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Acute Inflammatory Pain Induces Sex-different Brain Alpha Activity in Anesthetized Rats Through Optically Pumped Magnetometer Magnetoencephalography*

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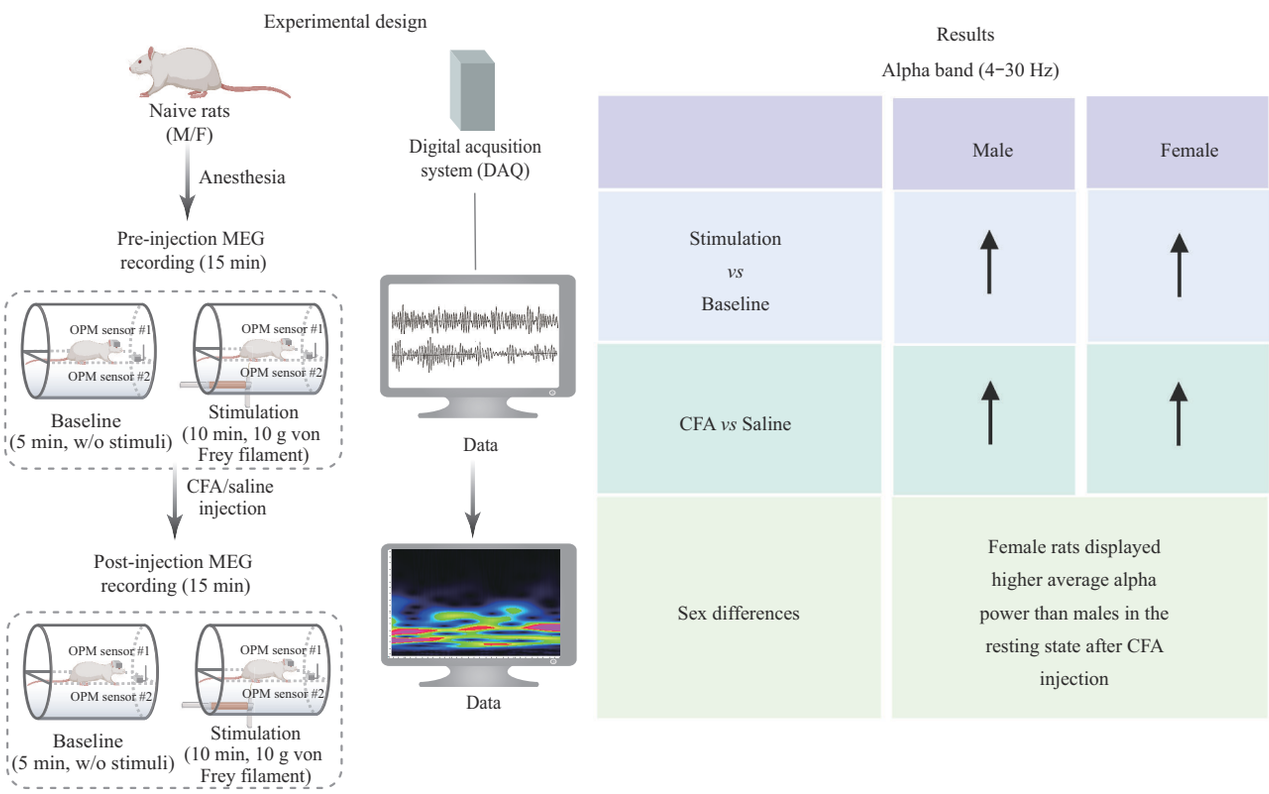
Graphical abstract



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Abstract **Objective** Magnetoencephalography (MEG), a non-invasive neuroimaging technique, meticulously captures the magnetic fields emanating from brain electrical activity. Compared with MEG based on superconducting quantum interference devices (SQUID), MEG based on optically pump magnetometer (OPM) has the advantages of higher sensitivity, better spatial resolution and lower cost. However, most of the current studies are clinical studies, and there is a lack of animal studies on MEG based on OPM technology. Pain, a multifaceted sensory and emotional phenomenon, induces intricate alterations in brain activity, exhibiting notable sex differences. Despite clinical revelations of pain-related neuronal activity through MEG, specific properties remain elusive, and comprehensive laboratory studies on pain-associated brain activity alterations are lacking. The aim of this study was to investigate the effects of inflammatory pain (induced by Complete Freund's Adjuvant (CFA)) on brain activity in a rat model using the MEG technique, to analysis changes in brain activity during pain perception, and to explore sex differences in pain-related MEG signaling. **Methods** This study utilized adult male and female Sprague-Dawley rats. Inflammatory pain was induced *via* intraplantar injection of CFA (100 μl, 50% in saline) in the left hind paw, with control groups receiving saline. Pain behavior was assessed using von Frey filaments at baseline and 1 h post-injection. For MEG recording, anesthetized rats had an OPM positioned on their head within a magnetic shield, undergoing two 15-minute sessions: a 5-minute baseline followed by a 10-minute mechanical stimulation phase. Data analysis included artifact removal and time-frequency analysis of spontaneous brain activity using accumulated spectrograms, generating spectrograms focused on the 4−30 Hz frequency range. **Results** MEG recordings in anesthetized rats during resting states and hind paw mechanical stimulation were compared, before and after saline/CFA injections. Mechanical stimulation elevated alpha activity in both male and female rats pre- and post-saline/CFA injections. Saline/CFA injections augmented average power in both sexes compared to pre-injection states. Remarkably, female rats exhibited higher average spectral power 1 h after CFA injection than after saline injection during resting states. Furthermore, despite comparable pain thresholds measured by classical pain behavioral tests post-CFA treatment, female rats displayed higher average power than males in the resting state after CFA injection. **Conclusion** These results imply an enhanced perception of inflammatory pain in female rats compared to their male counterparts. Our study exhibits sex differences in alpha activities following CFA injection, highlighting heightened brain alpha activity in female rats during acute inflammatory pain in the resting state. Our study provides a method for OPM-based MEG recordings to be used to study brain activity in anaesthetized animals. In addition, the findings of this study contribute to a deeper understanding of pain-related neural activity and pain sex differences.

Key words magnetoencephalography, pain, sex differences, alpha activity, inflammation

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Pain, a multidimensional experience encompassing sensory and emotional aspects, is often defined as an unpleasant sensation linked to actual or potential tissue damage by the International Association for the Study of Pain (IASP)[1]. Despite this definition, the intricacies of pain processing and the perception of noxious stimuli remain a scientific challenge.

