MiR-30a-5p Downregulation Induces Neuronal Autophagy by Activating AMPK After 2856MHz Microwave Radiation

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Research

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Abstract

Background With the development of science and technology, microwaves are being widely used. More and more attention has been paid to the potential health hazards of microwave exposure. The regulation of miR-30a-5p (miR-30a) on autophagy is involved in the pathophysiological process of many diseases. Our previous study found that 30 mW/cm² microwave radiation could reduce miR-30a expression and activate neuronal autophagy in rat hippocampus. However, the roles played by miR-30a in microwave-induced neuronal autophagy and related mechanisms remain largely unexplored.

Results In the present study, we established neuronal damage models by exposing rat hippocampal neurons and rat adrenal pheochromocytoma (PC12) cell-derived neuron-like cells to 30 mW/cm² microwave, which resulted in miR-30a downregulation and autophagy activation in vivo and in vitro. Bioinformatics analysis was conducted, and Beclin1, Prkaa2, Lrs1, Pik3r2, Rras2, Ddit4, Gabarapl2 and autophagy-related gene 12 (Atg12) were identified as potential downstream target genes of miR-30a involved in regulating autophagy. Based on our previous findings that microwave radiation can cause a neuronal energy metabolism disorder, Prkaa2, encoding adenosine 5'-monophosphate-activated protein kinase α2 (AMPKa2, an important catalytic subunit of energy sensor AMPK), was selected for further analysis. Dual-luciferase reporter assay results showed that Prkaa2 is a downstream target gene of miR-30a. Microwave radiation increased the expression and phosphorylation (Thr172) of AMPKa both in vivo and in vitro. Moreover, the transduction of cells with miR-30a mimics suppressed AMPKa2 expression, inhibited AMPKa (Thr172) phosphorylation and reduced autophagy flux in neuron-like cells. Importantly, miR-30a mimics abolished microwave-activated autophagy and inhibited microwave-induced AMPKa (Thr172) phosphorylation.

Conclusions AMPKa2 was a newly founded downstream gene of miR-30a involved in autophagy regulation, and miR-30a downregulation after microwave radiation could promote neuronal autophagy by increasing AMPKa2 expression and activating AMPK signaling.

Background

Microwave use is widespread in modern life. Radiofrequency (RF), including microwaves, is classified as 2B carcinogens in 2013. Moreover, the National Toxicology Program of USA have reported the latest evidence that microwave radiation at a frequency of 900MHz can cause cancers such as glioma in rats[1]. The brain is considered to be sensitive to microwave radiation, as many studies have reported the damaging effects of microwave exposure on the brain [2–5]. Therefore, it is important to study the biological effects and underlying mechanisms of microwave-induced neuronal damage. Autophagy, typically referred to as macroautophagy, is a cellular process that sequesters, removes and recycles unwanted macromolecules and damaged organelles and is of great importance in maintaining cellular homeostasis [6]. Numerous studies have shown that autophagy has diverse functions in the pathophysiological processes of various diseases, including tumors and neurodegenerative disorders [7–11]. In our previous study, we established a 30 mW/cm² microwave radiation in vivo model that induced
cognitive impairment and synaptic plasticity damage in rats. Autophagy was previously shown to be activated in rat hippocampal neurons following microwave exposure [12]. Although neuronal autophagy appears to primarily be a protective process in the nervous system, autophagy may represent a cell death mechanism under specific pathophysiological conditions. Thus, paradoxically, autophagy can also play a role in neuronal death. However, as the mechanisms involved in regulating autophagy after microwave radiation remain unclear, it is of great importance to further investigate microwave-induced autophagy and related mechanisms, to elucidate the biological effects and make better use of microwaves.

MicroRNAs (miRNAs) are 20-24 nucleotide noncoding RNAs that can affect protein expression at the posttranscriptional level by modulating the stability and translation of corresponding messenger RNAs (mRNAs) [13]. The roles that miRNAs play in various physiological and pathological processes have received increasingly widespread attention. In recent years, our group has focused on investigating the roles and mechanisms of miRNAs in microwave-induced neuronal injuries. In our previous study, several differentially expressed miRNAs were screened from the rat hippocampus after 30 mW/cm² microwave exposure [14], among which miR-30a attracted our attention due to its strong regulatory effects on autophagy. Studies have reported that miR-30a negatively regulates Beclin1-mediated autophagy, which has been emerged as a promising therapeutic target for multiple diseases, such as cerebral ischemic stroke, infection and cancers [15–19]. However, little attention has focused on the route by which miR-30a affects autophagy. As an evolutionarily conserved serine/threonine-protein kinase, AMPK acts as an energy sensor that plays key roles in maintaining the metabolic balance in cells. AMPK functions as a heterotrimer comprising a catalytic subunit α (including two isoforms α1 and α2) and two regulatory subunits β and γ. The N-terminal kinase domain of the α subunit is central to AMPK activity, and its phosphorylation at Thr172 site is necessary for AMPK activation[20]. There is strong consensus that AMPK signaling is one of the most important in autophagy regulation[21]. However, the regulatory effects of miR-30a on AMPK signal have yet to be reported.

