



iTRAQ quantitatively proteomic analysis of the hippocampus in a rat model of accumulative microwave-induced cognitive impairment

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Abstract

Central nervous system is sensitive and vulnerable to microwave radiation. Numerous studies have reported that microwave could damage cognitive functions, such as impairment of learning and memory ability. However, the biological effects and mechanisms of accumulative microwave radiation on cognitive functions were remained unexplored. In this study, we analyzed differential expressed proteins in rat models of microwave-induced cognitive impairment by iTRAQ high-resolution proteomic method. Rats were exposed to 2.856 GHz microwave (S band), followed by 1.5 GHz microwave exposure (L band) both at an average power density of 10 mW/cm² (SL10 group). Sham-exposed (control group), 2.856 GHz microwave-exposed (S10 group), or 1.5 GHz microwave-exposed (L10 group) rats were used as controls. Hippocampus was isolated, and total proteins were extracted at 7 days after exposure, for screening differential expressed proteins. We found that accumulative microwave exposure induced 391 differential expressed proteins, including 9 downregulated and 382 upregulated proteins. The results of GO analysis suggested that the biological processes of these proteins were related to the adhesion, translation, brain development, learning and memory, neurogenesis, and so on. The cellular components mainly focused on the extracellular exosome, membrane, and mitochondria. The molecular function contained the protein complex binding, protein binding, and ubiquitin-protein transferase activity. And, the KEGG pathways mainly included the synaptic vesicle cycle, long-term potentiation, long-term depression, glutamatergic synapse, and calcium signaling pathways. Importantly, accumulative exposure (SL10 group) caused more differential expressed proteins than single exposure (S10 group or L10 group). In conclusion, 10 mW/cm² S or L band microwave induced numerous differential expressed proteins in the hippocampus, while accumulative exposure evoked strongest responses. These proteins were closely associated with cognitive functions and were sensitive to microwave.

Keywords iTRAQ · Proteomic analysis · Hippocampus · Rat · Accumulative microwave exposure

Introduction

Recently, concerns about microwave-induced potential health hazards have been growing. The nervous system, especially

hippocampus, is sensitive and vulnerable to microwave (Qiao et al. 2014). Studies from our group and others have showed that S band microwave (frequency of 2.856 GHz) could induce structural and functional injuries of hippocampus, which resulted in impaired ability of learning and memory, and abnormal brain electric activities. Moreover, we previously found that the 10 mW/cm² microwave exposure at the frequency of 2.856 GHz could cause significant hippocampal injuries, obvious deficits in spatial learning and memory, as well as impairment of long-term potentiation (LTP) (Wang et al. 2013). Declining cognitive functions also could be detected after exposure to 900, 1800, and 2450 MHz microwave, accompanying with increased HSP70 and cytokine expression, and DNA damage in brain (Deshmukh et al. 2016; Deshmukh et al. 2015; Megha et al. 2012; Megha et al. 2015). However,

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most of the researches focused on the biological effects of single frequency microwave exposure. During occupational and accidental microwave exposure, people were always affected by various frequency microwaves. Therefore, it will be significant to study the accumulative or interactive effects from different frequency microwaves.

Screening sensitive biomarkers of microwave-induced injuries has been emerged as an important task in electromagnetic biology. Single nucleotide polymorphism (SNP) sites, microRNAs, *N*-methyl-D-aspartate (NMDAR) pathway, and GABA receptor are potential markers in microwave-induced injuries and have been attracting much more attention (Zhao et al. 2014; Mausset-Bonnefont et al. 2004; Wang et al. 2016; Xiong et al. 2015; Xu et al. 2006). In rat model of long-term exposure under extremely low frequency electromagnetic fields (ELF-EMF), the differential proteomic analysis from SDS-PAGE suggested that synaptic transmission, oxidative stress, protein degradation, energy metabolism, Tau aggregation, and inflammation were involved in injury processes (Liu et al. 2015). Isobaric tags for relative and absolute quantitation (iTRAQ) is a stable and efficient tool to study quantitative changes of proteins and to screen differential expressed proteins by tandem mass spectrometry (Velez-Bermudez et al. 2016). Recent years, the iTRAQ proteomic analysis had been widely used in the biological and medical fields (Guo et al. 2015; Slomnicki et al. 2016; Sterley et al. 2016). However, application of high-throughput proteomic methods to find microwave-sensitive biomarkers still has not been reported.

In this study, we established an accumulative microwave exposure model and screened differential expressed proteins by iTRAQ-based quantitatively proteomic analysis. Bioinformatic analysis was conducted to classify the differential expressed proteins according to significant biological processes, cellular components, molecular functions, and pathways. Finally, the valuable differential expressed proteins were identified, which might be the potential biomarkers for diagnosis and assessment.

Materials and methods

Animals and microwave exposure

Forty male Wistar rats (200 ± 20 g), provided by the Laboratory Animal Center (Beijing Institute of Radiation Medicine, Beijing, China), were housed in constant temperature (22 ± 1 °C) and relative humidity (60%) under a regular dark-light schedule (lights on from 7 a.m. to 7 p.m.). All rats were divided into four groups ($n = 10$ per group): the control group (C), the 2.856 GHz microwave exposure group with the average power density of 10 mW/cm^2 (S10), the 1.5 GHz microwave exposure group with the average power density of 10 mW/cm^2 (L10), and the 2.856 GHz and 1.5 GHz

accumulative microwave exposure with the stable average power density of 10 mW/cm^2 (SL10).

Two microwave sources, at the frequency of 2.856 GHz and 1.5 GHz, respectively, were placed next to each other in an electromagnetic shield chamber. In the accumulative exposures, rats were firstly exposed under 2.856 GHz antenna (belonged to S band), then were parallel moved to the 1.5 GHz antenna (belonged to L band) by a conveyor belt. The interval time between the two exposures was very short and negligible. The specific absorption rate (SAR) values were calculated specifically in our previous paper (Wang et al. 2013). The animal groups, microwave exposure methods, and SAR values are detailed described in Table 1.

iTRAQ proteome analysis

Protein extraction

At 7 days after microwave exposure, the rats from each group ($n = 3$) were sacrificed and the hippocampus was separated immediately. All samples were stored at -80 °C for further use. The hippocampal samples were homogenized by the lysis buffer (7 M urea, 1.4 M thiourea, 4% CHAPS). The protein concentration was measured using the Bradford method (Bradford 1976). The protein concentration is shown in Table 2.