Magnetoencephalography (MEG) is a non-invasive, silent, completely passive neurophysiological imaging technique that directly measures the magnetic field generated by synchronized ionic neural currents in the brain. Compared with traditional optical brain imaging techniques, such as functional magnetic resonance imaging (fMRI), and positron emission tomography (PET), MEG directly captures the brain's neural electrophysiology and therefore independent of a signal transduction model. Moreover, optically pump magnetometer (OPM)-MEG can be placed closer to the scalp surface, improving sensitivity, spatial resolution, and the uniformity of coverage. MEG has a millisecond temporal resolution of signal dynamics across the entire brain[2-4]. Its capability to measure synchronized neural electrical signals renders MEG valuable for studying large-scale brain activity dynamics in systems and behavioral neuroscience.

While both MEG and electroencephalography (EEG) are sensitive to electrical currents, MEG's unique ability to detect magnetic fields arising from these currents provides direct and non-invasive access to the brain's electrophysiological activity. Previous studies[5] used EEG to assess electrical potentials involved in pain, but MEG is much less frequently used in pain research[6]. To note, MEG has a better combination of direct and noninvasive access to the electrophysiological activity of entire brain, with great spectral differentiation and minimum bias. This

specificity positions MEG as a powerful tool for understanding pain-related neural processes.

Despite the use of MEG in clinical research, there exists a notable gap in our understanding due to the scarcity of MEG setups in animal models. Because MEG can detect neural signals from deep brain structures, such as subcortical regions, this feature helps to investigate the involvement of these regions in pain modulation, providing valuable insights into the subcortical mechanisms underlying pain. To address this gap, our study introduces a rat model of Complete Freund's Adjuvant (CFA)-induced pain, a well-established model for studying inflammatory pain. Through a combination of MEG and behavioral assessments in both female and male rats, we explore brain activities in resting and stimulated states.

Sex differences in chronic pain conditions have been documented, underscoring the importance of considering sex as a biological variable in pain research. It has been reported that the prevalence of chronic pain is higher in women than men in most chronic pain conditions[7-8]. Prior research has elucidated sex-specific functional brain abnormalities in chronic pain, particularly in patients with neuropathic pain, where abnormalities in alpha oscillations within the dynamic pain connectome have been identified[9-10]. Therefore, incorporating sex as a biological variable is crucial for enhancing the clinical translation of preclinical findings. However, the specific sex-related differences in brain activity, particularly in the context of inflammatory pain, remain inadequately explored, especially in animal models using neurophysiological imaging techniques, such as MEG or EEG.

In this study, we establish a rat model of CFA-induced pain, a widely used animal model for studying inflammatory pain. Using MEG and behavioral tests in both female and male rats, we investigated the brain activities recorded by MEG in anesthetized female and male rats in the resting or stimulation state (by applying mechanical stimuli to the injected paw). We also compared the brain activities after inflammatory insult (CFA) or non-harmful injection (saline) in rats of both sexes. A primary focus of our investigation was discerning potential sex-specific differences in the brain activities recorded by MEG during physiological state and inflammatory pain. The utilization of non-invasive MEG technology provides a complementary understanding of neural activity associated with pain, promising insights that can contribute to the development of more effective treatments for chronic pain conditions. This study sheds light on the intricate interplay between sex differences and neural responses in the context of inflammatory pain, thereby advancing our understanding of pain mechanisms and therapeutic strategies.

1 Materials and methods

1.1 Animals

Male and female Sprague-Dawley rats (6−8 weeks old) were utilized in this study. The rats were housed in a temperature-controlled animal facility under a 12 h light-dark cycle, with free access to water and food in their home cages. All procedures adhered to the guidelines outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the Animal Care and Use Committee of Ningbo University, Ningbo, China (Approval Number: NBU20220153).

1.2 Establishment of CFA−induced inflammatory pain

For MEG recording experiments, rats were anesthetized with pentobarbital sodium (60 mg/kg, i. p.), and CFA emulsion (100 μl, 50% in saline) or saline (100 μl) was injected into the plantar of the left hind paw. A total of 8 male and 8 female rats were randomly assigned to CFA-treated pain and saline-treated control groups (n=4 per group). In CFA-treated pain groups, 4 male and 4 female rats received intraplantar injections of CFA. In saline-treated control groups, 4 male and 4 female rats received injections of saline, serving as the vehicle.

1.3 Pain behavioral tests

Pain behavior tests were performed before CFA or saline injections (baseline) and at 1 h after injections. To measure the mechanical thresholds, male and female rats were put in separate chambers on a perforated sheet (Cat. No. 38450, Ugo Basile, Italy) and adapted for at least 30 min. In the behavioral tests, rats were anesthetized with 2.5% isoflurane and received CFA (100 μl, 50% in saline) or saline (100 μl) injection in the hind paw. The mechanical thresholds of nociceptive reflexes were tested by von Frey filaments (Bioseb, United States).

The mechanical withdrawal latencies were calculated by the up-and-down method[11-12].

1.4 MEG recording

MEG recording was performed under sedation and anesthesia in a modified recording system (Figure 1a). Rats in each group were anesthetized with pentobarbital sodium (60 mg/kg, i. p.). The OPM sensor 1 was placed directly on the central surface of the anesthetized rat head (Figure 1a). The rat was then transferred to a multi-layered cylindrical magnetic shield (CMS, Figure 1b) manufactured by Magnetic Wave Intelligence Technology (Magnetic Wave Intelligence Technology Co. Lt, Ningbo, China). The CMS comprises 4 layered high-permeability permalloys, which create a clean and stable magnetic environment by canceling external magnetic fields through the shell material. The shielding factor at 1 Hz was measured to be above 106 and the magnetic noise is less than 15 fT/Hz$^{1/2}$ within the center of the CMS. The rat was placed at the center of the CMS with a non-magnetic stage. Figure 1b shows the settings of the placement of the rat and the non-magnetic stage. As shown in Figure 1a, magnetic signals were recorded with two OPMs. The OPMs were two QuSpin Zero-Field Magnetometers (QZFMs) manufactured by QuSpin (QuSpin, Inc., Louisville, CO, USA). Each OPM was self-calibrating and had a compact electronics module for measuring. The filed sensitivity was ~15 fT/ Hz$^{1/2}$ in 3−100 Hz band. The dynamic range was ±5 nT. The full technical details of the OPMs have been described in previous reports[13-14].

