



Research report

Microwave radiation leading to shrinkage of dendritic spines in hippocampal neurons mediated by SNK-SPAR pathway



Wei-Jia Zhi^a, Rui-Yun Peng^a, Hai-Juan Li^a, Yong Zou^a, Bin-Wei Yao^a, Chang-Zhen Wang^a, Zong-Huan Liu^a, Xiao-Hui Gao^a, Xin-Ping Xu^a, Ji Dong^a, Li Zhao^a, Hong-Mei Zhou^b, Li-Feng Wang^{a,*}, Xiang-Jun Hu^{a,*}

^a Laboratory of Experimental Pathology, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China

^b Laboratory of Radiation Protection and Health, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China

ARTICLE INFO

Article history:

Received 9 February 2017

Received in revised form 21 October 2017

Accepted 20 November 2017

Available online 24 November 2017

Keywords:

Microwave radiation

Dendritic spines

Learning and memory

SNK-SPAR pathway

ABSTRACT

The popularization of microwave raised concerns about its influence on health including cognitive function which is associated greatly with dendritic spines plasticity. SNK-SPAR is a molecular pathway for neuronal homeostatic plasticity during chronically elevated activity. In this study, Wistar rats were exposed to microwaves (30 mW/cm² for 6 min, 3 times/week for 6 weeks). Spatial learning and memory function, distribution of dendritic spines, ultrastructure of the neurons and their dendritic spines in hippocampus as well as the related critical molecules of SNK-SPAR pathway were examined at different time points after microwave exposure. There was deficiency in spatial learning and memory in rats, loss of spines in granule cells and shrinkage of mature spines in pyramidal cells, accompanied with alteration of ultrastructure of hippocampus neurons. After exposure to 30 mW/cm² microwave radiation, the up-regulated SNK induced decrease of SPAR and PSD-95, which was thought to cause the changes mentioned above. In conclusion, the microwave radiation led to shrinkage and even loss of dendritic spines in hippocampus by SNK-SPAR pathway, resulting in the cognitive impairments.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

Microwave has been universally applied in communication, medical treatment, military and many other fields, consequently, the concern about its biological effects raised as well. Microwave radiation can be absorbed by organisms causing a series of physiological function changes. There are many intricate electrical activities in central nervous system, such as learning and memory, which makes it vulnerable to microwave radiation (Vorobyov et al., 2010). It is known that central nervous system is one of the most sensitive targets of microwave radiation (Eliyahu et al., 2006; Vorobyov et al., 2010). As known to all, hippocampus plays an important role in learning and memory and synapses make it possible for information transfer between neurons. Synaptic plasticity, as well as the dendritic spine plasticity is the foundation for normal operation in learning and memory. Dendritic spines are small dynamic protrusions on the dendritic shafts of principal neurons which are mediated by many signaling pathways (Cahill et al., 2013; Chutabhakdikul and Surakul, 2013; Hruska and

Dalva, 2012; Inestrosa and Varela-Nallar, 2015). They comprise the postsynaptic compartments of most glutamatergic synapses in the mammalian brain (Sui et al., 2010). Ning (Ning et al., 2007) reported that chronic exposure to 2.4 W/kg GSM 1800 MHz microwaves led to a significant decrease in the mobility of dendritic filopodia and the density of mature spines in the neurons. Shahin (Shahin et al., 2015) exposed Swiss strain male mice to microwave radiation (2.45 GHz, 0.0248 mW/cm², SAR (specific absorption rate) = 0.0146 W/kg) found that the decrease in hippocampal subfield neuronal arborization and dendritic spines were correlated with increased duration of microwave exposure. However, the mechanism for abnormality of spine plasticity induced by microwave is still unclear. SNK (serum inducible kinase)–SPAR (spine-associated Rap guanosine triphosphatase activating protein) is a molecular pathway for neuronal homeostatic plasticity during chronically elevated activity (Pak and Sheng, 2003; Seeburg et al., 2008). Induced by synaptic activity and targeted to dendritic spines, activated SNK leads to the elimination of SPAR, depletes the core postsynaptic scaffolding molecule PSD-95 and results in loss of mature dendritic spines and synapses (Sui et al., 2010). In this study, after Wistar rats exposed to microwave, the capacity of spatial learning and memory, the morphology and density of dendritic spines in hippocampus and the expression and

* Corresponding authors.

E-mail addresses: fangchang_14@hotmail.com (L.-F. Wang), xjhu2003@vip.sina.com (X.-J. Hu).

interaction of the involved molecules in SNK-SPAR pathway were detected to explore the role of SNK-SPAR pathway in the abnormality of spine plasticity induced by microwave radiation.

2. Results

2.1. Spatial memory ability of rats decreased after microwave exposure

The Morris Water Maze was applied as the schedule shown in Fig. 2.A. Afterwards, the swimming speed, average escape latency (AEL) in navigation test, the percentage of time spent in target quadrant and average crossing times in probe test were recorded and analyzed. The swimming speeds between control group and exposure group were no difference and didn't change throughout the test (Fig. 2.B). In navigation test (Fig. 2.C), the AEL decreased in each group as the training times went on indicating that the rats in both groups gradually got the ability to find the platform; besides, the AEL in exposed group was significantly longer than control group ($p < .01$), and the AEL was longer at each corresponding time points ($p < .05$) except the first day and the fourth day. In probe test, at 14d after microwave exposure, the percentage of time in target quadrant and average crossing times in control group were both higher than that in exposure group (Fig. 2.D and E).

2.2. Microwave exposure shifted spine distribution in Golgi-stained hippocampus

After microwave radiation, the density of dendritic spines in granule cells decreased at 6 h, 14d and 28d ($p < .05$) (Fig. 3. A and B). The morphology of dendritic spine embraces mushroom ones which characterized by an intumescent head and a thin neck, stubby ones which had a neck the same width with its head and long thin spines. It is reported that the larger the heads are, the more mature and stable the spines get. So the percentage of mushroom spines among all spines had been calculated as follow: the percentage of mushroom spines in pyramidal cells had decreased at each detecting time especially at 28 d after exposure ($p < .05$) (Fig. 3. D and F).

2.3. Ultrastructure alteration of hippocampal neurons after microwave exposure

The ultrastructure of hippocampal neurons was observed using a TEM to detect the alteration of ultrastructure in dendritic spines

and neurons at 6 h, 7d, 14d and 28d after exposed to microwave respectively, the detecting time points was chosen according to former studies (Wang et al., 2013b; Xiong et al., 2015). In exposure group, dilation of endoplasmic reticulum and swelling of mitochondria in neurons could be observed (Fig. 4. B). The overwhelming majority of excitatory synapse are composed of dendritic spines and presynaptic elements (Bourne and Harris, 2007) which are characterized by asymmetric structure, in other words, these synapses contain apparent postsynaptic density (PSD) which was identified as a fuzzy electron-dense structure stretching into cytoplasm beneath the plasma membrane (Gulley and Reese, 1981; Landis and Reese, 1983), and round synaptic vesicles different from symmetric synapses (Fig. 4.C–F). There were sporadic polyribosomes (polyrib) in spines (Fig. 4.D). Mitochondria (mit) in dendritic spines (Fig. 4.C–E) and round synaptic vesicles (sv) could be easily observed as well (Fig. 4.C–F). In exposure group, the endocytosis via spinules on spines labeled by purple were observed at 6 h, 7d and 14d after exposed to microwave (Fig. 4.G–I). This is a phenomenon happened when part of dendritic spine was engulfed by presynaptic axons or other parts (Harris and Weinberg, 2012).