Filter-aided sample preparation

Each sample, containing $100 \mu\text{g}$ protein solution, in a centrifuge tube was added $4 \mu\text{L}$ 1 M DTT at 37 °C for 1 h and then was added $20 \mu\text{L}$ 1 M IAA at room temperature for 40 min in a dark box. The alkylated protein solution was diluted five times, transferred to the 10 K ultrafiltration tube, and centrifuged at $12,000 \times g$ for 20 min. The bottom solution in the tube was collected, added with $200 \mu\text{L}$ of UA buffer (8 M urea,

Table 1 Animal groups, microwave exposure methods, and SAR values for each group (Tan et al. 2017)

Groups	Average power density of S band microwave (mW/cm^2)	Average power density of L band microwave (mW/cm^2)	SAR values (W/kg)
C ^a	0	0	0
S10 ^a	10	0	3.3
L10 ^a	0	10	3.7
SL10 ^b	10	10	3.3 for the first 6 min + 3.7 for the last 6 min

^a Rats in the C, S10, and L10 were exposed for 6 min

^b The rats in the SL10 group were firstly exposed in the 2.856 GHz microwave for 6 min and then were immediately exposed to the 1.5 GHz microwave for 6 min

Table 2 Protein concentration of each sample ($\mu\text{g}/\mu\text{L}$)

Groups	Rep1	Rep2	Rep3
C	6.69	4.44	5.17
S10	4.16	5.29	5.98
L10	5.71	6.47	6.17
SL10	9.43	4.21	5.18

100 mM Tris-HCl), and then centrifuged at $12,000\times g$ for 20 min. This step was repeated twice. Then, 200 μL of 200 mM triethylammonium bicarbonate (TEBA) buffer was added to the filters to replace the UA buffer, $12,000\times g$ for 20 min, and washed twice. Proteins were digested overnight by using 2 μg trypsin (AB Sciex, USA) at an enzyme-to-protein ratio of 1:50 at 37 °C. The released peptides were collected by centrifugation at $12,000\times g$ for 20 min followed by two washes with 100 μL pure water.

iTRAQ labeling

The peptides were labeled with an iTRAQ-8 plex kit (AB Sciex, USA) according to the manufacturer's protocol. Samples from the C, S10, L10, and SL10 were labeled with 115, 116, 117, and 118 tags, respectively. The incubation was proceeded at room temperature for 2 h, and then, the samples were dried in a centrifugal concentrator to terminate the labeling reaction.

High pH reverse phase chromatography

Firstly, the mobile phase A (98% ddH₂O, 2% acetonitrile, pH 10) and mobile phase B (98% acetonitrile, 2% ddH₂O, pH 10) were prepared. Secondly, 40 μg BSA (Sigma-Aldrich, USA) was separated (column temperature 4 °C, detection wavelength 214 nm) to check the detection system. The labeled samples were dissolved with 40 μL mobile phase A and centrifuged at $14,000\times g$ for 20 min. Twenty microliters from each sample was mixed and then separated. The flow rate was 0.7 mL/min, and the separation gradient was as follows (Table 3). At 15 min,

Table 3 The separation gradient for high pH reverse phase chromatography

Time (min)	Ratio of mobile phase B (%)
0	5
5	8
35	18
62	32
64	95
68	95
72	5

the samples began to collect (every 1.2 min per tube). The shunt samples were mixed into ten fractions.

Nano-reversed phase liquid chromatography-Q Exactive for protein analysis

The parameters for ion source were set as follows: spray voltage 2002.7v and capillary temperature 320 °C. Parameters for full MS were containing the 70,000 FWHM resolution, 3e6 for the full-scan AGC target, 60 ms for the full-scan max.IT, and 300–1400 m/z for scan range. The parameters of dd-MS2 were as follows: 17,500 FWHM for the resolution, 5e4 for the AGC target, 80 ms for the maximum IT, 6.3e3 for the intensity threshold, HCD for the fragmentation methods, 30% for NCE, and 20 for top N. Mobile phase A contained 100% ddH₂O and 0.1% formic acid, and mobile phase B contained 100% acetonitrile and 0.1% formic acid. The fractions collected from the high pH reserved phase chromatography were centrifuged at $14,000\times g$ for 20 min. Sample volume was 2 μL of each sample, and the flow rate of loading pump was 350 nL/min. Moreover, the separation gradient was as follows (Table 4).

Bioinformatic analyses

The database used in this study was downloaded from UniProt. The mass spectrometric analysis of iTRAQ was performed by Thermo Q-Exactive mass spectrometry. The original document generated by mass spectrum was processed by the software Proteome Discoverer 1.4. The search parameters were set as follows: enzyme: trypsin; static modification: C carboxyamidomethylation (57.021 Da); dynamic modification: M oxidation (15.995 Da); N-terminal, K, iTRAQ 8 plex; FDR determination: false discovery rate (FDR) of all peptide and protein identifications < 1%; precursor ion mass tolerance ± 15 ppm; fragment ion mass tolerance ± 20 mmu; and max missed cleavages 2.

Table 4 The separation gradient for nano-reversed phase liquid chromatography

Time (min)	Ratio of mobile phase B (%)
0	4
5	15
40	25
65	35
70	95
82	95
85	4
90	4

Western blot

Western blot was performed on samples from rats in each group ($n = 5$) at 6 h and 7 days. Total protein was extracted from the hippocampal tissues. The samples (30 μg) were subjected to % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, USA). Then, the PVDF membranes were blocked by 5% nonfat milk and incubated overnight at 4 °C with primary antibodies: anti-Braf (1:1000, Abcam, USA), anti-COX IV-1 (1:1000, Cell Signaling, USA), anti-LGP85 (1:1000, Abcam, USA), anti-N-Sec1 (1:5000, Abcam, USA), anti-Orai2 (1:5000, Abcam, USA), and anti-SDHA (1:1000, Abcam, USA). The secondary antibodies were as follows: HRP-conjugated anti-rabbit or anti-mouse antibody (1:5000, Zhongshan Biotechnology, Beijing, China). Relative density of the resultant protein immunoblot images was semi-quantitatively analyzed using the Alpha Imager HP analysis system (Protein Simple, Silicon Valley, CA, USA).

Data were shown as mean (\pm standard deviation). The 2×2 factorial design was applied on the proteins after S and L band microwave radiation. The regression analysis would find the major factor between S band or L band. The software was SPSS 19. Differences at $P < 0.05$ were considered to be significant.

Results

Protein identification and differential protein screening

At 7 days after accumulative exposure to 10 mW/cm² 2.856 GHz and 1.5 GHz microwave, 5289 differential expressed proteins were identified in rat hippocampus by three repeated proteomic experiments using iTRAQ (Fig. 1). The normality test strongly fitted the frequency distribution of normalized log-transformed median values of expression ratios of the 5289 proteins. Proteins featuring a change ratio of ≥ 1.5 -fold or ≤ 0.67 -fold were regarded as significant differential expressed proteins ($P < 0.05$). Comparison between each two groups showed that most differential proteins were found between control and SL10 groups, indicating that accumulative exposure could induce strongest responses in rat hippocampus. All the differential expressed proteins are presented in Table 5, and most of them were upregulated by microwave.

