Original Article

Microwave Exposure Impairs Synaptic Plasticity in the Rat Hippocampus and PC12 Cells through Over-activation of the NMDA Receptor Signaling Pathway*

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Abstract

Objective The aim of this study is to investigate whether microwave exposure would affect the N-methyl-D-aspartate receptor (NMDAR) signaling pathway to establish whether this plays a role in synaptic plasticity impairment.

Methods 48 male Wistar rats were exposed to 30 mW/cm² microwave for 10 min every other day for three times. Hippocampal structure was observed through H&E staining and transmission electron microscope. PC12 cells were exposed to 30 mW/cm² microwave for 5 min and the synapse morphology was visualized with scanning electron microscope and atomic force microscope. The release of amino acid neurotransmitters and calcium influx were detected. The expressions of several key NMDAR signaling molecules were evaluated.

Results Microwave exposure caused injury in rat hippocampal structure and PC12 cells, especially the structure and quantity of synapses. The ratio of glutamic acid and gamma-aminobutyric acid neurotransmitters was increased and the intracellular calcium level was elevated in PC12 cells. A significant change in NMDAR subunits (NR1, NR2A, and NR2B) and related signaling molecules (Ca^{2+}/calmodulin-dependent kinase II gamma and phosphorylated cAMP-response element binding protein) were examined.

Conclusion 30 mW/cm² microwave exposure resulted in alterations of synaptic structure, amino acid neurotransmitter release and calcium influx. NMDAR signaling molecules were closely associated with impaired synaptic plasticity.

Key words: Microwave; Rat hippocampus; PC12; Synaptic plasticity; NMDA receptor signaling pathway

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INTRODUCTION

Microwave technology has been applied in many fields, including telecommunications, medicine, and household appliances. Meanwhile, the potential risks of microwave exposure have raised attention in society[1-3]. The central nervous system (CNS), especially the hippocampus, is sensitive to microwave exposure[4-5]. During the past few years, epidemiological and experimental research has provided a large amount of evidence to indicate that microwave exposure can result in harmful biological effects in the brain, such as sleep disturbance, headache[6-7], cognitive alteration, or molecular expression changes[8-10]. With regards to cognitive

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function, learning and memory impairments have been reported following microwave exposure[5,11].

The synapse is a particular structure for transmitting electrochemical signals among nerve cells in the CNS. Synaptic plasticity is a unique function of synapses, and a key player in learning and memory. It can be regulated by altering: the release of neurotransmitters, the expression of receptors located on the synapses, and the activation of intracellular signaling cascades[12-14]. Microwave radiation may reduce the formation and the number of excitatory synapses in cultured rat hippocampal neurons[15]. The findings of our previous study indicated that microwave exposure could induce the disruption of amino acid neurotransmitters, which might be involved in the cognitive dysfunction[16]. However, the underlying mechanisms remain poorly understood. The N-methyl-D-aspartate receptor (NMDAR), one of the ionotropic glutamate receptor complexes, is widespread in the CNS and is involved in excitatory synaptic transmission. The alterations of NMDAR-related signaling pathways are closely associated with various physiological and pathological processes, especially changes in synaptic plasticity[17-19]. However, little is known about the effects of microwave radiation on the NMDAR-related signaling pathway.

The object of this study was to identify the molecular mechanism associated with microwave-induced synaptic plasticity impairment. In order to investigate functional and structural changes in synaptic plasticity, and to examine the effect of microwaves on the NMDAR signaling pathway, we chose microwaves with an average power density of 30 mW/cm^2. Two models were developed for synaptic plasticity. The expression levels of several key NMDAR signaling molecules were evaluated, including NR1, NR2A, NR2B, Ca^{2+}/calmodulin-dependent kinase II gamma (CaMKIIγ), cAMP-response element binding protein (CREB), and phosphorylated CREB (p-CREB). The release of neurotransmitters and calcium influx were also measured in the cell model.

MATERIALS AND METHODS

Animals and Microwave Exposure

Animal protocols were approved by the Institutional Animal Care and Use Committee. 48 male Wistar rats (Laboratory Animal Center of Beijing Institute of Radiation Medicine, Beijing, China), weighing an average of 220 g, were maintained under controlled environmental conditions (22 °C, 12 h light/dark cycle), and allowed ad libitum access to food and water. They were randomly divided into an experimental group and a sham group. Based on our previous studies, we chose microwaves with an average power density of 30 mW/cm²[14,20]. The microwave source is a klystron amplifier model JD 2000 (Vacuum Electronics Research Institute, Beijing, China), which could generate pulsed microwaves with the frequency of 2.856 GHz. A rectangular waveguide and A16-dB standard-gain horn antenna was used for transmitting the microwave energy to an electromagnetic shield chamber[21]. The average power density was measured using a waveguide antenna, the GX12M1CHP power meter (Guanghua Microelectronics Instruments, Hefei, China), and GX12M30A power heads. We determined the distance from the antenna to the top of the animal cage was 1.4 m, because the peak field power density was required to 200 W/cm². It was an approximate far field zone. The microwave pulse was delivered at 300 pps, with a pulse width of 500 ns. The experimental group was placed in a plastic cage inside a Plexiglas cylinder which contained twenty radial boxes for placing the rats, and exposed to microwaves for 10 min every other day for three days (Twelve rats were evenly distributed in the cage for exposing at one time). We calculated the SAR in different locations of the cage and provided 0.33 W/kg as the average value of the whole body per unit power density (1 mW/cm²). Accordingly, the average SAR of the whole body was calculated to be 9.9 W/kg for the 30 mW/cm² exposed group. The change of the body temperature before and after exposure was measured by infrared imager (ThermaVision A40, FLIR systems, Wilsonville, OR, USA), and there was no significant temperature elevation. The sham group was treated the same way, but without any microwave exposure.

Hematoxylin and Eosin (H&E) Staining

Rats were sacrificed at 1, 7, 14, and 28 d after microwave exposure (n=6). Brains were removed and fixed in 10% buffered formalin solution. Tissue blocks containing the hippocampus were embedded in paraffin and cut at 5 μm thick in the coronal plane. The sections were then stained with H&E and observed blindly under a light microscope (Leica DM6000, Leica, Wetzlar, Germany) for histological examination.