In the present study, we showed that microwave radiation activates autophagy and reduces miR-30a expression in both rat hippocampal neurons and PC12 cell-derived neuron-like cells. In addition, bioinformatics analysis suggested that miR-30a may regulate autophagy via the AMPK signaling pathway, and the results of subsequent analyses confirmed that Prkaa2, encoding AMPKα2, is the downstream target gene of miR-30a. In neuron-like cells, miR-30a overexpression reduced AMPKα2 expression, inhibited AMPK activation, and abolished microwave-induced autophagy.

Methods

Animals and microwave exposure

Sample size was arbitrarily set to 48 (2 groups with 24 animals each). Male Wistar rats (212.5 ± 7.1 g, 8-week-old, specific pathogen-free), provided by the Laboratory Animal Center of Beijing Institute of Radiation Medicine, were maintained in an animal facility at 22 ± 2°C, 55 ± 10% humidity and with a 12 h light-dark cycle. Food and water were freely available. The rats were randomly divided into 2 groups
based on weight: the microwave-exposed (MW) and sham-exposed (SH) groups. The rats in the MW group were placed in fan-shaped boxes made of plexiglass and free of metal just below the microwave source and exposed to 2856 MHz microwaves with an average power density of 30 mW/cm² for 15 min once every other day for three exposures. The rats in the SH group were processed in parallel with those in the MW group, but with the microwave source was switched off. The microwave exposure system was described in detail in our previous study [12]. No animals died during experiments.

At appointed time points, rats were anesthetized with 1% pentobarbital sodium (30 mg/kg) by intraperitoneal injection to minimize animal suffering during the procedure. The rats were then decapitated and hippocampi were isolated and used for further tests.

**Cell culture and microwave exposure**

PC12 cells, provided by China Infrastructure of Cell Line Resource, were cultured in RPMI 1640 basic medium (Gibco, Waltham, MA) supplemented with 10% horse serum (HS; Gibco) and 5% fetal bovine serum (FBS; Gibco). To induce the formation of neuron-like cells, PC12 cells were maintained in RPMI 1640 basic medium supplemented with 10 ng/mL nerve growth factor (NGF) (Sigma, St. Louis, MO) and 1% HS for 5 days. Subsequently, the neuron-like cells with neuronal phenotype (an extension of neurites) were observed.

The neuron-like cells were randomly divided into the MW and SH groups. The cells in the MW group were exposed to microwave radiation at an average power density of 30 mW/cm² for 15 min. Similar processing was conducted on cells in SH group but without microwave radiation. Thereafter, the cells were harvested for analysis at specific time points.

**Transmission electron microscopy (TEM)**

At 7 d, 14 d and 1 mo., rat hippocampi were isolated and 1 mm³ tissue blocks were collected. At 6 h, neuron-like cells cultured on removable 96 well plate were harvested. Then, the samples were successively fixed in 2.5% glutaraldehyde and 1% osmium acid, processed with graded ethyl alcohols and embedded in EPON618. After being cut into ultrathin (70 nm) sections, the samples were subsequently stained with uranyl acetate and lead citrate. The ultrastructure of rat hippocampal neurons and neuron-like cells, especially autophagosomes and autolysosomes, was observed under TEM (Hitachi, Japan).

**Western blotting**

Total proteins were extracted from rat hippocampus at 7 d, 14 d and 1 mo. after exposure from neuron-like cells at 6, 12 and 24 h after exposure. Beclin1, microtubule-associated protein light chain 3 (MAP/LC3 or LC3), Atg5, Atg7, Atg9, AMPKα2, p-AMPKα (Thr172), AMPKα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with a rabbit anti-Beclin1 antibody (1:1,000 dilution; #3,495; Cell Signaling Technology, CST), rabbit anti-LC3A/B antibody (1:1,000 dilution; #12,741; CST), rabbit anti-Atg5 antibody (1:1,000 dilution; #12,994; CST), rabbit anti-Atg7 antibody (1:1,000 dilution; #8,558; CST), rabbit anti-Atg9A antibody (1:1,000 dilution; #13,509; CST), rabbit anti-AMPKα2 antibody (1:1,000 dilution; #3,760; Abcam), rabbit anti-phospho-AMPKα (Thr172) antibody (1:1,000 dilution; #2,535; CST), rabbit
anti-AMPKα antibody (1:1,000 dilution; #5,832; CST), and mouse anti-GAPDH antibody (1:5,000 dilution; #8,245; Abcam), respectively. After incubating with the corresponding goat anti-rabbit IgG-horseradish peroxidase (HRP) (1:5,000 dilution; #2,004; Santa Cruz) and goat anti-mouse IgG-HRP (1:5,000 dilution; #2,005; Santa Cruz), the protein bands were recorded using an X-OMAT BT Film imaging system (Carestream, Rochester, NY). Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD) was used to analyze the integrated optical density (IOD) of protein bands.