The thickness and length of PSD were measured in Photoshop 7.0 software and then analyzed. The PSD length at 6 h in exposure group was significantly decreased compared to control group ($p < .01$) (Fig. 5.B); the thickness of PSD at 6 h, 7d, 14d and 28d after exposure was significantly lower than that in control group respectively ($p < .05$) (Fig. 5.C).

2.4. Expression fluctuation of SNK, SPAR and PSD-95 in hippocampus after microwave exposure

The level of SNK mRNA was displayed as Fig. 6.A, the mRNA in exposure groups was significantly up-regulated than that in control groups. The protein of SNK at 6 h and 28d after exposure was significantly lower, while at 14d, it was higher than control group. The expression of SPAR showed a great alteration during a month after exposure: at 6 h and 14d, the exposure groups were lower than control. And there was significant discrepancy in the expression of PSD-95 at 28d between exposure group and control group (Fig. 6.B–E).

2.5. The interaction of SPAR and PSD-95 decreased in hippocampus after microwave exposure

SPAR can recognize cytoskeletal protein and submit PSD-95 to post synaptic structure, regulating the plasticity of dendritic

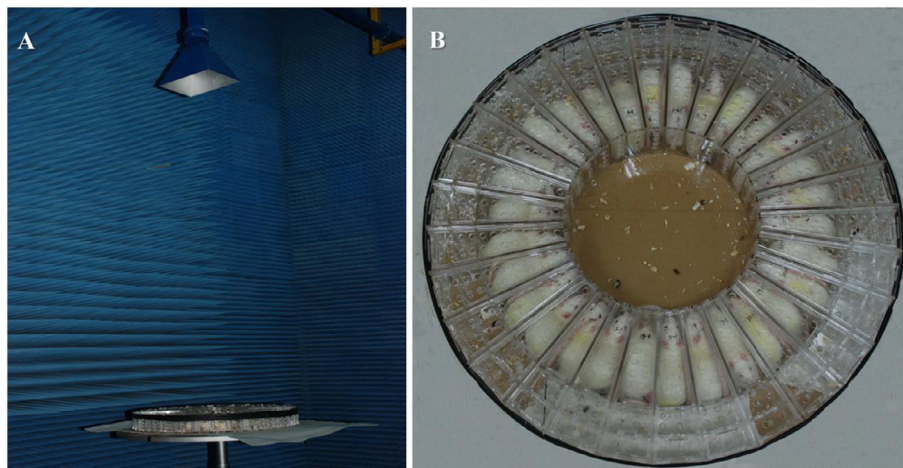


Fig. 1. A: Radiation equipment; B: Fixing device of rats.

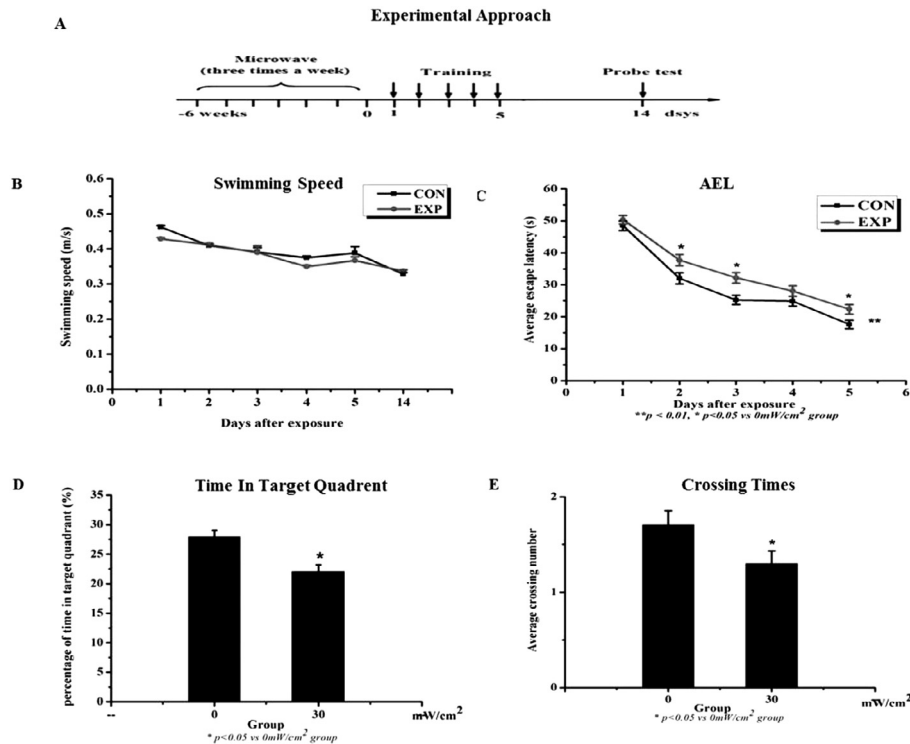


Fig. 2. Performances of rats in Morris water maze test. A: Time schedule of experiments; B: Swimming speed of rats; C: Average escape latency during navigation test. Statistical significances (repeated measures ANOVA): * $p < .05$ versus control group, ** $p < .01$ versus control group at corresponding time point after exposure; D: Percentage of time in the target quadrant in probe test. * $p < .05$ versus control group; E: Average crossing times in probe test. * $p < .05$ versus control group.

spines. Thus the co-localization of SPAR and PSD-95 in hippocampus was detected by double-label immunofluorescence could be the corroborative evidence for interaction of these two proteins. In this study we use Manders' overlap coefficient (R) to indicate the actual overlap of green and red signals, which was considered to represent the true degree of co-localization (Zinchuk and Grossenbacher-Zinchuk, 2009). For SPAR and PSD-95, the value of R in exposure group showed significant difference at 6 h ($p < .01$) and 28d ($p < .05$) respectively compared to control group (Fig. 7).

3. Discussion

Studies have reported that long-term microwave radiation can cause cognitive deficiency which is largely associated with hippocampus and its synaptic plasticity (Li et al., 2015, 2012; Lu et al., 2012; Wang et al., 2013b). The most excitatory synapses are composed of dendritic spines as postsynaptic elements and the presynaptic parts such as axons of other neurons. SNK-SPAR pathway plays an important role in maintaining homeostatic status of synapses (Pak and Sheng, 2003; Wu et al., 2007) by synaptic remodeling and function regulating. To explore the effects of microwave radiation on cognitive ability and neuronal structure, and the role of SNK-SPAR pathway during this process, several indexes were tested at 6 h, 7 d, 14 d and 28 d after microwave exposure.

Morris water maze has been widely used in neurobehaviour to test the spatial learning and memory of experimental animals (Morris, 1984). Therefore, this classic method is often adopted to test cognitive ability after microwave radiation. In present study, the AEL in navigation test and the indicators such as crossing times and the percentage of time spent in targeted quadrant in probe test all indicated that microwave radiation caused dysfunction of spatial learning and memory ability in exposed rats, which was consistent with the results of researches mentioned above. Besides, the

previous study also showed memory retard of rats at 28d after microwave radiation under the same experiment condition (Li et al., 2015).

