

Changes in rat spatial learning and memory as well as serum exosome proteins after simultaneous exposure to 1.5 GHz and 4.3 GHz microwaves

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ABSTRACT

This study aimed to elucidate the effects and biological targets sensitive to simultaneous 1.5 and 4.3 GHz microwave exposure in rats. A total of 120 male Wistar rats were divided randomly into four groups: the sham (S group), 1.5 GHz microwave exposure (L group), 4.3 GHz microwave exposure (C group) and simultaneous 1.5 and 4.3 GHz microwave exposure (LC group) groups. Spatial learning and memory, cortical electrical activity, and hippocampal ultrastructure were assessed by the Morris Water Maze, electroencephalography, and transmission electron microscopy, respectively. Additionally, serum exosomes were isolated by ultracentrifugation and assessed by Western blotting, nanoparticle tracking and transmission electron microscopy. The serum exosome protein content was assessed by label-free quantitative proteomics. Impaired spatial learning and memory decreased cortical excitability, and damage to the hippocampal ultrastructure were observed in groups exposed to microwaves, especially the L and LC groups. A total of 54, 145 and 296 exosomal proteins were differentially expressed between the S group and the L, C and LC groups, respectively. These differentially expressed proteins were involved in the synaptic vesicle cycle and SNARE interactions during vesicular transport. Additionally, VAMP8, Syn7 and VMAT are potential serum markers of simultaneous microwave exposure. Thus, exposure to 1.5 and 4.3 GHz microwaves induced impairments in spatial learning and memory, and simultaneous microwave exposure had the most severe effects.

1. Introduction

With the rapid development of communication networks, scientists have begun to examine the health hazards of increasing use of microwave communication equipment (Hardell and Nyberg, 2020; Liu et al., 2021). According to the literature, the central nervous system (CNS) is one of the most vulnerable systems to microwave exposure (Chauhan et al., 2017; Kesari et al., 2013; Maaroufi et al., 2014; Narayanan et al., 2018; Suhova et al., 2013). In addition, studies have examined the effects of single-frequency microwave exposure, indicating that the degree of harm was largely related to microwave frequency (Fragopoulou et al., 2018; Zhang et al., 2017). However, although multiple microwave frequencies are used in daily life, few studies have evaluated the effects of simultaneous exposure to multiple frequencies of microwaves.

Learning and memory, two of the most important brain functions, as well as their associated brain region (the hippocampus) (Zola-Morgan

and Squire, 1993) are of particular interest and have attracted substantial attention in studies of the effects of microwave exposure. Chauhan's team found damaged cortical and hippocampal pyramidal neurons in the brains of rats exposed to 2.45 GHz microwave radiation for 2 h/d at 0.2 mW/cm² over 35 d (Chauhan et al., 2017). Li et al. (2015) reported impaired performance on the Morris Water Maze (MWM) in rats exposed to 2.856 GHz microwave radiation for 6 weeks at different intensities administered three times a week for 6 min each (Li et al., 2015). Narayanan et al. found that rats exposed to 900 MHz microwave radiation for 1 h/day over 28 d showed impaired performance in the MWM as well as structural damage to hippocampal neurons (Narayanan et al., 2015).

However, previous studies have mainly focused on changes in neurons, synaptic plasticity, and related receptors (Wang et al., 2017a; Zhao et al., 2012). Recent studies have shown that interneuronal communication (consisting of neurons and other cells in the brain, especially glial

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cells) is particularly important for learning and memory as it regulates the formation and clearance of synapses, while this regulation is reflected in different learning styles such as sensory experience, social experience, and motor learning. (Parkhurst et al., 2013; Wilton et al., 2019; Xin and Chan, 2020). Due to the ease and relatively non-invasive nature of obtaining serum samples, biomarkers isolated from serum samples have been widely used to diagnose diseases; however, serum markers that reflect changes underlying microwave-induced brain injuries have rarely been reported. Exosomes contain substances released by cells to transmit information. These substances can enter the bloodstream (and hence, serum) from the brain through the blood-brain

barrier; therefore, we predicted that the molecules released in exosomes could be biological targets and fill gaps in this field.

Extracellular membrane vesicles (EMVs), including exosomes and microvesicles, are nanometre-sized vesicles (50–100 nm in diameter) that transfer functional biomolecules (including proteins, messenger RNAs (mRNAs), microRNAs, noncoding RNAs, DNA and lipids) among cells without necessitating direct cell-to-cell contact; thus, they represent a novel form of intercellular communication. Many cells in the nervous system release exosomes involved in neuron-glia communication (Men et al., 2019), neurodegenerative diseases (Janas et al., 2016), and self-protection following neurotrauma (Jiang et al., 2020).

