Relationship between Cognition Function and Hippocampus Structure after Long-term Microwave Exposure*

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Abstract

Objective To analyze the effects of long-term microwave exposure on hippocampal structure and function in the rat.

Methods Experiments were performed on 184 male Wistar rats (three exposure groups and a sham group). Microwaves were applied daily for 6 min over 1 month at average power densities of 2.5, 5, and 10 mW/cm². Learning and memory abilities were assessed by Morris water maze. High performance liquid chromatography was used to detect neurotransmitter concentrations in the hippocampus. Hippocampal structures were observed by histopathological analysis.

Results Following long-term microwave exposure there was a significant decrease in learning and memory activity in the 7 d, 14 d, and 1 m in all three microwave exposure groups. Neurotransmitter concentrations of four amino acids (glutamate, aspartic acid, glycine, and gamma-aminobutyric acid) in hippocampus were increased in the 2.5 and 5 mW/cm² groups and decreased in the 10 mW/cm² group. There was evidence of neuronal degeneration and enlarged perivascular spaces in the hippocampus in the microwave exposure groups. Further, mitochondria became swollen and cristae were disordered. The rough endoplasmic reticulum exhibited sacculated distension and there was a decrease in the quantity of synaptic vesicles.

Conclusion These data suggest that the hippocampus can be injured by long-term microwave exposure, which might result in impairment of cognitive function due to neurotransmitter disruption.

Key words: Microwave; Hippocampus; Learning and memory; Neurotransmitter

INTRODUCTION

In the modern era, microwaves are widely used in various industrial, communications, medical, and domestic applications. However, there is evidence that microwaves may produce adverse biological effects in the nervous system[1-5], even at low levels of radiation power[15-16]. The hippocampus is suggested to be especially vulnerable to microwave radiation, which may result in memory and learning deficits. Nevertheless, the long-term effects of microwaves on cognitive function remain controversial[6-8]. The neurotransmitters glutamate (Glu), aspartic acid (Asp), glycine (Gly), and gamma-aminobutyric acid (GABA) play critical roles in processes such as associative activity (learning and memory)[9-10], sleep-wake cycle[11], reaction time in behavioral response[12], induction of pain[13], and brain plasticity[14], and are also implicated in various central nervous system (CNS) diseases. In the present study, we examined

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the effects of long-term microwave exposure on hippocampus structure and neurotransmitter content, as well as cognitive function and behavior in rats.

MATERIALS AND METHODS

Experimental Groups and Exposure

One hundred and eighty four male Wistar rats (180±20 g) were obtained from the Laboratory Animal Center (Beijing, China), and were maintained at 22±2 °C with a 12 h light-dark cycle. Food and water were freely available. All protocols were approved by the Institutional Animal Care and Use Committee. Rats were divided randomly into four groups with three exposure groups and a sham group, as follows: (1) Long-term exposure groups (n=138): animals were exposed to microwaves with the average power density of 2.5, 5, or 10 mW/cm² for 6 min daily up to 1 month. The average calculated SAR was 1.05, 2.1, and 4.2 W/kg, respectively. Animals in the exposure groups were subjected to the microwave exposure in individual polypropylene cages. (2) Sham control group (n=46): animals were handled and processed in parallel to the exposure groups, but without microwave exposure.

Morris Water Maze Behavioral Task

There were 10 rats in each group used for behavioral tests. Assessment of behavior was performed on 1 d, 2 d, 3 d, 4 d, 7 d, 14 d, 1 m, 2 m, and 6 m after exposure. Behavioral alterations in different psychophysiological parameters were assessed using behavioral testing equipment, as previously reported [17].

Apparatus

The water maze task was performed in a circular pool (150 cm in diameter) filled with water maintained at 23±0.5 °C in a suitably equipped room with constant temperature, humidity, and brightness. The surface of a clear ferric movable escape platform (12×15 cm) was submerged 1.5 cm below the water surface at a specific location for the entire session. The pool was surrounded by thick curtains to avoid extra maze visual cues for the rats.

Training and Testing Procedures

Rats were trained to find a submerged escape platform, located in a fixed position relative to the extra maze visual cues, during four consecutive daily sessions. Each session consisted of four trials. Four different starting positions, equally spaced around the perimeter of the pool, were used in a fixed order. Each animal was released in the water and positioned to face the wall of the pool. Each trial had a maximum duration of 60 s. During the training sessions, the rats that did not find the platform within 60 s were placed on the platform for 20 s, while during the memory sessions these rats were continued to the next trial.

Recording of Behavior

The behavior in the Morris water maze (MWM) experiments during the training and memory-testing procedures was digitally recorded using the SLY-MWM system (Beijing Sunny Instrument Co. Ltd., Beijing, China), and average escape latency (AEL) was used as the final index.