**Autophagic flux analysis after microwave exposure**

To analyze autophagic flux in neuron-like cells after microwave exposure, 50 µM chloroquine (CQ, #142116, Abcam) was added 1 h before microwave radiation to inhibit the lysosome-mediated degradation of autophagosomes. LC3-II and LC3-I expression in cells pretreated with or without chloroquine was detected by western blot analysis at 6, 12 and 24 h after microwave exposure, using rabbit anti-LC3A/B antibody (1:1,000 dilution; #12,741; CST), and the ratio of LC3-II to LC3-I was calculated and statistically analyzed.

The expression and localization of LC3 protein in neuron-like cells treated with or without chloroquine was detected by immunofluorescence (IF) staining at 6 h after microwave radiation. Briefly, neuron-like cells grown on coverslips were harvested at 6 h after exposure and fixed with a mixture of methanol and acetone (1: 1) for 10 min at room temperature. Then, the cells were labeled with rabbit anti-LC3A/B antibody (1:100 dilution; #12,741; CST) overnight at 4°C. After being washed with phosphate buffered saline (PBS) containing 0.1% Tween-20 three times, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution; #0311; ZSGB-BIO; Beijing, China) for 1 h at room temperature. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Finally, the expression and localization of LC3 in cells was observed using a fluorescence microscope (DM6000, Leica, Wetzlar, Germany).

**MiR-30a expression in rat hippocampi and neuron-like cells**

At 7 d, 14 d and 1 mo. after microwave exposure, rat hippocampi were collected, while neuron-like cells were harvested at 6, 12, and 24 h after exposure. Total RNA was isolated from rat hippocampi and neuron-like cells using an mirVana miRNA Isolation kit (Ambion, Waltham, MA), according to the manufacturer’s instructions. Then, complementary DNA (cDNA) was synthesized using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, ABI, Waltham, MA). The expression of miR-30a was quantified by real-time RT-PCR using TaqMan Universal Master Mix II (with UNG) and TaqMan MicroRNA assays (ABI). Finally, miR-30a was normalized to that of U6.

**In situ hybridization (ISH)**

At 7 and 14 d after microwave radiation, rat hippocampus-containing brain were removed and fixed in 10% buffered formalin solution, embedded in paraffin and cut into 3-µm-thick sections. Then, miR-30a expression was assessed by ISH. Briefly, the sections were hybridized with miR-30a, U6, or scramble miRNA probes (Exiqon, Vedbaek, Denmark). Then, after being labeled with anti-DIG (Boster Biological
Technology, China), and stained with diaminobenzidine (DAB) (ZSGB-Bio), miR-30a and U6 expression was assessed in the miR-30a, U6, and scrambled probe treatment groups in a blind manner under a light microscope (Leica). To quantify miR-30a expression, Image-Pro Plus 6.0 was used to analyze the IOD, and miR-30a was normalized to that of U6.

**Bioinformatics analysis of downstream target genes of miR-30a**

To predict the target genes of miR-30a, miRanda (Version 1.9, http://cbio.mskcc.org/miRNA2003/miranda.html), miRDB (Version 5.0, http://mirdb.org/index.html), TargetScan (Version 7.1, http://www.targetscan.org/mmu_71/), and miWalk (Version 2.0, http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) analysis was conducted, and only the genes predicted at least by three databases were selected as candidates for further analysis. Gene naming was standardized using NCBI Entrez Gene ID. The functional enrichment analysis of the target genes of miR-30a was conducted using clusterProfiler [22] with the Kyoto Encyclopedia of Genes and Genomes.db (KEGG.db; Version 83.1, http://www.genome.jp/kegg/)[23]. Significance was assessed using hypergeometric tests and Benjamini-Hochberg correction, with a significance threshold of p < 0.05.

**Luciferase reporter vectors and luciferase assay**

HEK-293T cells were transiently transfected with pmirGLO-rPrkaa2 3’-untranslated region (3’UTR) wild-type (WT, 5’-CCUUCUGUUACUUUUAGAA-3’), pmirGLO-rPrkaa2 3’UTR mutant type (MUT, 5’-CCUUCCAUAGAUUUUAGAA-3’) and empty pmirGLO vectors (GenePharma, Shanghai, China) in combination with miR-30a mimics/negative control miRNA (Ambion) using Lipofectamine 2000 (Invitrogen). Cells were lysed with Passive Lysis Buffer (Promega, Madison, WI) for 15 minutes. Then, firefly and Renilla luciferase activities were detected using a Dual Luciferase Assay kit (Promega) on a luminometer (Thermo Fisher Scientific).