Structure is the foundation of function regulating. Distribution and morphology of dendritic spines play a vital role in learning and memory. Synaptic strengthening and LTP, induced by stimulation of hippocampal afferents, such as learning and memory, are associated with either increase in spine density or spine head diameter or both of them (Chang and Greenough, 1984; Desmond and Levy, 1986; Fifková and Anderson, 1981; Sala and Segal, 2014; Zee, 2014). While synaptic overstimulation can adversely result in spine shrinkage to prevent damage from excess calcium influx (Paulin et al., 2016). What's more, the structure plasticity of dendritic spines can in turn influence behaviors (Gipson and Olive, 2016). To explore the reason of disability of rats in learning and memory after microwave exposure, the change in spine plasticity was observed by Golgi stain. Considering that the hippocampus was mainly composed of two kinds of neurons: granule and pyramidal cells, the outcomes also included the two kinds of neurons. Interestingly, the effects of microwave radiation varied between two areas: in granule cells, it appeared to be the density decrease of dendritic spines, which could be interpreted as loss of spines, while in pyramidal cells, it showed synaptic remodeling: the increase of immature (non-mushroom) spines and decrease of mature (mushroom) ones, but the density of spines didn't change. That is to say that the mature spines shrank into the immature ones. These results indicated that microwave radiation influenced the distribution of dendritic spines in both granule cells and pyramidal cells, leading to shrinkage and even loss of dendritic spines, restrained the LTP induced by learning and memory process (Wang et al., 2013b). The results suggested that the dendritic spines in granule cells might be more vulnerable to microwave radiation than pyramidal neurons. It is reported that the structure damage could be observed especially in dentate gyrus of hippocampus by

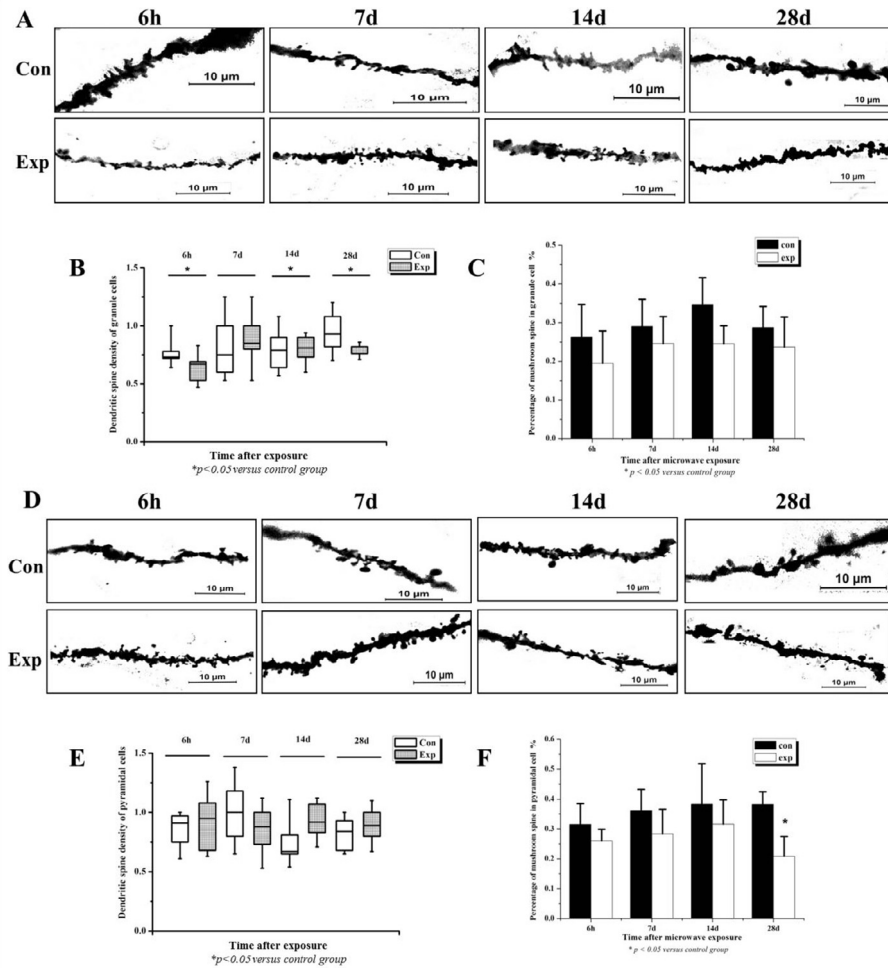


Fig. 3. Shifts of dendritic spine distribution after microwave exposure in granule and pyramidal cells. A: Shifts of dendritic spine distribution in granule cells; B: Density of dendritic spine in granule cells ($p < .05$ versus control group); C: Percentage of mushroom spine in granule cells ($p < .05$ versus control group); D: Shifts of dendritic spine distribution in pyramidal cells; E: Density of dendritic spine in pyramidal cells ($p < .05$ versus control group); F: Percentage of mushroom spine in pyramidal cells ($p < .05$ versus control group).

Hematoxylin-Eosin staining (Li et al., 2015), the decrease of population spike (PS) amplitudes of LTP in the medial perforant path (MPP)-dentate gyrus (DG) pathway was also been detected (Wang et al., 2013b), which was consistent with our outcomes.

Despite the alteration in distribution of dendritic spines in hippocampus, the abnormal phenomena of ultrastructure in these neurons/spines also suggested the damage in cognitive ability after microwave radiation. The dilation of endoplasmic reticulum, swelling of mitochondria in neurons of hippocampus were observed after microwave radiation, which were also reported elsewhere (Li et al., 2012; Xiong et al., 2015), implying the neurons edema in hippocampus after microwave exposure. The endoplasmic reticulum participates in calcium regulation, protein synthesis, protein modification after translation, along with mitochondria as energy supplier, contributing to normal function of neurons in hippocampus (Deller et al., 2000; Spacek and Harris, 1997). Therefore, the abnormality of neurons might indicate that microwave radiation resulted in reduction of protein synthesis. Polyribosome can be unambiguously identified as a cluster of three or more ribosomes. It is more prevalent in spines after LTP induced stimuli and synaptic growth, which synthesize cytoplasmic proteins such as PSD-95 (Bourne and Harris, 2007; Bourne and Harris, 2011; Ostroff et al., 2002). The location of mitochondria might predict enhanced

synaptic activity for its crucial role in Ca^{2+} regulation and generation of ATP indispensable for synaptic plasticity (MacAskill et al., 2010). They were usually found in large and complex spines such as mushroom or branched spines located on proximal dendrites of CA3 pyramidal cells after LTP (Chicurel and Harris, 1992). However, these two organelles were rarely observed after microwave radiation, which was implying inhibition of LTP. The trans-endocytosis via spinules is another way for signaling between neurons except synaptic communication, it may also help to redistribute membrane resources locally and cause remodeling of dendritic spines and synapse morphology (Harris and Weinberg, 2012), which happened at 6 h, 7d and 14d after microwave radiation as a symbol of the shrinkage and even loss of dendritic spines.