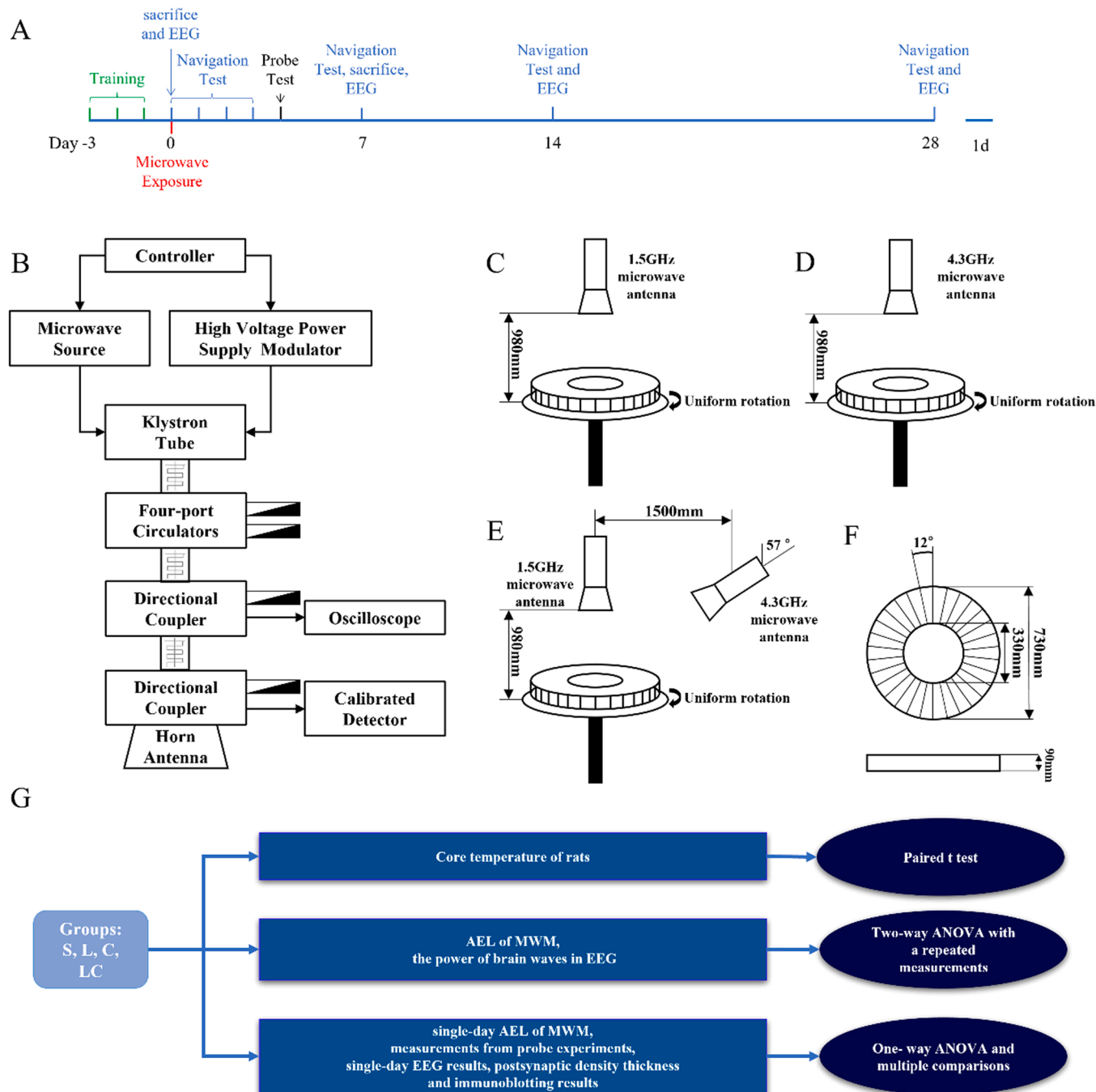


Fig. 1. Schematic diagram of the microwave radiation experimental procedure, equipment and analyses performed. (A) Schematic diagram of the whole experiment. (B) Schematic diagram of the microwave radiation source. (C) Schematic diagram of the L-band microwave radiation process. (D) Schematic diagram of the C-band microwave radiation process. (E) Schematic diagram of the simultaneous L- and C-band microwave radiation process. (F) Schematic diagram of the rat container. (G) Schematic diagram of the analyses performed.

Additionally, researchers are actively exploring applications of these exosomes for therapeutic purposes (Lai and Breakefield, 2012). Exosomes can easily penetrate the blood–brain barrier and enter the peripheral blood and thus serve as useful biological targets (Loov et al., 2016; Wood, 2015). Given the rapid advances in proteomics technology, both qualitative and quantitative characterization of exosomal proteins is possible (Tate et al., 2013).

The aims of this study were to evaluate changes in cognitive function in rats after simultaneous exposure to two microwave frequencies and to identify possible biomarkers of microwave exposure among serum exosomes by proteomics to better understand the biological effects of this exposure and its underlying mechanism.

2. Materials and methods

2.1. Animals and microwave exposure

A total of 120 male Wistar rats (200 ± 20 g, 6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained in a specific pathogen-free (SPF) animal facility. All rats were maintained at 22 ± 2 °C and 55 ± 10 % humidity on a 12 h light-dark cycle (lights on at 8 a.m.). Food and distilled water were freely available. All experiments were performed between 8 a.m. and 6 p.m. Throughout the experiment, 2–3 rats were kept in each cage. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC-AMMS-2020–780).

To exclude the possible influence of body weight, the rats were categorized according to their weight by stratified sampling before microwave exposure and placed into the following groups: the sham group (S group); the 1.5 GHz microwave exposure group subjected to microwave exposure at an average power density of 10 mW/cm^2 for 6 min (L group); the 4.3 GHz microwave exposure group subjected to microwave exposure at an average power density of 10 mW/cm^2 for 6 min (C group); and the 1.5 GHz and 4.3 GHz microwave exposure group subjected to simultaneous microwave exposure at 10 mW/cm^2 for 6 min (LC group).

The sham group was processed in parallel with the exposure groups but did not receive microwave radiation. A flow chart describing the whole experiment is shown in Fig. 1A; the establishment of the microwave exposure system, dosimetry and animal container are presented in detail in Fig. 1B–F.

The special absorption rate (SAR) of each rat was calculated under plane wave exposure, which was performed with the finite difference time domain (FDTD) method and the formula $\text{SAR} = \sigma E^2 / \rho$ (W/kg). In the formula, E represents the electric field strength (V/m), σ represents the electrical conductivity (S/m), and ρ represents the density of the rat (kg/m^3) (Zhu et al., 2021). For a typical 230-g rat, the SARs were as follows: 3.3 W/kg, 2.5 W/kg, and 5.8 W/kg in the L, C, and LC groups, respectively.

2.2. Body temperature monitoring

Four rats from each group were randomly selected and their temperature was measured with a portable thermometer (JM222, Beijing ZhongWang Electronic Technology Co., Ltd. Beijing, China) before and immediately after microwave exposure to investigate changes in core temperature due to exposure.

2.3. Electroencephalogram (EEG) recording

An MP-150 multichannel physiological recording and analysis system (BIOPAC Company, USA) was used to record rat EEGs at 6 h, 7 d, 14 d, and 28 d after microwave exposure.

Twenty rats ($n = 5$ per group) were injected with 1 % pentobarbital sodium (0.5 g/100 g body weight, intraperitoneal). Under this mild anaesthesia, the fur on the top of the rat's head was removed, and the

scalp was disinfected with 75 % alcohol. The recording electrodes were fixed on the scalp at the top of the head on both sides of the centreline, and the reference electrodes were placed on the right ear lobe. All electrodes were connected to the amplifier, and signals were recorded for 3 min in a quiet state. The rats underwent EEG recording in random order.

2.4. Morris Water Maze test

The MWM test is a behavioural experiment widely used to examine spatial learning and memory in rodents (Vorhees and Williams, 2006). A circular pool (150 cm in diameter) filled with water at 23 ± 2 °C was divided into four equal quadrants. The platform (12 cm in diameter and 15 cm in height) was submerged 1 cm below the water surface in the target quadrant. The swimming trials of all rats were digitally recorded by the Anymaze system (Stoelting Co., Illinois, USA). Before microwave exposure, forty rats ($n = 10$ per group) were trained to locate the submerged platform over three consecutive days, in trials with a maximum duration of 60 s. If the rat found the platform within 60 s, it was allowed to stay on the platform for 15 s; if it did not, it was placed on the platform for 15 s facilitate learning of the platform location. After microwave exposure, the navigation tests were performed at 6 h, 1 d, 2 d, 3 d, 7 d, 14 d and 28 d. The movement path, duration in each quadrant, swimming speed and other parameters were recorded by the recording software. The average escape latency (AEL), defined as the average time that the rat took to reach the escape platform over four trials, was used to evaluate spatial learning and memory. Probe trials (in which the platform was removed) were conducted 4 d after microwave exposure, and the number of crossings of the previous platform location was recorded (Fig. 2A).