**RNA transfection**

At 24 h before microwave exposure, neuron-like cells were transiently transfected with 12.5 nM miR-30a mimics and negative control (Ambion) using Lipofectamine RNAiMAX (Invitrogen). Neuron-like cells were harvested at 6 h after microwave exposure, after which the miR-30a was analyzed by real-time RT-PCR, and Beclin1, LC3-I, LC3-II, AMPKα2, p-AMPKα (Thr172), AMPKα levels were detected by western blot analysis.

**Statistical analysis**

Data are presented as the mean ± standard error of the mean (SEM). All experiments were performed with a minimum of three independent replicates. One-way ANOVA followed by Bonferroni post hoc tests were performed to analyze multiple groups using SPSS software (IBM, Armonk, NY, USA). The differences were considered significant at the level of a two-sided p < 0.05.

**Results**
Microwave radiation induces autophagy in rat hippocampal neurons and PC12 cell-derived neuron-like cells.

First, the ultrastructure of rat hippocampal neurons was observed by TEM at 7 d, 14 d and 1 mo. after 30 mW/cm\(^2\) microwave radiation. The number of autophagosomes and autolysosomes in neurons notably increased from 7 d to 1 mo. after microwave exposure. Double membrane autophagosomes, encapsulating mitochondria, synaptic vesicles and other cytosolic constituents, were primarily distributed at the synaptic terminal of hippocampal neurons. Monolayer autolysosomes, black granular or amorphous aggregates, and degraded cytoplasmic components at different stages were typically located in cell bodies of hippocampal neurons (Fig. 1A). To analyze the dynamic regulation of microwave radiation on autophagy, Beclin1 and LC3, two well-known and widely used molecular markers of autophagy, were analyzed in rat hippocampi. Beclin1 levels were increased at 14 d after microwave radiation but downregulated at 1 mo., suggesting that a regulatory adjustment occurred (Fig. 1B). The expression of LC3-II, which is positively correlated to the number of autophagosomes, was also measured. We observed that the LC3-II was significantly increased at 14 d and 1 mo. after microwave exposure, indicating an increased number of autophagosomes (Fig. 1C). Additionally, Atg5 and lysosomal associated membrane protein 1 (LAMP1) levels in rat hippocampi were upregulated after microwave exposure, suggesting an increase in autophagy and lysosomal activity, which was reported in our previous investigation with the same radiation conditions as those used in the present study [12]. These results suggested that 30 mW/cm\(^2\) microwave radiation promoted the occurrence of autophagy in rat hippocampal neurons.

In addition, the level of autophagy in PC12 cell-derived neuron-like cells was detected at the indicated time points after microwave radiation. To evaluate “autophagic flux”, the LC3-II and LC3-I levels in neuron-like cells were detected in cells with or without chloroquine (a lysosomal inhibitor) pretreatment from 6 to 24 h after microwave radiation. In the untreated cells, the LC3-II content showed no difference between SH and MW groups. When the degradation of autophagosomes by lysosomes was inhibited by chloroquine, the expression of LC3-II in neuron-like cells from the microwave-exposed group was increased at 6 h but restored to basal levels 12 h and 24 h after exposure, indicating the enhanced “autophagic flux” in neuron-like cells induced by microwave radiation (Fig. 2A-2B). Additionally, the expression of autophagy markers including Beclin1, Atg5, Atg7 and Atg9 increased at 6 h and 12 h after microwave radiation, indicating the enhanced expression of autophagy related genes induced by microwaves. However, the expression of Beclin1, Atg5, Atg7 and Atg9 downregulated at 24 h after microwave treatment, which might be caused by a regulatory adjustment (Fig. 2A-2B). Besides, we observed that autophagosomes and autolysosomes in neuron-like cells showed an increasing trend at 6 h after microwave exposure by TEM. The autophagosomes were morphologically identified by the presence of double membranes and an intact cytoplasm, and the autolysosomes were identified by having a single membrane and a partially degraded, electron-dense cytoplasm (Fig. 2C). Through immunofluorescence staining, an increase in the number of LC3 puncta (punctate fluorescent pattern of LC3) was observed in neuron-like cells pretreated with chloroquine at 6 h after microwave radiation.
(Fig. 2D). Our data suggested that the 30 mW/cm² microwave exposure promoted functional autophagy in neuron-like cells.