The length and thickness was an indirect reflection of PSD area, additionally, the area was almost perfectly correlated with spine head volume, total number of presynaptic vesicles and the area of active zone which the vesicles docked at (Schikorski and Stevens, 2001). The PSD contains a variety of receptors, scaffolding proteins and signaling complexes involved in synaptic transmission and plasticity (Sheng and Kim, 2011). Thus the longer and thicker PSD indicated larger head of spines, better function of transformation and more compact subcellular organization of constituent proteins (Tao-Cheng et al., 2007), which was observed in

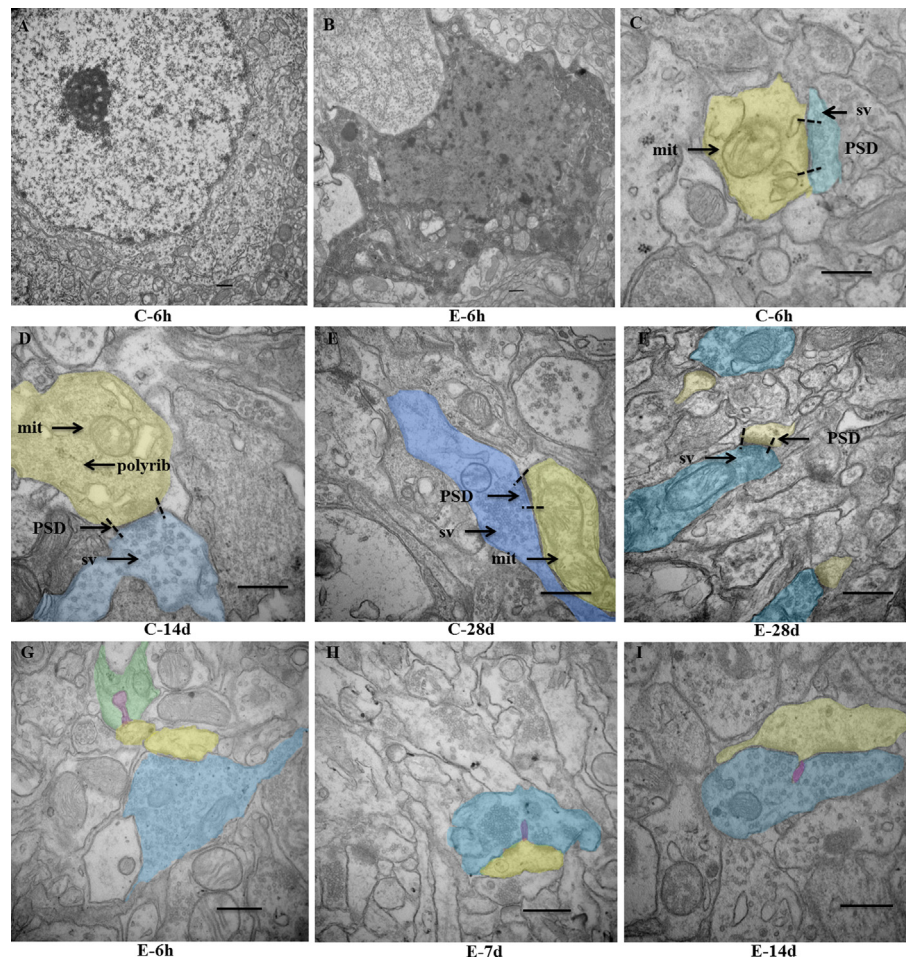


Fig. 4. Ultrastructure of hippocampal neurons and dendritic spines at 6 h and 7d after microwave exposure (C: Control groups, E: Exposure groups). A: The neuron at 6 h in control group (scale bar = 100 μ m); B: The neuron at 6 h in exposure group (scale bar = 100 μ m); C, D, E and F: Amplified typical axospinous synapses which postsynaptic element was a dendritic spine; the pseudocolor was used to elaborate the ultrastructure of dendritic spines more clearly, yellow indicated the dendritic spines in hippocampus, while blue was the presynaptic elements (scale bar = 500 nm); G, H and I: Endocytosis via spinules in exposure group, the pseudocolor of yellow and blue represent the same as above, purple indicated the spinules, green was the other cells in hippocampus (scale bar = 500 nm).

unexposed groups. The decrease of PSD length and thickness after microwave exposure, to some extent, echoed the alteration in distribution of dendritic spines and main protein expression of SNK-SPAR pathway, explained the learning and memory disability in rats. As to the inconformity of the change between the PSD length and thickness, it might implying the thickness of PSD was more sensitive to the microwave exposure than length. Meanwhile, it might also be contributed to the fact that the fields TEM observed were much limited compared to the whole hippocampus.

The plasticity of dendritic spines was regulated by many signaling pathways, among which SNK-SPAR is a molecular pathway mediating neuronal homeostatic plasticity during chronically elevated activity. Induced by synaptic activity and targeted to dendritic spines, SNK activity caused the elimination of SPAR protein and itself by phosphorylation and subsequently ubiquitin proteasome pathway, depleted PSD-95 and caused loss of mature dendritic spines and excitatory synapses which was indispensable for memory formation and maintenance. SNK is an immediate-early gene, which mRNA is rapidly up-regulated following increased neuronal activity (Kauselmann et al., 1999). Activity induced gene expression is critical for formation of synaptic plasticity in the brain. Depending on the time which synaptic alteration takes, different forms of synaptic plasticity were mediated by variety molecular mechanisms and depended on gene expression. Short-term synaptic alterations are regulated mainly by post-

translational modification and regulation of existing proteins (Izquierdo et al., 2001), while new gene transcription and protein synthesis are required for long-term synaptic changes to take place (Bozon et al., 2003; Frey et al., 1988). The up-regulated SNK mRNA in this study indicated that the microwave radiation elevated the activity of neurons in hippocampus and the effects lasted not only for several hours, but for a longer period. The results of western blot in present study displayed the dynamic equilibrium of protein synthesis and degradation. SNK was well suited to participate in longer lasting forms of synaptic plasticity. At 6 h after exposure, the level of protein expression might mainly attribute to stress reaction. The expression of SNK at 14d was significantly increased owing to the protein synthesis consistent with the level of mRNA. At 28d, the decrease of SNK after exposure might largely be due to the self-phosphorylation and its own degradation through ubiquitin-proteasome pathway (Pak and Sheng, 2003), which played a key role in homeostatic mediation of synaptic plasticity. SPAR is selective for spiny neurons and is present in about 65% of the mature mushroom-shaped spines, inducing an increase in complexity of spine shape when it is overexpressed. The expression of SPAR showed a decrease at 14d after exposure indicating the loss of spiny neurons in hippocampus which was induced by increase of SNK. While at 28d after exposure, the SPAR has recovered to normal level, which was also consistent with the decrease of SNK. PSD-95 was one of the dominant cytoskeleton protein in

