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Acute exposure to high-peak-power pulsed microwaves affecting the histamine H_3 receptor expression in rat hippocampus

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Abstract In the Morris Water Maze test, high-peak-power pulsed microwave (MW)-exposed rats displayed some learning and memory behavior dysfunctions, and their escape time and swimming distance to the submerged platform were longer than those of the sham-exposed rats. To understand the molecular mechanism involved, the reverse transcription-polymerase chain reaction (RT-PCR) and the Western-blotting technique were used for investigating the mRNA and protein expression patterns of the histamine H_3 receptor (H_3R) in rat hippocampus. High-peak-power pulsed microwave-exposure did not remarkably lead to the change in expression of H_3R mRNA in rat hippocampi; however, it promoted the up-regulatory expression of the H_3R protein, which was possibly triggered through the mitogen-activated protein kinase (MAPK) pathways. Therefore, further investigation of the molecular mechanism of the MW effects on the learning and memory behaviors is required.

Key words Histamine H_3-receptor, Rat hippocampus, Microwave, Expression

CLC numbers R122, R122.4

1 Introduction

Numerous *in-vivo* and *in-vitro* studies have been performed to investigate the effects on the biological system resulting from exposure to microwaves (MW), including the use of animal models, the cultured cell model, as well as human volunteers [1]. Some of these were focused on the central nerve system (CNS), especially on the learning and memory behaviors of MW-exposed rats. Similarly, MW at different doses reported by many laboratories using the diverse methods and experimental designs [2] may affect the learning and memory behaviors in rats and mice [3]. These will require considerably more researches for a complete understanding of the molecular mechanisms involved in the learning and memory effects of the MW exposure.

The hippocampus is directly involved in synaptic plasticity and cognitive functions[4]. The N-methyl D-aspartate (NMDA) receptor is a crucial receptor in the production of long-term potential (LTD) in post-synapse, but the formation of the learning and memory behaviors requires more complex interactions among histaminergic, cholinergic, dopaminergic, GABAergic, and other systems [5,6]. In the recent years, many studies have reported that as one of the four histamine receptors (H_1R, H_2R, H_3R, and H_4R), in rat hippocampi, H_3R plays an important role in the learning and memory behaviors[7,8]. The development of H_3R knockout mice has identified the importance of H_3R in the memory processes [9].

Since H_3R predominantly located presynaptically as an autoreceptor was originally discovered in 1983[10], it has been shown to be involved in the regulation of the release of various neurotransmitters (his-

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tamine, γ-aminobutyric acid, noradrenaline, 5-HT, and acetylcholine). As a member of the G-Protein-coupled receptor family of proteins [11], when H_3R was coupled to Gαί, it inhibited the production of cAMP[12]. Rat H_3R cDNA was successfully cloned by Lovenberg *et al* and Drutel *et al*[13,14] in 2000, and therefore, it is now feasible to investigate the mRNA and protein expressions of the H_3R gene in rat hippocampi.

In the present study, the expression pattern of the H_3R gene in rat hippocampi at different times after acute exposure to pulsed MW was investigated at the molecular level. The H_3R mRNA and its proteins were analyzed at different times by the reverse transcription-PCR (RT-PCR) and Western-blotting analysis, and their expression patterns were compared after acute exposure to pulsed MW.

2 Methods and materials

2.1 Animals

Male Sprague–Dawley rats (2 months old, 200—220 g) were purchased from the Experimental Animal Center of Third Military Medicine University, Chongqing, China. The rats were housed in a room with a 12-h light–dark cycle (light on from 7:00 to 19:00 h) at an ambient temperature of 22°C. The animals were provided with food and water during the experiment.

2.2 MW exposures

Thirty rats were simultaneously exposed to pulsed MW, and 10 rats were exposed to sham for 20 min. The MW-exposed rats were exposed to 2.45 GHz pulsed microwaves with a pulse duration of 50 ns and a peak power density of 3.5–6.0 $kW \cdot cm^{-2}$. Owing to the extremely short duration of the pulses, the specific absorption rate (SAR) of the local and whole body could not be measured [15]. The rats were decapitated at different times of 0.08 h, 3 h, 6 h, 12 h, and 24 h post-exposure. During the sham exposures, the animals were placed in similar waveguides for the same periods of time as the microwave-exposed animals, except that the waveguides were not activated.