**Microwave radiation reduces miR-30a expression in rat hippocampi and neuron-like cells.**

It has been widely reported that miRNAs play important roles in physiological and pathological events in the central nervous system [24–26]. In our previous study, we showed that microwave radiation with the same parameters as those described in the present study can induce cognitive dysfunction and damage hippocampal neurons in male Wistar rats [12]. Additionally, we previously screened differentially expressed miRNAs in the rat hippocampus after 30 mW/cm² microwave radiation, among which miR-30a attracted our attention due to its potential regulatory roles on autophagy (Fig. 3A) [14]. Based on these results, in the present study, we assessed the changes in miR-30a levels in both in the rat hippocampus and neuron-like cells at the indicated time points after microwave radiation. The real-time reverse transcription polymerase chain reaction (RT-qPCR) results suggested that microwave radiation reduced miR-30a expression in rat hippocampi from 7 d to 1 mo. after exposure and in neuron-like cells at 6 h after exposure (Fig. 3B-3C). Decreased miR-30a in rat hippocampi was subsequently confirmed by ISH, at 7 and 14 d after microwave exposure (Fig. 3D-3E). These results indicated that microwave radiation inhibited miR-30a both in vivo and in vitro, which may be involved in the regulation of microwave-induced autophagy in neurons.

**AMPKa2 is one of the downstream genes of miR-30a, microwave radiation increases AMPKa2 expression and AMPK (Thr172) phosphorylation both in vivo and in vitro.**

miR-30a has been shown to be an important molecule in the regulation of autophagy [15–18, 27]. In the present study, we screened for predicted target genes of miR-30a using 4 databases, including miRanda, miRDB, TargetScan and miWalk. Subsequently, 365 target genes of miR-30a predicted using at least three databases were selected for KEGG functional enrichment analysis (Fig. 4A). Our data showed that the predicted target genes of miR-30a are involved in multiple signaling pathways, such as cellular senescence, cytokine-cytokine receptor interactions, the cyclic guanosine monophosphate-dependent protein kinase (cGMP-PKG) signaling pathway, the apelin signaling pathway, and autophagy (Fig. 4B). Among the predicted pathways, autophagy attracted our interest and was further analyzed. As the predicted downstream target genes of miR-30a, Beclin1, Prkaa2, Irs1, Pik3r2, Rras2, Ddit4, Gabarapl2 and Atg12 appeared to play important roles in regulating autophagy, among which Beclin1, Gabarapl2 and Atg12 directly affect the induction and formation of autophagosomes. The AMPK signaling pathway and the mammalian target of rapamycin (mTOR) signaling pathway are two key pathways that regulate autophagy and are associated with cellular energy and nutrition status, respectively [28, 29]. Prkaa2, the encoding gene of AMPKα2, has been shown to be involved in autophagy regulation through the AMPK signaling pathway. REDD1 appears to directly modulate mTOR signaling activity to regulate autophagy, while Irs1, Pik3r2, and Rras2 indirectly affect mTOR signaling via the insulin signaling and mitogen-activated protein kinase (MAPK) signaling pathways (Fig. 4C). We previously showed that microwave radiation can induce abnormal energy metabolism in hippocampal neurons and neuron-like cells [30, 31].
Accordingly, in the present study, we further assessed the roles of AMPK signaling in the miR-30a-mediated regulation of autophagy in neurons, which has yet to be reported.

The expression of AMPKa2, a possible target of miR-30a, in rat hippocampi from 14 d to 1 mo. after microwave exposure as well as in neuron-like cells 12 h after microwave exposure (Fig. 4D-4E). AMPKa phosphorylation at Thr172, which lies in the activation loop, is required for AMPK signaling, the level of which can better reflect the changes in AMPK signaling pathway activity [20]. Therefore, we analyzed the effects of microwave radiation on the phosphorylation level of AMPKa (p-AMPKa) at Thr172. In rat hippocampi, p-AMPKa (Thr172) levels increased 14 d after microwave exposure (Fig. 4D). In neuron-like cells, microwave radiation induced increased p-AMPKa (Thr172) levels at 6 h and 12 h after exposure (Fig. 4E). Considering the previously observed changes in autophagy, AMPK signaling appeared to exhibit the same pattern as that observed for autophagy after microwave radiation, both in vivo and in vitro. These results suggested that AMPK signaling activation may be closely associated with the enhanced autophagy in neurons induced by microwave radiation. In addition, we observed that miR-30a overexpression reduced the luciferase activity of Prkaa2 3’UTR WT reporter-transfected cells but not in Prkaa2 3’UTR MUT reporter-transfected cells, suggesting an upstream regulatory role of miR-30a on AMPKa2 (Fig. 4F). Accordingly, we hypothesized that miR-30a downregulation after microwave radiation may promote neuronal autophagy by regulating AMPKa2 and activating AMPK signaling.

