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RESEARCH ARTICLE

Multi-frequency electromagnetic radiation induces anxiety in mice via inflammation in the cerebral cortex

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Abstract

Modern life is filled with radiofrequency electromagnetic radiation (RF-EMR) in various frequency bands, while the health risks are not clear. In this study, mice were whole-body exposed to 0.9/1.5/2.65 GHz radiofrequency radiation at 4 W/kg for 2 h per day for 4 weeks to investigate the emotional effects. It was found that the mice showed anxiety but no severe depression. The ELISA results showed a significant decrease in amino acid neurotransmitters (GABA, DA, 5-HT), although acetylcholine (ACH) levels were not significantly altered. Furthermore, Western blot results showed that BDNF, TrkB, and CREB levels were increased in the cerebral cortex, while NF-κB levels were decreased. In addition, pro-inflammatory factors (IL-6, IL-1β, TNF-α) were significantly elevated, and anti-inflammatory factors (IL-4, IL-10) tended to decrease. In conclusion, multi-frequency electromagnetic radiation induces an inflammatory response through the CREB-BDNF-TrkB and NF-κB pathways in the cerebral cortex and causes a decrease in excitatory neurotransmitters, which ultimately causes anxiety in mice.

Keywords Multi-frequency RF-EMR · Anxiety · BDNF · CREB · TrkB · Inflammation

Introduction

Modern life is inseparable from the electromagnetic environment, which is an important carrier of information. Some studies have confirmed that radiofrequency electromagnetic radiation (RF-EMR) at certain frequencies has certain effects on the brain or central nervous system, such as damaging the cerebral cortex (Saikhedkar et al.2014; Salford et al. 2003) or altering the blood–brain barrier (Stam 2010; Sirav and Seyhan 2009). Some researchers have also found that RF-EMR has a clear effect on emotions, such as causing anxiety and depression (Narayanan et al. 2013; Obajuluwa et al. 2017; Zhang et al. 2017; Xia et al. 2022). However, few studies have investigated the effects of multi-frequency RF-EMR on overall emotions in the body.

The cerebral cortex, as the supreme nerve hub, controls most bodily functions and is also linked to learning, emotion, memory, and other advanced cognitive activities. Although most studies have focused on the hippocampus (Engin and Treit 2007; Tu et al. 2014), the cerebral cortex has received minimal attention. Recent studies have shown that the frontal cortex plays a crucial role in emotion regulation (Hu and Dolcos 2017; Jacobs and Moghaddam 2021; Maayan et al. 2006). Salford et al. (2003) found significant neuronal damage in the cerebral cortex after a 2-h exposure to 900 MHz electromagnetic fields. In addition, a report showed that exposure to 1800 MHz can affect microglia and neuronal activity in the primary auditory cortex (Occelli et al. 2018).

It is well known that neurotransmitters play a crucial role in the regulation of emotions (Liu et al. 2020; Matiushenko et al. 2020). When stimulated, the electrical signals are converted into neurotransmitters in the presynaptic membrane, released into the synaptic space, and once again converted back into electrical signals in the postsynaptic membrane, completing the transmission of information between neurons. An imbalance in neurotransmitter metabolism can lead

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to the manifestation of negative emotions such as anxiety and depression in the body (Clement and Chapouthier 1998). In addition, research has shown that exposure to RF-EMR can alter neurotransmitter levels in several brain regions (Narayanan et al. 2019).

Brain-derived neurotrophic factor (BDNF) and its specific receptor tyrosine kinase B (TrkB) are widely distributed throughout the nervous system, with particularly high concentrations in the cerebral cortex and hippocampus (Lindholm and Castrén 2014; Musumeci and Minichiello 2011; Jin 2020). They play a critical role in neuronal growth, development, information transmission, and repair. Recent research has shown that when the body's neurotransmitter metabolism is disrupted, BDNF levels in the brain are significantly reduced (Czira et al. 2012; Sleiman et al. 2016). Cyclic adenosine monophosphate effector binding protein (CREB) is a widely distributed protein in cells that regulates gene transcription. Its primary function is to activate specific genes, increase protein expression, and subsequently regulate bodily functions. In recent years, numerous studies have shown a correlation between CREB and the activities of higher order neurons, such as those associated with emotion (Pandey 2003; Wallace et al. 2004). It has been reported that the upregulation of neurotransmitters can lead to increases in CREB and BDNF (Xhakaza et al. 2021). In certain neurological disorders, such as Alzheimer's disease and anxiety disorder, the levels of CREB, BDNF, and other proteins have been found to be reduced to varying degrees. The CREB-BDNF-TrkB pathway is one of the most extensively studied pathways in anxiety emotion research. Several studies have shown a decrease in the protein levels of this pathway in anxious individuals, suggesting that the body may regulate emotions through this pathway (Jiang et al. 2022; Młyniec and Nowak 2015).

Inflammatory factors refer to various cytokines involved in the inflammatory response, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), and others. Inflammation is the body's self-defense response to external stimuli. Some studies have shown that the levels of several inflammatory factors are increased to varying degrees in individuals with anxiety (Shan et al. 2022; Song et al. 2020). Gene knockout experiments have confirmed that the expression of inflammatory factors is indeed a factor contributing to anxiety (Zhuang et al. 2022). Research indicates that BDNF may inhibit inflammation, regulate anti-inflammatory factors, and be involved in the brain's immune response (Papathanassoglou et al. 2015). While inflammation is the body's defense mechanism, excessive stimulation can lead to the body's "inflammatory storm" and cause damage to the body. Increased levels of inflammatory factors in the cerebral cortex can damage surrounding neurons and cause anxiety, depression, and other disorders.