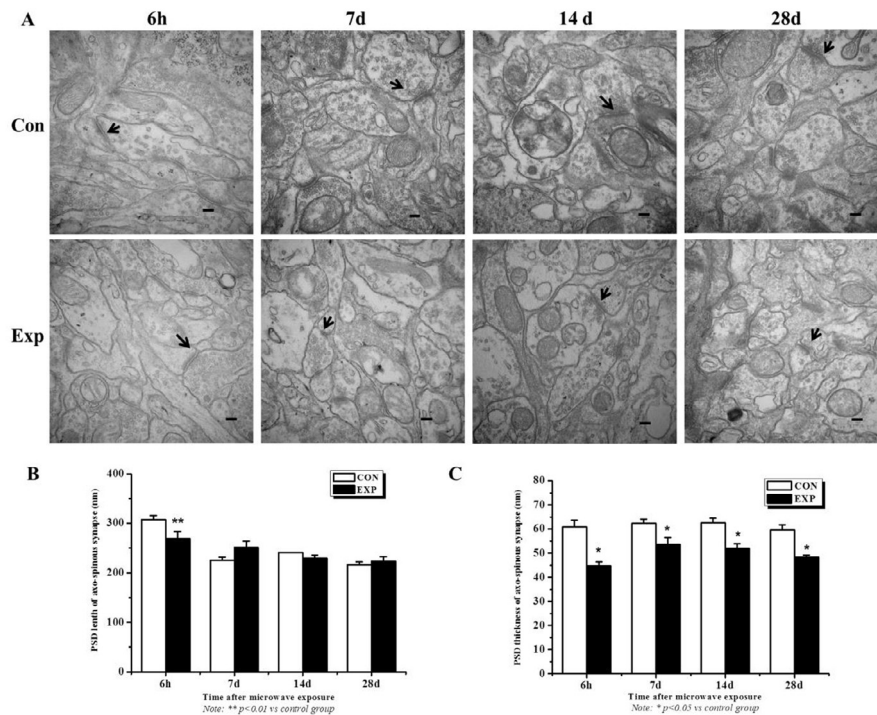


Fig. 5. Effects of microwave on length and thickness of PSD in hippocampal neurons. A: Representative TEM picture of PSD on dendritic spines of each group (6h, 7d, 14d and 28d), labeled by arrow (Con: Control groups, Exp: Exposure groups) (scale bar = 100 nm); B: PSD length of dendritic spines in each group (**p < .01 versus control group); C: PSD thickness of dendritic spines in each group (*p < .05 versus control group).

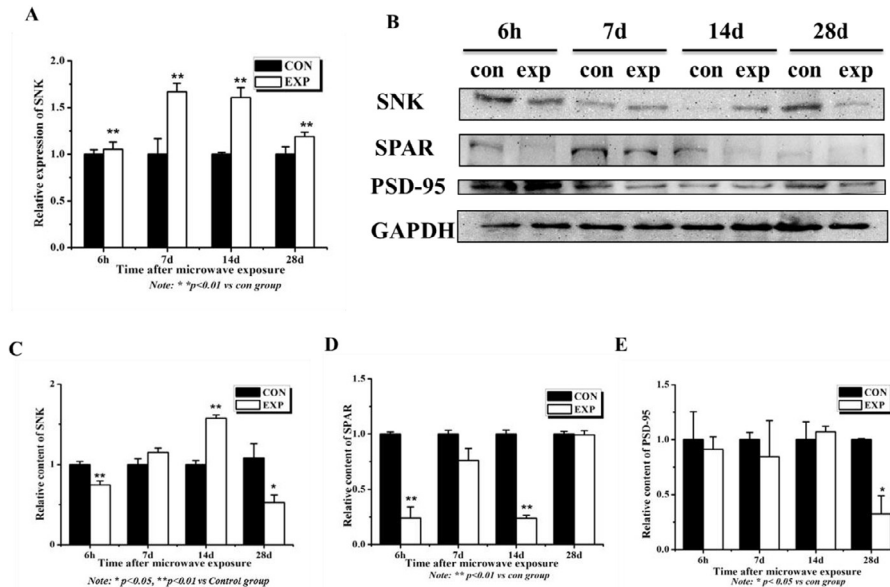


Fig. 6. Expression fluctuation of SNK, SPAR and PSD-95 A: The mRNA level of SNK in hippocampus (**p < .01 versus control group); B-E: Fluctuation of protein expression of SNK, SPAR, PSD-95 (*p < .05 versus control group, **p < .01 versus control group).

dendritic spines mediated by numerous signal pathways, the reduction at 28d after microwave exposure might be the delay effect of increased SNK followed by the triggered reduction of SPAR in earlier period, and the consequence mediated by other associated pathways. The lower expression of PSD-95 in exposure group also implied the shrinkage even loss of mature spines after microwave exposure.

Both SPAR and PSD-95 are indispensable for spine plasticity, thus lack of either of them could cause shrinkage or even loss of

spines, which was consistent with the outcomes in Golgi stain that the density of dendritic spines of granule cells decreased at 14 d and 28 d after exposure or the percentage of mature spines of primary cells decreased at 28d after microwave exposure. As to the decrease of spine density in granule cells at 6 h might be transient stress reaction, while the spine density reduction at 14d was the effects of long term microwave radiation mediated by SNK-SPAR pathway resulted from the decrease of SPAR. And the abnormality of spine plasticity at 28d was the consequences of reduced PSD-95.

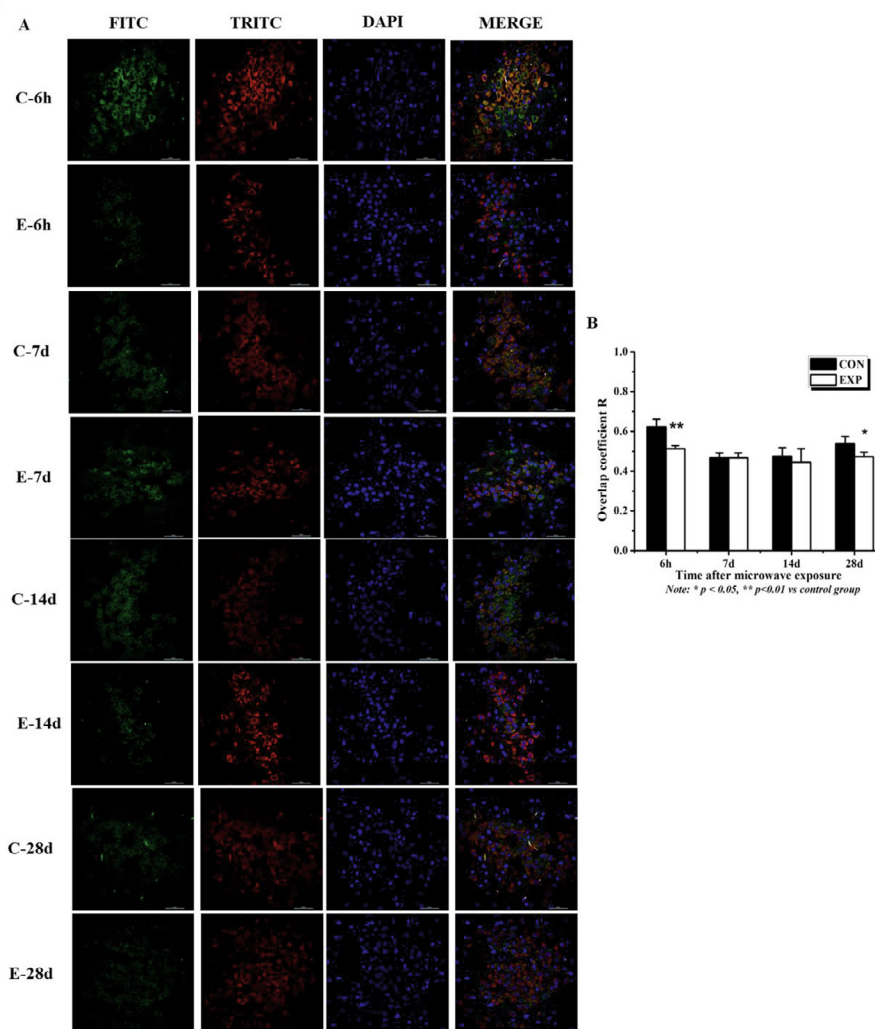


Fig. 7. The colocalization of SPAR and PSD-95 in hippocampus. A: Immunofluorescence photomicrographs of SPAR and PSD-95 in hippocampus (scale bar = 50 μ m). B: The quantification of colocalization. The vertical coordinates of histogram is Manders' overlap coefficient R (** $p < .01$, * $p < .05$ versus control group).