2.5. Observation of the hippocampal ultrastructure

The sampling time was determined according to the literature (Wang et al., 2017a). Hippocampal samples (1 mm³ in size) were dissected from the dentate gyrus (DG) area of the four groups at 7 d after microwave exposure. After being fixed in 2.5 % glutaraldehyde (Merck, Darmstadt, Germany), the specimens were sequentially processed with 1 % osmium tetroxide (AppliChem, Gatersleben, Germany) graded ethyl alcohol and embedded in EPON618 (TAAB Laboratories Equipment, Berks, UK). The thin sections were stained with uranyl acetate and lead citrate (Advanced Technology & Industrial Co. Ltd., Hong Kong, China) on copper meshes for contrast. After drying, the grids were viewed by transmission electron microscopy (TEM) (H-7650, Tokyo, Japan).

The thickness of the postsynaptic density (PSD) was measured by the reconstruct software ([Version 1.1.0.0, 2007], available from <https://synapseweb.clm.utexas.edu/software-0>) (FIALA, 2005). In brief, after correction to the scale ruler, the thickness of the PSD of all synapses was measured.

2.6. Exosome isolation

At 6 h after microwave exposure, serum from venous blood was collected and placed at -80 °C ($n = 4$ rats per group). Then, exosomes were extracted by gradient centrifugation with the following steps: (1) the serum was thawed in a water bath at 25 °C and placed on ice; (2) the serum was centrifuged at 2,000 g at 4 °C for 10 min, and the supernatant was aspirated; (3) the serum was centrifuged at 10,000 g at 4 °C for 30 min, and the supernatant was retained; (4) the serum was centrifuged at 110,000 g at 4 °C for 75 min, and the supernatant was discarded; (5) the pellet was resuspended with 1 mL of phosphate buffer solution (PBS), diluted with PBS after resuspension, and filtered with a 0.22- μm membrane; (6) the sample was centrifuged at 110,000 g at 4 °C for 75 min, and the supernatant was discarded; and (7) the pellet was resuspended in PBS, and the sample was stored at -80 °C.

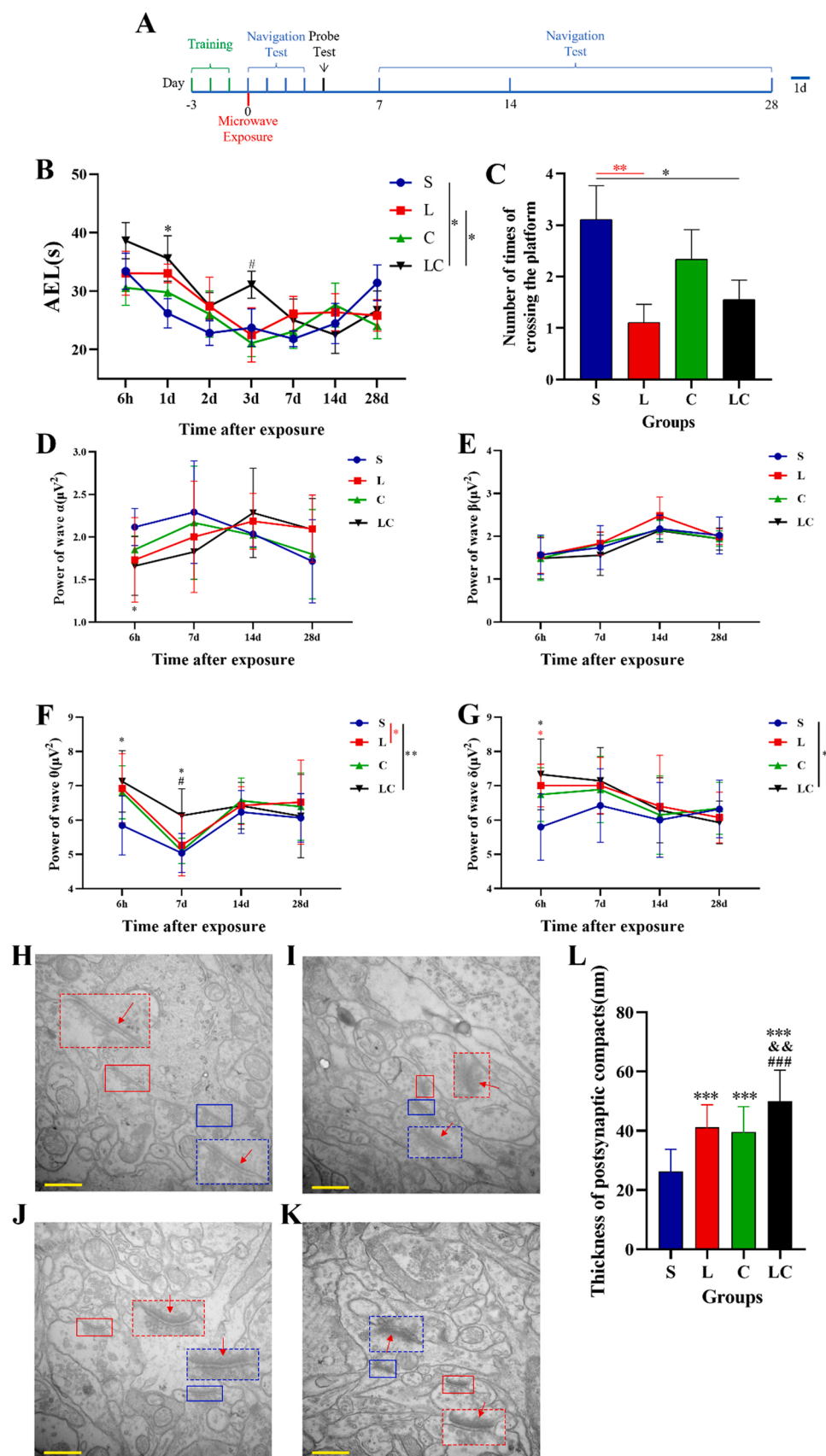


Fig. 2. (A) Morris water maze experimental procedure; (B) learning curves obtained from different groups of rats; and (C) number of platform crossings of different groups in the probe test. (D) α wave power at different time points after microwave exposure; (E) β wave power at different time points after microwave exposure; (F) θ wave power at different time points after microwave exposure; and (G) δ wave power at different time points after microwave exposure. Compared with the S group: * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$. Compared with the L group: && indicates $p < 0.01$. Compared with the C group: # indicates $p < 0.05$, and ### indicates $p < 0.001$. (H-K) Ultrastructure of the hippocampus of different groups at 7 d after microwave exposure (TEM with $40,000 \times$ magnification). (H) S group; (I) L group; (J) C group; (K) LC group, (L) quantitative analysis of the thickness of the PSD. Scale bar for H-K, 500 nm. The dashed box in each figure presents a $2 \times$ magnified image of the solid box in the same colour in the original picture; red arrows indicate the PSD.

2.7. Exosome identification

Fractions of isolated exosomes from each group were used for protein measurements, Western blotting, nanoparticle tracking analysis (NTA), morphology analysis by TEM and proteomic analysis.

Proteins in the isolated exosome fractions were extracted by radio-immunoprecipitation assay (RIPA) lysis buffer, and the concentrations were measured by a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, USA) according to the manufacturer's instructions. Isolated exosomes were tested for the positive exosomal markers CD9, CD63 and TSG 101 (Abcam, UK) and the negative marker calnexin (Abcam, UK) by Western blotting.