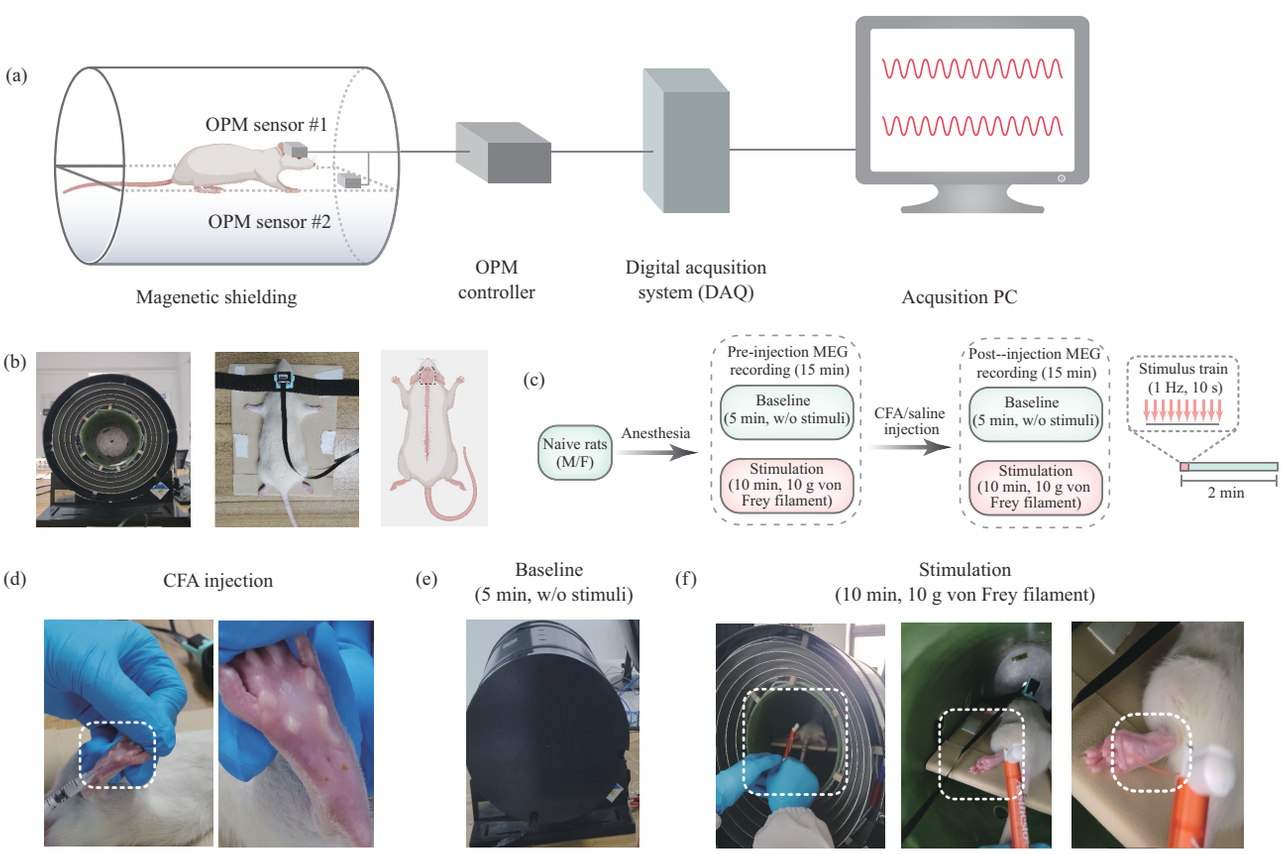

Fig. 1 The modified rodent MEG recording systems and the design of MEG recording experiments

(a) Schematic diagram of the animal magnetoencephalography system. Magnetic signals were recorded with two OPMs, which were QuSpin Zero-Field Magnetometers (QZFMs). The optically pumped magnetometer (OPM) sensor 1 was placed directly on the central surface of the anesthetized rat head. (b) Schematic diagram illustrating the multi-layered cylindrical magnetic shield created by Magnetic Wave Intelligence Technology. The CMS comprises 4 layered high-permeability permalloys, which create a clean and stable magnetic environment by canceling external magnetic fields through the shell material. The rat was placed in the center of cylindrical magnetic shield for MEG recordings. (c) The schematic design of MEG experiments. The MEG recording module contains a 5 min baseline recording epoch and a 10 min mechanical stimulation epoch. During the stimulation epoch, a train of 10 mechanical stimuli was manually applied on the rat plantar surface at a frequency of 1 Hz using a 10 g von Frey filament every 2 min, repeated 5 times in the stimulation epoch. (d) The illustration of plantar CFA injection. (e) The illustration of a 5 min baseline MEG recording. (f) The illustration of applying stimulation to the plantar of rats by von Frey filaments.

1.5 Protocols of MEG recording experiments

The schematic design of MEG experiments is shown in Figure 1c. Each rat received two modules of 15 min MEG recording before or after CFA or saline injection (Figure 1d). The MEG recording module contains a 5 min baseline recording epoch (Figure 1e) and a 10 min mechanical stimulation epoch (Figure 1f). During the stimulation epoch, a train of 10 mechanical stimuli [15] was manually applied on the rat plantar surface at a frequency of 1 Hz using a 10 g von Frey filament (Figure 1f) every 2 min, repeated five times in the stimulation epoch (Figure 1c). Empty recordings (5 min) without rats were conducted before the rat brain recording experiments.

1.6 MEG data analyses

Artifacts were sought out through visual inspection of the MEG data. Waveforms of MEG that contained artifacts that could be identified (amplitude greater than 6 pT) were not included in the data analysis. Accumulated spectrograms were utilized in order to conduct an analysis of the frequency characteristics of the spontaneous brain activity. For the purpose of converting data from the time domain to the frequency domain, the Morlet continuous wavelet transform was utilized. In low-frequency, wavelets are sensitive to the frequency, whereas in high-frequency, they are sensitive to the time. This is because wavelets have a fixed sigma value, which represent the number of oscillations. To get around this shortcoming and concentrate on the frequency characteristics of spontaneous brain activity, we utilized two sigma values for time-frequency analysis: one small value for low-frequency signals and one large value for high-frequency signals. This allowed us to focus on the frequency aspects of spontaneous brain activity. The sigma level was set to 6 for neuromagnetic signals that fell within the 0.5−100 Hz band, whereas the sigma level was adjusted to 48 for signals that were within the 100−2 000 Hz band. A single epoch of data, which lasted for 120 s, was broken up into 60-time windows, with each time window lasting for 2 s and containing 24 000 data points[16]. The computation of a single spectrogram was carried out for each time window for every physical sensor. One epoch of data was used to construct 60 spectrograms for each sensor, and these spectrograms were used to analyze low-frequency signals. After putting all 60 spectrograms together (accumulation), one accumulated spectrogram was produced for each sensor. This accumulated spectrogram was produced for one epoch of data. A global field spectral power was computed from all the MEG measuring sensors by computing the mean value for each time and frequency point[17]. This was done to determine the entire brain's frequency profile.