SPAR can recognize the actin cytoskeleton and recruits PSD-95 to F-actin as a bridging molecule between PSD-95 and F-actin which is the prominent cytoskeleton in dendritic spines, and then control spine shape by regulating arrangement of actin (Ehlers, 2002; Pak and Sheng, 2003; Segal, 2001). Either reduction of SPAR or PSD-95 could result in the decrease of overlap coefficient and the extent of interaction of these two proteins. In our study, the interaction of these two proteins was attenuated at 6 h and 28 d after exposure. Despite the shortly decrease at 6 h owing to stress, the reduction at 28 d implying that less PSD-95 was submitted to enlarge the dendritic spines which explained the change of spine distribution of neurons in Golgi stain and echoed the level of protein expression of PSD-95 described above. However, the decrease at 14 d of SPAR expression and density in granule cells was not reflected in immunofluorescence, which might be contributed to the different subregions the pictures were taken.

In summary, this study suggested that long-term exposure to microwave (average power density of 30 mW/cm², SAR = 15 W/kg) induced dysfunction of SNK-SPAR pathway, which was manifested as the abnormal expression or interaction of SNK, SPAR and PSD-95, leading to loss of spines in granule cells and decrease of mature dendritic spines in pyramidal cells, as well as the alteration of ultrastructure such as decrease of PSD length and thickness of dendritic spines in hippocampus neurons, which

conducted to the deficiency of cognitive ability ultimately. However, further studies are still needed to clarify the detailed mechanisms.

4. Experimental procedure

4.1. Animal groups and microwave exposure system

Male Wistar rats which weight ranged in 140–160 g (4 weeks old) were bought from Laboratory Animal Center of Beijing Institute of Radiation Medicine (Beijing, China) and reared in alternate 12 h light/dark mode at 22 ± 2 °C. The animals were treated complying with rules of the Institutional Animal Care and Use Committee. 100 rats were randomly divided into control and microwave exposure groups with 50 rats in each group.

The details of microwave generator had been elaborated in previous report (Li et al., 2015; Wang et al., 2013a). As the previous study have shown the dose-effect relationship between microwave exposure (5, 10, 20, 30 mW/cm²) and cognitive deficiency (Li et al., 2015), thus in this study rats were fixed in radial pattern (Fig. 1) and exposed to 30 mW/cm² microwaves for 6 min (3 times a week, up to 6 weeks). To negate any other type of psychophysiological effects, animals in the sham group were processed in parallel to

the exposed groups, but without microwave exposure. The SAR calculation was based on the finite difference time domain (FDTD) method. In our study, the software for calculating SAR of rats was the simulation platform Empire: IMST-Empire v-4.10 (GmbH, Kamp-Lintfort, Germany). The average SAR of whole-body was calculated to be 15 W/kg.

4.2. Morris water maze (MWM) behavioral test

Morris water maze is a classic approach to evaluate the cognitive capacity of rats (spatial learning and long-term memory). It has been described elsewhere (Li et al., 2015; Qiao et al., 2014). The MWM system was composed of a black round pool (diameter in 150 cm) filled with clear water ($23 \pm 0.5^\circ\text{C}$). The pool was covered by thick curtains in order to hide the visual cues outside from rats in the pool. A movable platform (diameter in 12 cm) was placed at the center of a quadrant defined randomly, the platform was 1.5 cm below the surface of water and fixed in the same position throughout the test. Once the microwave exposure lasted for 6 weeks ended, the MWM training sessions started. In five consecutive days' training, rats were forced to swim in the pool until they found the platform. Each rat could get four trials each day which was begun from four starting positions in a fixed order. Rats were released into water facing the wall of swimming pool. Each trial lasted for 60 s at most, if the rats failed to find the platform in 60 s, they would be guided to the platform. All rats could stay on platform for 5 s before next trial. The behavior of rats and relevant data were recorded using a computer-assisted tracking system (SLY-MWM system, Beijing Sunny Instrument Co. Ltd., Beijing, China), and the average escape latency was analyzed to evaluate the learning ability of rats. To test the long-term effects of microwave on spatial memory, 14 days after exposure, the probe test proceeded with the escape platform removed. The rats were placed in the start point farthest to the quadrant which platform was located in navigation test. The time for probe trials was 60 s. Percentage of time in the target quadrant and the times across the position where the platform located in training session were recorded and analyzed. The time schedule and results of MWM test are shown in Fig. 2A.

4.3. Golgi stain and dendritic spine analysis

FD Rapid Golgi Stain kit (FD Neuro Technologies) was adopted for Golgi stain of brain. According to the former studies (Li et al., 2015), 6 h, 7 d 14 d and 28 d after 6 weeks microwave exposure have been chosen as the detecting points to explore the effects within one month of microwave exposure and the mechanisms associated. 3 rats in each group were anaesthetized by injecting sodium pentobarbital (80 mg/kg) into the peritoneum with a 27 gauge, half-inch needle. Brain tissues were quickly removed on ice. Golgi staining was performed according to manufacturer's instructions. In brief, brains were impregnated into solution A and B for 17 days at room temperature shielded from light. 72 h after placing in solution C (in the dark), coronal sections (100 μm) were cut on a freezing microtome (Leica, Wetzlar, Germany) and mounted onto gelatinized slides. After drying in the dark, sections were rehydrated in solutions D and E, then followed dehydration of gradient ethanol of 50%, 75%, 95%, and 100% respectively. Finally, sections were cleared in xylene and covered with resinous mounting media. For quantitative analysis, at least fifteen neurons from three animals of each group were analyzed. Spines were counted at high magnification (100 $\times$ oil objective). Spine density was calculated per 10 μm of dendritic length.

4.4. Transmission electron microscopy (TEM) observation

5 rats in each group were anesthetized as above, 1 mm³ cube of the hippocampus was dissected from the dentate gyrus in the right hemisphere of brain at 6 h, 7 d, 14 d and 28 d after the 6 weeks microwave exposure, in order to detect the morphology alteration after exposure. After fixation in 2.5% glutaraldehyde (Merck, Darmstadt, Germany), the samples proceeded with 1% osmium tetroxide (AppliChem, Gatersleben, Germany) graded ethyl alcohol and then embedded with EPON618 (TAAB Laboratories Equipment, Berks, UK). The sections on copper meshes were stained by uranyl acetate and lead citrate (Advanced Technology & Industrial Co. Ltd, Hong Kong, China) for contrast. After getting dried, the specimens were observed on a TEM (H-7650, Tokyo, Japan) (Wang et al., 2013b). For the thickness and length of PSD quantitative analysis, 50 dendritic spines of each group were analyzed.

4.5. Total RNA isolation and real-time RT-PCR

Total RNA of hippocampal tissue was isolated from the left hemisphere of 5 rats' brains which were the same ones used in TEM in each group sacrificed as described above at 6 h, 7 d, 14 d and 28 d after microwave exposure, and extracted with Trizol, which was then reverse transcribed using a RevertAid first strand cDNA synthesis kit (Thermo Scientific, USA). Real-Time RT-PCR was carried out using a thermocycler (Applied Biosystems 7300 Real-Time PCR System, USA). The reaction parameters were as follows: the denaturing step at 95°C for 10 min, followed by 40 cycles annealing step at 95°C for 15 s and 60°C for 30 s. GAPDH gene was the internal reference. The relative quantitative value was calculated by comparing target gene expression with control group, and used for statistical analysis. The primer sequences were as follow (5'-3'): SNK: forward: CTTTCAACAACGGCGCTCA, reverse: ATTGTTCGGGGCATCTGTT; GAPDH: forward: GCTACTGGGAGACGAGTTC, reverse: CATGGTGGAGACGTGGAACA.