High Performance Liquid Chromatography (HPLC)

Instrumentation

HPLC experiments were conducted on a model 1050 pump (Hewlett-Packard Components Group, Palo Alto, USA). The injection volume was 20 μL. The column was an Agilent Hypersil (20 cm×2.1 mm) (Agilent Technologies, Santa Clara, USA).

Reagent Preparation

The mobile phase was 0.1 mol/L phosphate buffer solution (pH 6.8) containing 30% methanol, which was delivered at a flow rate of 0.4 mL/min. Water and methanol were HPLC grade (Fisons Corporation-Medical, Fairfax, USA). The mobile phase was filtered through a 0.45 μm Millipore filter and degassed for 15 min. All experiments were performed at room temperature and the pH value was calibrated with a pH meter (Sartorius AG, Denver, Germany). Aspartic acid (Asp), Glutamic acid (Glu), glycine (Gly), and gamma-aminobutyric acid (GABA) were analytical grade (Sigma-Aldrich Co., St. Louis, USA). The derivatizing agent was based on a previously reported method [18]. O-phthalaldehyde (OPA) (22 mg) was dissolved in 0.5 mL of sodium sulfite (1 mol/L), which was then supplemented with 0.5 mL of absolute ethanol and 0.9 mL of sodium tetaborate buffer (0.1 mol/L) adjusted to pH 10.4 with 5 mol/L sodium hydroxide. We found that the most suitable source of OPA was from Sigma, as the OPA from alternative sources contained impurities that contaminated the samples. The reagent was prepared daily and remained stable throughout the working day if kept in a darkened vial. All chemicals were obtained from Sigma, unless otherwise stated.

Sample Processing and Application

To investigate changes in amino acids in the hippocampus, regional samples were hand homogenized in 1 000 μL of ice-cold phosphate buffered solution (PBS) and then centrifuged at 4 °C for 8 min at 1 000 rpm. The supernatant was removed, and 10% salicylsulfonic acid was added at the same volume as the precipitate, and the samples were rehomogenized and incubated on ice for 15 min, then centrifuged at 4 °C for 10 min at 12 000 rpm. Twenty microlitres of supernatant were then added to 100 μL of OPA reagent, and 20 μL
of this solution was transferred into HPLC vials.

**Hematoxylin and Eosin (H&E) Staining**

At 6 h, 7 d, 14 d, 1 m, 2 m, and 6 m after microwave exposure, rats (n=144) were anesthetized (sodium pentobarbital, 50 mg/kg IP), and the brains were removed and fixed in 10% buffered formalin solution, then embedded in paraffin. Five coronal brain sections (5 μm) were prepared including the hippocampal area. The sections were dipped in hematoxylin for 3 min, washed in running tap water for 30 min, and de-stained in warm water for several seconds. The sections were washed again in running water for 15 min, dipped in eosin for 15 s prior to washing again for 20 min, and then dehydrated in an alcohol gradient, followed by xylene clearance and coverslipping. The stained sections were observed under a light microscope, and the hippocampus was photographed at a 200× magnification.

**Transmission Electron Microscopy**

Hippocampal samples (1 mm³ cubes) were dissected from the CA3 area under an anatomical microscope. The samples were then fixed in 2.5% glutaraldehyde, sequentially processed with 1% osmium tetroxide, graded ethyl alcohols, and embedded in EPON618. Ultrathin sections cut onto copper mesh grids were stained with the heavy metals, uranyl acetate, and lead citrate for contrast. After drying, the grids were then viewed on a transmission electron microscope (TEM; HITACHI Ltd, Tokyo, Japan).

**Temperature**

The rectal temperature of rats was measured before and after microwave exposure in the 1 d, 7 d, 14 d, and 1 m during experiments just after the removal of the subjects from the animal holder for both sham and microwave exposure groups.

**Data Analysis**

Data are presented as means±SEM. Differences between the groups were analyzed by analysis of variance (ANOVA) using Statistical Package for the Social Sciences software. Differences at P<0.05 were considered significant.

**RESULTS**

Long-term microwave exposure (6 min daily for 1 m) at 2.5, 5, or 10 mW/cm² had no effect on body temperature at 1 d, 7 d, 14 d, or 1 m when compared to the corresponding sham group animals, suggesting that thermal effects were unlikely in our study.

**Learning and Memory Ability**

Animals exposed to microwaves were unable to retrieve the location of the submerged platform that was learned during the training days. By contrast, sham-exposed rats exhibited a clear preference for the quadrant in which the platform was located during training, showing that they had consolidated the learned information and they could effectively retrieve it. Compared to the sham groups, the AEL of the rats were significantly longer in the 2.5 mW/cm² group at 1 m (P<0.05), in the 5 mW/cm² group at 14 d (P<0.05) and 1 m (P<0.01), and the 10 mW/cm² group at 7 d (P<0.05), 14 d (P<0.05), and 1 m (P<0.01) (Figure 1).