All samples were thawed in 25 °C water and diluted with PBS. NTA was performed using a nanoparticle tracking analyser (Particle Metrix, Germany) and ZetaView 8.04.02 software (Particle Metrix, Germany).

For each of the four groups, five microlitres of exosome samples were taken and dropped onto a copper net. After the samples on the copper net incubated for 5 min at room temperature, excess liquid was absorbed with absorbent paper. Then, 2 % uranyl acetate was added to the copper net and incubated for 1 min. Excess liquid was absorbed. The samples were observed by TEM (Tecnai G2 Spirit BioTwin, FEI, USA).

2.8. Label-free proteomics

1) Pretreatment of exosome samples

An equal volume of protein lysate (7 M urea, 2 % sodium dodecyl-sulfate [SDS] and 1 × protein inhibitor cocktail) as the sample was added to each sample. All samples were treated with an ultrasonic cell crusher while on ice. Then, we centrifuged the pyrolysis products at 13,000 rpm for 20 min and transferred the supernatant to a new Eppendorf (EP) tube. Six volumes of 100 % acetone were added and the samples were incubated at 20 °C overnight. The precipitate was obtained by centrifugation. Then, 500 µL of precooled washing solution (ethanol:acetone:acetic acid = 50:50:0.1) was added to the precipitate and the solution was centrifuged at 13,000 rpm for 15 min. To this solution 6 M guanidine hydrochloride and 300 mM triethylammonium bicarbonate (TEAB) were added to redissolve and precipitate.

2) Filter-aided proteome preparation

A total of 37.7 µg of protein solution was obtained from each sample, and the volume was adjusted to 100 µL by adding a 25 mM ammonium bicarbonate solution. Two microlitres of dithiothreitol (DTT; 1 M) was added, and the solution was mixed and incubated at 57 °C for 1 h. Next, ten microlitres of an iodoacetamide (1 M) solution was added, and the solution was placed at room temperature in the dark. Then, the reduced alkylated protein was added to a 10-K ultrafiltration tube and centrifuged at 12,000 rpm, and the bottom liquid was collected. Ammonium bicarbonate was used as dissolution buffer and added to the ultrafiltration tube for washing. Trypsin prepared with buffer was added for enzymatic hydrolysis at 37 °C overnight. The next day, the peptides were collected and dried by centrifugation.

3) Desalination

The dried polypeptides were concentrated by centrifugation, desalinated by a monospin desalting column, and dried for mass spectrometry. The detailed methods are as follows. A 0.1 % trifluoroacetic acid (TFA) solution was used to dissolve the mixed peptide. Then, 100 % acetonitrile and 0.1 % TFA were separately used to activate and balance the desalting column, and the redissolved samples were added to the desalting column and centrifuged. The 0.1 % TFA solution was added to clean the desalting column, and a 50 % acetonitrile solution was added for the next centrifuge step. The eluted solution was collected in new EP tubes and then centrifuged to remove acetonitrile.

4) Liquid chromatography–mass spectrometry (LC–MS) analysis

Formic acid (FA; 0.1 %) was added to the dried samples for subsequent LC–MS analysis. An EASY-nLC 1200 HPLC (Thermo Scientific, USA) was used for separation by the analytical column (C18, 1.9 µm, 75 µm × 20 cm, 200 nL/min). The mass spectrometer used was an Orbitrap Fusion Lumos (Thermo Scientific, USA). Tandem mass spectrometry was used in the mode of data-dependent acquisition (DDA). The full scan resolution was 60,000 (full-width at half-maximum; FWHM), the mass charge ratio range was set to m/z 375–1600, and the collision energy was set to 30 % in the higher energy collisional dissociation (HCD) fragmentation mode.

The mass spectrometry data analysis software used was Maxquant 1.5.8.3 (Max Planck Institute for Biochemistry, Germany). The parameters are shown in Table 1.

2.9. Differentially expressed proteins

The differentially expressed proteins between the S group samples and the L, C and LC group samples were identified. According to the chi-square test and one-way analysis of variance (ANOVA), the proteins that differed in expression between the groups were screened, and those with significant differences were considered differentially expressed (ratio ≥ 1.5 or ≤ 0.667 , $p < 0.05$). According to the results of the Gene Ontology (GO) analysis, including biological processes (BP), cellular components (CC), and molecular functions (MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, we selected exosomes with functions related to neuron structure, neuron function, synaptic plasticity, learning and memory.

2.10. Identification of differentially expressed proteins by Western blotting

The remaining protein lysates were used in the Western blot analysis. The primary antibodies used were as follows: GAPDH (ab181602, Abcam), VAMP8 (ab76021, Abcam), Syn7 (ab224223, Abcam), and VMAT2 (ab19121, Abcam).

2.11. Statistical analysis

Data are shown as the mean $\pm$ standard deviation. SPSS 25 was used to analyse all experimental data.

Paired *t* tests were used to analyse the effect of exposure to microwave radiation on the core temperature of rats.

Repeated measures analysis (specifically, two-way ANOVAs and multiple comparisons) were used to evaluate the MWM test and EEG data.

To analyse single-day AEL of MWM, measurements from probe experiments, single-day EEG results, thickness of the PSD, and immunoblotting results, a one-way ANOVA and multiple comparisons were used.

Table 1
Parameters for mass spectrometry.

Item	Parameter
Database	UniProt
Taxonomy	Rattus norvegicus
Enzyme	Trypsin
Fixed modifications	Carbamidomethyl (C)
Variable modifications	Oxidation (M), acetyl (Protein N-term)
Maximum missed cleavages	2
Peptide charge state	2 + , 3 + , 4 +
Peptide spectrum match (PSM) false discovery rate (FDR)	0.01
Protein FDR	0.05
Site FDR	0.01
Minimum peptide length	7
MS/MS tolerance (FTMS)	20 ppm
Decoy mode	Revert

to analyse differences among groups. The significance threshold was set at $p < 0.05$. The above description is summarized in Fig. 1G.

3. Results

3.1. Core temperature did not increase after microwave exposure

There were no significant differences in rat rectal temperature ($p = 0.573$, $p = 0.914$, $p = 1.000$, $p = 0.833$) before and after microwave exposure among the four groups, implying that microwave radiation predominantly exerted nonthermal effects (Supplementary Table 1).

3.2. Spatial learning and memory were impaired after microwave exposure

Repeated-measures analysis and the learning curves obtained from different groups of rats indicated that the performance of the LC group on the MWM was obviously different from that of the S group ($p = 0.029$) and C group ($p = 0.038$). Meanwhile, there was no significant difference in swimming speed between the groups of rats, indicating no difference in their locomotor ability (Fig. S1). These results implies that microwave radiation impairs spatial learning and memory in rats (Fig. 2B).