Transmission Electron Microscopy

The hippocampal specimens (1 mm^3) were dissected from the CA3 area 7 d and 14 d after microwave exposure. Briefly, the samples were placed in 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide. After graded ethyl alcohols, the cubes were embedded in EPON618. Thin sections laid on copper mesh were stained with heavy metals, uranyl acetate, and lead citrate for contrast. A Hitachi-H7650 transmission electron microscope (TEM; Hitachi, Japan) was used to observe the hippocampal ultrastructure.

Cell Culture and Microwave Exposure

Rat pheochromocytoma (PC12) cells, a neuron precursor cell line, were used to study neurite development. PC12 cells were seeded and cultured in 6-well plates coated with poly-L-lysine (Sigma, St. Louis, MO, USA) in Dulbecco's Modified Eagle Medium supplemented with 10% horse serum (Gibco, Grand Island, NY, USA) and 5% fetal bovine serum (Kang Yuan Biology, Tianjin, China). PC12 cells were primed with 5 ng/mL nerve growth factor (NGF; Sigma, St. Louis, MO, USA) 6 h after they had been seeded. They were maintained in the medium supplemented with 5 ng/mL NGF and 1% horse serum for 5 d. The cells responded to NGF by differentiating into the neuronal phenotype (an extension of neurite). They were randomly divided into an experimental group and a sham group. The experimental group was exposed to microwaves with an average power density of 30 mW/cm^2 for 5 min, and the sham group was treated at the same place for 5 min, but without microwave exposure. The cells were harvested 1, 6, 12, and 24 h after the exposure.

Evaluation of Neuron-like PC12 Cells by Immunocytochemistry and Laser Confocal Microscopy

As the characteristic markers, both neuron-specific enolase (NSE) and nerve factor (NF) levels were determined to evaluate the neuronal features of PC12 cells by immunocytochemistry and laser confocal microscopy, respectively. The following antibodies were used: rabbit anti-NSE and anti-NF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-rabbit antibody conjugated to horseradish peroxidase (HRP; Zhongshan Biotechnology, Beijing, China) for NSE, and Alexa fluor 488 (Molecular Probes, Eugene, OR, USA) for NF. PC12 cells were grown on plastic tissue culture coverslips (Thermo Fisher Scientific, Waltham, MA, USA) coated with poly-L-lysine. The cells were fixed directly onto the culture coverslips in 4% paraformaldehyde for 15 min and then rinsed in 0.1 mol/L phosphate-buffered saline (PBS). The primary antibodies were incubated overnight at 4 °C. The secondary antibodies were applied for 1 h at room temperature. Immunostaining was observed under a light microscope or laser confocal microscope.

Scanning Electron Microscopy

Six hours after exposure treatment, NGF-differentiated PC12 cells, grown on plastic tissue culture coverslips, were rapidly washed twice with PBS and fixed in 2.5% glutaraldehyde at 4 °C for 2 h. After being washed four times with glucoside-PBS, they were post-fixed in 2.5% osmium tetroxide, and dehydrated through graded ethanol. The cells were then critical-point dried and sputter-coated with gold palladium before being observed with a Hitachi S-3400 scanning electron microscope (SEM).

Atomic Force Microscopy

The PC12 cells were seeded on plastic coverslips coated with poly-L-lysine. At the termination of experiments, the medium was removed and the cells were washed three times with PBS and immediately fixed with 2.5% glutaraldehyde at 4 °C for 2 h. The cells were then rinsed with distilled water, and blown dry with air. Synapse morphology was visualized with an atomic force microscope (AFM; Nano Wizard, JPK Instruments, Berlin, Germany). Images were acquired with a TESP probe scanner (Veeco Instruments, Plainview, NY, USA), which was operated in an intermittent contact mode under room temperature.

Sample Preparation and High Performance Liquid Chromatography (HPLC) Analysis

To determine the release of amino acid neurotransmitters, PC12 cells were stimulated in 30 mmol/L KCl for 2 min, and centrifuged for 10 min at 3000 rpm at 4 °C. The supernatant was collected and incubated with 10% salicylsulfonic acid on ice for 10 min, and centrifuged for 20 min at 15,000 rpm at 4 °C. The supernatant (20 μL) was transferred to HPLC vials. HPLC analysis was conducted on an Agilent system equipped with a model 1050 pump, Agilent Hypersil column, autosampler, and

fluorescence detector. The mobile phase consisted of 0.1 mmol/L NaH_2PO_4 and 30% methanol, which was delivered at a flow rate of 0.9 mL/min. Aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), and gamma-aminobutyric acid (GABA) were of analytical grade (Sigma, St. Louis, MO, USA); these were dissolved in 0.1 mol/L HCl and mixed together as standard to normalize amino acid neurotransmitters in the samples. Preparation of the derivatization reagent O-phthalaldehyde (OPA) was based on the protocol of Abdel Ali Belaidi[22]. OPA (50 mg) was dissolved in 1 mL methanol, mixed with 9 mL of 0.4 mol/L borate buffer (pH 9.5) and 40 mL 2-mercaptoethanol. Precolumn derivatization was carried out with an autosampler, which was programmed to mix 1 μL of the sample with 5 μL of the derivatization reagent. After 1 min incubation, the derivatized mixture was injected into the HPLC system for analyzing.

Determination of Calcium Influx by Laser Confocal Microscopy and Flow Cytometry

Fluo-3-AM (Molecular Probes, Eugene, OR, USA) was used to assess intracellular calcium ion levels in NGF-differentiated PC12 cells. Immediately following 5 min microwave exposure, the cells were incubated for 30 min at 37 °C in 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 10 μmol/L Fluo-3-AM (protected from light), and washed twice with HEPES. The image was taken at room temperature, with illumination at argon laser 488 nmol/L, using the LSM 510 META (Carl Zeiss, Jena, Germany) laser confocal microscope. The cells were also quantitated by flow cytometry.