Bioinformatic analysis

According to GO functional enrichment analysis, the distribution of differential expressed proteins in various biological processes (BPs), cellular components (CCs), molecular functions (MFs), and KEGG pathways was figured out. The results of

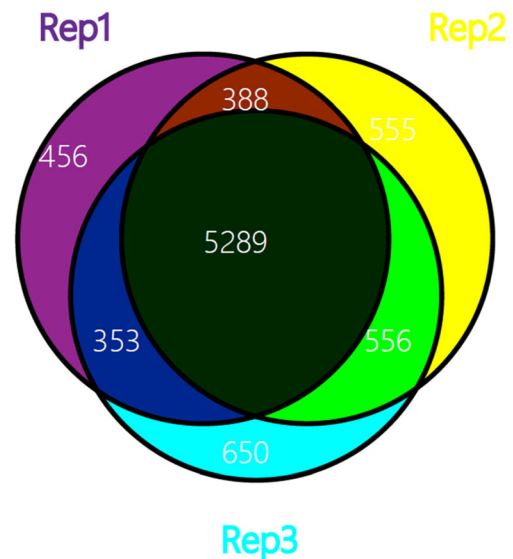


Fig. 1 Number of proteins after repeated experiments

hypergeometric distribution test indicated the functional enrichment degree of the protein. The smaller the P value, the greater the enrichment and more reliable of the results. In our study, the P value < 0.05 was taken into consideration.

L10 vs. C

Differential expressed proteins between L10 and control groups mainly located in small GTPase-mediated signal transduction, protein transport, intracellular protein transport, translation, cell-cell adhesion, and other complex biological processes (Fig. 2a). The cellular components mainly focused on the cytoplasm, nucleus, extracellular exosome, membrane, and cytosol (Fig. 2b). The molecular functions included the poly(A) RNA, GTP, protein and protein complex binding, and structural molecule activity (Fig. 2c). The KEGG pathway analysis suggested that synaptic vesicle cycle, long-term depression, endocrine, and other factor-regulated calcium reabsorption were involved. The related genes and proteins are listed in Fig. 2d.

S10 vs. C

As described above, 1.5 GH microwave exposure could induce 311 differential expressed proteins in rat hippocampus. These proteins play pivotal roles in various biological processes, including the translation, response to drug, aging, positive regulation of GTPase activity, and cell-cell adhesion (Fig. 3a). And, the cellular components mainly focused on the cytoplasm, extracellular exosome, nucleus, membrane, and mitochondrion (Fig. 3b). Moreover, the molecular functions included poly(A) RNA, protein and ATP binding, structural constituent of ribosome, and protein complex binding (Fig. 3c). Furthermore, the KEGG pathway analysis suggested that differential proteins belonged to endocrine and other factor-

Table 5 Number of differential proteins among any two groups

Groups	Number of all differential proteins	Number of downregulated proteins	Number of upregulated proteins
L10/C	295	20	275
S10/C	311	21	290
S10/L10	246	16	230
SL10/C	391	9	382
SL10/L10	238	8	230
SL10/S10	253	3	250

regulated calcium reabsorption, GABAergic synapse, long-term depression, synaptic vesicle cycle, glutamatergic synapse, long-term potentiation, and calcium signaling pathways. The involved genes and proteins are listed in Fig. 3d.

SL10 vs. C

We found that accumulative exposure to 2.856 GHz and 1.5 GHz microwave induced most differential expressed proteins in rat hippocampus. These proteins participated in various biological processes, such as positive regulation of GTPase activity, brain development, small GTPase-mediated signal transduction, protein transport, and intracellular protein transport (Fig. 4a). The cellular components mainly located in cytoplasm, extracellular exosome, membrane, mitochondrion, and cytosol (Fig. 4b). The molecular functions suggested that differential expressed proteins play important roles in protein ATP, poly(A) RNA, and GTP binding (Fig. 4c). Moreover, the KEGG pathway analysis indicated that those proteins are closely associated with glutamatergic synapse, GABAergic synapse, long-term potentiation, calcium

signaling pathway, synaptic vesicle cycle, long-term depression, and neurotrophin signaling pathways. The involved genes and proteins are listed in Fig. 4d.

S10 vs. L10

Different frequency microwaves could induce diverse biological effects. Therefore, we compared the expression of proteins between 10 mW/cm² S band (S10) and L band (L10) microwaves. The biological processes related differential expressed proteins mainly located in the process of oxidation-reduction, translation, phosphorylation, learning and memory, and so on (Fig. 5a). The cellular components mainly focused on the cytoplasm, extracellular exosome, membrane, mitochondrion, and cytosol (Fig. 5b). The molecular function-associated differential expressed proteins included protein, ATP, and poly(A) RNA binding, identical protein binding, and structural constituent of ribosome (Fig. 5c). The KEGG pathway of differential proteins between S10 and L10 included the Alzheimer's disease,

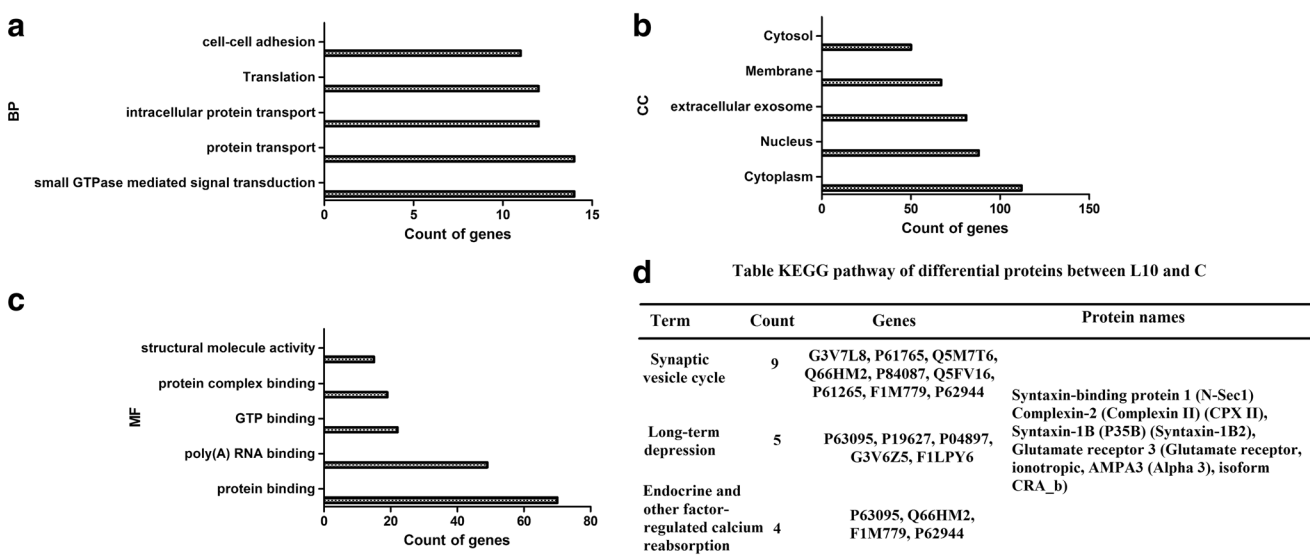


Fig. 2 The GO analysis between L10 and C. **a–d** The BP, CC, MF, and KEGG pathway