Moreover, the multiple comparisons analysis indicated a significant difference between the S group and the LC group at 1 d after microwave exposure ($p = 0.029$, Fig. 2B). The AEL of the rats in the LC group was significantly longer than that in the C group at 3 d after microwave exposure ($p = 0.038$, Fig. 2B). In the probe test, the number of times the rats crossed the platform in the L and LC groups was significantly lower than that in the S group ($p = 0.009$, $p = 0.038$, Fig. 2C).

The above results indicate that simultaneous exposure to 1.5 GHz and 4.3 GHz microwave radiation and single exposure to 1.5 GHz microwave radiation impaired spatial learning and memory. In addition, the simultaneous microwave exposure caused more severe impairments than exposure to a single microwave frequency.

3.3. EEG changes caused by microwave exposure

The repeated-measures analysis did not detect significant differences among the groups in terms of changes in the power of α and β waves from 6 h to 28 d after microwave exposure (Fig. 2D-E). The power of the θ wave in the L and LC groups and the power of the δ wave in the LC group were significantly higher than those observed in the S group ($p = 0.034$, $p = 0.007$, Fig. 2F; $p = 0.049$, Fig. 2G). Moreover, multiple comparisons at each time point revealed that the power of the α wave in the LC group was significantly lower than that in the S group at 6 h after microwave exposure ($p = 0.044$, Fig. 2D). The power of the θ wave in the LC group was significantly higher than that in the S group at 6 h ($p = 0.036$) and 7 d after microwave exposure ($p = 0.023$) (Fig. 2F). In addition, the power of the θ wave in the LC group was significantly higher at 7 d after exposure than that observed in the C group ($p = 0.03$, Fig. 2F). We also found that the power of the δ wave in the L ($p = 0.042$) and LC groups ($p = 0.013$) was significantly higher than that of the S group at 6 h after exposure (Fig. 2G).

The above results indicate that cortical EEG signals exhibited decreased excitability after exposure, especially for rats subjected to simultaneous exposure to 1.5 and 4.3 GHz microwave frequencies.

3.4. Microwave exposure damaged the hippocampal ultrastructure

Examinations of the hippocampal ultrastructure mainly focused on synaptic changes, as these are important for synaptic plasticity as well as learning and memory. The synaptic clefts in the S group were clear, and abundant synaptic vesicles were present (Fig. 2H). However, the synaptic structure was significantly altered in the exposure groups. An

increase in the thickness of the PSD and a blurring of synaptic clefts were observed in the L, C and LC groups (Fig. 2I-K). Quantitative analysis showed that the thickness of the PSD in all exposure groups was significantly greater than that observed in the S group ($p = 0.000$, $p = 0.000$, and $p = 0.000$, respectively; Fig. 2L). In addition, the PSD in the LC group was significantly thicker than that observed in the L group ($p = 0.004$) and the C group ($p = 0.000$, Fig. 2L).

Overall, we found that microwave radiation damaged the synaptic structure of neurons, and that the LC group showed the most severe damage.

3.5. Serum exosomes were successfully isolated and identified

Serum exosomes from the four groups were separated and Western blotting was used to detect the presence of exosome markers (positive markers: CD9, CD63 and TSG101; negative marker: calnexin; Fig. 3A). After fixation, the exosomes were observed to be cup-shaped by TEM (Fig. 3B-E).

The NTA showed that the diameters of the exosomes of all groups were similar (mainly concentrated at approximately 100 nm, Fig. 3F-I). In addition, we compared the number of exosome particles by NTA, finding that simultaneous 1.5 and 4.3 GHz microwave irradiation significantly increased the number of exosomes released ($p = 0.006$, Fig. 3J). The numbers of exosomes in the L ($p = 0.03$) and C groups ($p = 0.024$) were significantly lower than those observed in the LC group (Fig. 3J).

3.6. Bioinformatics analysis showed that microwave irradiation caused differential expression of serum exosome proteins

The number of differentially expressed proteins among the groups was determined with the chi-square test (Fig. 4A).

The GO functional analysis (Fig. 4B-D) included three categories: BPs, MFs, and CCs. The differentially expressed proteins were involved in BPs including negative regulation of endopeptidase activity, proteolysis, drug response, inflammatory response, and ageing. The differentially expressed proteins were involved in MFs including protein binding, calcium ion binding, protein heterodimerization, ATP binding and DNA binding. The differentially expressed proteins were involved in CCs mainly including extracellular exosomes, extracellular space, cytoplasm, nucleus and plasma membrane.

The KEGG pathways in which the differentially expressed proteins were involved are shown in Fig. 4E. As our focus was on impairments in learning and memory, we mainly investigated the signalling pathways closely related to cognitive function, including synaptic vesicle circulation and SNAP receptor (SNARE) interactions in vesicle transport.

After considering our previous research on microwave-induced damage to hippocampal function along with the results of GO analysis and KEGG pathway analysis, three differentially expressed proteins closely associated with functional deficits were selected for validation in this study. Functional annotations of these three proteins are provided in Table 2.

3.7. Validation of several differentially expressed proteins

The Western blotting verified that the expression levels of VAMP8 in the LC group were significantly lower than those in the S group ($p = 0.009$, Fig. 5A-B) and the L group ($p = 0.017$, Fig. 5A-B). The expression levels of Syn7 in the LC group were significantly lower than those in the L, C, and LC groups ($p = 0.001$, $p = 0.003$, and $p = 0.04$, respectively; Figs. 5A and 5C), and the expression levels of VMAT in the LC group were significantly lower than those in the S group ($p = 0.039$, Fig. 5A and 5D). The Western blot analysis indicated that the relative protein levels of VAMP8, Syn7 and VMAT exhibited the same downwards trends as those observed in the proteomics results, verifying the proteomic results. Thus, VAMP8, Syn7 and VMAT might be serum