Western Blot

Total protein was extracted from hippocampal tissues or PC12 cells with RIPA buffer containing protease inhibitor (Applygen Technologies Inc., Beijing, China). The concentration was determined using a bicinchoninic acid assay (Pierce, Rockford, IL). The samples (30 μg) were subjected to 8%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The blotting membranes were blocked with 5% (w/v) nonfat milk. They were then incubated overnight at 4 °C with the following primary antibodies: anti-NR1, anti-CREB, anti-p-CREB (rabbit monoclonal 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-NR2A (rabbit monoclonal 1:1000, Millipore, Billerica, MA, USA), anti-NR2B (rabbit monoclonal 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-CaMKII gamma (mouse polyclonal 1:1000, Abcam, Cambridge, MA, USA). The membranes were then incubated with the following secondary antibodies: HRP-conjugated anti-rabbit or anti-mouse antibody (1:5000, Zhongshan Biotechnology, Beijing, China), and processed with the super enhanced chemiluminescence plus Western blotting detection system (Applygen Technologies Inc., Beijing, China). For the internal control, the same membrane was probed with an antibody against glyceraldehyde 3-phos- phate dehydrogenase (GAPDH; mouse monoclonal 1:10,000, KangChen, Shanghai, China) after being stripped. The relative density of the resultant protein immunoblot images were semi-quantitatively analyzed using the Alpha Imager HP analysis system (Protein Simple, Silicon Valley, CA, USA).

RNA Extraction, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), and Real-time PCR

Total RNA was extracted from hippocampal tissues or PC12 cells using Trizol (Invitrogen, Carlsbad, CA, USA), and reverse transcribed with a AMV First Strand cDNA Synthesis Kit (Sangon Biotech, Shanghai, China). The Real Master Mix SYBR Green Kit (Tiangen, Beijing, China) was used to run real-time PCR. GAPDH expression was used as an internal control. The sequences of the gene-specific primers were as follows: GluN1 (NM_017010), forward 5'-GATCGCCTACAAGCGACACAA-3' and reverse 5'-TTAGGGTCGGGCTCTGCTCTAC-3'; GluN2A (NM_012573), forward 5'-GCTTGTGGTGATCGTGCTG AA-3' and reverse 5'-AATGCTGAGGTGGTTGTCATC TG-3'; GluN2B (NM_012574), forward 5'-TGGCTAT CCTGCAGCTGTTTG-3' and reverse 5'-TGGCTGCTCA TCACCTCATTC-3'; and GAPDH (NM_017008), forward 5'-GTGCTGAGTATGTCGTGGAG-3' and reverse 5'-CGGAGATGATGACCCTTTT-3'. Forty cycles at 95 °C for 15 s and 60 °C for 1 min were performed with an ABI Prism 7300 sequence detection system (Applied Biosystems, Grand Island, NY, USA). The formula $2^{-\triangle\triangle}$Ct was used for normalization. Gene expression was calculated as the ratio of the experimental group to the sham group.

Statistical Analysis

Data were expressed as the mean±standard error of the mean. One-way ANOVA was applied for statistical analysis, followed by Student's *t*-tests to compare the difference between sham group and

experimental group at the same time point after exposure by using SPSS software 18.0 (SPSS Inc, IL, USA). A *P* value of <0.05 was considered statistically significant.

RESULTS

Microwave Exposure Induced Significant Damage to Hippocampal Tissue

In order to assess morphological changes in synaptic plasticity associated with learning and memory, histological examination of the rat hippocampus was carried out. There was no significant degeneration of neurons in the sham group. The hippocampal neurons presented in the regular arrangement, with distinct edges, and clear nucleus and nucleolus in the sham group (Figure 1A). After microwave exposure, the hippocampal neurons were significantly injured; symptoms included degeneration, irregular arrangement, enlarged pericellular space, and karyopyknosis with dark staining (Figure 1B-C). The injury was apparent at 1 d and 7 d after exposure, and signs of recovery appeared at 14 d after exposure (Figure 1D-E).

The pathological features of hippocampal ultrastructure, especially synapse structure, were observed with TEM. In the sham group, normal synapse ultrastructure was observed in the hippocampus: a clear synaptic cleft and postsynaptic density (PSD), and certain numbers of synaptic vesicles in the presynaptic membrane (Figure 1F). However, the number of synaptic vesicles and the thickness of PSD in the synapses decreased after microwave radiation, and the synaptic cleft became blurred (Figure 1G). The structural injury of synapses partially recovered 14 d after microwave exposure, but the mitochondria stayed swollen and vacuolated, which are symptoms associated with cristae disruption (Figure 1H).

Multiple NMDAR-related Signaling Pathways in Hippocampal Tissues were Affected by Microwave Radiation

Compared with the sham rat hippocampus, the expression of NR1, NR2A, NR2B, and CaMKIIγ increased in hippocampal tissues 1 d after exposure (*P*=0.034, 0.016, 0.028, and 0.035, Figure 2). This indicated that over-activation of NMDAR subunits and related signaling molecules might be a cause of synaptic plasticity impairment induced by microwave exposure. The mRNA levels of NR2A at 7 d (Figure 3B) and NR2B at 1 d (Figure 3C) also increased after exposure (*P*=0.004 and 0.033, respectively), although there was no change in NR1 (Figure 3A). Interestingly, the expression of p-CREB significantly decreased 7 d after exposure (*P*=0.024, Figure 2), suggesting that multiple signaling pathways might be affected by microwave radiation.