Currently, few studies have investigated the effects of exposure to multiple RF-EMRs on the body's mood and the relevant mechanisms in the cerebral cortex. In this study, we investigated the emotional changes in mice exposed to a complex RF-EMR environment of 0.9/1.5/2.65 GHz and explored the molecular mechanism underlying these changes. We also analyzed the expression of inflammatory factors and measured neurotransmitter levels in the cerebral cortex. Our results suggest that exposure to multi-frequency RF-EMR environments can affect mood in mice, possibly through mechanisms related to changes in inflammatory factor expression and neurotransmitter levels in the cerebral cortex.

Results

Multi-frequency electromagnetic exposure induces anxiety but not depression in mice

The anxious and depressive behavioral responses of the two groups were analyzed using the open field test (OFT, Fig. 1a): the elevated plus maze test (EPM, Fig. 1c) and the tail suspension test (TST, Fig. 1e) after mice were exposed to 0.9/1.5/2.65 GHz RF-EMF at a SAR value of 4.0 W/kg for 2 h per day, with 40 min of exposure per frequency per day. The OFT results showed a significant reduction in both the time spent in the center region and the distance travelled within this region after 4 weeks of exposure (Fig. 1b). The number of entries into the open arms and the time spent in the open arms were also significantly reduced in the EPM compared to the control group (Fig. 1d). The above results indicate that the mice developed significant anxiety after exposure. The TST is a classic experiment used to detect depression in animals. The results showed that there was no significant difference between the RF-EMR group and the control group in terms of various indicators, including exercise time and immobility time (Fig. 1f). This suggests that the mice did not develop depression.

Amino acid neurotransmitters significantly reduced after exposure

The behavioral changes in the mice led us to investigate changes in neurotransmitters. Neurotransmitter levels in the cerebral cortex were measured by enzyme-linked immunosorbent assay (ELISA). Consistent with the behavioral changes in the mice, we observed a significant reduction in excitatory neurotransmission (Fig. 2). The results showed

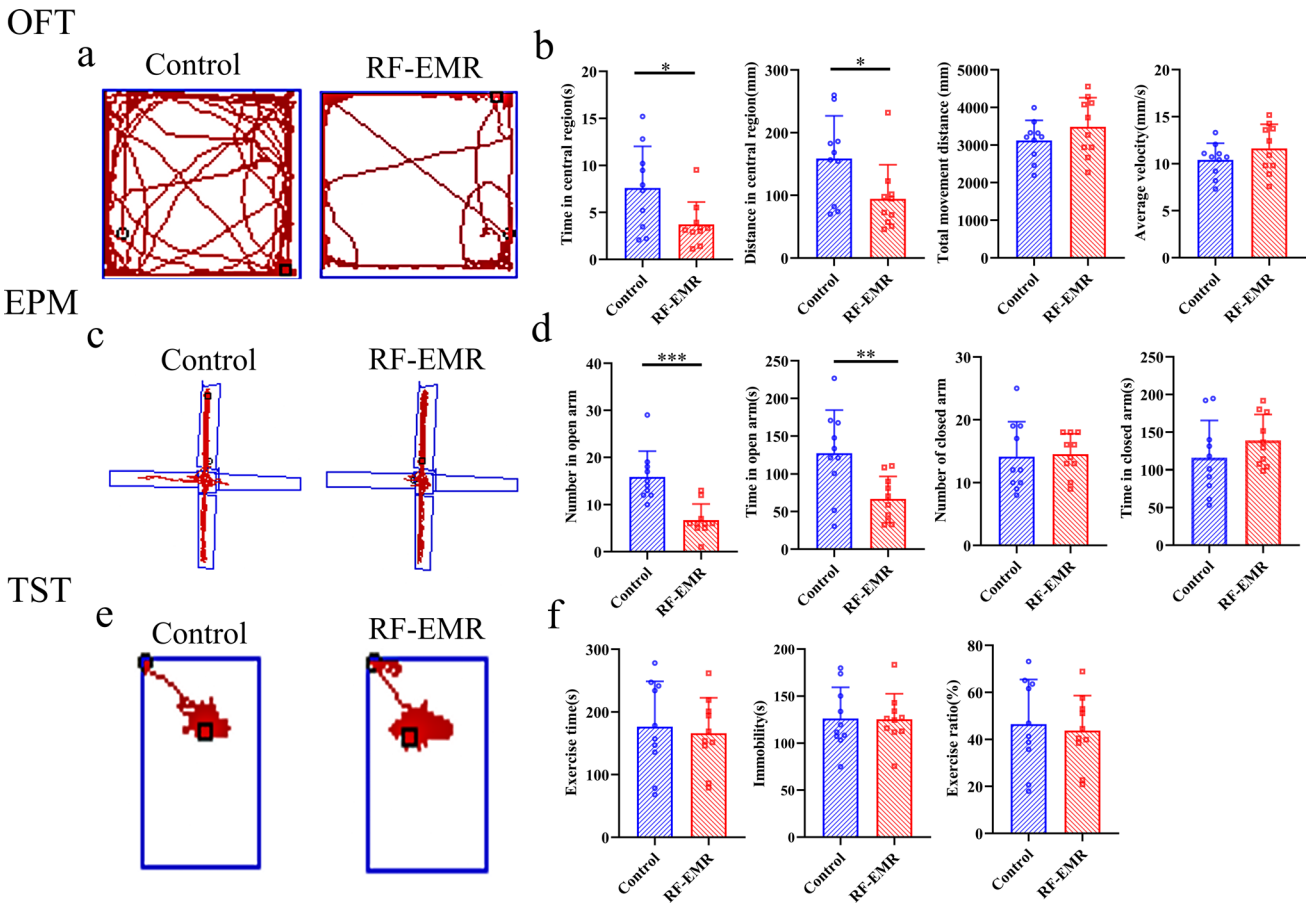


Fig. 1 Effects of exposure to multi-frequency RF-EMR on the behaviors of mice tested with OFT, EPM, and TST. OFT: **a** the movement trajectory of mice in OFT; **b** time in the central region, distance in the central region, average velocity, and total movement distance. EPM: **c** the movement trajectory of mice in EPM; **d** number in open arm, time in open arm, number in closed arm, time in closed arm. TST: **e** the movement trajectory of mice in TST; **f** exercise time, immobility, exercise radio. Histograms represent mean values ± SEM ($n = 10$). Unpaired two-tailed Student's *t*-test was used for statistical analysis of all data, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