4.6. Protein extraction and western blot

The hippocampus used in this part was the same ones used in RNA extraction, which was excised from the left hemisphere of brain in 5 rats. Samples were homogenized in 200 μl RIPA lysate (Applygen Technologies, BJ, China) with 1% (v/v) protease inhibitor cocktail (Selleck Chemicals, USA) and lysed for 45 min on ice. Then the samples were centrifuged at 12,000 rpm for 15 min at 4°C . A bicinchoninic acid (BCA; Roche Applied Science) protein assay was adopted for quantification of protein. Then the protein was denatured for 10 min at 100°C in $4 \times$ sample buffer.

Proteins (50 μg) from each group were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at the concentration of 8% and then transferred onto polyvinylidene fluoride (PVDF) membranes (Merk Millipore, Billerica, MA, USA). The membrane was processed in 5% low fat milk for 1 h and probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10,000 dilution, Bioworld) rabbit polyclonal primary antibody, anti-SNK (E-10) antibody (1:100 dilution, Santa Cruz), anti-SPAR (N-20) antibody (1:500 dilution; Santa Cruz Biotechnology), anti-PSD-95 (N-18) antibody (1:500 dilution, Santa Cruz Biotechnology). Bands were developed by an enhanced chemiluminescence detection system with Fluor Chem FC2 (Alpha Innotech) and quantified by AlphaVIEW SA. The intensities of target bands were analyzed refer to the GAPDH intensities from the same sample.

4.7. Immunofluorescence

5 rats in each group was anesthetized as above at 6 h, 7 d, 14 d and 28 d after microwave exposure, and then perfused by heparin

in 0.1 M phosphate buffer saline (PBS, pH 7.4) and then fixed with 4% paraformaldehyde (PFA) from the left ventricle using a peristaltic pump. Then the brain was removed and post-fixed in PFA for 2 h at 4 °C, followed by 30% sucrose PBS solution. Subsequently, the brain was cut into transverse slices (20 µm). With a fine brush, sections were mounted on adhering slides dried at room temperature and then transferred into acetone methanol (1:1) solution for 10 min. Repair the antigen in citrate buffer for 15 min in microwave oven, permeabilize with 0.2 % Triton X-100 in PBS for 30 min at room temperature, block with blocking buffer (10% BSA, 0.2% Triton X-100 in PBS) for 1 h at room temperature in slide holder, incubate with primary antibodies diluted in blocking buffer (as below): anti-SPAR (N-20) antibody (1:300 dilution, Santa Cruz Biotechnology), anti-PSD-95 antibody (1:100 dilution, Cell Signaling Technology). Keep in black slide holder, covered at 4 °C overnight. Incubate with secondary antibody diluted in PBS for 30 min at room temperature. Then mount slides with DAPI mounting media (Vectashield Laboratories, USA), detect signals with laser scanning confocal microscope (Nikon Ti A1). For quantitative analysis, about 10 fields of each group were analyzed.

4.8. Data analysis

Data were analyzed by SPSS 16.0 software. Repeated-measures ANOVA and Post-hoc test was adopted for the analysis of AEL in MWM test. One-way ANOVA (with Post-hoc multiple comparisons) was used to compare the differences among control and exposure groups for data obeying normal distribution. Mann-Whitney *U* test and Kruskal-Wallis test were used to compare the other data. The accepted level of significance for all tests was $p < .05$.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [grant number: 61501492].

Disclosure

The authors declare no conflict of interest.