![Figure 1. AELs of rats in the Morris water maze. Compared to the sham group, there was a significant increase in the AEL of the rats in the 2.5 mW/cm² group at 1 m (P<0.05), the 5 mW/cm² group at 14 d (P<0.05) and 1 m (P<0.01), and in the 10 mW/cm² group at 7 d (P<0.05), 14 d (P<0.05), and 1 m (P<0.01). Note that although all groups of animals showed the same overall learning curve, their performance during the first trial of each day indicated that exposed animals exhibited consolidation and/or recall deficits. Group effect: *P<0.05, **P<0.01.](image-url)
Neurotransmitter Content in the Hippocampus

The mean concentrations of Asp, Glu, Gly, and GABA (μg×10^{-2}/mg of brain wet tissue) were determined in rat hippocampus homogenates (n=6) at 6 h, 14 d, and 2 m after exposure. The mean Asp content was increased in the 2.5 mW/cm\(^2\) group at 6 h, 14 d, and 2 m (P<0.05 or P<0.01) and in the 5 mW/cm\(^2\) group at 14 d and 2 m (P<0.01), but was decreased in the 10 mW/cm\(^2\) group at 2 m (P<0.05), after microwave exposure. The mean Glu content was increased in the 2.5 mW/cm\(^2\) and 5 mW/cm\(^2\) groups at 14 d and 2 m (P<0.05 or P<0.01), but was decreased in the 10 mW/cm\(^2\) group at 2 m (P<0.05), after microwave exposure. The mean Gly content was increased in the 2.5 mW/cm\(^2\) and 5 mW/cm\(^2\) groups at 14 d and 2 m (P<0.05 or P<0.01), but did not change in the 10 mW/cm\(^2\) group, after microwave exposure. The mean GABA content was increased in the 2.5 mW/cm\(^2\) and 5 mW/cm\(^2\) groups at 6 h, 14 d, and 2 m (P<0.05 or P<0.01), but was decreased in the 10 mW/cm\(^2\) group at 6 h (P<0.05), after microwave exposure (Table 1).

Table 1. Neurotransmitter Content in the Rat Hippocampus after Microwave Treatment (n=6/group)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Days after Exposure</th>
<th>Groups</th>
<th>Contents (μg×10^{-2}/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>6 h</td>
<td>0</td>
<td>16.86±2.79</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>20.28±1.49\textsuperscript{*}</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>17.46±1.38</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>14.51±2.70</td>
</tr>
<tr>
<td>14 d</td>
<td>0</td>
<td>11.34±0.42</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>18.54±1.53\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>19.08±1.97\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13.16±1.28</td>
</tr>
<tr>
<td>2 m</td>
<td>0</td>
<td>13.74±1.66</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>18.79±2.47\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>18.85±1.52\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.46±0.51\textsuperscript{2}</td>
</tr>
</tbody>
</table>

Note. \textsuperscript{a}Values were calculated from the calibration curves and represent means (n=6) for each case.

Hippocampal Structure

To determine the effect of microwave treatment on hippocampal morphology, we examined the hippocampus by histology. In the sham group, hippocampal neurons exhibited a regular arrangement, with distinct edges, and a clear nucleus and nucleolus, and there was no significant necrosis of pyramidal neurons (Figures 2A and 2C). By contrast, in the microwave exposure groups the hippocampus exhibited edema and raritas. There was also a significant decrease in numbers of hippocampal neurons, and neurons were irregularly arranged with evidence of karyopyknosis (Figures 2B). The vessels in the microwave groups were congestion and showed signs of hemorrhage with an enlarged perivascular space (Figures 2D). The degree of injury peaked at 1 m, and then showed signs of recovery at 6 m, and there was a dose-dependent response to the degree of microwave radiation.

Pathological changes in the hippocampal ultrastructure were observed by TEM. Normal neurons in the hippocampus exhibited a large nucleus with a distinct nuclear envelope and nucleoli, and numerous rough endoplasmic reticulum and mitochondria in the cytoplasm (Figure 3A). In the hippocampus of microwave-exposed rats, individual shrunken cells could be seen with condensed cytoplasm and nucleus. The mitochondria were swollen and vacuolized, and the cristae were disordered and fewer in number. The rough endoplasmic reticulum also exhibited sacculated distension (Figure 3B). Compared with the synapses of hippocampal neurons in sham rats, injured animals exhibited a decrease in the quantity of synaptic vesicles in the synapse, and the synaptic clefts were wider or blurred (Figure 3C). These ultrastructural changes were increased with increasing dose of microwave exposure.
Figure 2. Effect of microwave treatment on morphological changes in the hippocampus. A) and C) Sham group. The hippocampal neurons and vessels exhibited a regular arrangement, with distinct edges, and a clear nucleus and nucleolus, and no significant necrosis of pyramidal neurons. B) 5 mW/cm² group. The hippocampus showed edema, and neurons exhibited pyknosis and anachromasis at 7 d after exposure. D) 10 mW/cm² group. The perivascular spaces in the hippocampus were widened, with signs of hemorrhage (HE staining, original magnification ×200).