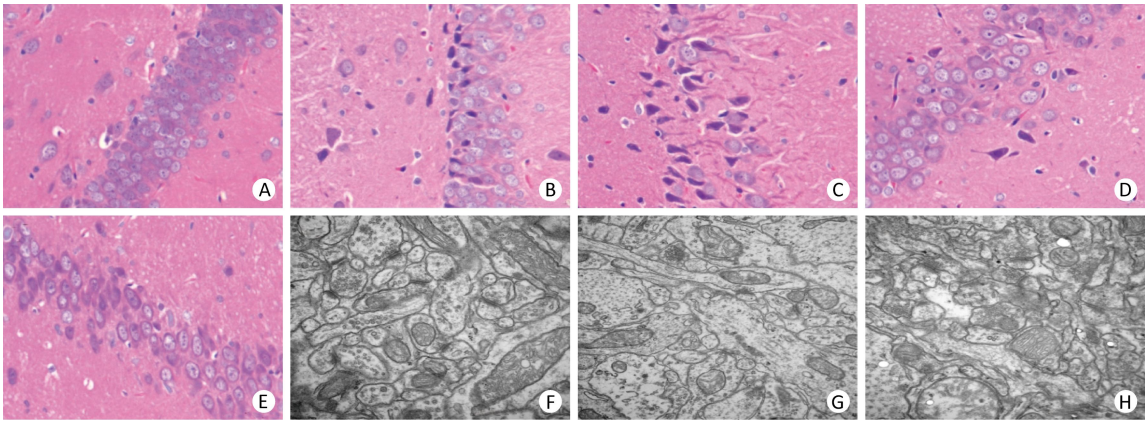

Figure 1. Effect of microwave exposure on histology of rat hippocampal structure (A-E) and ultrastructure (F-H). A and F: sham group. There was no degeneration of the neurons. B-E, G, and H: experimental group. The damages of the hippocampal neurons were showed at 1 d (B) and 7 d (C) after microwave exposure in the H&E staining slides. There were signs of recovery at 14 d (D) and 28 d (E) after exposure. The hippocampal ultrastructure was observed by transmission electron microscope (TEM). Compared with normal synapse ultrastructure in the sham group (F), the synapse ultrastructure damages were observed at 7 d after microwave exposure (G). And the structural injury of synapses partially recovered at 14 d after microwave exposure (H). (H&E scale bar =50 μm, TEM scale bar =500 nm).

Microwave Exposure Could Damage Structures Associated with Synaptic Plasticity in NGF-differentiated PC12 Cells

Both immunocytochemistry and laser confocal microscopy revealed neuronal features in NGF-differentiated PC12 cells. Using the SEM and AFM, we also observed that the synapses of NGF-differentiated PC12 cells grew longer than those without NGF differentiation, and they were interlaced with other synapses, confirming that PC12 cells could serve as a valid cell model to study synaptic plasticity (figures not shown). Six hours after microwave exposure, the synaptic length of NGF-differentiated PC12 cells shortened and the number of synapses decreased (Figure 4C-D). The membrane surfaces of

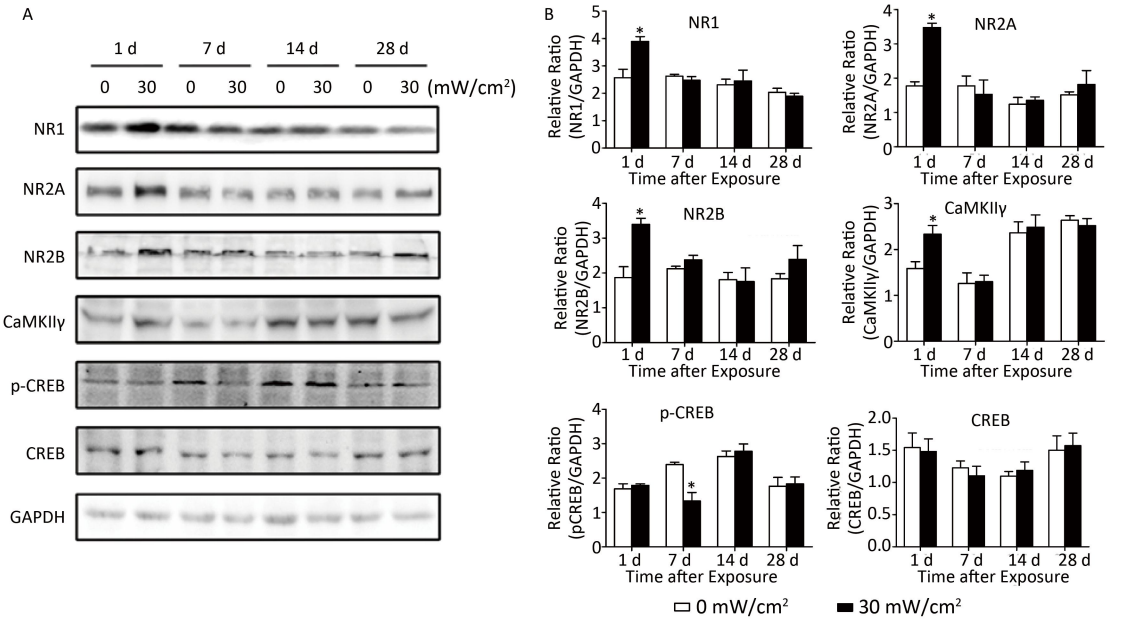

Figure 2. Protein expression of N-methyl-D-aspartate receptor subunits and related signaling molecules in hippocampal tissues. The protein expression levels of NR1 (P=0.034), NR2A (P=0.016), NR2B (P=0.028), and CaMKIIγ (P=0.035) were notably up-regulated in hippocampal tissues at 1 d after 30 mW/cm² microwave exposure. Interestingly, the expression of p-CREB was significantly attenuated in the experimental group at 7 d after exposure (P=0.024). A: Western blot images; B: semi-quantitation of the immunoblots. CaMKIIγ=Ca^{2+}/calmodulin-dependent kinase II gamma; CREB=cAMP-response element binding protein; p-CREB=phosphorylated CREB; GAPDH=glyceraldehyde 3-phosphate dehydrogenase. Group effect: $^{*}P$<0.05.