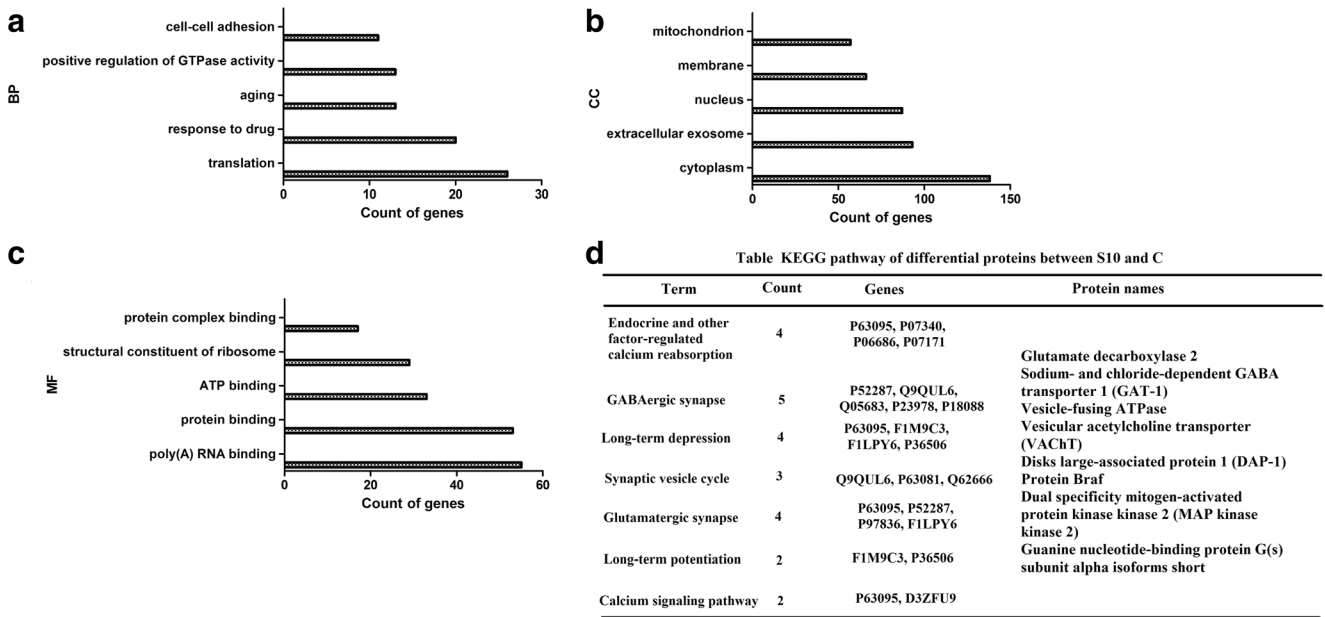


Fig. 3 The GO analysis between S10 and C. a–d The BP, CC, MF, and KEGG pathway

MAPK signaling pathway, cAMP signaling pathway, long-term potentiation, mTOR signaling pathway, long-term depression, GABAergic synapse, and neurotrophin signaling pathways. The involved genes and proteins are listed in Fig. 5d.

SL10 vs. L10

Although the expression of numerous proteins were influenced significantly both by single frequency and accumulative microwave exposure, the changes were at different

level. Therefore, we still found 238 differential expressed proteins between SL10 and L10 groups. These proteins play pivotal roles in regulating several biological processes, such as protein ubiquitination, protein transport, intracellular protein transport, central nervous system development, and neurogenesis (Fig. 6a). The cellular components mainly focused on the cytoplasm, extracellular exosome, mitochondrion, nucleoplasm, and cytosol (Fig. 6b). The molecular functions included ATP binding, zinc ion binding, ubiquitin-protein transferase activity, RNA binding, and structural molecule activity (Fig. 6c). The