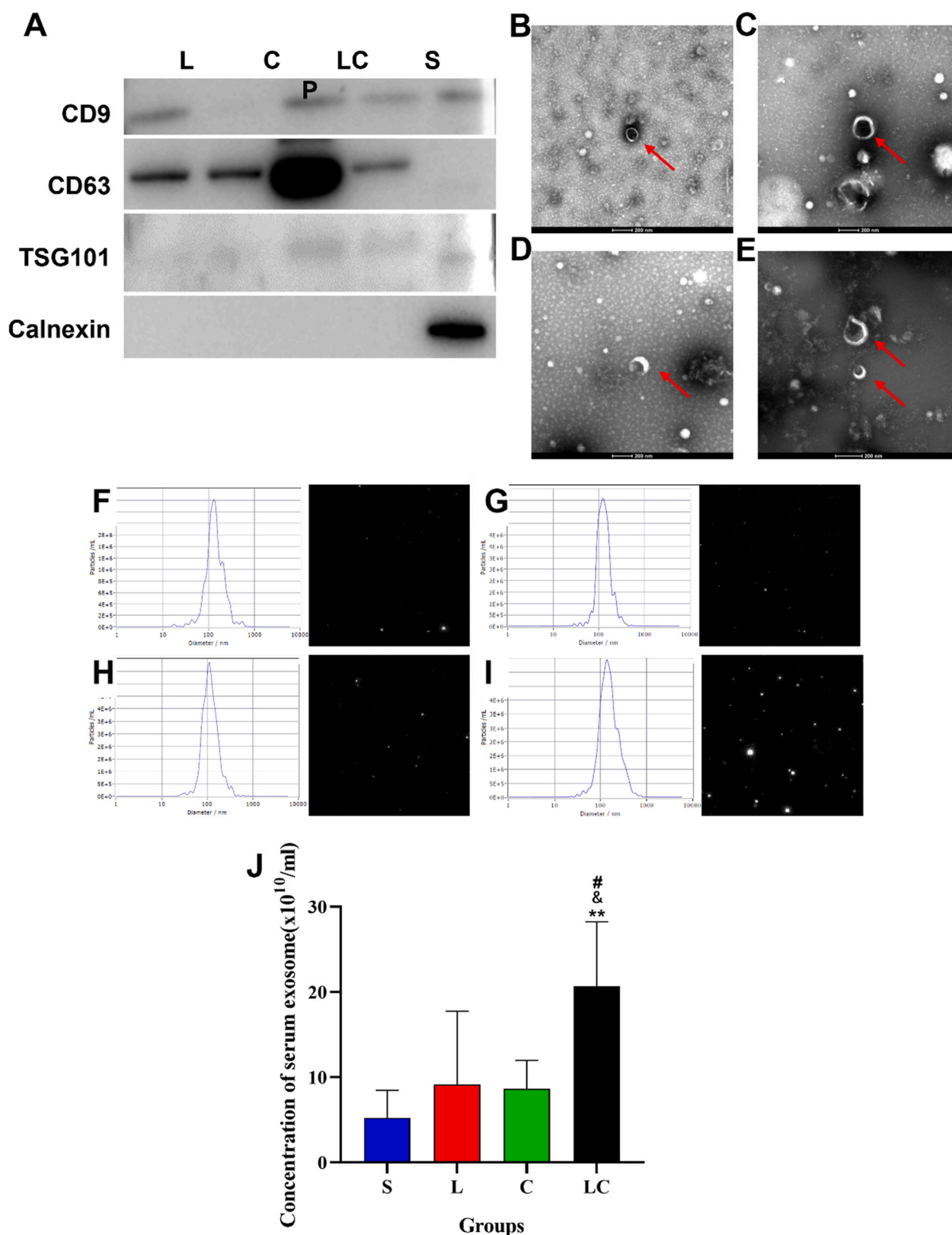


Fig. 3. Isolated and identified serum exosomes. (A) Western blotting results of different markers (positive markers: CD9, CD63, and TSG101; negative marker: calnexin.); (B) TEM image of the S group after microwave exposure at 1,500,000 × magnification; (C) TEM image of the L group after microwave exposure at 1,500,000 × magnification; (D) TEM image of the C group after microwave exposure at 1,500,000 × magnification; (E) TEM image of the LC group after microwave exposure at 1500,000 × magnification; (F) diameter of exosomes and sample graph of the S group; (G) diameter of exosomes and sample graph of the L group; (H) diameter of exosomes and sample graph of the C group; (I) diameter of exosomes and sample graph of the LC group; and (J) exosome concentration in rat serum 6 h after microwave exposure. Scale bar, 200 nm for B-E; red arrows point to exosomes. ** indicates $p < 0.01$ compared with S group; & indicates $p < 0.05$ compared with L group; # indicates $p < 0.05$ compared with C group.

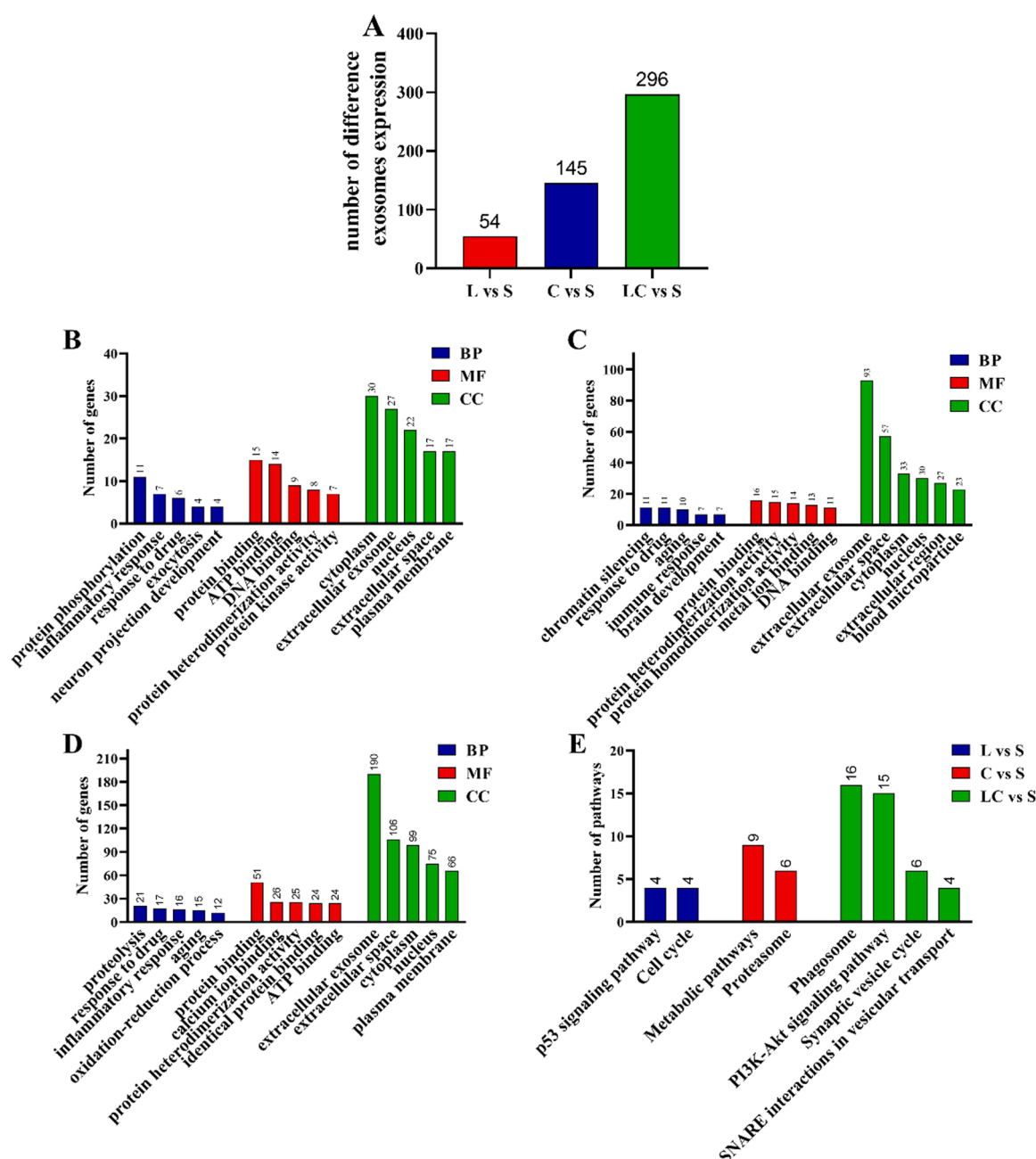


Fig. 4. Results of the GO and KEGG pathway analysis exploring the associations of differentially expressed proteins among the groups. (A) Number of differentially expressed proteins among groups after microwave exposure; (B) GO analysis of differentially expressed proteins between the L and S groups; (C) GO analysis of differentially expressed proteins between the C and S groups; (D) GO analysis of differentially expressed proteins between the LC and S groups; (E) KEGG pathways associated with differentially expressed proteins among the groups.

molecules sensitive to microwave exposure.