References

- Bourne, J., Harris, K.M., 2007. Do thin spines learn to be mushroom spines that remember? *Curr. Opin. Neurobiol.* 17, 381–386.
- Bourne, J.N., Harris, K.M., 2011. Coordination of size and number of excitatory and inhibitory synapses results in a balanced structural plasticity along mature hippocampal CA1 dendrites during LTP. *Hippocampus* 21, 354–373.
- Bozon, B., Kelly, A., Josselyn, S.A., Silva, A.J., Davis, S., Laroche, S., 2003. MAPK, CREB and zif268 are all required for the consolidation of recognition memory. *Philos. Trans. R. Soc. London B: Biol. Sci.* 358, 805–814.
- Cahill, M.E., Remmers, C., Jones, K.A., Xie, Z., Sweet, R.A., Penzes, P., 2013. Neuregulin1 signaling promotes dendritic spine growth through kalirin. *J. Neurochem.* 126, 625–635.
- Chang, F.L.F., Greenough, W.T., 1984. Transient and enduring morphological correlates of synaptic activity and efficacy change in the rat hippocampal slice. *Brain Res.* 309, 35–46.
- Chicurel, M.E., Harris, K.M., 1992. Three-dimensional analysis of the structure and composition of CA3 branched dendritic spines and their synaptic relationships with mossy fiber boutons in the rat hippocampus. *J. Comp. Neurol.* 325, 169–182.
- Chutabhakdikul, N., Surakul, P., 2013. Prenatal stress increased Snk Polo-like kinase 2, SCF β -TrCP ubiquitin ligase and ubiquitination of SPAR in the hippocampus of the offspring at adulthood. *Int. J. Dev. Neurosci.* 31, 560–567.
- Deller, T., Merten, T., Roth, S.U., Mundel, P., Frotscher, M., 2000. Actin-associated protein synaptopodin in the rat hippocampal formation: Localization in the spine neck and close association with the spine apparatus of principal neurons. *J. Comp. Neurol.* 418, 164–181.
- Desmond, N.L., Levy, W.B., 1986. Changes in the numerical density of synaptic contacts with long-term potentiation in the hippocampal dentate gyrus. *J. Comp. Neurol.* 253, 466–475.
- Ehlers, M.D., 2002. Molecular morphogens for dendritic spines. *Trends Neurosci.* 25, 64–67.
- Eliyahu, I., Luria, R., Hareuveni, R., Margalio, M., Meiran, N., Shani, G., 2006. Effects of radiofrequency radiation emitted by cellular telephones on the cognitive functions of humans. *Bioelectromagnetics* 27, 119–126.
- Fifková, E., Anderson, C.L., 1981. Stimulation-induced changes in dimensions of stalks of dendritic spines in the dentate molecular layer. *Exp. Neurol.* 74, 621–627.
- Frey, U., Krug, M., Reymann, K.G., Matthies, H., 1988. Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. *Brain Res.* 452, 57–65.
- Gipson, C.D., Olive, M.F., 2016. Structural and functional plasticity of dendritic spines – root or result of behavior? *Genes Brain Behav.*
- Gulley, R., Reese, T., 1981. Cytoskeletal organization at the postsynaptic complex. *J. Cell Biol.* 91, 298–302.
- Harris, K.M., Weinberg, R.J., 2012. Ultrastructure of synapses in the mammalian brain. *Cold Spring Harbor Perspect. Biol.* 4, 570–581.
- Hruska, M., Dalva, M.B., 2012. Ephrin regulation of synapse formation, function and plasticity. *Mol. Cell. Neurosci.* 50, 35–44.
- Inestrosa, N.C., Varela-Nallar, L., 2015. Wnt signalling in neuronal differentiation and development. *Cell Tissue Res.* 359, 215–223.
- Izquierdo, P., Esquena, J., Tadros, T.F., Dederen, C., Garcia, M.J., Azemar, N., Solans, C., 2001. Formation and stability of nano-emulsions prepared using the phase inversion temperature method. *Langmuir* 18, 26–30.
- Kauselmann, G., Weiler, M., Wulff, P., Jessberger, S., Konietzko, U., Scafidi, J., Staubli, U., Bereiter-Hahn, J., Strebhardt, K., Kuhl, D., et al., 1999. The polo-like protein kinases Fnk and Snk associate with a Ca²⁺- and integrin-binding protein and are regulated dynamically with synaptic plasticity. *Embo J.* 18, 5528–5539.
- Landis, D., Reese, T.S., 1983. Cytoplasmic organization in cerebellar dendritic spines. *J. Cell Biol.* 97, 1169–1178.
- Li, H.-J., Peng, R.-Y., Wang, C.-Z., Qiao, S.-M., Yong, Z., Gao, Y.-B., Xu, X.-P., Wang, S.-X., Dong, J., Zuo, H.-Y., 2015. Alterations of cognitive function and 5-HT system in rats after long term microwave exposure. *Physiol. Behav.* 140, 236–246.
- Li, Z., Peng, R.Y., Wang, S.M., Wang, L.F., Gao, Y.B., Ji, D., Xiang, L., Su, Z.T., 2012. Relationship between cognition function and hippocampus structure after long-term microwave exposure. *Biomed. Environ. Sci.* 25, 182–188.
- Lu, Y., Xu, S., He, M., Chen, C., Lei, Z., Liu, C., Fang, C., Yu, Z., Zhou, Z., Min, Z., 2012. Glucose administration attenuates spatial memory deficits induced by chronic low-power-density microwave exposure. *Physiol. Behav.* 106, 631–637.
- MacAskill, A.F., Atkin, T.A., Kittler, J.T., 2010. Mitochondrial trafficking and the provision of energy and calcium buffering at excitatory synapses. *Eur. J. Neurosci.* 32, 231–240.
- Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* 11, 47–60.
- Ning, W., Chiang, H., Yang, W., 2007. Effects of GSM 1800 MHz on dendritic development of cultured hippocampal neurons. *Acta Pharmacol. Sin.* 28, 1873–1880.
- Ostroff, L.E., Fiala, J.C., Allwardt, B., Harris, K.M., 2002. Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. *Neuron* 35, 535–545.
- Pak, D.T., Sheng, M., 2003. Targeted protein degradation and synapse remodeling by an inducible protein kinase. *Science* 302, 1368–1373.
- Paulin, J.J., Haslehurst, P., Fellows, A.D., Liu, W., Jackson, J.D., Joel, Z., Cummings, D. M., Edwards, F.A., 2016. Large and small dendritic spines serve different interacting functions in hippocampal synaptic plasticity and homeostasis. *Neural Plast.* 2016, 1–11.
- Qiao, S., Peng, R., Yan, H., Gao, Y., Wang, C., Wang, S., Zou, Y., Xu, X., Zhao, L., Dong, J., 2014. Reduction of phosphorylated synapsin I (Ser-553) leads to spatial memory impairment by attenuating GABA release after microwave exposure in wistar rats. *PLoS One* 9, e95503.
- Sala, C., Segal, M., 2014. Dendritic spines: the locus of structural and functional plasticity. *Physiol. Rev.* 94, 141–188.
- Schikorski, T., Stevens, C.F., 2001. Morphological correlates of functionally defined synaptic vesicle populations. *Nat. Neurosci.* 4, 391–395.
- Seeburg, D.P., Feliu-Mojer, M., Gaiottino, J., Pak, D.T., Sheng, M., 2008. Critical role of CDK5 and Polo-like kinase 2 in homeostatic synaptic plasticity during elevated activity. *Neuron* 58, 571–583.
- Segal, M., 2001. New building blocks for the dendritic spine. *Neuron* 31, 169–171.
- Shahin, S., Banerjee, S., Singh, S.P., Chaturvedi, C.M., 2015. 2.45 GHz Microwave radiation impairs learning and spatial memory via oxidative/nitrosative stress induced p53-dependent/independent hippocampal apoptosis: molecular basis and underlying mechanism. *Toxicol. Sci.* 148, 380–399.
- Sheng, Morgan, Kim, Eunjoon, 2011. The postsynaptic organization of synapses. *Neurobiology.*
- Spacek, J., Harris, K.M., 1997. Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. *J. Neurosci.* 17, 190–203.
- Sui, H., Lu, X.G., Zhan, L.B., Jiang, W.Z., Qi, X., Gong, X.Y., Niu, X.P., 2010. Decreased expression of spine-associated Rap guanosine triphosphatase-activating protein (SPAR) in glutamate-treated primary hippocampal neurons. *J. Clin. Neurosci.* 17, 1042–1046.
- Tao-Cheng, J.H., Gallant, P.E., Brightman, M.W., Dosemeci, A., Reese, T.S., 2007. Structural changes at synapses after delayed perfusion fixation in different regions of the mouse brain. *J. Compar. Neurol.* 501, 731–740.
- Vorobyov, V., Janac, B., Pesic, V., Prolic, Z., 2010. Repeated exposure to low-level extremely low frequency-modulated microwaves affects cortex-hypothalamus interplay in freely moving rats: EEG study. *Int. J. Radiat. Biol.* 86, 376–383.

- Wang, H., Peng, R., Zhou, H., Wang, S., Gao, Y., Wang, L., Yong, Z., Zuo, H., Zhao, L., Dong, J., 2013a. Impairment of long-term potentiation induction is essential for the disruption of spatial memory after microwave exposure. *Int. J. Radiat. Biol.* 89, 1100–1107.
- Wang, H., Peng, R., Zhou, H., Wang, S., Gao, Y., Wang, L., Yong, Z., Zuo, H., Zhao, L., Dong, J., Xu, X., Su, Z., 2013b. Impairment of long-term potentiation induction is essential for the disruption of spatial memory after microwave exposure. *Int. J. Radiat. Biol.* 89, 1100–1107.
- Wu, L., Sun, C., Zhang, Y.M., Fan, M., Xu, J., Ma, H., Zhang, J., 2007. Involvement of the Snk-SPAR pathway in glutamate-induced excitotoxicity in cultured hippocampal neurons. *Brain Res.* 1168, 38–45.
- Xiong, L., Sun, C.F., Zhang, J., Gao, Y.B., Wang, L.F., Zuo, H.Y., Wang, S.M., Zhou, H.M., Xu, X.P., Ji, D., 2015. Microwave exposure impairs synaptic plasticity in the rat hippocampus and PC12 cells through over-activation of the NMDA receptor signaling pathway. *Biomed. Environ. Sci.* 28, 13–24.
- Zee, E.A.V.D., 2014. Synapses, spines and kinases in mammalian learning and memory, and the impact of aging. *Neurosci. Biobehav. Rev.* 50, 77–85.
- Zinchuk, V., Grossenbacher-Zinchuk, O., 2009. Recent advances in quantitative colocalization analysis: Focus on neuroscience. *Prog. Histochem. Cytochem.* 44, 125–172.