuroblastoma cells. It demonstrated for the first time that inhibition of the AMPK signaling pathway reduced the level of melatonin in alleviating ER stress in neuroblastoma.

The present study is a preliminary exploration of the effects of melatonin on autophagy in neuroblastoma cells. It revealed for the first time its novel therapeutic effects in *in vitro* experiments on neuroblastoma cells and delineated its mechanism by alleviating the ER stress pathway. However, the present study has its limitations. The results were only verified in *in vitro* tumor cell experiments and further related research is needed to confirm its effectiveness and safety in clinical applications. In addition, although the present study confirmed the therapeutic effect of melatonin on neuroblastoma cells, the specific therapeutic mechanism still needs to

be verified through further research in animal models and clinical trials.

Taken together, the present study concluded that melatonin induced autophagy in neuroblastoma by alleviating Pak2-mediated ER stress (Fig. 6).

In conclusion, the present study demonstrated for the first time to the best of the authors' knowledge that melatonin has a therapeutic effect on neuroblastoma by alleviating Pak2-mediated ER stress and inducing autophagy. Therefore, the present study hypothesized that melatonin is a promising new drug candidate for the treatment of neuroblastoma. In the future, more effective combined treatment methods can be explored by combining melatonin with other drugs or treatments, thereby improving the comprehensive treatment effect of neuroblastoma.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

QQ and XS designed the study. QQ, NZ, YX, JQ, GY and XS performed the literature review, experiments and statistical analysis. QQ, NZ and XS edited the manuscript. QQ and XS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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