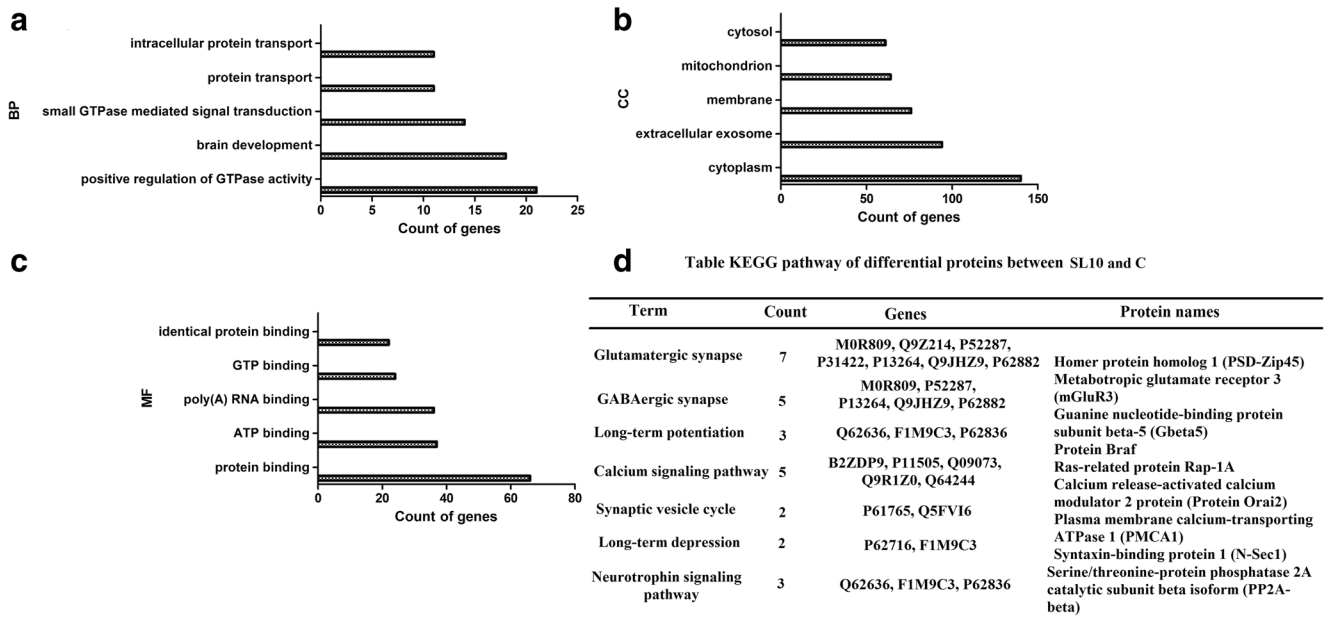


Fig. 4 The GO analysis between SL10 and C. a–d The BP, CC, MF, and KEGG pathway

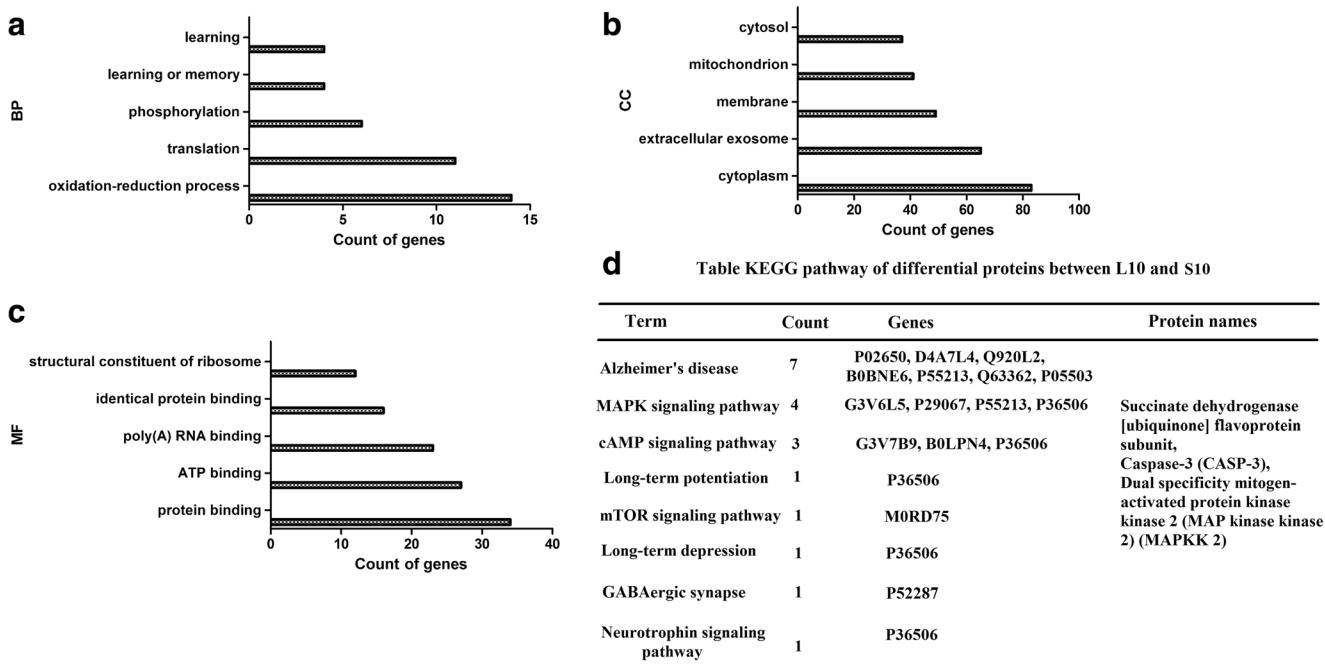


Fig. 5 The GO analysis between S10 and L10. a–d The BP, CC, MF, and KEGG pathway

KEGG pathway analysis suggested that serotonergic synapse, dopaminergic synapse, AMPK signaling pathway, long-term depression, long-term potentiation and calcium signaling pathway, GABAergic synapse, glutamatergic synapse, and Alzheimer's disease progression were involved. The involved genes and proteins are listed in Fig. 6d.

SL10 vs. S10

The biological processes mainly included the oxidation-reduction process, translation, phosphorylation, learning or memory, and learning (Fig. 7a). The cellular components mainly focused on the cytoplasm, extracellular exosome, membrane, mitochondrion, and cytosol (Fig. 7b). The

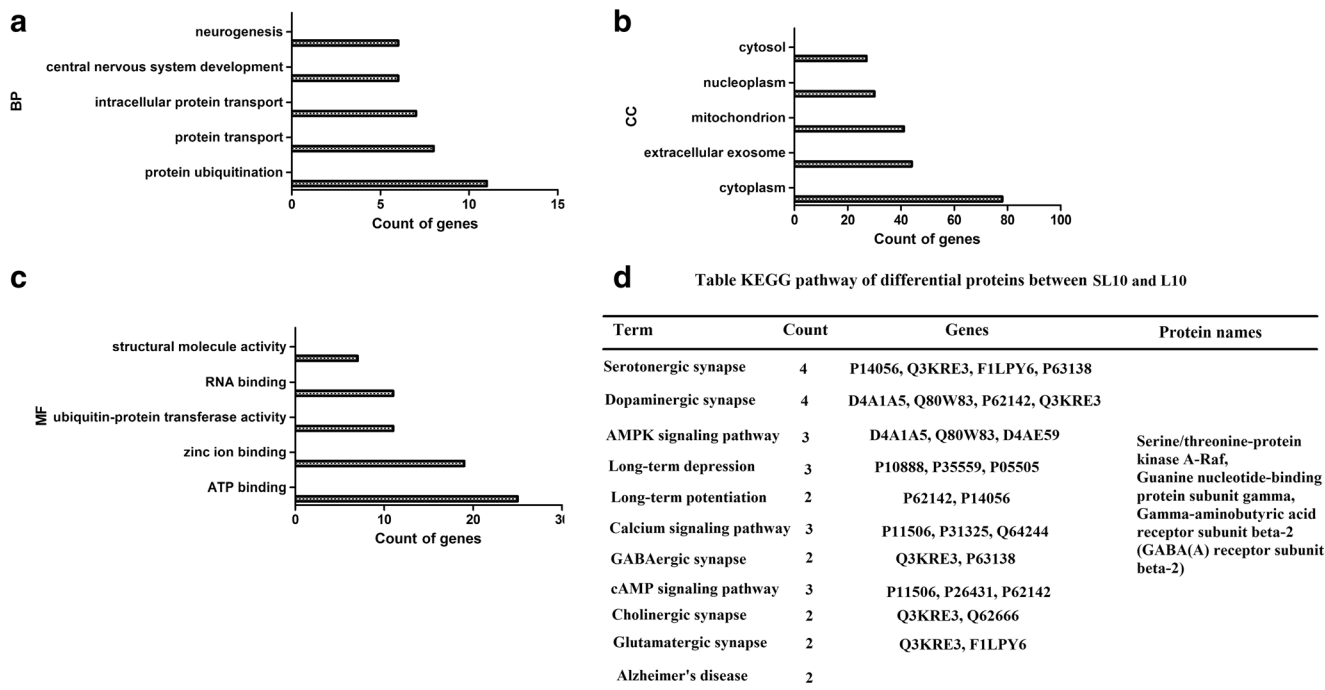


Fig. 6 The GO analysis between SL10 and L10. a–d The BP, CC, MF, and KEGG pathway