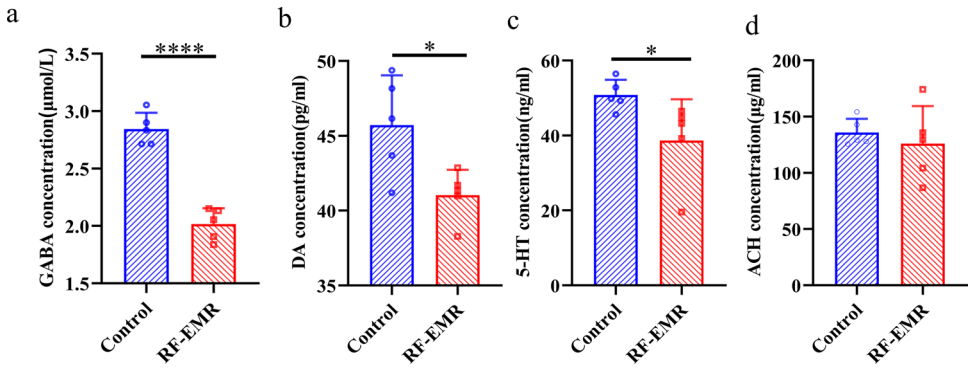


Fig. 2 Effects on neurotransmitters after RF-EMR exposure measured by ELISA. The levels of GABA (**a**), DA (**b**), 5-HT (**c**), and ACH (**d**) in cerebral cortex. Histograms represent mean values ± SEM ($n = 5$). Unpaired two-tailed Student's *t*-test was used for statistical analysis of all data, $^{*}P < 0.05$, $^{****}P < 0.0001$

a significant decrease in the levels of gamma-aminobutyric acid (GABA Fig. 2a), dopamine (DA, Fig. 2b), and 5-hydroxytryptamine (5-HT, Fig. 2c) in the cerebral cortex of RF-EMR mice. Acetylcholine (ACH) levels tended to decrease in the exposed group, although there was no significant difference between the two groups (Fig. 2d).

Effects of multi-frequency electromagnetic exposure on the CREB-BDNF-TrkB and NF-κB signalling pathways

To further investigate the molecular mechanism underlying the behavioral and neurotransmitter changes in mice exposed

to multi-frequency RF-EMR, we conducted a more detailed study focusing on CREB-BDNF-TrkB. Compared with the control group, the levels of BDNF, TrkB, and CREB were significantly decreased in the cerebral cortex as measured by Western blot (Fig. 3a). The above results suggest that radiofrequency electromagnetic irradiation causes dysregulation of the BDNF/CREB signalling pathway in the cerebral cortex and may lead to an inflammatory response in mice. The expression and levels of genes associated with inflammatory pathways were also analyzed by Western blot and RT-PCR. The results showed a significant increase in the level of NF-κB compared to the control group (Fig. 3b). Akt gene expression was significantly upregulated in the RF-EMR group using RT-PCR. There was a slight increase and decrease in IGF-1 and PGC-1α expression between the control and RF-EMR groups, but the difference was not statistically significant (Fig. 3c).

Effects of multi-frequency electromagnetic exposure on inflammatory cells and inflammatory factors

To investigate whether multi-frequency RF-EMR can cause inflammation of the cerebral cortex, inflammatory cells were first observed by hematoxylin–eosin staining, and the expression of IL-6, IL-1β, IL-4, IL-10, and TNF-α was analyzed by RT-PCR. The results showed a significant increase in the number of inflammatory cells in the cerebral cortex (Fig. 4a). The expression of the pro-inflammatory factors IL-6, IL-1β, and TNF-α was significantly upregulated. In addition, the expression of the anti-inflammatory factor IL-4 was significantly downregulated, while there was a non-significant decrease in IL-10 gene expression (Fig. 4b). To verify the reliability of the above results, the levels of inflammatory factors were further determined by ELISA. The results also showed that the levels of TNF-α and IL-1β were significantly increased compared to the control group. IL-6 levels were also elevated, although not statistically different. At the same time, the expression of the anti-inflammatory factors IL-4 and IL-10 was significantly downregulated (Fig. 4c).