**MiR-30a overexpression inhibits AMPKa2 expression and phosphorylation (Thr172) and reduces autophagic flux in neuron-like cells**

To investigate the underlying mechanisms associated with how the reduced miR-30a levels affect autophagy in microwave-irradiated neurons, miR-30a mimics were transfected into neuron-like cells 24 h before microwave exposure. We observed that miR-30a mimics significantly increased miR-30a levels in neuron-like cells, showing that the intervention measures were effective (Fig. 5A). In addition, miR-30a overexpression decreased AMPKa2 expression and inhibited AMPKa (Thr172) phosphorylation, indicating the impairment of AMPK signaling. Moreover, miR-30a inhibited the microwave-induced increase in AMPKa2 and p-AMPKa (Thr172) levels (Fig. 5B-5C). These results suggested that miR-30a negatively regulates AMPK signaling in neuron-like cells. Accordingly, microwave radiation could modulate the activity of AMPK signaling by suppressing miR-30a, at least in part.

Furthermore, miR-30a overexpression reduced Beclin1 levels in neuron-like cells and inhibited the microwave-induced upregulation of Beclin1 expression in rat hippocampi (Fig. 5D). miR-30a overexpression reduced “autophagic flux” in neuron-like cells, and completely abolished microwave-induced enhancement of autophagy (Fig. 5E-5F). Taken together, miR-30a-induced autophagy was abrogated in neurons by inhibiting Beclin1 and AMPK signaling after microwave radiation.

**Discussion**
For decades, our group has focused on investigating the biological effects and mechanisms by which microwave exposure alters the central nervous system [5, 14, 31–37]. In the present study, we observed the following: i) 30 mW/cm² microwave exposure induces autophagy in both hippocampal neurons and PC12 cell-derived neuron-like cells; ii) downregulation of miR-30a promotes neuronal autophagy after microwave radiation; and iii) miR-30a regulates microwave-induced autophagy, in part, via the AMPK signal pathway.

Autophagy has been reported to play dual roles in pathological processes of neurodegenerative diseases. At the early stage, autophagy can enhance the degradation of denatured proteins to maintain neuronal functions, whereas at the late stage, continuous activation of autophagy ultimately induces the autophagic cell death of neurons [38–42]. In the present study, we observed that microwave radiation with an average power density of 30 mW/cm² promoted autophagy in both rat hippocampal neurons and PC12 cell-derived neuron-like cells. The results of our previous study showed that hippocampal neurons have the ability to initiate self-recovery after 30 mW/cm² microwave exposure [31]. We speculated that neuronal autophagy was most likely a protective response to microwave radiation. At higher exposure levels, microwave radiation may overactivate autophagy, resulting in an unbalanced cellular homeostasis and ultimately leading to the irreversible injury of neurons, although further study was noted to be required [32, 43, 44]. Importantly, microwaves have potential clinical applications both in diagnosis and therapy, and several groups have reported the therapeutic value of microwave in preventing cognitive impairment in Alzheimer’s disease (AD) patients [45, 46]. The aggregation of Aβ peptides is the fundamental pathogenic mechanism of AD, which can result in damage to neurons. We hypothesized that microwave therapy may induce autophagy to initiate Aβ clearance in neurons. Thus, it will be of great importance to investigate microwave-induced autophagy and related mechanisms to guide microwave therapy and decrease potential adverse effects.