molecular functions included protein binding, ATP binding, poly(A) RNA binding, identical protein binding, and structural constituent of ribosome (Fig. 7c). The KEGG pathway of differential proteins between SL10 and S10 included protein processing in endoplasmic reticulum, lysosome, PPAR signaling pathway, and Alzheimer’s disease. The involved genes and proteins are listed in Fig. 7d.

Validation of several differential expressed proteins at different exposure time

Combined our previous work on microwave-induced hippocampal functional injuries and KEGG pathway analysis, five differential expressed proteins that closely associated with the functional injuries were selected for validation in this study. The functional annotations of those five proteins are specifically listed in Table 6.

To validate the results of iTRAQ proteomic analysis, the expression of five proteins, including protein Braf, lysosome membrane protein 2 (85 kDa lysosomal membrane sialoglycoprotein) (LGP85), syntaxin-binding protein 1 (N-Sec1), calcium release-activated calcium modulator 2 protein (protein Orai2), and succinate dehydrogenase [ubiquinone] flavo protein subunit, was detected by western blotting at 6 h and 7 days after microwave exposure. Generally, the results were in accordance with proteomic results (Fig. 8).

Interestingly, after factorial design, we also found that there were significant differences between 1.5 GHz and 2.856 GHz microwave exposure in the Braf expression at 6 h, LGP 85 expression at 6 h and 7 days, Orai2 expression at 7 days, and SDHA expression at 6 h ($P < 0.05$). Then, the regression analysis

was conducted. As for the expression of SDHA at 6 h, the absolute value of coefficient in the S10 group (0.679) was greater than that of L10 group (0.216). However, as for the Braf at 6 h, the absolute value of standardized regression coefficient in the S10 group (0.371) was less than that of the L10 group (0.607), which indicated that the L band microwave radiation played a major role on the expression of Braf. As for the Orai2 at 7 days, the absolute value of standardized regression coefficient in the S10 group (0.638) was lower than that of the L10 group (0.681), which indicated that the L band microwave radiation played a major role on the expression of Orai2. As for the LGP 85 at 6 h, the value of coefficient in the S10 (0.476) was less than that of L10 (0.488). As for the LGP 85 at 6 h, the value of coefficient in the S10 (0.261) was less than that of L10 (0.554).

The regression results indicated that in the expression of SDHA, the S band microwave radiation played a major role on the protein expressions, while in the expression of Orai2, LGP85, and Braf, the L band microwave radiation played a major role.

Discussion and conclusion

Proteomic techniques have been emerged as effective tools to reveal differential expressed proteins, which was associated with indicated physical and pathological processes (Pandey and Mann 2000; Wasinger et al. 1995). In model of microwave-induced testis injury, proteomic techniques and bioinformatic analysis were conducted to analyze differential expressed proteins. And, proteins that related to misfolding of proteins or stress, such as heat shock

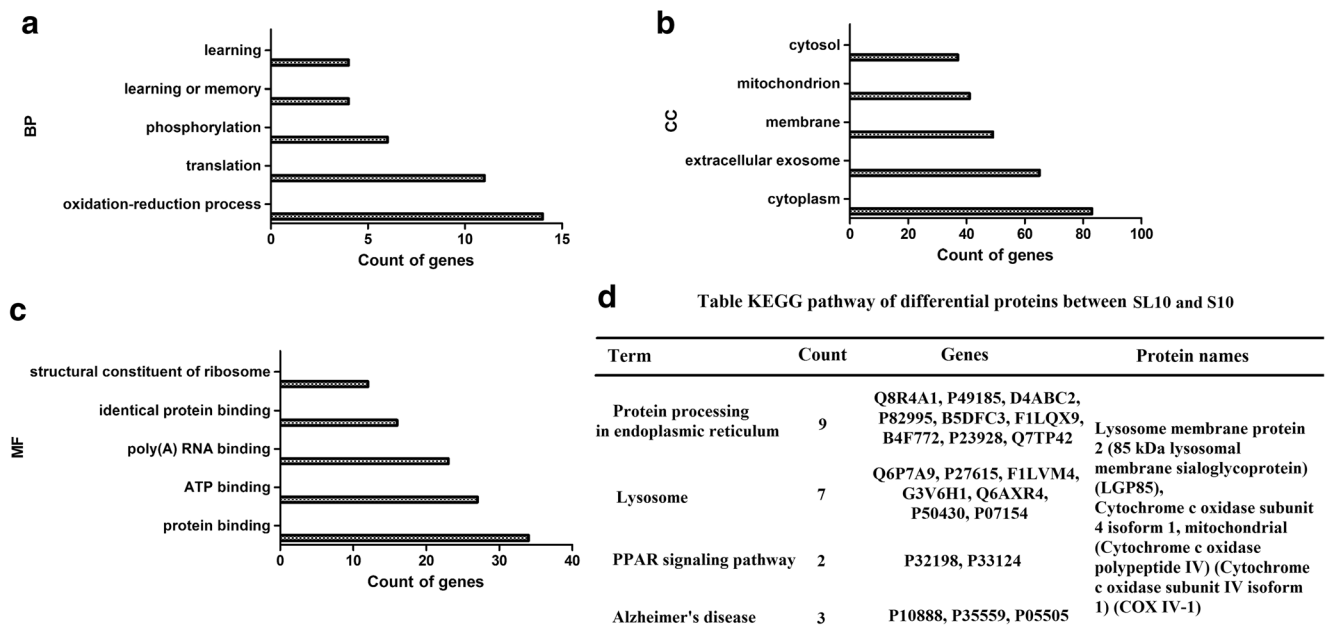


Fig. 7 The GO analysis between SL10 and S10. a–d The BP, CC, MF, and KEGG pathway