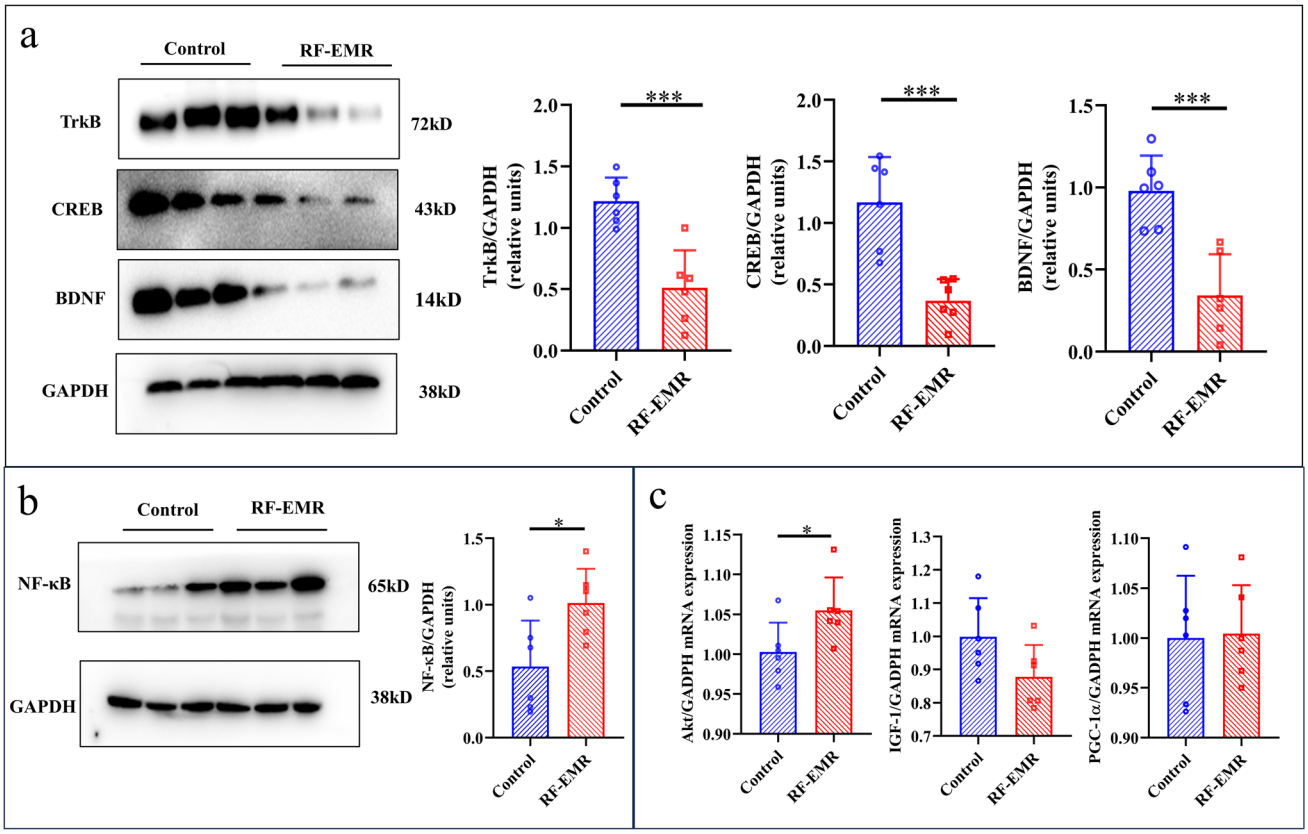


Fig. 3 Effects on CREB-BDNF-TrkB and NF-κB signalling pathways in mice after exposure to multi-frequency RF-EMR. **a** Western blot showing the levels of TrkB, CREB, BDNF, and GAPDH in the cerebral cortex. **b** The levels of NF-κB were tested by Western blot. **c** Gene expressions of Akt, PGC-1α, and IGF-1 were tested by RT-PCR. Histograms represent mean values ± SEM ($n = 6$). Unpaired two-tailed Student's *t*-test was used for statistical analysis of all data, $^{*}P < 0.05$, $^{**}P < 0.01$