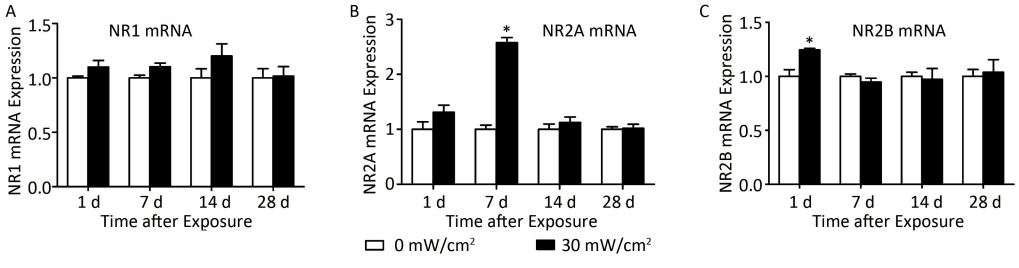

Figure 3. mRNA expression of N-methyl-D-aspartate receptor subunits in hippocampal tissues. Real-time polymerase chain reaction did not show a significant difference in the mRNA expression of NR1 between the sham and experimental groups (A). However, the mRNA expression of NR2A notably increased at 7 d (B), and for NR2B it increased at 1 d (C) after 30 mW/cm² microwave exposure (P=0.004 and 0.033, respectively). Group effect: $^{*}P$<0.05.

synapses became rough (Figure 4H-J). The results clearly revealed structural damage that would affect synaptic plasticity.

Release of Amino Acid Neurotransmitters in NGF-differentiated PC12 Cells was Affected by Microwave Exposure

The release of each amino acid from NGF-differentiated PC12 cells was measured 6 h and 24 h after microwave exposure. Although the release of Asp did not change (Figure 5A), the release of Gly, Glu, and GABA decreased at 6 h after exposure (P=0.002, 0.000, and 0.000, Figure 5B-D). However, the ratio of Glu and GABA significantly increased (P=0.000, Figure 5E), indicating that the excitatory neurotransmitters might play a key role in activating NMDAR-related signal cascades after microwave exposure. There was no significant difference between the two groups at 24 h after exposure.

Microwave Exposure Increased the Calcium Influx in NGF-differentiated PC12 Cells

Calcium entry can trigger an intracellular signaling cascade and affect synaptic plasticity. Intracellular calcium levels in NGF-differentiated PC12 cells were markedly elevated after microwave exposure (P=0.000, Figure 6), demonstrating that calcium influx was involved in microwave-mediated synaptic plasticity changes.

Expression of NMDAR Subunits and Related Signaling Molecules Increased in NGF-differentiated PC12 Cells after Microwave Exposure

Protein immunoblot analysis showed a significant increase in the expression of NR1 (P= 0.038 and 0.032) and NR2B (P=0.036 and 0.031) at 1 h and 6 h, and in NR2A (P=0.028) at 6 h after microwave exposure. The expression of NMDAR-related signaling

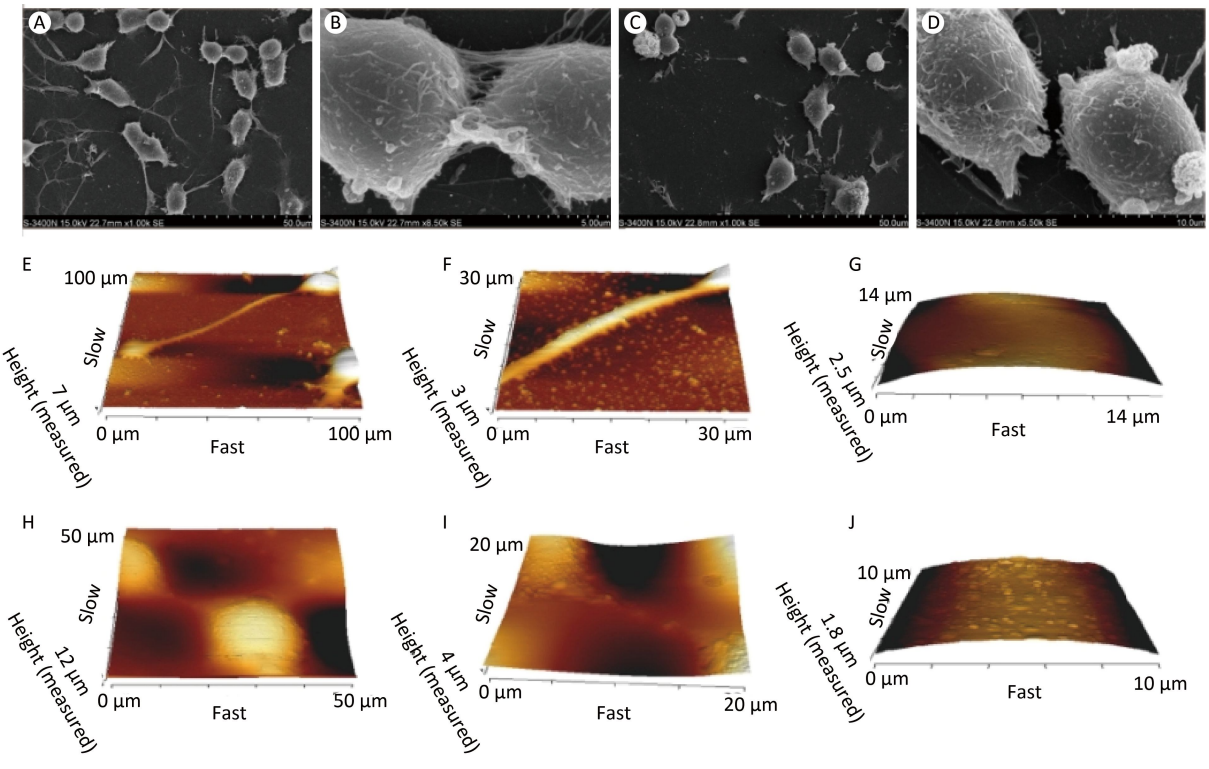

Figure 4. Effect of microwave exposure on the structure affecting synaptic plasticity in nerve growth factor (NGF)-differentiated PC12 cells. A scanning electron microscope (SEM) (A-D) and atomic force microscope (AFM) (E-J) were used to assess these structural changes. Compared with the long and interlaced synapses in the sham NGF-differentiated PC12 cells, we observed the synaptic length shortened and the numbers of synapses decreased 6 h after 30 mW/cm² microwave exposure using the SEM (C, D). Compared with the sham cells (E-G), the membrane surfaces of synapses from the exposed cells appeared rough using the AFM (H-J).