Table 6 Differential proteins for validation and their functional annotations

Protein names	Biological process	Molecular function	Cellular component
Protein Braf	Long-term synaptic potentiation, MAPK cascade, negative regulation of endothelial cell apoptotic process, negative regulation of fibroblast migration, negative regulation of neuron apoptotic process, negative regulation of synaptic vesicle exocytosis, positive regulation of axonogenesis, positive regulation of axon regeneration, positive regulation of ERK1 and ERK2 cascade, positive regulation of gene expression, positive regulation of stress fiber assembly, regulation of cell proliferation, response to cAMP, response to peptide hormone	ATP binding, calcium ion binding, MAP kinase kinase kinase activity, mitogen-activated protein kinase kinase binding, protein heterodimerization activity, protein serine/threonine kinase activity	Ciliary basal body, cytoplasmic vesicle
Lysosome membrane protein 2 (85 kDa lysosomal membrane sialoglycoprotein) (LGP85)	Positive regulation of neuron projection development, protein targeting to lysosome	Receptor activity	Heterochromatin, nuclear replication fork, nucleoplasm, nucleus, site of double-strand break
Syntaxin-binding protein 1 (N-Sec1)	Axon target recognition, long-term synaptic depression, negative regulation of neuron apoptotic process, negative regulation of protein complex assembly, negative regulation of SNARE complex assembly, negative regulation of synaptic transmission, GABAergic, neuromuscular synaptic transmission, neurotransmitter secretion, positive regulation of calcium ion-dependent exocytosis, positive regulation of exocytosis, regulation of synaptic vesicle priming, synaptic vesicle maturation, vesicle docking involved in exocytosis	Identical protein binding, poly(A) RNA binding, protein domain specific binding, protein kinase binding, protein N-terminus binding, SNARE binding, syntaxin-1 binding, syntaxin binding	Cytosol
Calcium release-activated calcium modulator 2 protein (protein Orai2)	Store-operated calcium entry	Store-operated calcium channel activity	–
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	Anaerobic respiration; mitochondrial electron transport, succinate to ubiquinone; nervous system development; respiratory electron transport chain; succinate metabolic process; tricarboxylic acid cycle	Electron carrier activity, flavin adenine dinucleotide binding, succinate dehydrogenase (ubiquinone) activity	Mitochondrial inner membrane, mitochondrial respiratory chain complex II, succinate dehydrogenase complex (ubiquinone), myelin sheath

proteins, superoxide dismutase, and peroxiredoxin-1, were identified. In this study, novel proteomic technique with higher resolution, iTRAQ, was used to analyze differential expressed proteins in rat model of microwave-induced learning and memory impairment. In rat hippocampus, we found that both S and L band microwaves induced numerous differential expressed proteins. However, comparing with control group, accumulative exposure resulted in much more differential expressed proteins than single

exposed groups, indicating that accumulative exposure might induce the strongest biological response.

Bioinformatic analysis suggested that most of the differential expressed proteins are (1) pivotal molecules participating in several biological processes, such as cell adhesion, translation, brain development, learning and memory, neurogenesis, and so on; (2) composing cellular components in extracellular exosome, membrane, and mitochondria; (3) important molecules exerting diverse functions,

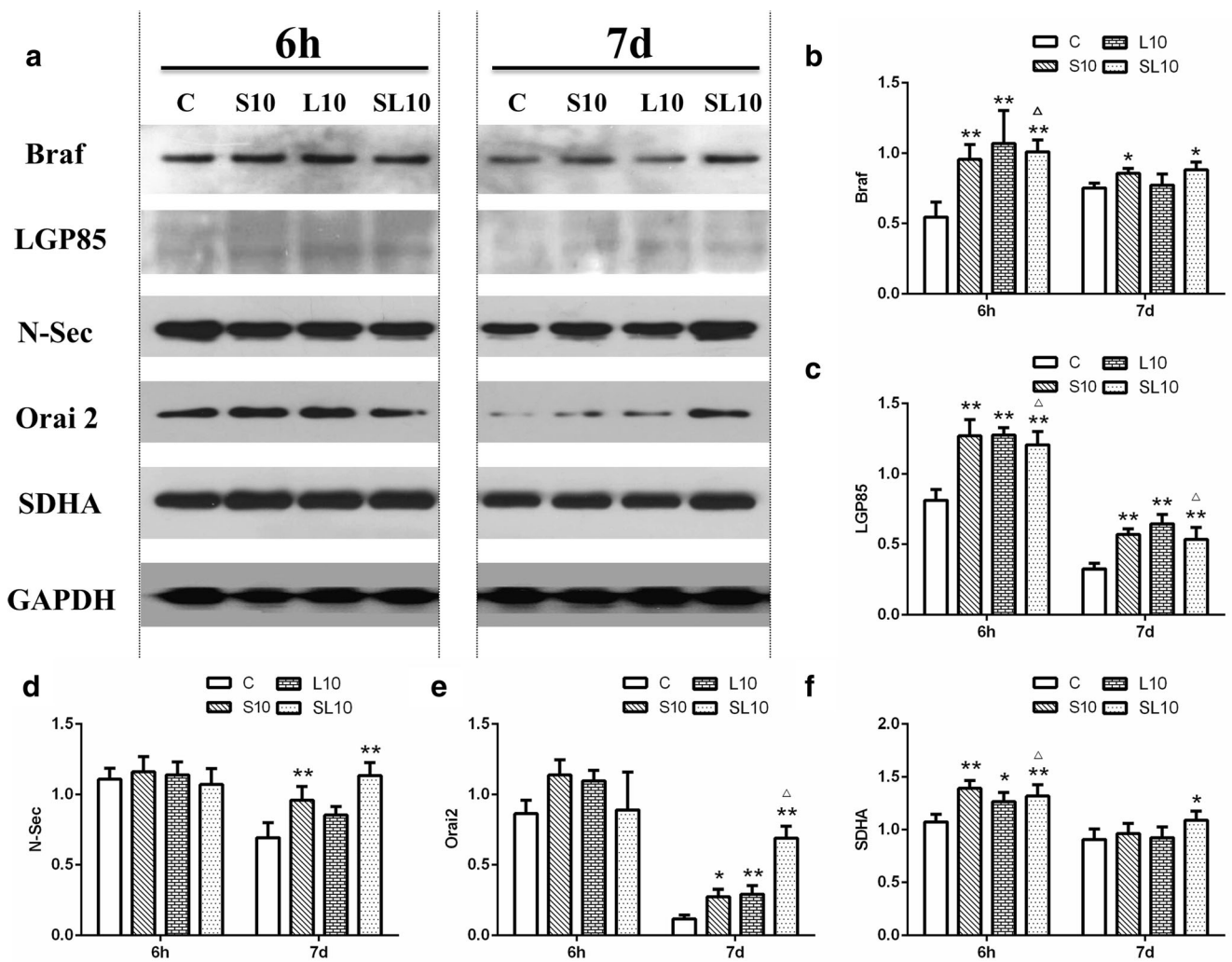


Fig. 8 The level of proteins was normalized to that of GAPDH at 6 h and 7 days after microwave exposure ($n = 5$). **a** The level of protein Braf, LGP 85, N-Sec1, Orai2, and SDHA. **b–f** Statistical results of the above