The MEG data were preprocessed with the digital filter *via* a band-stop filter to remove power-line interference (50 Hz). The deviated trials and channels were removed. The MEG Processor software then extracted and fragmented the MEG data from the resting state condition into epochs. To eliminate interference caused by system and motion artifacts, the epochs of MEG data were visually examined. For MEG burst detection in each epoch, MEG signals from sensors placed on the head and reemergence sensors monitoring noise were screened. Sharp signals (>6 pT) that were clearly distinguishable from ongoing background activity were rejected and regarded as probable MEG noise or artifacts. In addition to artifact and noise elimination, we rejected epochs with sharp signals due to a clear contribution from heartbeats, eye movements, or other physiological signals. Previous evidence suggested that OPMs might be sensitive to low-frequency noise; the present study focused on signals above 4 Hz. Although the MEG system could digitize at a high sample rate (or sample frequency), the OPM sensors appeared to be mainly sensitive to signals below 75 Hz. To completely exclude power-line noise around 50 Hz, the present study focused on waveform signals at 4−30 Hz.

The frequency features of spontaneous brain activity were analyzed with accumulated spectrograms. The Morlet continuous wavelet transform was used for transforming time-domain data to frequency-domain data. A Morlet wavelet has a Gaussian window shape in both time and frequency, while maintaining a sinusoidal underlying structure from a mathematical point of view. This wavelet structure provides easily interpretable results in both the time and frequency domains, as it produces qualitatively similar data to those obtained from a time-frequency analysis using a Fourier transform. With a fixed sigma value (number of oscillation), wavelets are sensitive to the frequency in low-frequency while sensitive to the time in high-frequency. To overcome this weakness and focus on

the frequency characteristics of spontaneous brain activity, we adjusted sigma values for time-frequency analysis: one small value for low-frequency signals and one large value for high-frequency signals. In addition, a correction was necessary to make the time-frequency data zero-mean. Consequently, the current study employed the following equation:

$$g(t,f) = c_\sigma \pi^{-\frac{1}{4}} e^{-\frac{1}{2}t^2} (e^{-iot} - k_\sigma) \quad (1)$$

In the equation, t indicates time; f indicates a specific frequency; k_σ indicates the admissibility and c_σ indicates a normalized constant for frequency f. g (t,f) indicates wavelet coefficients for a given frequency bin. If signals appeared in the given sensitive time (a small sigma value) and sensitive frequency (a large sigma value) ranges, they would be enhanced[18]. The time-frequency representations of waveform data of all sensors could be computed with the same coefficients and stored in the same format (the same numbers of rows and columns). The magnitude of magnetic signals was quantified with power. MEG data with clear background and no obvious noise were retained. To analyze the frequency specific neural activities, MEG data were transferred to time-frequency representation in 4−30 Hz frequency bands. The spectral power was quantified with accumulated spectrogram. Accumulated spectrogram is a method to summarize long-time recordings as a short-time window spectrogram. Since the results were computed with time-frequency and statistical analyses, the unit is typically ignored. In the present study, 60 spectrograms were computed for one epoch of data for each sensor for low-frequency signals. One accumulated spectrogram was produced by adding all 60 spectrograms together (accumulation) for one epoch of data for each sensor[19].

1.7 Data acquisition

Synchronized data acquisition from the OPMs was accomplished with a National Instruments (NI) card cDAQ-9171 (Austin, TX, USA). The OPMs (magnetometers) were linked to the NI card, which was then connected to a Windows computer (Dell workstation, Microsoft Corporation, Redmond, WA, USA). Building on previous reports[20-22], custom software was developed to perform data acquisition and real-time data processing. The sampling rate of all MEG recordings was set at 500 Hz[13]. The waveforms of magnetic data at sensor levels were visually identifiable and were different from the empty recordings. Artifacts and noise were pre-dominantly identified in a low-frequency range (<1 Hz) and identified at the beginning of baseline, stimulation, and around the injection.

1.8 Statistical analysis

All data are presented as mean±SEM and analyzed by GraphPad Prism 8 (GraphPad Software, San Diego, CA). Student's *t*-test, two-way or three-way analysis of variance (ANOVA), followed by Tukey's post hoc as indicated in figure legends. A significance level of $P<0.05$ was applied for all analyses.

2 Results

2.1 MEG recordings showed that mechanical stimulation increased spectral power in naive rats of both sexes

We first conducted MEG recordings on naive (pre-injection) female and male rats to investigate neural activity during resting (pre-injection baseline) and mechanical stimulation states (pre-injection stimulation). Specifically, we focused on detecting alpha activity, which was analyzed using time-frequency analyses. Figure 2 illustrates the waveforms and brain magnetic data (Figure 2a, b, female; Figure 2c, d, male) recorded from 8 female and 8 male rats during both states.

In the pre-injection (naive) state, we observed clear alpha band (8−13 Hz) activity and significant increases in spectral power. Application of mechanical stimuli by 10 g von Frey filament to the rats' plantar area led to a significant rise in average spectral powers (post-injection stimulation) compared to pre-injection baseline levels in both female and male rats (Figure 2e, stimulation: $F(1, 14)=144.8$, $P<0.000\ 1$; female: $P<0.000\ 1$, male: $P<0.000\ 1$, baseline *vs* stimulation). Sex differences were not found in the average MEG spectral power either in baseline or stimulation recordings (Figure 2e, gender: $F(1, 14)=4.424$, $P=0.054\ 0$; baseline: $P=0.332\ 5$, stimulation: $P=0.070\ 7$, female *vs* male).

Further analysis of peak spectral power (4−30 Hz) before and after mechanical stimuli revealed increased peak powers during stimulation recordings in both female and male rats, compared to baseline levels (Figure 2f, stimulation: $F(1, 14)=89.36$, $P<0.000\ 1$; female: $P<0.000\ 1$, male: $P=0.000\ 1$,

baseline *vs* stimulation). While no sex differences were observed in average MEG spectral power during baseline recordings, female rats displayed higher peak spectral powers in response to repeated external mechanical stimuli (Figure 2f, gender: $F(1, 14) = 4.516$; P=0.051 9; baseline: P=0.691 1, stimulation: P=0.032 9, female *vs* male).

Notably, conventional behavioral tests evaluating the paw withdrawal thresholds by the von Frey filament set showed a slight decrease in the female's mean mechanical threshold relative to the male's (Figure 2g, P=0.046 7, female *vs* male). It is crucial to mention that the mechanical stimulus (10 g) applied during MEG recording remained lower than the average paw withdrawal thresholds of both sexes, ensuring the stimuli were almost innocuous. Collectively, our MEG recordings, in conjunction with behavioral data, demonstrated that repeated mechanical stimuli significantly increased average and peak brain activities, especially in the alpha band, in anesthetized rats. Moreover, female rats exhibited higher peak powers than their male counterparts in response to repetitive external mechanical stimuli.