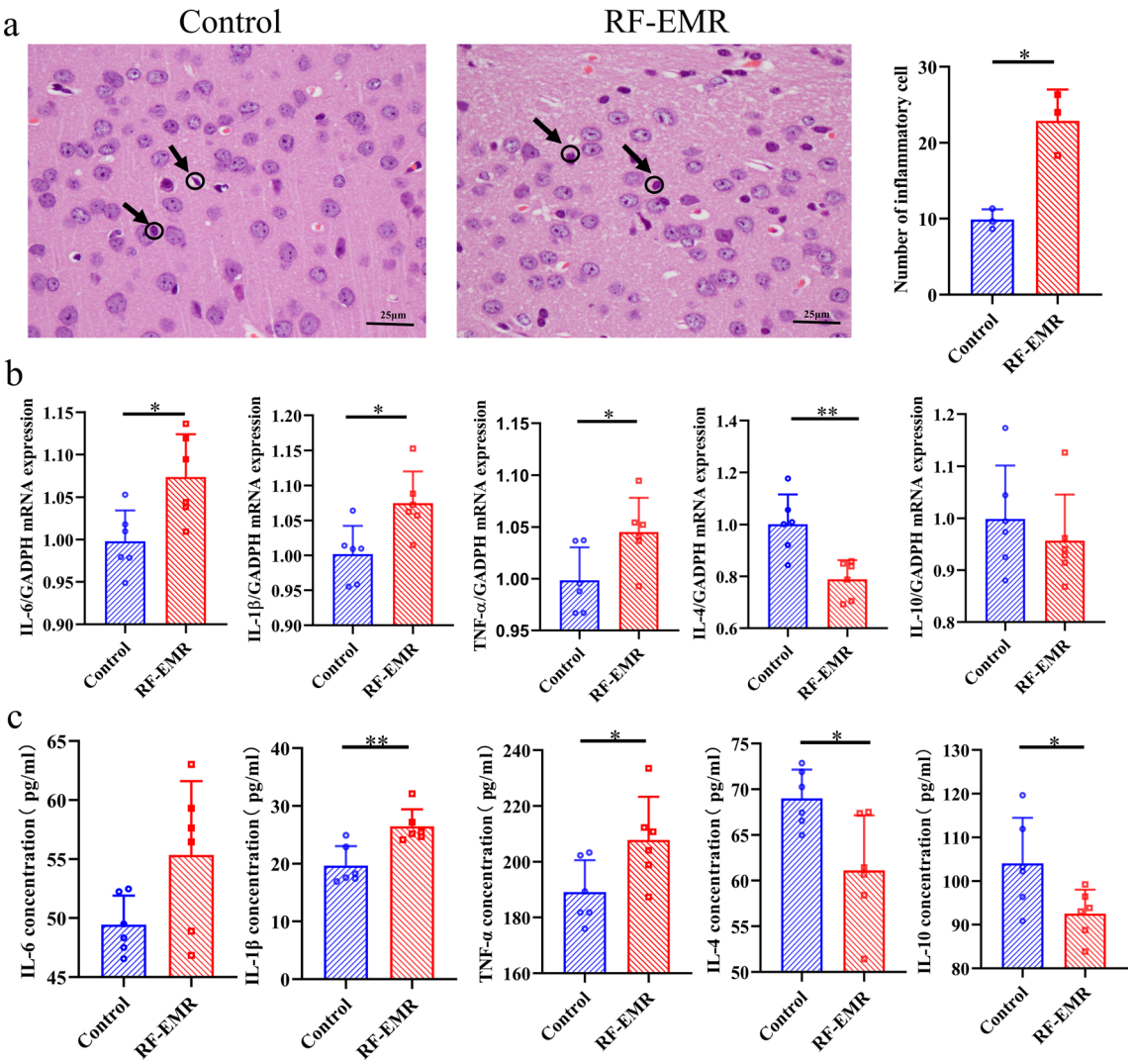


Fig. 4 Effects on inflammatory cells and factors in cerebral cortex after exposure to multi-frequency RF-EMR. **a** The number of inflammatory cells. **b** Gene expression of IL-6, IL-1β, TNF-α, IL-4, and IL-10 were detected with RT-PCR ($n=6$). **c** The content of TNF-α, IL-6, IL-1β, IL-4, and IL-10 were tested by ELISA ($n=6$). Histograms represent mean values ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis of all data, bar = 25 μm, $^{*}P<0.05$, $^{**}P<0.01$

Discussion

There have been long-standing concerns about the adverse health effects of various types of RF-EMR (Li and Wu 2023; Yang et al. 2019). Research has shown that RF-EMR can accelerate glucose metabolism in the brain (Volkow et al. 2011), weaken the blood–brain barrier (Tang et al. 2015), cause changes in the morphology and structure of cells in the hippocampus and cerebral cortex, and subsequently damage the brain's central nervous system, leading to various neurological disorders such as anxiety and memory impairment (Aldad et al. 2012; Li et al. 2012; Ntzouni et al. 2011; Sharma et al. 2017). Anxiety is a common emotion in everyday life. Excessive anxiety can lead to irritability and impaired learning and memory. Saikhedkar et al. found that rats exposed to mobile phone radiation for half a month (4 h per day) showed increased anxiety in the EPM experiment. Similarly, Sokolovic et al. observed anxiety-related behavior in rats exposed to GSM mobile phone radiation at 900 MHz for 4 h per day after 10 days of exposure (Sokolovic et al. 2012). However, few studies have investigated the effects of mixed multi-frequency radiation on the general emotional state.

In a previous study, we have observed that multi-frequency electromagnetic radiation was more likely to induce anxiety than single-frequency electromagnetic radiation in mice (data unpublished). Here, we employed three frequencies commonly used in cellular phone communication—0.9 GHz, 1.5 GHz, and 2.65 GHz—to irradiate mice for 40 min per day at each frequency with a SAR of 4 W/kg

for 4 weeks. The complex electromagnetic environment of modern life is more accurately reflected by multi-frequency electromagnetic radiation. The aim was to investigate the impacts on the mice's mood and a feasible and stable pathway mechanism. The interesting finding is that the mice exposed to multi-frequency RF-EMR showed significant anxious behavior in both the OFT and EMP experiments compared to the control group. However, it should be noted that the TST test did not show significant depression-like behavior in mice after long-term exposure.

Neurotransmitter metabolism and inter-neuronal signalling have been shown to be disturbed in patients with anxiety disorders (Ding et al. 2021; Taylor et al. 2005). Neurotransmitters play a crucial role in neuronal signalling and information transmission. These chemicals, such as amino acids, monoamines, choline, and neuropeptides, have a wide range of chemical properties (Borodinsky et al. 2014; Teleanu et al. 2022). Noor et al. found that RF-EMR can disrupt the balance of amino acid neurotransmitters in different regions of the brain in animals (Noor et al. 2011), and this phenomenon has also been observed in humans (Ferreri et al. 2006). It has now been reported that after long-term exposure to RF-EMR, neurotransmitters such as GABA were significantly decreased in the RF-EMR group (Zhang et al. 2017; Ahmed et al. 2007; Khadrawy et al. 2009). In this study, after 4 weeks of exposure, DA, GABA, and 5-HT were significantly decreased, while ACH did not change significantly. Based on this, we speculate that multi-frequency RF-EMR may reduce the levels of amino acid neurotransmitters in the brain, making it a significant inducer of anxiety.

In recent years, the CREB-BDNF-TrkB signalling pathway has been frequently implicated in studies exploring the mechanisms underlying anxiety. Several studies have shown that the levels of BDNF and its receptor TrkB are significantly reduced in individuals experiencing anxiety (Loewenstern et al. 2019; Shen et al. 2019), while CREB has also been observed to decrease during anxious states (Espasandín et al. 2022). BDNF and its specific receptor TrkB help the brain to make new nerve connections, increase the number of synapses, repair aging brain cells, and protect healthy neurons. CREB, a trans-acting factor found within neurons, can bind to the cis-acting element of associated genes, activate the gene, initiate the transcription program, and produce corresponding proteins, acting as a "relay station" in various major signal pathways. Although Johansson's study did not find that 0.9 GHz RF-EMR at a SAR value of 1 W/kg could cause atopic dermatitis, it unexpectedly found that serum BDNF levels in the subjects were significantly reduced after exposure (Johansson et al. 2008). Kim et al. also discovered that the decrease in BDNF content occurred in the cortical area 1 (CA1) and dentate gyrus (DG) regions of hippocampal neurons (Kim et al. 2021). By measuring the protein levels of CREB, BDNF, and TrkB in the cerebral cortex, this study showed that after long-term exposure to mixed RF-EMR, the protein levels of the three (especially TrkB) decreased significantly. Combined with previous behavioral experiments, we speculated that mixed RF-EMR might induce anxiety through the CREB-BDNF-TrkB signalling pathway. This reduction in CREB levels subsequently leads to a reduction in BDNF and TrkB levels in the cerebral cortex, which impairs neuronal repair and synaptic regeneration, ultimately leading to anxiety.