2.3 Morris Water Maze

The water maze was a steel circular pool filled with water (22°C) to a depth of 28 cm. A steel platform was placed at the center of the S-E quadrant of the maze and was submerged 5 cm below the surface of the water. In each training session, the animals were exposed to MW ($n=10$ rats) or were sham-exposed ($n=10$ rats) for 20 min in the waveguides. The sessions and modes of the training set of rats were as described by Wang *et al* [3]. The traces were recorded using a video recorder, and the escape time was measured using a stop watch.

2.4 RT-PCR

At different time phases of 0.08 h, 3 h, 6 h, 12 h, and 24 h after exposure to pulsed MW, all rats were decapitated and their hippocampi were isolated; the intact rat hippocampi were then homogenized in a glass homogenizer on ice. RNA was extracted with Tripure (Roche Co., Germany), and the concentration of RNA was evaluated by the ratio of the A260/A280 OD values on the spectrophotometer (DU640, Beckman Co., USA). For each sample, the expression of H_3R mRNA was analyzed using PCR with the primers: 5'-ACAGGTATGGGGTGGGTGAG-3' (forward) and 5'-TGTAGTGGCACAGTGGGTAG-3' (reverse). The length of the H_3R PCR products was 435 bp. From the control β-actin mRNA, a 250-bp region was amplified with the primers: 5'-TAAAGACCTCTATGCCAACACAGT-3' (forward) and 5'-CACGATGGAGGGGCCGGACTCATC-3' (reverse). The reaction mixtures were all used at a 25 μL reaction volume (each sample contained 4 μg mRNA). The mixtures contained 2.5 μL PCR buffer, 5 μL $MgCl_2$ (25 mmol/L), 2.5 μL dNTPs (10 mmol/L), 0.5 μL RNAase inhibitor (40 U/μL), 0.5 μL AMV Rtase XL (5 U/μL), 0.5 μL AMV- optimized Taq polymerase (5 U/μL), 25 pmole H_3R primers (or 10 pmole β-actin primers), and 20 μL of RNase-free dH_2O (TaKaRa RNA PCR Kit, TaKaRa Biotechnology Co., Japan). The RT-PCR conditions were: reverse transcription at 50°C for 30 min, denaturation at 94°C for 3 min, 35 cycles at 94°C for 45 s/58°C for 45 s/72°C for 45 s, and the final elongation at 72°C for 10 min.

The electrophoresis of the PCR products was performed on a 1.5% agarose gel stained with EB. The ratio of the H_3R products to the β-actin products was

evaluated using digital photographs (inverted phase) prepared using a Gel Doc2000 scanner system (Bio-Rad, USA) and was calculated according to the following formula: (mean intensity of the H_3R band − mean background)/(mean intensity of the β-actin band − mean background).

2.5 Western-blotting

The expression of H_3R proteins in rat hippocampus at 0.08 h, 3 h, 6 h, 12 h, and 24 h after exposure to pulsed MW was examined using Western-blotting analysis. At different times after exposure, the rats were decapitated, and their hippocampi were isolated and immediately lysed in the membrane protein lysis solution containing 50 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 32 mmol/L sucrose, 1 mmol/L EDTA, 1% NP-40 (Sigma Chemical Co., USA), 1% sodiumdesoxycholate, 0.1% SDS, 1 μg/mL leupeptin (Sigma), 1 μg/mL aprotinin (Sigma), and 1 mmol/L phenylmethylsulfonyl fluoride, and then synchronously homogenized in a glass homogenizer on ice. The lysates were incubated for 30 min on ice and then centrifuged for 10 min at 800g at 4°C. The supernatant was then centrifuged for 20 min at 20000 g at 4°C. The sediments were resoluted in 100 μL lysis buffer and stored at -70°C. The protein concentration was determined by the Lowry method using BSA (Sigma) as a standard. Twenty milligrams of proteins from each sample were separated using 10% SDS–PAGE, which was subsequently followed by electroblotting onto a PVDF membrane (Roche Co., Germany) for 3 h at 4°C at 150 mA. The PVDF membranes were then saturated with 5% non-fat dried milk, 1.5% BSA in TBS (20 mmol/L Tris–HCl, 137 mmol/L NaCl, pH 7.6) − 0.1% Tween-20 (TBS-T; Sigma) for 1 h at room temperature. The PVDF membranes were then rinsed in TBS-T and incubated overnight at 4°C with 1:300 diluted polyclonal anti-H_3R antibody (Santa Cruz Biotechnology, USA). The membranes were washed thrice (each for 5 min) with TBS-T to remove the unbound antibody and were incubated for 1 h in the presence of biotinylated anti-goat IgG (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) diluted 1:300 at 37°C. The membranes were washed thrice (each for 5 min) with TBS-T and were incubated for 1 h in horseradish peroxidase (HRP) Streptavidin anti-mouse antibody (Beijing Zhongshan Golden Bridge Biotechnology, China). After several washings, the HRP activity was detected using the DAB kit, and the density of each band was scanned and analyzed using a Gel Doc2000 scanner system (Bio-Rad, USA).