proteins. Compared with the C group, * $P < 0.05$, ** $P < 0.01$. $\Delta P < 0.05$ means differences after the factor analysis

including protein complex binding, protein binding, and ubiquitin-protein transferase activity; and (4) critical proteins locating in the signaling pathways of synaptic vesicle cycle, long-term potentiation, long-term depression, glutamatergic synapse, and calcium signaling. According to our previous data and KEGG pathway analysis, we selected six differential expressed proteins for verification, which are potential molecules in regulating injury of learning and memory ability after microwave exposure. Those proteins included protein Braf, LGP 85, N-Sec1, Orai2, and SDHA.

Protein Braf, also named serine/threonine-protein kinase B-raf, plays important roles in mitogen-activated protein kinase (MAPK) signal transduction in the post synaptic hippocampal neurons. Braf deficiency in mouse hippocampus resulted in abnormal behaviors, including autoaggression and loss of balance fitness. Moreover, absence of Braf not only inhibited structural development of

neurons, but also partially disturbed neuronal circuits in cerebellum and hippocampus (Pfeiffer et al. 2013). During development of nervous system, Braf silence markedly attenuated baseline phosphorylation of Erk in neural tissues and led to growth retardation (Zhong et al. 2007). On the other hand, in mouse embryonic neurons, conditional activation of Braf kinase alone is sufficient to drive the growth of long-range peripheral sensory axon projections in vivo, although lacking upstream neurotrophin signaling (O’Donovan et al. 2014). Therefore, we believed that protein Braf played positive roles in neuronal development. In this study, we found that the expression of Braf increased at 6 h and 7 days after microwave exposure, which could contribute to the process of tissue repair.

Lysosomal membrane glycoprotein termed LGP85 (also named LIMP II), a type III transmembrane glycoprotein, located mainly in membrane of lysosomes and late

endosomes. LGP85 plays important roles in the biogenesis, maintenance, and reorganization of endosomes/lysosomes (Kuronita et al. 2002; Tabuchi et al. 2000). And, LGP85 deficiency was closely associated with degeneration of the striavascularis and/or demyelination of peripheral nerves (Gamp et al. 2003). Microwave radiation could not induce permanent damage. In most cases, the damage and repair coexisted. LGP85 plays important roles in the biogenesis, maintenance, and reorganization of endosomes/lysosomes. In our experiment, the LGP85 significantly increased after microwave exposure. We believed that the biogenesis endosomes/lysosomes indeed occurred. The lysosomes could clear damaged organelle and promote hippocampal repair. Therefore, we believed the existence of active repair in hippocampus after microwave radiation.

N-Sec1, a neural-specific syntaxin-binding protein, was tightly associated with syntaxin via N-type calcium channels and participated in regulating synaptic vesicle docking and fusion. Studies also suggested that *Drosophila* syntaxin 1A played multiple regulatory roles in neurotransmission in vivo (Wu et al. 1999). Moreover, tomosyn-formed genuine SNARE core complexes, composed of SNAREs, syntaxin 1, and SNAP-2, played important roles in downregulating exocytosis (Hatsuzawa et al. 2003). In hippocampus, downregulation of vesicle-associated membrane protein 3 (VAMP3) and syntaxin-1A resulted in difficulty in forming SNARE complex and maintaining GABA/Glu balance (Wang et al. 2015). Our study demonstrated that microwave exposure increased N-Sec1 expression in rat hippocampus, which might represent a recovery mechanism after microwave exposure.

It has been reported that Orai1 or Orai2 resulted in a substantial increase in calcium release-activated calcium (CRAC) current (DeHaven et al. 2007). Studies showed that fast Ca^{2+} -dependent inactivation was mediated by three conserved glutamates in the C termini of Orai2 and Orai3 (Lee et al. 2009). Orai2 knockout attenuated antigen stimulation induced Ca^{2+} release from Ca^{2+} store (Ikeya et al. 2014). Besides, the Orai1, Orai2, and STIM1 formed functional CRAC channels in OUMS-27 cells which were responsible for sustained Ca^{2+} entry. In this study, we showed that microwave exposure could upregulate Orai2 expression at 7 days after exposure, which suggested the increases in CRAC and CRAC channels.

Succinate dehydrogenase complex flavoprotein subunit A (SDHA), located in the mitochondrial inner membrane to catalyze succinic acid-ubiquinone oxidoreductase, plays pivotal roles in mitochondrial electron transport, respiratory electron transport chains, succinic acid metabolic processes, and tricarboxylic acid cycles. The enzymatic function of succinate dehydrogenase was dependent on covalent attachment of FAD (flavin adenine dinucleotide) on the ~ 70 kDa flavo protein subunit Sdh1 (Kim

et al. 2012). SDHA mutations were associated with gastrointestinal stromal tumors and mitochondrial complex II deficiency (Alston et al. 2012; Belinsky et al. 2013; Dwight et al. 2013). In our study, we found that microwave increases expression of SDHA at 6 h and 7 days after exposure, which suggested enhanced oxygen utilization rate in mitochondria. This might be a compensatory change in the structure and function of mitochondria after microwave interference.

We also analyzed the accumulative effects between S band and L band microwave exposure in this study. Our results suggested that S band microwave radiation played a major role in the regulation of SDHA expression, while the L band microwave radiation played a major role in the regulation of Braf, Orai2, and LGP85 expression. Therefore, under accumulative microwave exposure, we believed that the injuries were obviously different from the single band exposure and L band microwave played important roles in changes of most proteins. This might be because of the lower frequency and greater penetration of L band microwave than those of S band microwave.

In conclusion, the S and L band microwave with the average power density of 10 mW/cm^2 could induce numerous differential expressed proteins in rat hippocampus. The accumulative exposure of the S and L band microwave could induce similar responses. Those differential expressed proteins mainly focused in the synaptic vesicle cycle, long-term potentiation, long-term depression, glutamatergic synapse, and calcium signaling pathway. Among those proteins, protein Braf, LGP85, N-Sec1, Orai2, and SDHA were validated and were sensitive to microwave radiation.

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