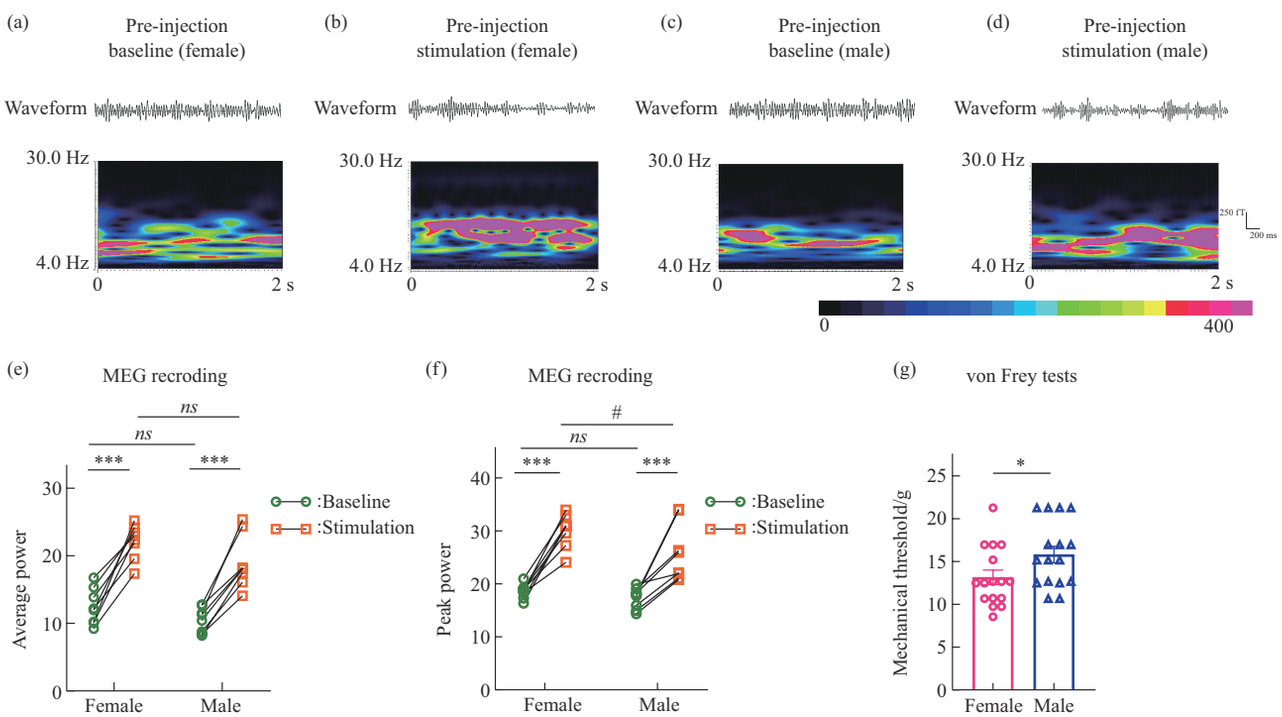

Fig. 2　Mechanical stimulation increased spectral power in both female and male naive rats

(a, b) The representative MEG waveforms and time-frequency spectrograms of pre-injection baseline (a) and pre-injection stimulation (b) in female naive rats. (c, d) The representative MEG waveforms and time-frequency spectrograms of pre-injection baseline (c) and pre-injection stimulation (d) in male naive rats. (e, f) The average (e) and peak (f) power (4−30 Hz) of pre-injection baseline and pre-injection stimulation in female and male rats. Data are presented as mean±SEM. Two-way ANOVA and Tukey's multiple comparisons test (n=8 per group). *, baseline *vs* stimulation; #, female *vs* male; ***P< 0.001; #P<0.05; *n*s, not significant. (g) Mechanical threshold of naive female and male rats. Data are presented as mean± SEM. An Unpaired t-test was used (n=16 per group). * P<0.05 (female *vs* male).

2.2　Effects of saline injection on brain activity by MEG recordings in female and male rats

In pain research, CFA is commonly employed to induce inflammatory pain, with saline serving as a control reagent[23-24]. Despite its frequent use, the effects of non-harmful solutions like saline on brain activity remain understudied. In the present study, we administered saline into the rat plantar area as a control treatment, and recorded brain activities before and after saline treatment using MEG.

Figure 3 displays the waveforms and brain magnetic data (Figure 3a, b, female; Figure 3c, d, male) recorded 1 h after saline injection for both female and male rats. Time-frequency analyses

revealed a significant increase in average powers (4−30 Hz) in both sexes during baseline and stimulation recordings following saline injection (Figure 3e, saline injection: $F(1, 12)=44.41$, $P<0.000\ 1$; female: $P=0.001$, male: $P=0.001\ 7$, pre-saline baseline *vs* post-saline baseline; female: $P=0.013\ 5$, male: $P=0.001\ 8$, pre-saline stimulation *vs* post-saline stimulation). Furthermore, mechanical stimuli intensified these average powers in both sexes of saline-treated rats (Figure 3e, stimulation: $F(1, 12)=203.9$, $P<0.000\ 1$; female: $P=0.001\ 2$, male: $P<0.000\ 1$, post-saline baseline *vs* post-saline stimulation). Notably, no gender differences were discerned in the average spectral power of saline-treated rats (Figure 3e, gender: $F(1, 12)=0.020\ 24$, $P=0.889\ 2$).

Additionally, the effects of saline injection and stimulation on peak powers were evaluated, revealing a significant impact of both factors, while gender differences were not evident (Figure 3f, saline injection: $F(1, 12)=7.84$, $P=0.001\ 2$; stimulation: $F(1, 12)=40.52$, $P<0.000\ 1$; gender: $F(1, 12)=0.231\ 3$, $P=0.639\ 2$). Interestingly, stimulation increased peak spectral power in female, but not male, saline-treated rats (Figure 3f, female: $P=0.030\ 0$, male: $P=0.737\ 2$, post-saline baseline *vs* post-saline stimulation).

On the other hand, pain behavioral tests indicated a decrease in mechanical thresholds post-saline injection in both female and male rats, without exhibiting any sex differences (Figure 3g, saline injection: $F(1, 14)=45.35$; $P<0.000\ 1$; gender: $F(1, 14)=1.703$, $P=0.213\ 0$; female: $P=0.009\ 9$, male: $P<0.000\ 1$). Taken together, our findings demonstrate that saline injection not only altered pain thresholds at the behavioral level but also influenced the pattern of MEG recordings in both sexes.