4. Discussion

Numerous studies have shown that microwave irradiation induces varying degrees of impairment in the CNS and functional deficits in other systems (Altun et al., 2017; Kamali et al., 2018; Narayanan et al., 2019; Othman et al., 2017). Moreover, many studies have shown the degree of damage might depend on the microwave frequency that rats are exposed to (Wang et al., 2017b; Zhang et al., 2017; Zhu et al., 2021), but few reports have examined the effects of simultaneous exposure to multiple microwave frequencies. In this study, we focused on cognitive impairments induced by simultaneous exposure to 1.5 GHz and 4.3 GHz

microwave radiation, frequencies that are commonly used in the communication field; with the aim of identifying potential serum biomarkers for microwave exposure.

In our study, exposure to a single microwave frequency (1.5 GHz) and simultaneous exposure to multiple microwave frequencies (1.5 and 4.3 GHz) prolonged the AEL and decreased the number of crossings of the target platform, indicating that both kinds of exposure impaired learning and memory. In addition, simultaneous exposure resulted in more severe impairments than single-frequency microwave exposure. Thus, two microwave frequencies can exert synergistic effects. The results of the EEG, a commonly used method for assessing the physiological state of the cerebral cortex (Fell et al., 2011; Vakalopoulos, 2014), were consistent with those on the MWM. The α and β waves

Table 2
Differentially expressed proteins and their functional annotations.

Gene name	Protein name	Function	L/S ratio	C/S ratio	LC/S ratio
VAMP8	Vesicle-associated membrane protein 8 (VAMP8)	Protein transport, protein complex assembly, vesicle-mediated transport, vesicle fusion, regulation of protein localization to plasma membrane, exocytosis, regulation of endocytosis, SNARE complex assembly, etc.	1.02	0.21	0.00
STX7	Syntaxin-7 (Syn7)	Membrane fusion, intracellular protein transport, vesicle-mediated transport, vesicle fusion, regulation of protein localization, vesicle docking, synaptic vesicle exocytosis, positive regulation of receptor localization to synapse, etc.	0.65	0.82	0.13
SLC18A2	Synaptic vesicular amine transporter (VMAT)	Endocytic recycling, response to zinc ion, chemical synaptic transmission, aminergic neurotransmitter loading into synaptic vesicle, serotonin transport, neurotransmitter transport, negative regulation of neurotransmitter transport, etc.	0.92	0.42	0.00

mainly appear during states of higher cortical excitability, whereas increases in the power of θ and δ waves indicate that cortical excitability is suppressed. According to our results, exposure to a single microwave frequency (1.5 GHz) and simultaneous exposure to multiple microwave frequencies (1.5 and 4.3 GHz) decreased in electrical excitability. This result is consistent with the previously observed tendency of slow-wave specific gravity or power in rat EEGs to increase after long-term exposure to 2.856 GHz microwaves at different powers (5/10/20/30 mW/cm² applied for 6 min/d, 3 d/w, over 6 w; 10 mW/cm² applied for 6 min/d, 5 d/w, over 6 w) (Li et al., 2015; Wang et al., 2017a).

According to the MWM and EEG data, microwave exposure impaired spatial learning and memory and decreased brain electrical activity, respectively. We also aimed to identify the underlying mechanism of these changes. Many studies have described the relationship between learning and memory and the hippocampus (Gulyaeva, 2018; Hughes and Herron, 2019; Xia et al., 2021). Studies of the biological effects of microwave radiation also revealed hippocampal injuries (Altun et al., 2017; Hussein et al., 2016; Narayanan et al., 2015). We found that damage to rat brain tissue often appeared 7 d after short-term exposure; therefore, we selected this time point for tissue observation (Zhu et al., 2021). Synapses are essential for synaptic plasticity and memory (Luchkina and Bolshakov, 2019; Martin et al., 2000; Neves et al., 2008). However, few quantitative studies have examined changes in synaptic structure. In this study, we found that the thickness of the PSD was significantly increased in the three exposure groups, indicating that microwave exposure affected the synapse structure and induced abnormal recruitment of postsynaptic proteins. When combined with the results of our previous research on long-term potentiation, this change in the PSD might be due to the compensatory mechanism caused

by the decreased synaptic plasticity (Tan et al., 2017; Wang et al., 2017a; Zhu et al., 2021).

In the nervous system, most cells (such as neural stem/progenitor cells, neurons, astrocytes, and microglia) are believed to release exosomes. Several studies have implicated exosomes in neuronal development, synaptic activity and nerve regeneration (Adolf et al., 2019; Huang et al., 2018; Wang et al., 2011). As exosomes are capable of crossing the blood-brain barrier and transferring proteins and genetic information, researchers have studied their potential role as biological markers in nervous system diseases (Goetzl et al., 2020; Hamlett et al., 2018; Mustapic et al., 2017). Exosomes, transmitters of information between cells, might cause a wide range of physiological reactions. In addition, serum exosomes are easy to sample, and differences in serum exosomes among groups could serve as markers for assessing microwave-induced damage.

Therefore, combined with previous findings concerning the biological function of exosomes, we identified three representative differentially expressed proteins in serum exosomes: VAMP8, Syn7 and VMAT. VAMP8 has roles in protein transport, protein complex assembly, vesicle-mediated transport, vesicle fusion, regulation of protein localization to the plasma membrane, exocytosis, regulation of endocytosis, and SNARE complex assembly. VAMP8 has been reported to increase the levels of tau and α -synuclein clearance to prevent intracellular accumulation in Alzheimer's disease (Pilliod et al., 2020). In addition, VAMP8 phosphorylation reduces levels of vesicle fusion in vitro and suppresses secretion in living cells, allowing vesicles to dock but preventing their fusion with the plasma membrane (Malmersjo et al., 2016). The above results indicate that VAMP8 plays an important role in neuronal function and vesicle release. In our study, VAMP8 levels in serum exosomes decreased after microwave exposure, especially in the group subjected to simultaneous exposure, indicating a decrease in vesicular membrane exposure, which might induce abnormalities in synaptic vesicle release.