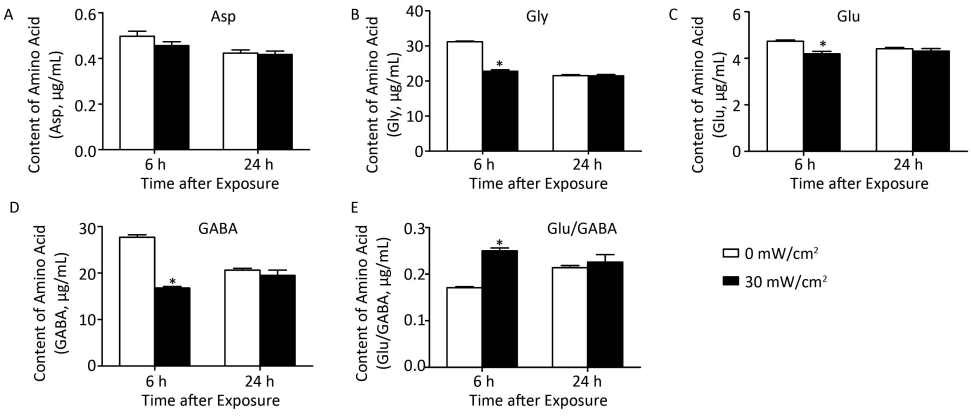

Figure 5. Effect of microwave exposure on the release of amino acid neurotransmitters in nerve growth factor differentiated PC12 cells. There were no significant changes in the release of Asp between the sham and the experimental groups (A). The release of Gly, Glu, and GABA decreased at 6 h after exposure (P=0.002, 0.000, and 0.000, B-D). However, the ratio of Glu and GABA showed a remarkable increase after 30 mW/cm² microwave exposure (P=0.000, E). Asp=aspartic acid; Gly=glycine; Glu=glutamic acid; GABA=gamma-aminobutyric acid. Group effect: $^{*}P$<0.05.

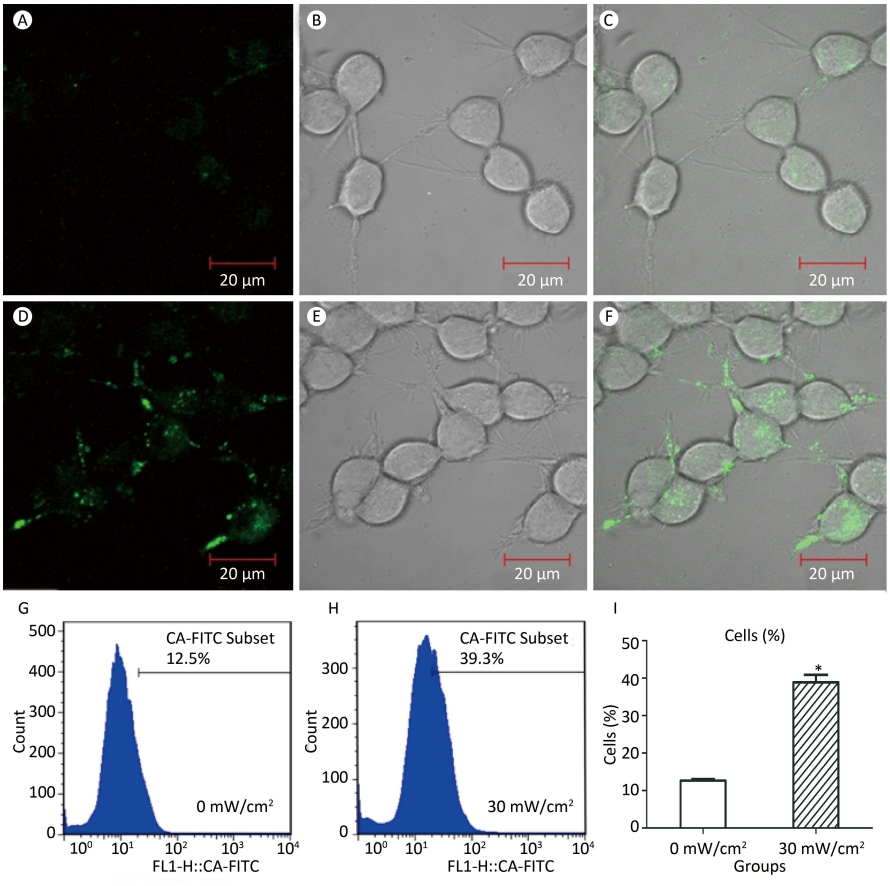

Figure 6. Effect of microwave exposure on the level of calcium influx in nerve growth factor (NGF)-differentiated PC12 cells. Compared with the sham group (A-C), the intracellular calcium level in NGF-differentiated PC12 cells increased after 30 mW/cm² microwave exposure (assessed using laser confocal microscopy; D-F). We also detected the intracellular calcium levels in sham group (G) and experimental group (H) using flow cytometry. The quantitation of flow cytometry also showed elevated intracellular calcium levels (P=0.000, I). Group effect: $^{*}P$<0.05.

molecules, including CaMKIIγ (*P*=0.037 and 0.044) and p-CREB (*P*=0.044 and 0.030), also increased at 1 h and 6 h after exposure (Figure 7). Real-time PCR revealed remarkable up-regulation of NR1 at 6 h and 12 h (*P*=0.004 and 0.001, Figure 8A), of NR2A at 6 h (*P*=0.034, Figure 8B), and of NR2B at 1, 6, and 12 h (*P*=0.033, 0.001, and 0.036, Figure 8C) after microwave exposure.