Numerous studies have demonstrated that miR-30a negatively regulates autophagy, a process that is closely associated with the pathophysiological processes of multiple diseases, such as cerebral ischemic stroke, cancer and hepatic fibrosis [35, 47–49]. The result of our previous showed that miR-30a was significantly downregulated in rat hippocampi after a 30 mW/cm² microwave exposure [14]. However, the roles played by miR-30a in microwave induced hippocampal injury had yet to be explored. In the present study, we observed that 30 mW/cm² microwave radiation activated autophagy both in vivo and in vitro. Based on these results, we speculated that miR-30a may participate in the regulation of microwave-induced autophagy in neurons. The results of the present study showed that miR-30a levels were significantly downregulated in rat hippocampal neurons from 7 d to 1 mo. after microwave radiation, at which time autophagy was notably activated. In addition, similar results were obtained for neuron-like cells. Furthermore, miR-30a overexpression completely abolished microwave-induced autophagy in neuron-like cells. Taken together, these results suggested that reduced miR-30 levels are an underlying mechanism involved in the activation of autophagy in neurons after microwave radiation.
As an important molecular target that regulates autophagy, the role of miR-30a in Beclin1-mediated autophagy has been widely studied and reported for a variety of diseases [35, 47–49]. The results of the present study also support that decreased miR-30a levels positively regulates autophagy by promoting Beclin1 expression in neurons exposed to microwaves. However, the potential downstream target genes of miR-30a involved in regulating autophagy have yet to be systematically studied. Through bioinformatics analysis, we predicted the target genes of miR-30a that may be involved in autophagy regulation, including Beclin1, Prkaa2, Irs1, Pik3r2, Rras2, Ddit4, Gabarapl2 and Atg12. The results of our previous study demonstrated that microwave radiation induces abnormal energy metabolism in hippocampal neurons and neuron-like cells [30, 31]. In the present study, we showed that microwave radiation activates AMPK signaling, acellular energy sensor, in both rat hippocampi and neuron-like cells. There is consensus that AMPK signaling mediates the activation of autophagy in various cells [50]. We observed that Prkaa2, encoding AMPKa2, is a downstream target gene of miR-30a, which was verified by double luciferase reporter assay results. Taken together, these results suggested that miR-30a may regulate autophagy in neurons by modulating the AMPK pathway. In contrast to the observed changes in miR-30a expression, microwave radiation increased AMPKa2 expression and phosphorylation (Thr172) both in vivo and in vitro. Moreover, miR-30a overexpression partly inhibited the microwave-induced activation of AMPKa2 and p-AMPKa and abolished microwave-induced autophagy in neuron-like cells. With the exception of Beclin1, we hypothesize that the reduced miR-30a levels after microwave exposure promotes the occurrence of autophagy partly by activating AMPK signaling, which had not been previously reported. However, the in vivo interaction between miR-30a with Prkaa2 requires additional study for verification. Furthermore, the results of the present study were primarily obtained from rat-derived cells, and whether a conserved mechanism is used among species, especially humans, requires further analysis. Other downstream target genes of miR-30a, such as Atg12, Atg8, REDD1, phosphatidylinositol 3-kinase (PI3K) and MAPK, may be involved in the miR-30a-mediated regulation of autophagy, but additional research is required to test this possibility.

**Conclusions**

AMPKa2 was a newly founded downstream gene of miR-30a involved in autophagy regulation, and miR-30a downregulation after microwave radiation could promote neuronal autophagy by increasing AMPKa2 expression and activating AMPK signaling.

**Declarations**

**Ethics approval.** All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the Beijing Institute of Radiation Medicine.

**Consent for publication.** Figure 4C is quoted from KEGG pathway database, and have obtained a formal permission from Kanehisa laboratories.
Availability of data and materials. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests. The authors declare no conflict of interest.

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Authors' contributions. R.P. and L.Z. conceived and designed the experiments. Y.H., W.L., H.W. (Hui Wang), J.Z. and H.W. (Haoyu Wang) performed the experiments. J.D., B.Y. and X.X. contributed reagents/materials/analysis tools. Y.H., W.L. and L.Z. analyzed the data. Y.H. and L.Z. wrote the main manuscript text.

Acknowledgement. Not applicable.

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Microwave promotes autophagy in rat hippocampal neurons. Adult male Wistar rats were exposed to microwaves with an average power density of 30 mW/cm² for 15 min/d, once every other day for a total of three treatments. At specific time points after radiation, 3 rats from each group were euthanized, and the hippocampal tissues were isolated. The ultrastructure of hippocampal neurons (Scale bar = 500 nm) was observed by transmission electron microscopy (TEM). Representative images are presented in A. The
autophagosomes are indicated by arrows without tails, while the autolysosomes are indicated by arrows with tails. In addition, the protein levels of Beclin1, LC3-I and LC3-II in rat hippocampus were detected by western blotting. The Beclin1 bands in immunoblots and the associated statistical results, where Beclin1 expression was normalized to that of GAPDH, are presented in B. The LC3-II and LC3-I protein bands are shown in C, and relative values of LC3-II to GAPDH was statistically analyzed. SH = Sham-exposed Group, MW = Microwave-exposed Group. The data are presented as the means±s.e.m. *, p < 0.05; **, p < 0.01 vs the SH group at the same time point after radiation.
Microwave radiation increases autophagic flux in PC12 cell-derived neuron-like cells. PC12 cells were induced by nerve growth factor (NGF) to generate neuron-like cells with a neuronal phenotype. Then, the neuron-like cells were exposed to microwaves with an average power density of 30 mW/cm² for 15 min. To assess “autophagic flux”, neuron-like cells were pretreated with chloroquine (CQ) 1 h before microwave exposure. At 6, 12 and 24 h after exposure, LC3-I and LC3-II expression was assessed in neuron-like cells with and without chloroquine pretreatment by western blot analysis. Additionally, general autophagy markers including Beclin1, Atg5, Atg7 and Atg9 in neuron-like cells without chloroquine treatment were also detected by western blotting. Representative western blot images were shown in A, and relative values normalized by GAPDH were presented in B. At 6 h after exposure, neuron-like cells without chloroquine pretreatment were fixed and stained with heavy metals. The ultrastructure was observed by transmission electron microscope (TEM), and representative images are shown in C. The autophagosomes are indicated with arrows without tails, while the autolysosomes are indicated with arrows with tails. At 6 h after exposure, the LC3-I and LC3-II levels from neuron-like cells pretreated with or without chloroquine were assessed by immunofluorescence, and representative images are presented in D. SH = Sham-exposed Group, MW = Microwave-exposed Group, CQ = Chloroquine. The data are presented as the means±s.e.m. *, p < 0.05; **, p < 0.01, compared to the SH group.
Figure 3