Inflammation is also closely linked to anxiety. NF-κB is a key protein that regulates the transcription of inflammatory factors. Its transcriptional regulatory site is located in the promoter region of pro-inflammatory and immunoregulatory factors and plays a critical role in the induction of immune responses in tissues and organs. Previous studies have reported a significant elevation of NF-κB associated with severe inflammatory responses in anxiety-like behaviors (Fan et al. 2016). Meanwhile, many researchers have found that Akt can activate the transcription of inflammatory factors and promote the occurrence of inflammatory responses (He et al. 2020), while IGF-1 and PGC-1α play a crucial role in inhibiting inflammatory responses (Chen et al. 2020; Fu et al. 2021). Our results showed a significant increase in NF-κB levels and Akt expression, a decrease in IGF-1, and no apparent change in PGC-1α expression after exposure. It is speculated that multi-frequency RF-EMR may activate pro-inflammatory response-related pathways, trigger inflammatory responses in the brain, and inhibit the expression of anti-inflammatory response-related pathways in the brain.

Inflammation is a defense mechanism of the body triggered by stimuli. Inflammatory cytokines are a general term for the mediators involved in this process. An excessive inflammatory response can damage normal cells, tissues, and organs, and even trigger cytokine storms that can lead to death (Tang et al. 2021). There is increasing evidence suggesting that negative emotions such as anxiety and depression are associated with inflammatory responses, and that BDNF can inhibit inflammation in the brain (Papathanassoglou et al. 2015; Wang and Tian 2021; Won and Kim 2020). Beumer et al. (2012) provided a detailed review of the relationship between immune inflammation and psychiatric disorders, suggesting that inflammation may be a contributing factor to these disorders, with inflammatory and anti-inflammatory factors playing either a positive or negative role. Using gene knockout experiments, Zhuang et al. demonstrated that IL-33 is a key player in the anxiety circuit and can potentially cause anxiety by reducing the expression of BDNF (Zhuang et al. 2022). Recent studies have reported that RF-EMR can cause an increase in ILs and IL-1β in the brain, and in behavioral experiments, the radiation group displayed notable anxiety-like behaviors (Bouji et al. 2012, 2016). Our results showed a significant increase

in the inflammatory factors IL-6, IL-1β, and TNF-α, while the expression of IL-4 and IL-10 decreased but not significantly. Based on these findings, we speculate that RF-EMR may induce mild inflammation in the cerebral cortex and regulate BDNF levels through specific signalling pathways, ultimately leading to anxiety in the body.

In conclusion, these findings suggest that multi-frequency RF-EMR may induce anxiety by decreasing brain neurotransmitter levels and triggering inflammatory responses via the CREB-BDNF-TrkB pathway (Fig. 5). Currently, there are few studies on the relationship between multi-frequency electromagnetic radiation and mood changes, and this study provides some data support and a direction for future research. However, further in-depth investigations are needed to elucidate the specific mechanism of whether multi-frequency RF-EMR can induce negative emotions.

Materials and methods

Animals

Eight-week-old male C57BL/6 J mice (average weight ± standard deviation: 20 ± 0.9 g) were purchased from SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China). Five mice were housed per cage and maintained in a standard environment (room temperature, 22–24 °C; 12-h light/dark cycle, lights on from 09:00 to 21:00; relative humidity, 50–60%). They were fed ad libitum with standard food (SPF (Beijing) Biotechnology Co., Ltd., Beijing, China) and water. After 7 days of adaptive feeding, mice were divided into two groups ($n = 20$): the control ($n = 10$) and multi-frequency RF-EMR groups ($n = 10$).

RF-EMR exposure

The experimental irradiation system includes an RF Signal Generator (RIGOL Technologies, Inc., China), a radiofrequency power amplifier (Nanjing Nate Communication Electronics Co., Ltd., China), an antenna selector, a control computer, and an irradiation box (Fig. 6). The experimental irradiation system is capable of conducting whole-body irradiation experiments on animals within the 0.8–4 GHz frequency band. The shield walls are made of metallic mesh allowing for ventilation and heat dissipation. And the experimental irradiation system can be found in published literature (Zheng et al. 2023). Six antennas were connected to different positions of the signal converter (R16-SN12T18-D-BP, China), and the microwave beam was sequentially outputted from each antenna to the irradiation box. These antennas could illuminate the irradiation box alternatively and randomly, with a 1-s interval between each switch.

Mice were whole-body exposed to 0.9/1.5/2.65 GHz RF-EMR with a SAR of 4.0 W/kg. Each frequency was irradiated for 40 min for a total of 2 h per day for 4 weeks. Mice were placed in an acrylic box containing two rows of 10 small compartments during RF-EMR exposure ($8.4 \times 6.7 \times 5.4$ cm^3). The control group was treated in the same way but without exposure. After the 4-week exposure, mice were subjected to 3-day behavioral experiments and then sacrificed for morphological and biochemical studies.