2.6 Data analysis

Statistical analysis was performed using the SPSS software. All data were expressed as mean ± standard error. Each experimental group was compared with its matched control group by using the paired *t*-test. The values of $P < 0.05$ were regarded as significant based on the ANOVA test.

3 Results

3.1 Morris Water-Maze

The results of the escape time during the six sessions are shown in Fig. 1. It shows a significant decrease in the escape time with the training sessions in each group ($P < 0.05$), that is, a significant session effect. Besides, the mean escape time over the training sessions of the MW-exposed animals was found to be significantly longer than that of the sham-exposed animals ($P < 0.05$). It was observed that during the training sessions, the MW-exposed rats had a tendency to spend considerably more time in intentionally climbing up the wall or swimming along the wall of the pool. The tracings of the swimming pattern of the MW-exposed animals and the sham-exposed animals were different from each other. The MW-exposed animals searched less for the submerged platform in the S-E quadrant and swam more over the other area of the maze.

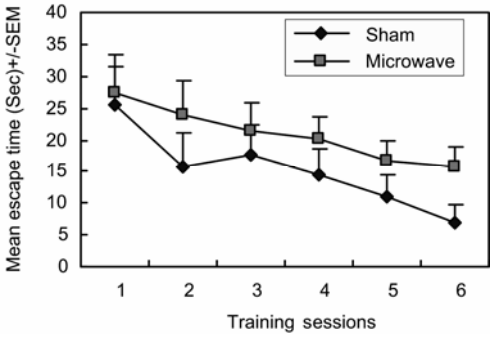

Fig.1 Average escape time (time to reach the submerged platform after being sent into the water) of the microwave-exposed and the sham-exposed rats. The number of rats in each group was 10.

3.2 Effect of the pulsed MW exposure on the H_3R mRNA expression in rat hippocampi

The product of the H_3R gene fragment amplified using RT-PCR was 435 bp, which appeared on 1.5% agarose electrophoresis and was identical to the fragment length designed before the experiment (Fig.2). When compared with the sham-exposed, at times of 0.08 h, 3 h, 6 h, 12 h, and 24 h after exposure to pulsed MW for 20 min, the mRNA expressions of the H_3R gene in rat hippocampi did not change significantly ($p > 0.05$) although they all tended to be lower (Fig.2).

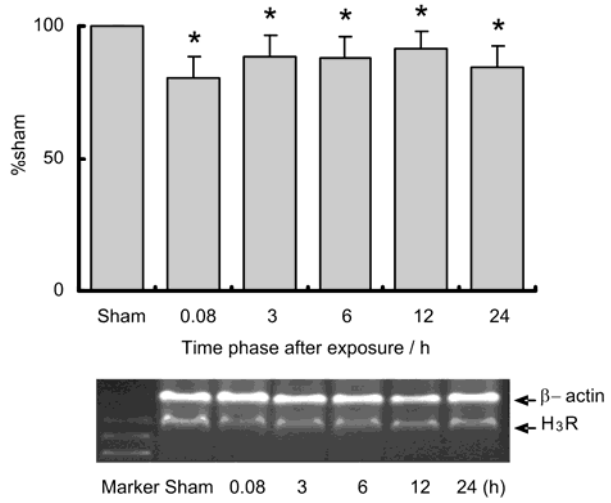

Fig.2 Effect of the pulsed microwave on the mRNA expression of the H_3R gene in rat hippocampi: the relative mRNA levels of the H_3R gene at the different time phases (0.08 h, 3 h, 6 h, 12 h, and 24 h) after exposure to pulsed MW or after sham exposure were analyzed by RT-PCR (n=6 experiments, with two parallels each). The data were plotted (in %) in ratios of H_3R to β-actin mRNA levels. The statistical significance was tested by the ANOVA for significance levels of 5% (*P > 0.05).