DISCUSSION

Microwave devices, such as the cell phone, wireless communication, and the microwave oven, have changed our lives in many ways. However, there has been growing public concern regarding the potential health risks associated with increased microwave exposure[2,23]. Epidemiological studies have

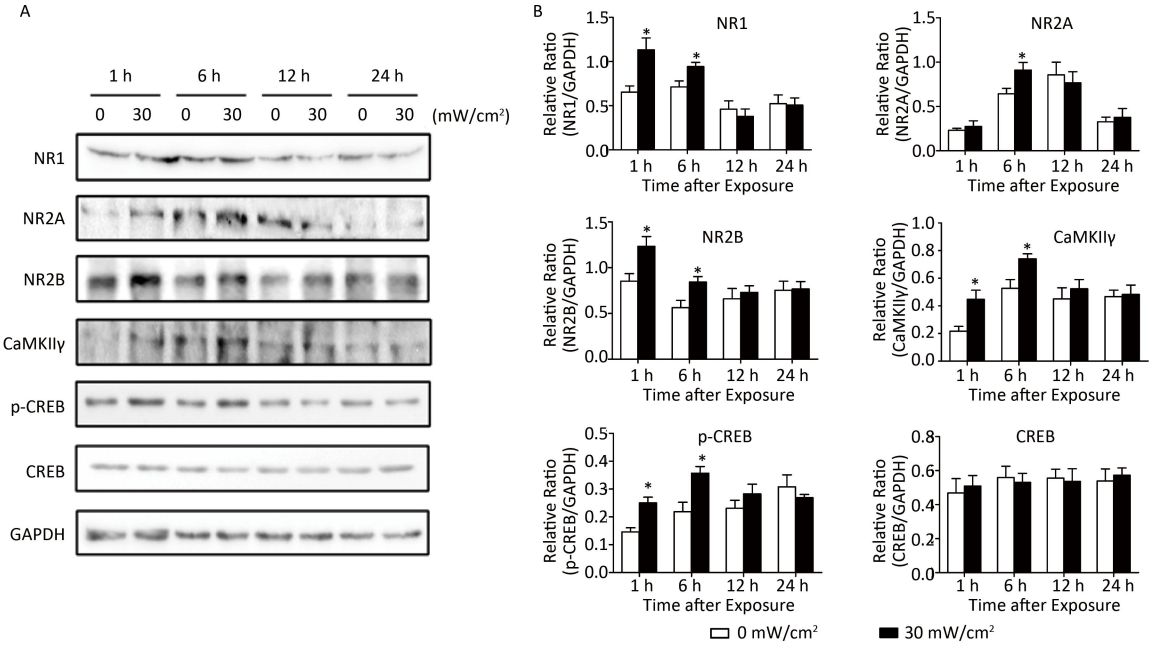

Figure 7. Protein expression of N-methyl-D-aspartate receptor subunits and related signaling molecules in nerve growth factor differentiated PC12 cells. Western blot showed over-expression of NR1 (*P*=0.038 and 0.032), NR2B (*P*=0.036 and 0.031), CaMKIIγ (*P*=0.037 and 0.044), and p-CREB (*P*=0.044 and 0.030) at 1 h and 6 h, and over-expression of NR2A (*P*=0.028) at 6 h after microwave exposure. A: Western blot images; B: semi-quantitation of the immunoblots. CaMKIIγ=Ca^{2+}/calmodulin-dependent kinase II gamma; CREB=cAMP-response element binding protein; p-CREB=phosphorylated CREB; GAPDH=glyceraldehyde 3-phosphate dehydrogenase. Group effect: $^{*}P$<0.05.

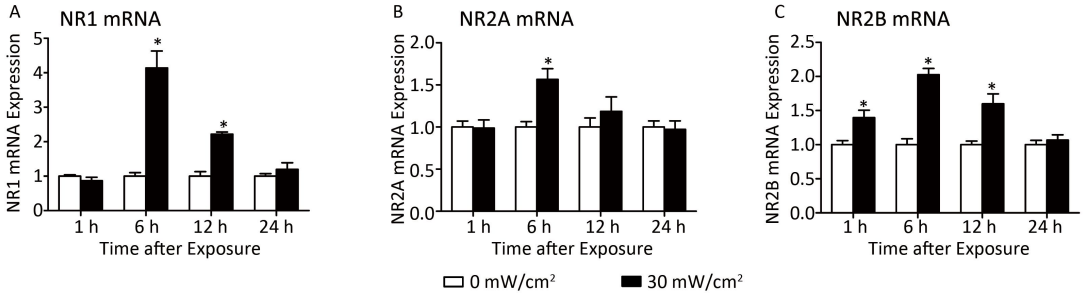

Figure 8. mRNA expression of N-methyl-D-aspartate receptor subunits in nerve growth factor differentiated PC12 cells. Real-time *polymerase chain reaction* showed that mRNA levels of NR1 significantly increased at 6 h and 12 h (*P*=0.004 and 0.001, A), NR2A increased at 6 h (*P*=0.034, B), and NR2B increased at 1, 6 and 12 h (*P*=0.033, 0.001, and 0.036, C) after microwave exposure. Group effect: $^{*}P$<0.05.

shown that the neurobehavioral effect is associated with mobile phone base station proximity. The major neurobehavioral symptoms, including headache, irritability, and lower cognitive performance, increased in populations living at distances less than 500 m from base stations[24]. Microwave exposure still might be the risk of cognitive disorders and brain tumors, however, recent studies revealed no or only scant evidence for the assumption that microwave radiation could undermine human health[25-26]. Experimental studies have also confirmed that microwave exposure can be detrimental to synaptic plasticity in animals and impair cognitive function, especially learning and memory[27-28].

Based on a large amount of research on microwaves, we proposed that microwave exposure might cause dysfunction of learning and memory as a result of synaptic plasticity impairment. Our previous research showed that 30 mW/cm² microwave radiation could induce the injury of synaptic structure and function of hippocampus, and induce the disorder of the learning and memory ability in rats[20]. However, the underlying mechanism is not yet clear. Learning and memory, which are advanced brain functions, are closely linked to the hippocampus. This is an important structure of the CNS and is extremely sensitive to microwave radiation[14,16]. In our study, we found that microwave exposure, with an average power density of 30 mW/cm², could notably damage the hippocampal structure in rats and cause the following symptoms in neurons: degeneration, irregular arrangement, enlarged pericellular space, and karyopyknosis with dark staining. In addition, the quantity of synaptic vesicles and the thickness of PSD in the synapses decreased, and the synaptic cleft became blurred. Furthermore, the length and number of synapses decreased, and the membrane surfaces of synapses became uneven. As amino acid neurotransmitters are the principal messengers in synaptic transmission, we analyzed the release of some major amino acid neurotransmitters associated with synaptic plasticity in neuron-like NGF-differentiated PC12 cells. The average release of Glu, Gly, and GABA were significantly attenuated after 30 mW/cm² exposure, confirming that microwave radiation can induce synaptic plasticity impairment. This led us to further investigate the potential mechanisms of microwave-related synaptic plasticity impairment via *in vivo* and *in vitro* models.