In this study, the targeted dosimetric value was set at 4 W/kg (WBSAR). There were two kinds of simulation-measurement methods used to obtain the necessary field (Chakarothai et al. 2013; Gong et al. 2017). In brief, the studies computed WBSAR in small animals using either 12 plane waves with specific configurations or random

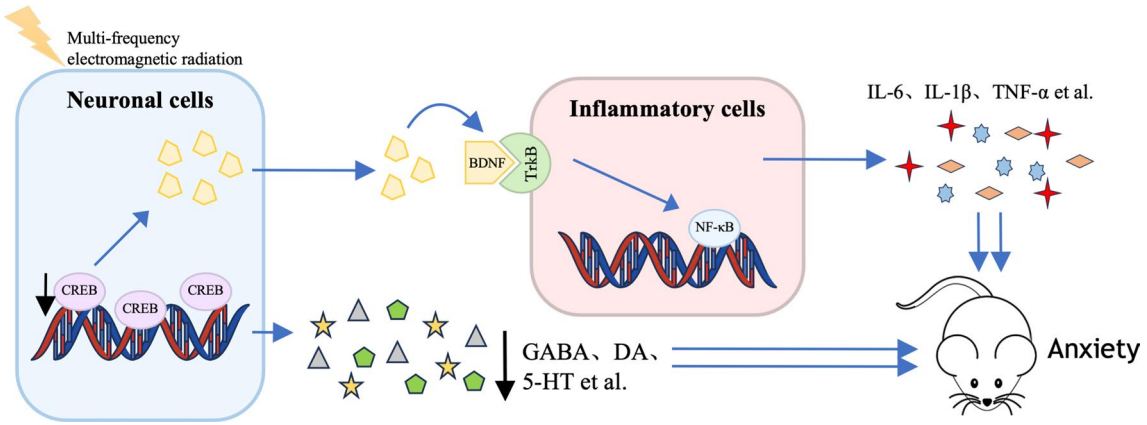


Fig. 5 Mechanistic diagram illustrating the anxiety induced by multi-frequency RF-EMR. Multi-frequency RF-EMR inhibited the transcription factor CREB, thereby reducing its levels. The reduced CREB could inhibit the production of BDNF and brain neurotransmitters (GABA, DA, 5-HT, et al.). BDNF consequently inhibited the levels of inflammatory factors. With the reduction in the BDNF levels, multi-frequency RF-EMR could activate the transcription factor NF-κB. Eventually, it could increase the content of inflammatory factors (IL-6, IL-1β, TNF-α, et al.), resulting inflammatory response. Decreased neurotransmitters and increased inflammatory factors, ultimately cause anxiety

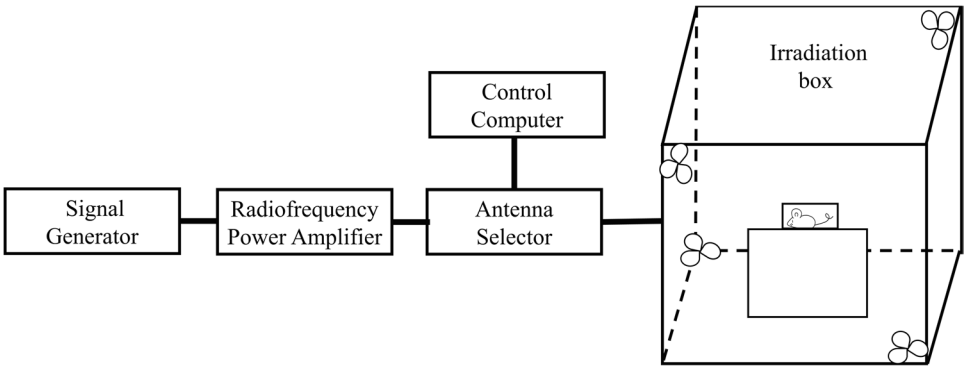


Fig. 6 Schematic diagram of RF-EMR radiation systems

incident waves. The ratio between the averaged E-field strength and WBSAR was therefore derived. By measurement, once the realistic E-field strength in the experiment was recorded, the realistic WBSAR could be obtained. We used an EF0391 electric field probe (Narda, Germany) to measure the field strength (V/m) during actual exposure to ensure that the expected field strength for WBSAR was achieved. In this exposure experiment, a field strength of 316.23 V/m was sufficient to generate WBSAR as 4.0 W/kg with 20 mice weighing 20.0 g for 0.9 GHz, a field strength of 182.12 V/m was sufficient to generate WBSAR as 4.0 W/kg with 20 mice weighing 20.0 g for 1.5 GHz, and a field strength of 161.00 V/m was sufficient to generate WBSAR as 4.0 W/kg with 20 mice weighing 20.0 g for 2.65 GHz.

OFT

The apparatus consisted of a 40 × 40 cm^2 square arena with 40 cm high walls. Each mouse was tested for 5 min, and the apparatus was wiped with 75% alcohol to remove any odors after each test. Anxiety was assessed by analyzing time spent in the center of the arena, average speed, total distance, and distance in the central zone.

EPM

The EPM consists of two opposite open arms (length, 35 cm × width 5 cm), two opposite closed arms (length, 35 cm × width 5 cm × height 15 cm), and a central square area (5 cm × 5 cm). Mice were placed in the maze from the central zone facing the closed arm. Each mouse was tested for 5 min, and the testing equipment was wiped with 75% alcohol to remove odors after each test. Open arm entry times, open arm dwelling time, closed arm entry times, closed arm dwelling time, and open arm distance were statistically analyzed.

TST

The TST is used to study depressive-like behavior in animals. Mice were suspended about 25 cm above the floor. Each mouse was tested for 6 min. Exercise time, immobility, and exercise ratio (exercise time/total time) were also recorded as indicators.

Hematoxylin–eosin staining

The whole brain tissue was fixed, dehydrated, and embedded in paraffin. Then, it was cut into 4-μm-thick sections, stained with hematoxylin and eosin, and observed under a 400 × microscope (OLYMPUS BX41; Olympus Corporation, Japan). The tissue sections were analyzed with NIS-Elements F 3.0 (Nikon Corporation, Japan) and Image J software (National Institutes of Health, USA).