Microwave radiation inhibits miR-30a expression in both rat hippocampal neurons and neuron-like cells. At 7 and 14 d after 30 mW/cm² microwave exposure, rats were euthanized and the hippocampi were removed. Differentially expressed miRNAs in the rat hippocampi were screened using a miRNA chip and are shown in A, with miR-30a highlighted with a red box. At specific time points after exposure, total RNA was extracted from rat hippocampi and PC12 cell-derived neuron-like cells. The expression of miR-30a
and U6 was detected by real-time RT-PCR, and the miR-30a content was normalized to that of U6. The relative values of miR-30a in animals and cells are presented in B and C, respectively. Furthermore, the expression of miR-30a expression in rat hippocampi at 7 and 14 d after microwave radiation was also evaluated by in situ hybridization (ISH). Representative images and the associated statistical results are shown in D and E, respectively. SH = Sham-exposed Group, MW = Microwave-exposed Group. The data are presented as the means±s.e.m. *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared to the SH group.

Figure 4
Prediction and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis of miR-30a downstream target genes, microwave radiation increases the expression of AMPKα2 and the phosphorylation (Thr172) of AMPKα in rat and PC12 cell models. Four databases (miRanda, miRDB, TargetScan, and miWalk) were used to predict the target genes of miR-30a, and the results are shown in A. Subsequently, the genes using at least three databases were used for further KEGG pathway analysis. The results revealed that miR-30a regulates multiple signaling pathways, including those involved in autophagy, as shown in B. Moreover, the details of the miR-30a target genes associated with the regulation of autophagy, including Beclin1, Prkaa2, Irs1, Pik3r2, Rras2, Ddit4, Gabarapl2, and Atg12, are presented in C and labeled in red. At specific time points after 30 mW/cm2 microwave exposure, total proteins were extracted from rat hippocampi and PC12 cell-derived neuron-like cells, and the total and phosphorylated (Thr172) levels of AMPKα were detected by western blot analysis. The representative protein bands of AMPKα2, p-AMPKα (Thr172), AMPKα and GAPDH in rat models are presented in D, while these of cell models are shown in E. AMPKα2 and p-AMPKα (Thr172) levels were normalized to that of GAPDH and AMPKα, and the statistical results are shown along the right side of the corresponding protein band images. Additionally, HEK-293T cells were transiently transfected with pmirGLO-rPrkaa2 3'UTR WT, pmirGLO-rPrkaa2 3'UTR MUT and empty pmirGLO vectors in combination with miR-30a mimics or corresponding negative control miRNAs. The cells were lysed, after which firefly and Renilla luciferase activities were detected using a luminometer. The ratio of firefly to Renilla luciferase activity was calculated, and the relative values are shown in F. SH = Sham-exposed Group, MW = Microwave-exposed Group. The data are presented as the means±s.e.m. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs the SH group.
miR-30a overexpression reduces AMPKα2 expression, suppresses AMPK phosphorylation, and inhibits autophagic flux in neuron-like cells. PC12 cell-derived neuron-like cells were transfected with miR-30a mimics or the negative control 24 h before 30 mW/cm² microwave radiation and were harvested 6 h after exposure. miR-30a was assessed by real-time RT-PCR, and the results are shown in A. AMPKα2 and p-AMPK (Thr172) levels in neuron-like cells transfected with miR-30a mimics and the corresponding negative control were assessed by western blot analysis, the bands of which and related statistical results are presented in B and C, respectively. Beclin1 expression in neuron-like cells without chloroquine pretreatment was analyzed by western blot analysis, and the results are shown in D. To inhibit the degradation of autophagosomes by lysosomes, neuron-like cells were pretreated with chloroquine 1 h before exposure. Neuron-like cells treated with or without chloroquine were collected 6 h after exposure, and LC3-II and LC3-I expression was detected by western blot analysis. The LC3-II (LC3-I) bands and related statistical results for cells treated with or without chloroquine are shown in E and F, respectively. CQ = Chloroquine. The data are presented as the means±s.e.m. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs corresponding control group.

Figure 5