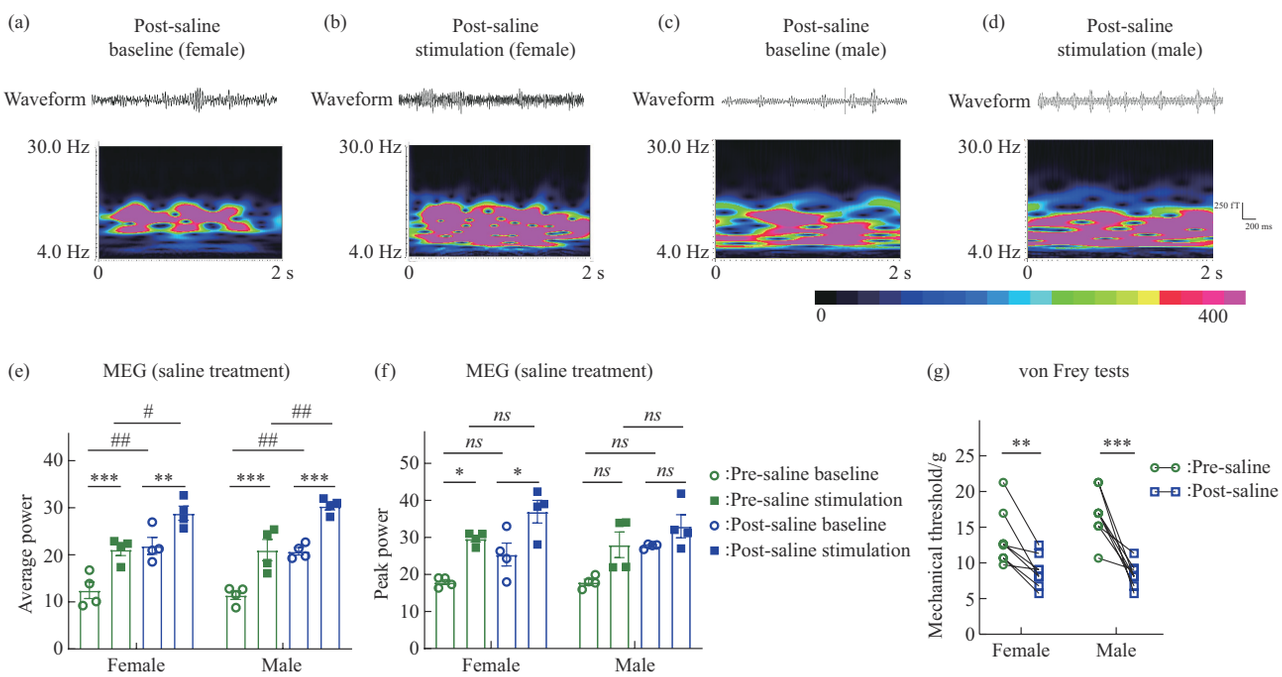

Fig. 3　Injection of saline increased average power and decreased mechanical pain threshold in both female and male in rats

(a, b) The representative MEG waveforms and time-frequency spectrograms of post-saline baseline (a) and post-saline stimulation (b) recorded 1 h after saline injection in female rats. (c, d) The representative MEG waveforms and time-frequency spectrograms of post-saline baseline (c) and post-saline stimulation (d) recorded 1 h after saline injection in male rats. (e, f) The average (e) and peak (f) power (4−30 Hz) of post-saline baseline and post-saline stimulation in female and male rats. Data are presented as mean±SEM. Three-way ANOVA and Sadik's multiple comparisons tests (n=4 per group). *, baseline *vs* stimulation; $^{\#}$, pre-saline *vs* post-saline; ***P<0.001; **, $^{\#\#}P$<0.01; *, $^{\#}P$<0.05; *ns*, not significant. (g) Mechanical threshold of female and male rats before and after saline injection. Data are presented as mean±SEM, two-way ANOVA and Tukey's multiple comparisons test. Pre-saline *vs* post-saline: ***P<0.001; **P<0.01.

2.3 Time−frequency analyses showed gender differences in the MEG recording of CFA−treated rats in the resting state （baseline）

We assessed the effects of CFA injection, mechanical stimulation, and gender on brain magnetic activities 1 h post-CFA administration. The waveforms and MEG (Figure 4a, b, female; Figure 4c, d, male) were presented.

Time-frequency analyses revealed that CFA injection greatly increased the average powers (4−30 Hz) in both sexes of rats during baseline and stimulation recordings (Figure 4e, CFA injection: $F(1, 12)=430.3$, $P<0.000\ 1$; female: $P<0.000\ 1$, male: $P<0.000\ 1$, pre-CFA baseline *vs* post-CFA baseline; female: $P<0.000\ 1$, male: $P<0.000\ 1$, pre-CFA stimulation *vs* post-CFA stimulation). Additionally, mechanical stimuli further augmented average powers in both genders of CFA-treated rats (Figure 4e, stimulation: $F(1, 12)=341.8$, $P<0.000\ 1$; female: $P=0.000\ 2$, male: $P<0.000\ 1$, post-CFA baseline *vs* post-CFA stimulation). To note, sex differences were observed in the average spectral power of CFA-treated rats during the resting state (Figure 4e, gender: $F(1, 12)=16.90$, $P=0.001\ 4$; post-CFA baseline: $P=0.006\ 6$, female *vs* male).

Subsequent analysis of peak powers revealed significant effects of CFA injection and stimulation on peak average power (Figure 4f, CFA injection: $F(1, 12)=117.1$, $P<0.000\ 1$; female: $P<0.000\ 1$, male: $P<0.000\ 1$, pre-CFA baseline *vs* post-CFA baseline; female: $P<0.000\ 1$, male: $P<0.000\ 1$, pre-CFA stimulation *vs* post-CFA stimulation). Mechanical stimuli further increased the peak powers in both female and male CFA-treated rats (Figure 4f, stimulation: $F(1, 12)=146.2$, $P<0.000\ 1$; female: $P=0.000\ 1$, male: $P=0.001\ 2$, post-CFA baseline *vs* post-CFA stimulation). No statistically significant gender differences were observed in the peak spectral power of CFA-treated rats during the resting or stimulated state (Figure 4f, gender: $F(1, 12)=4.001$, $P=0.068\ 6$; post-CFA baseline: $P>0.999\ 9$, post-CFA stimulation: $P=0.891\ 3$, female *vs* male).