3.3 Effect of the pulsed MW exposure on the H_3R protein expression in rat hippocampi

When compared with the standard protein molecular weight markers, the H_3R protein bands with molecular weights of about 60 kD were determined on SDS–PAGE; the Western-blotting was then performed, and its outcome is shown in Fig. 3. After exposure to pulsed MW for 20 min, the H_3R protein expression in rat hippocampi was significantly reduced immediately ($p < 0.05$) at 0.08 h after exposure. However, it was up-regulated significantly at all other times after exposure, and the highest level of its expression was observed at 24 h after exposure (Fig.3).

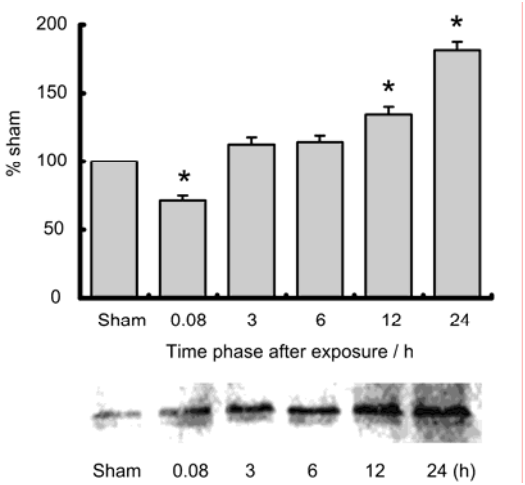

Fig.3 Effect of the pulsed microwave on the protein expression of the H_3R gene in rat hippocampi: the relative protein levels of the H_3R gene at the different time phases (0.08 h, 3 h, 6 h, 12 h, and 24 h) after exposure to pulsed MW or after sham exposure were analyzed by Western-blotting (n = 6 experiments, with 2 parallels each). The statistical significance was tested by the ANOVA for significance levels of 5% (* $P < 0.05$).

4 Disscusion

Others have reported that the MW exposure has some effect on the hippocampi in the rat's CNS, and it influences the learning and memory behaviors [3, 16]. It was also observed that the MW-exposed rats displayed some learning and memory behavior effects indicated by the fact that the escape time and the swimming distance to reach the submerged platform were longer than those of the sham-exposed in the Morris Water Maze test (Fig.1). However, the molecular mechanisms involved are not clearly known.

H_3R is a member of the G-Protein-coupled receptor family of proteins [11]. It couples with Gai and inhibits cAMP production [13] and has key modulatory roles in the release of neurotransmitters and has been implicated in arousal and cognition. The activation of the central H_3R using the selective agonist (R0-a-MeHA) inhibits the synaptic release of histamine and impairs cognition in object recognition and passive paradigms in the rodent [17,18].

When compared to the sham-exposed, at 0.08 h, 3 h, 6 h, 12 h, and 24 h after exposure to pulsed MW for 20 min, the mRNA expression of the H_3R gene in rat hippocampi did not change significantly ($p > 0.05$) although there was a slight difference between the sham-exposed and the MW-exposed (Fig.2). These data possibly showed that the exposure to pulsed MW

for 20 min on rats did not significantly affect the transcription of the H_3R gene in rat hippocampi, or atleast the H_3R gene structure was not impaired.

The protein expression of the H_3R gene in rat hippocampi was immediately depressed significantly ($p < 0.05$) at the time phase of 0.08 h (Fig. 3); however, after the 0.08-h phase, it was up-regulated significantly at the phases of 3 h, 6 h, and 12 h and best significantly at the phase of 24 h (Fig.3).These data indicated that the effect of the exposure to pulsed MW for 20 min on rats led to an abnormal translation level of the H_3R gene in rat hippocampi. After pulsed MW exposure, the protein expression of the H_3R gene in rat hippocampi was immediately down-regulated and later restored to the normal level or even above it; thus, the protein expression responded acutely to the pulsed MW exposure.

Activation of H_3R also leads to the activation of MAP kinase cascade via PTX-sensitive G proteins[14]. Linking H_3R to the MAPK pathway is believed to be important in neuronal plasticity and is activated in hippocampal long-term potentiation. H_3R is known to be involved in the learning and the memory processes[9]. Hence, the strong expression of H_3R in the hippocampi of MW-exposed rats is probably involved in the MAPK pathway. These results will result in additional complexity in understanding the role of H_3R in the process of MW exposure inducing learning and memory dysfunction.

In conclusion, pulsed MW at the above-mentioned dosage did not significantly led to the change in expression of H_3R mRNA in rat hippocampi, but promoted the up-regulatory expression of the H_3R protein, which was possibly triggered through the MAPK pathway. This requires further investigation of the molecular mechanisms of the MW effects on the learning and memory behaviors.

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