Interestingly, our data showed that the ratio of released Glu and GABA remarkably increased in neuron-like PC12 cells after 30 mW/cm² microwave exposure, suggesting that glutamate as a basic excitatory neurotransmitter might play a key role in microwave-associated synaptic plasticity impairment. In the CNS, NMDARs are critical glutamate receptors. They have a tetraheteromeric composition of NR1 subunit, NR2 subunits (NR2A-D), and NR3 subunits (NR3A and 3B), which also named GluN1, GluN2, and GluN3, respectively. NR1 is essential for the structure of NMDAR. And NR2 subunits, especially NR2A and NR2B, have a modulatory function on the targeting and stabilization of binding sites in the synapse[29-30]. The variable composition of NMDAR probably determines its multiple functional properties in synaptic transmission and synaptic plasticity[31].

The activation of NMDARs by glutamate neurotransmitters results in a calcium influx, which activates the plasticity-related protein network. However, NMDAR over-activation can also have neurotoxic consequences, leading to a variety of nervous system disorders, including schizophrenia, chronic pain, Huntington's disease, and Alzheimer's disease[17,31-32]. Yet little is known about the effects of microwaves on the NMDAR-related signaling pathway. Recent evidence suggests that some, or perhaps even most synaptic NMDARs contain NR1, NR2A, and NR2B[33]. Thus, we examined the expression of NR1, NR2A, and NR2B in rat hippocampal tissues and NGF-differentiated PC12 cells. We found that all three subunits were significantly up-regulated in hippocampal tissues 1 d after exposure to 30 mW/cm² microwaves, while in NGF-differentiated PC12 cells, this up-regulation occurred 1 h and/or 6 h after exposure. This suggests that over-activation of NMDAR subunits might be a cause of synaptic plasticity impairment associated with microwave exposure and might be directly related to glutamatergic excitotoxicity.

A remarkable increase of intracellular Ca^{2+} levels in the exposed PC12 cells was detected by laser confocal microscopy and flow cytometry in our study, which coincided with the over-activation of NMDAR. The Ca^{2+} influx signal through postsynaptic NMDARs can activate CaMKII, which plays a critical role in regulating the induction of synaptic plasticity modifications such as long-term potentiation (LTP)[34]. LTP, a physiological mechanism of learning and memory formation, could be inhibited by microwave exposure in our recent research[21]. CaMKII is an abundant and plasticity-associated protein kinase, known to have four highly related isoforms (α, β, γ,

and δ)[35]. While α and β isoforms are specifically localized in the brain, γ and δ isoforms are found ubiquitously in the developing nervous system, suggesting that the intracellular distribution of CaMKII is likely to be dependent on the protein-binding properties of γ and δ isoforms with NMDAR, particularly NR2B[36]. In comparison with the δ isoform, the γ isoform is enriched in both neurons and astrocytes, and splice variants of CaMKIIγ vary in their ability and sensitivity to respond to calcium-ion flux and/or calmodulin[37-38]. CaMKII activation evoked by glutamate-mediated Ca^{2+} entry may result in the activation of transcription factors including p-CREB[39]. Emerging evidence indicates that CREB is important in inducing LTP associated with neuronal plasticity, and is linked to several signaling pathways. It is associated with the conversion from short-term memory to long-term memory (LTM)[40]. Moreover, it is well known that gene transcription and protein synthesis are required for LTM formation[41]. Hence, the activation of CREB and CREB-dependent gene expression in the CNS affect various forms of synaptic plasticity, from altering the strength and number of synaptic connections, to modulating the synaptic functions during physiological and pathological processes[42]. However, the effects of microwave exposure on the expressions of CaMKIIγ and CREB, and on the phosphorylation of CREB, are still in debate. Our research data showed that the protein expression of CaMKIIγ was significantly up-regulated both in hippocampal tissues and NGF-differentiated PC12 cells, which might be induced by the elevated Ca^{2+} influx that occurred following microwave exposure. Interestingly, p-CREB notably increased in the PC12 cells after 30 mW/cm² exposure; however, p-CREB significantly decreased in the exposed hippocampal tissues, which seems inconsistent with the over-expression of NMDAR and CaMKIIγ. This different p-CREB expression pattern might result from the activation of diverse signaling pathways associated with 30 mW/cm² microwave radiation in the hippocampus. These complex signaling pathways may include protein kinase A (PKA), mitogen activated protein kinases (MAPK), and other Ca^{2+}/calmodulin kinases.

In conclusion, 30 mW/cm² microwave exposure can result in alterations of: synaptic structure, amino acid neurotransmitter release, calcium influx, and NMDAR signaling molecules. These alterations are closely associated with impaired synaptic plasticity. This is the first study to focus on the characteristic parameters of synaptic plasticity, both *in vivo* and *in vitro*, following microwave radiation. Our findings provide new evidence for the mechanisms of synaptic plasticity impairment induced by microwave exposure, which can be used to promote future research on protection against microwave exposure.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: PENG Rui Yun, ZHAO Li. Performed the experiments: XIONG Lu, SUN Cheng Feng. Analyzed the data: XIONG Lu. Contributed reagents/materials/analysis tools: ZHANG Jing, GAO Ya Bing, WANG Li Feng, ZUO Hong Yan, WANG Shui Ming, ZHOU Hong Mei, XU Xin Ping, DONG Ji, YAO Bin Wei. Wrote the paper: XIONG Lu.

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