Syn7 and VAMP8 are present in the neuronal endosomal SNARE complex, and it is currently believed that one member of each SNARE motif group is involved in the formation of a single SNARE complex (Besteiro et al., 2006). Syn7 is mainly localized in the vacuolar early endosomes (EEs) and may be involved in protein trafficking from the plasma membrane to the EE as well as in the homotypic fusion of endocytic organelles (Prekeris et al., 1999). In this study, we also found decreased levels of Syn7 in the group subjected to simultaneous exposure, consistent with the results of label-free proteomics, indicating that Syn7 might serve as a marker of damage caused by simultaneous microwave exposure.

Vesicular transport proteins package classic neurotransmitters for regulated exocytotic release and are localized to at least two distinct types of secretory vesicles. The vesicular acetylcholine transporter (VACHT) preferentially localizes with synaptic-like microvesicles (SLMVs), whereas the closely related vesicular monoamine transporters (VMATs) preferentially localize with large dense core vesicles (LDCVs) (Krantz et al., 2000). In our study, VMATs were found to exhibit decreased expression levels in the group subjected to simultaneous exposure, which indicates disorders of LDCVs. A combined assessment of the three differentially expressed proteins in serum exosomes indicated that Syn7, VAMP8 and VMAT levels decreased in the group subjected to simultaneous exposure. In addition, the three proteins all play roles in vesicle-mediated transport, vesicle fusion and chemical synaptic transmission, indicating that microwave exposure affected synaptic vesicle fusion and transmission. In the future, further attention should be devoted to synaptic vesicles in studies of microwave-induced injuries.

In this experiment, our initial aim was to identify exosomes that could serve as early indicators of microwave irradiation and to establish a link between acute changes in exosome levels and changes in learning and memory as well as brain structure. Moreover, we would analyze the levels of exosomes in the serum of rats at different times after microwave exposure in the further experiment.

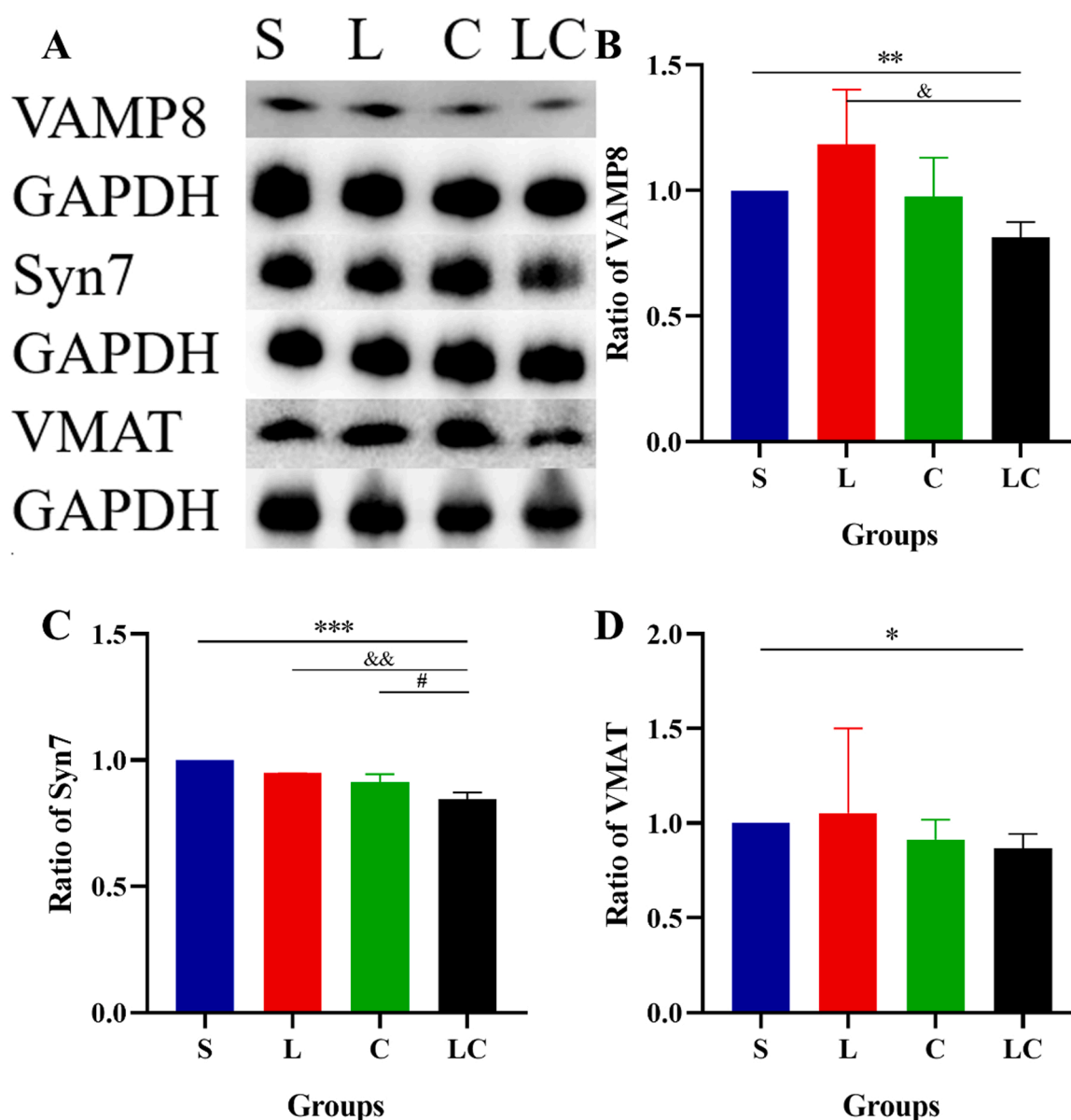


Fig. 5. Western blot verification of levels of serum exosome proteins. (A) Typical bands; (B) VAMP8; (C) Syn7; and (D) VMAT. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ compared with the S group; & indicates $p < 0.05$ and && indicates $p < 0.001$ compared with the L group; # indicates $p < 0.05$ compared with the C group.

5. Conclusion

In summary, exposure to a single microwave frequency (1.5 GHz, 10 mW/cm² for 6 min) and multiple microwave frequencies (1.5 GHz and 4.3 GHz, 10 mW/cm² for 6 min) impaired spatial learning and memory, induced EEG abnormalities, and damaged the hippocampal ultrastructure in rats. Simultaneous exposure to multiple microwave frequencies induced more severe impairments than exposure to a single microwave frequency. VAMP8, Syn7, and VMAT might be key molecules underlying the cognitive impairments caused by exposure to simultaneous microwave radiation.

CRediT authorship contribution statement

Hui Wang: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Yu Liu:** Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Yunbo Sun:** Conceptualization, Methodology. **Li Zhao:** Investigation,

Resources. **Ji Dong:** Investigation, Resources. **Xinping Xu:** Investigation, Resources. **Haoyu Wang:** Investigation, Resources. **Jing Zhang:** Investigation, Resources. **Binwei Yao:** Investigation, Resources. **Xue-long Zhao:** Methodology, Resources. **Shuchen Liu:** Supervision. **Ke Zhang:** Writing – review & editing. **Ruiyun Peng:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2022.113983](https://doi.org/10.1016/j.ecoenv.2022.113983).

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