Figure 3. Effect of microwave exposure on ultra-structural changes in the hippocampus. A) A normal hippocampal neuron in a sham rat. Numerous rough endoplasmic reticulum and mitochondria presented in the cytoplasm of hippocampal neurons in sham rats (TEM ×18000). B) 5 mW/cm² group. Example of a shrunken cell in the hippocampus of microwave exposed rats at 1 m. Note the appearance of swollen mitochondria with disordered and reduced numbers of cristae, and expanded rough endoplasmic reticulum (TEM ×15000). C) 5 mW/cm² group. Note the swollen synapses with fewer vesicles in the hippocampus of microwave exposed rats at 1 m (TEM ×50000).


DISCUSSION

Although it is well established that exposure to high intensity electromagnetic fields can exert detrimental effects on human health\(^{[19-20]}\), there is also growing evidence for a damaging role of long-term exposure to lower intensity electromagnetic fields. The nervous system, and in particular the hippocampus, is a sensitive target of microwave exposure. However, there are limited data on the risks of exposure to different sources of non-thermal microwaves on the brain. There is some evidence that long-term exposure to non-thermal microwave exposure can affect sensitive vital organs, which is dependent on exposure intensity, frequency of exposure, modulation frequency, and exposure duration\(^{[21]}\). In the present study, we demonstrated that exposure to low-level long-term microwave exposure produced marked alterations in the structure and function of the hippocampus in rats, that were non-thermal in nature.

Changes in behavior and cognition are important outcomes used to assess the effects of microwave exposure on the brain\(^{[22-26]}\). However, there are conflicting data on the effects of long-term microwave exposure on learning and memory abilities due to the variety of exposure parameters, research objects, and conditions used. As such, a detrimental action of long-term microwave exposure on learning and memory remains controversial. For example, chronic microwave exposure (0.05 W/kg daily, 45 min/day, 10 days) in mice was reported to have no effect on learning and memory assessed using the 8-arm radial maze behavioral task\(^{[8]}\), which may relate to the very low SAR value used. Nevertheless, SAR values from 0.02 to 4 W/kg have been previously used to induce and detect memory deficits\(^{[27-29]}\). In the present study, we demonstrated that exposure to low-level long-term microwave exposure produced marked alterations in the structure and function of the hippocampus in rats, that were non-thermal in nature.

The neurotransmitters Asp, Glu, Gly, and GABA are involved in multiple physiological and pathophysiological processes. For example, the inhibitory (Asp and Glu) and excitatory (Gly and GABA) neurotransmitters can regulate inhibitory or excitatory synaptic strength under normal and pathological conditions\(^{[30-31]}\). In the present study, long-term microwave exposure disrupted the normal levels of neurotransmitters in the hippocampus, which may relate to accumulation of metabolic products or damage to the internal environment. The changes observed in the 2.5 and 5 mW/cm\(^2\) groups may reflect an altered coordination between inhibitory and excitatory neurotransmitters, which may lead to excitotoxic neuronal death due to accumulation of excitatory neurotransmitters, or modulation of neuronal excitability due to increased inhibitory neurotransmitters. The decrease in neurotransmitters observed in the 10 mW/cm\(^2\) group suggests that consumption of excitatory neurotransmitters may outweigh that of production after microwave exposure, or that excitability is actively inhibited as an endogenous protective mechanism.

Since it is well established that performance in the MWM is dependent on the hippocampus, it was plausible to assume that exposure in our experiments affected this brain area, which is supported by the observation of tissue injury in the hippocampus of rats. Furthermore, the altered function of the hippocampus in response to microwave exposure may relate to the disruption of neurotransmitter levels.

Overall, we demonstrated that long-term microwave exposure could induce learning and memory disorders in rats, involving disrupted inhibitory and excitatory neurotransmitter systems and structural pathology in the hippocampus. Accurate analysis of neurotransmitter concentrations is required to further elucidate the underlying mechanisms of brain function and pathology\(^{[32]}\), which may help in the design of novel therapeutic approaches. Our future studies will examine the molecular proteomic impact of microwave treatment to determine the mechanisms of brain cell malfunction after radiation.

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