Western blot

The cerebral cortex was pestled in a grinder with ice-cold RIPA lysis buffer containing NaCl 150 mM, Tris–HCl 50 mM pH 7.4, EDTA 2 mM, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride (Beijing Biomed Genetic Technologies Limited, Beijing China), 1 × protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The samples were then centrifuged at 12,000 g for 15 min, 4 °C. The supernatant was quantified for protein content by BCA protein assay kit (Thermo Fisher Scientific Inc., USA). Equal amounts of protein were diluted in RIPA lysis buffer and then boiled with 1 × loading buffer. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes after electrophoresis, and then sealed with 5% of skim milk for 1 h at room temperature. The membranes were infiltrated with specific primary antibodies overnight at 4 °C, shaken for 1 h at room temperature, and incubated with secondary antibody for 1 h the next day. Specific protein bands were detected

by ECL Plus (Beijing Lablead Biological Technology Co., Beijing China) and Fluor Chem R Western blot detection system (Protein Simple, USA), and Image J software was used to quantify the bands. Primary and secondary antibodies for Western blot are listed in Table 1.

Real-time polymerase chain reaction (RT-PCR)

Total RNA for cerebral cortex was extracted using the Tissue RNA Kit (Omega Bio-Tek, USA), and total RNA samples were equilibrated in concentration and transcripted into cDNA using the RT System (T100TM Thermal Cycler, BIO-RAD, USA) and PrimeScript TM RT Reagent Kit (Takara, Japan). Real-time PCR reactions were performed using CFX Opus 96 Real-Time PCR System (BIO-RAD, USA) and SuperReal PreMix Plus-SYBR Green (TIANGEN Biotech Co. Ltd., Beijing, China). The thermal cycling profile included a pre-incubation step of 95 °C for 5 min, followed by 55 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 20 s), and extension (72 °C, 30 s). Table 2 lists the details of the oligonucleotide sequences and their annealing temperatures. Each sample was assayed in triplicate, and quantitative analysis was performed using the target gene/GAPDH gene mRNA expression method, then all data were standardized.

ELISA

According to the manufacturer's instructions, acetylcholine (ACH), dopamine (DA), 5-hydroxytryptamine (5-HT), and gamma-aminobutyric acid (GABA) of the cerebral cortex were detected by ELISA kit (Shanghai Meilian Biological Technology Co. Ltd). Data were analyzed using a SpectraMax iD3 multifunctional microplate reader (Molecular Devices, USA).

Table 1 Primary antibodies and secondary antibodies used for Western blot

Antibody name	Manufacturer	Dilution ratio	Molecular weight (kD)	Antibody species
BDNF	Cell Signaling Technology	1:1000	14	Rabbit
TrkB	Abcam	1:5000	75–85	Rabbit
CREB	Cell Signaling Technology	1:1000	43	Rabbit
NF-κB p65	Santa Cruz Biotechnology	1:500	65	Mouse
GAPDH	Beijing ZSGB-BIO	1:1000	38	Mouse
HRP-Goat Anti-Rabbit	Beijing ZSGB-BIO	1:2500		
HRP-Goat Anti-Rabbit	Beijing ZSGB-BIO	1:2500		

Table 2 Primer sequences and annealing temperature

Primer	Sequence (5′-3′)	Annealing temperature (°C)
IL-6 Forward	TCGTGGAAATGAGAAAAGAGTTGTG	57.21
IL-6 Reverse	GAGCATTGGAAATTGGGGTAGGA	56.21
IL-10 Forward	AATGCAGGACTTTAAGGGTTACT	55.63
IL-10 Reverse	TTGTAGACACCTTGGTCTTGGA	55.82
IL-4Forward	TCACAGCAACGAAGAACACCA	57.51
IL-4 Reverse	CAGGCATCGAAAAGCCCGAA	59.68
TNFα Forward	CCCAAAGGGATGAGAAGTTCC	55.47
TNFα Reverse	GCTACAGGCTTGTCACTCGAA	58.88
IL-1β Forward	TGCCACCTTTTGACAGTGATG	56.28
IL-1β Reverse	GTGCTGCTGCGAGATTTGAA	58.65
AKT Forward	CCGCCTGATCAAGTTCTCCT	57.45
AKT Reverse	TTCAGATGATCCATGCGGGG	57.45
PGC-1α Forward	GTCATTCGGGAGCTGGATGG	59.5
PGC-1α Reverse	CAACCAGAGCAGCACACTCTA	57.57
IGF-1 Forward	ATACCTGCCTGGGTGTCCA	57.32
IGF-1 Reverse	AGGGACGGGGACTTCTGA	57.18
GAPDH Forward	CTGACGTGCCGCCTGGAGAAAC	63.26
GAPDH Reverse	CCCGGCATCGAAGGTGGAAGAGT	63.13

Statistical analyses

All data in this study were analyzed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA), and the measurement data were expressed by mean ± standard deviation (SEM). Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare between the control and RF-EMR groups. A value of $P < 0.05$ was considered statistically significant.

Conclusions

In this study, we first investigate the effects of mixed multi-frequency RF-EMR on negative bodily emotions in mice. Through behavioral experiments, we found that after a long period of RF-EMR exposure, the RF-EMR group showed obvious anxiety, but not depression. At the same time, it was found that the levels of BDNF, TrkB, and CREB in the cerebral cortex decreased, the expression of inflammatory factors increased, the expression of anti-inflammatory factors decreased, and the contents of amino acid neurotransmitters decreased. Taken together, we speculate that multi-frequency RF-EMR may reduce the levels of amino acid neurotransmitters (GABA, DA, 5-HT) through the CREB- BDNF-TrkB signalling pathway and cause inflammation in the brain to cause anxiety.

Author contribution XJ and WC designed the experiments. LM, ZL, WQ, WX, ZX, LZ, and DG performed the experiments. XJ analyzed the data. XJ wrote the manuscript. XJ and WC edited the manuscript. All authors read and approved the final manuscript.

Data availability Not applicable.

Declarations

Ethical approval All studies involved in this work were approved by the Ethics Committee of National Beijing Center for Drug Safety Evaluation and Research (no. IACUC-2023-001D). All procedures were conducted on the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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