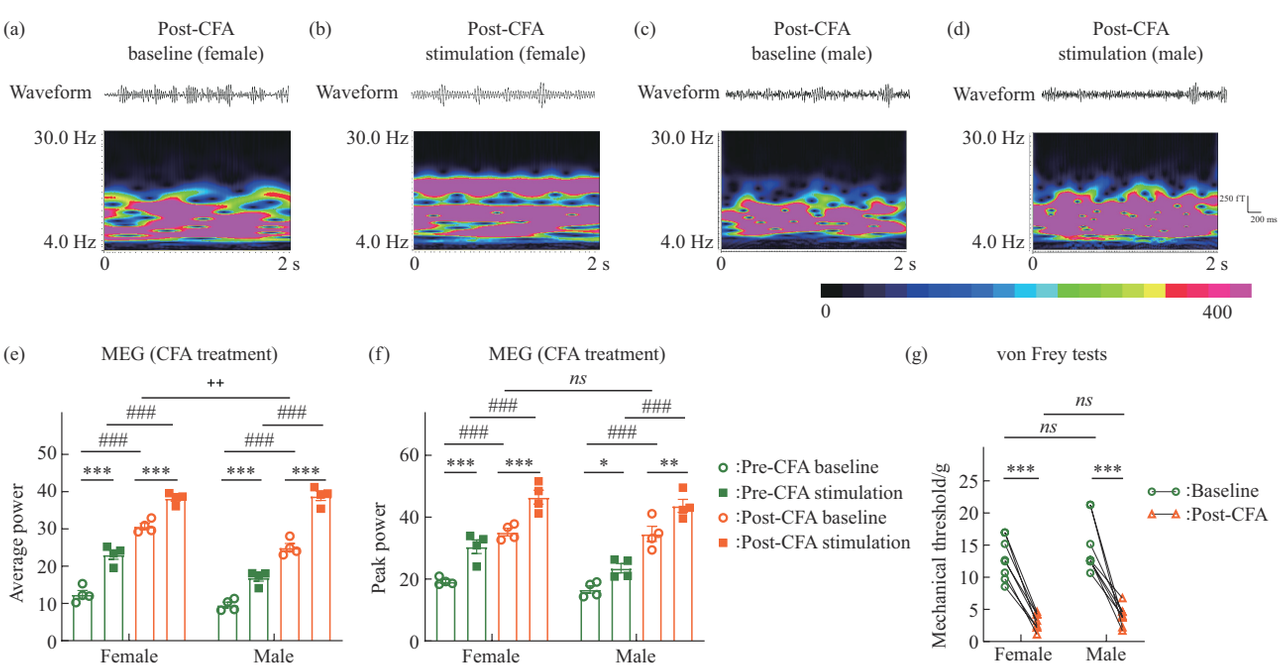

Fig. 4　CFA increased average and peak power in both sexes, and induced a sex difference in average power of post−CFA during baseline recording

(a, b) The representative MEG waveforms and time-frequency spectrograms of post-CFA baseline (a) and post-CFA stimulation (b) recorded 1 h after CFA injection in female rats. (c, d) The representative MEG waveforms and time-frequency spectrograms of post-CFA baseline (c) and post-CFA stimulation (d) recorded 1 h after CFA injection in male rats. (e, f) The average (e) and peak (f) power (4−30 Hz) of post-CFA baseline and post-CFA stimulation in female and male rats. Data are presented as mean±SEM. Three-way ANOVA and Sadik's multiple comparisons tests (n=4 per group). *, baseline *vs* stimulation; $^{\#}$, pre-saline *vs* post-saline; $^{+}$, female *vs* male; ***, $^{\#\#\#}P<0.001$; **, $^{++}P<0.01$; *$P<0.05$; *ns*, not significant. (g) Mechanical threshold of female and male rats before and after CFA injection. Data are presented as mean±SEM, two-way ANOVA and Tukey's multiple comparisons test (n=8 per group). * Baseline *vs* post-CFA; ***$P<0.001$.

In pain behavioral tests, CFA injection greatly decreased mechanical thresholds of both female and male rats (Figure 4g, CFA injection: $F(1, 14)=111.2$; $P<0.000\ 1$; female: $P<0.000\ 1$, male: $P<0.000\ 1$). However, no significant gender-related differences were observed (Figure 4g, gender: $F(1, 14)=1.951$, $P=0.184\ 2$). Taken together, our findings indicate that CFA injection altered the pattern of MEG recordings in both genders, with female rats exhibiting higher resting brain activities than male rats. Notably, this gender disparity was not evident in MEG data during the stimulated state.

2.4 Compared with saline injection, CFA injection increased a higher baseline spectral power in female rats in the resting state

We compared the effects of CFA/saline treatment, mechanical stimulation, and gender on brain magnetic activities (Figure 5a, b) and pain thresholds (Figure 5c) between saline-treated and CFA-treated rats. Treatment (saline or CFA) and stimulation significantly changed the average power (4−30 Hz) in both female and male rats (Figure 5a, treatment: $F(1, 12)=17.84$, $P<0.000\ 1$; stimulation: $F(1, 12)=1042$, $P<0.000\ 1$; gender: $F(1, 12)=1.080$, $P=0.319\ 3$).

Notably, during the resting state (baseline), CFA injection led to a significantly higher average spectral power in female rats compared to saline injection, while this effect was not observed in male rats (Figure 5a, female: $P=0.000\ 2$, male: $P=0.168\ 7$, post-saline baseline *vs* post-CFA baseline). In the stimulated state, CFA injection induced a higher average spectral power than saline injection in both female and male rats (Figure 5a, female: $P<0.000\ 1$, male: $P=0.000\ 3$, post-saline stimulation *vs* post-CFA stimulation).

Regarding peak power, no significant differences were observed between saline-treated and CFA-treated rats of both genders in either the baseline (Figure 5b, female: $P=0.093\ 3$, male: $P=0.476\ 6$, post-saline baseline *vs* post-CFA baseline) or stimulated state (Figure 5b, female: $P=0.112\ 8$, male: $P=0.052\ 6$, post-saline stimulation *vs* post-CFA stimulation). In pain behavioral tests, CFA injection led to a significant decrease in mechanical thresholds when compared with saline injection (Figure 5c, treatment: $F(1, 28)=69.43$; $P<0.000\ 1$; female: $P<0.000\ 1$, male:

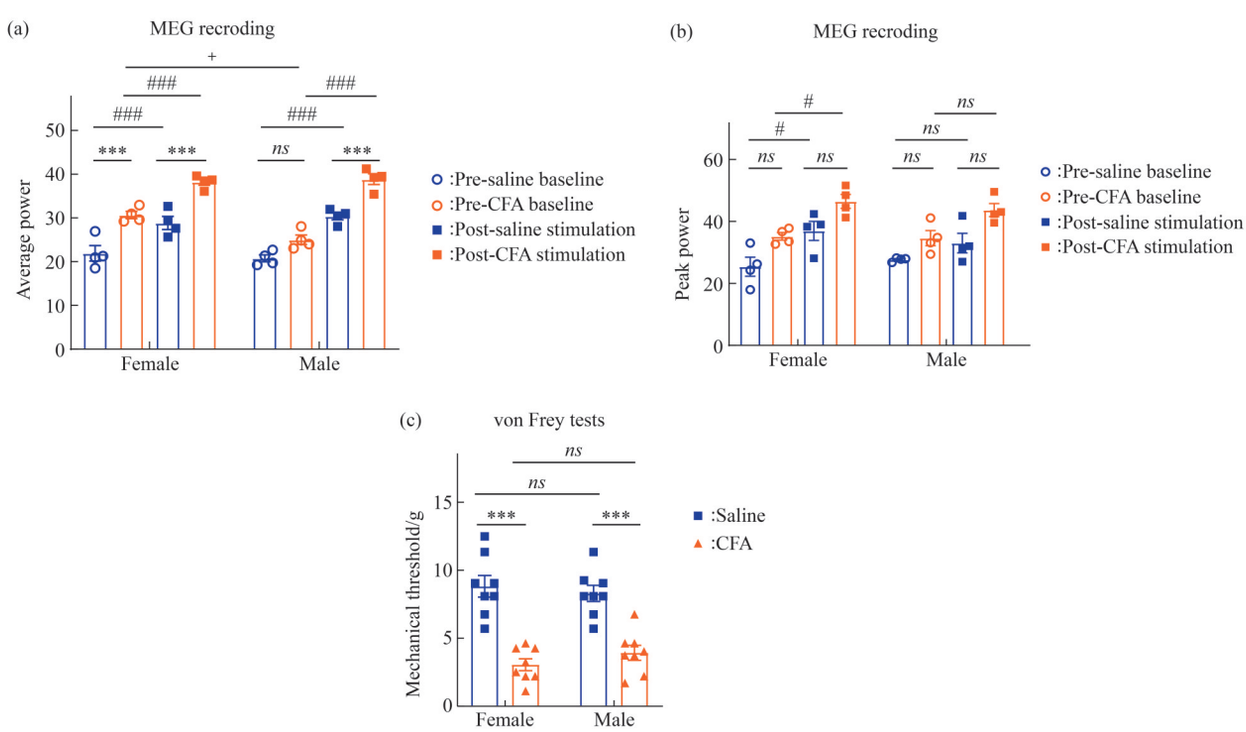

Fig. 5 Comparison of the spectral density and mechanical threshold between saline and CFA and between female and male rats

(a, b) The average (a) and peak (b) power (4−30 Hz) of post-saline/CFA baseline and stimulation in female and male rats. Data are presented as mean± SEM. Three-way ANOVA and Sadik's multiple comparisons tests (n=4 per group). *, saline *vs* CFA; $^{\#}$, baseline *vs* stimulation; $^{+}$, female *vs* male; ***, $^{\#\#\#}P<0.001$; $^{+}P<0.05$; *ns*, not significant. (c) Mechanical threshold of female and male rats after saline/CFA injection. Data are presented as mean± EM, Two-way ANOVA and Tukey's multiple comparisons tests (n=8 per group). * saline *vs* CFA; ***$P<0.001$.

P=0.000 1, saline *vs* CFA). Importantly, there was the absence of sex differences in mechanical thresholds in response to CFA treatments (Figure 5c, gender: $F(1, 28) = 0.081\ 02$; $P = 0.778\ 0$; saline: $P = 0.927\ 9$, CFA: $P = 0.743\ 3$, female *vs* male).

3 Discussion

The unique capability of MEG to detect neural signals from deep brain structures, including subcortical regions and the spinal cord, allows for a detailed exploration of the involvement of these regions in pain modulation and related processes. This sensitivity provides valuable insights into the subcortical mechanisms underlying pain, shedding light on the intricate neural pathways associated with pain perception and modulation. Previous studies utilizing MEG primarily have focused on identifying abnormal brain activity associated with neuropathic pain in humans[25-26]. The present study is an exploring study reporting MEG recording findings in animal models of pain. The application of MEG in animal studies would position it as a valuable translational tool for pain research. The commonalities in pain processing across species enable direct comparisons between findings from animal models and human pain perception. Because MEG is a non-invasive neuroimaging method, it reduces the risk of tissue damage, and enables longitudinal studies to be conducted on the same animal. Additionally, MEG in animal models can be further combined with powerful pharmacological and genetic techniques, to provide complementary information about pain-related neural activity. Integrating multiple modalities lead to a more comprehensive understanding of pain processing using animal models, enhancing our understanding of pain mechanisms and aiding in the development of innovative pain therapies.

In clinical EEG and MEG studies, it has been observed that individuals experiencing chronic pain exhibit heightened resting-state alpha-band spectral power and slower peak alpha frequency (PAF) [27-28]. These alterations have been proposed as potential markers for acute thermal pain sensitivity and as predictors for the development of chronic pain[29-30]. MEG, by detecting abnormalities in alpha oscillations across the dynamic pain connectome (DPC), can offer insights into aberrant neuronal communication in nociceptive processing and modulation.

Inflammation not only strengthens pain signals in the nervous system but also distorts the perception of pain, often leading to an exaggerated or inadequate response[31]. A recent study employing EEG technology investigated changes in EEG activity in rats following the injection of CFA into the hind paw. The results indicated significant alterations in rat EEG activity post-CFA injection, particularly in regions associated with pain such as the frontal lobe, cingulate gyrus, and central posterior gyrus[28, 32]. This abnormal neural electrical activity correlates with increased pain sensitivity in rats, highlighting the potential relevance of these findings to pain-related disorders[31]. Moreover, controlling rat pain behavior significantly influenced neural electrical activity in these regions, indicating a direct link between pain perception and neural responses.

Sex-specific differences in brain activity have been identified in previous EEG or MEG studies[8, 10, 27]. In our study, acute CFA injection altered MEG recordings in both male and female rats. Intriguingly, female rats exhibited heightened MEG activities in the resting state, contrasting with the absence of sex differences during painful stimulation or in behavioral thresholds for defensive paw withdrawal. Notably, this sex-specific difference in alpha brain activity was not observed after saline treatment, which is considered non-harmful. These findings indicate that acute inflammatory insult may induce heightened brain activity in female rats, especially in the absence of external painful stimulation. Spontaneous pain, characterized by the presence of pain without external stimuli, is frequently observed in patients with pain-related disorders[33-36]. Our findings contribute to the understanding of the higher sensitivity to pain in the female population.

Understanding sex disparities in pain perception is paramount given the higher prevalence and intensity of chronic neuropathic pain in women compared to men[33, 37-38]. These differences are influenced by various factors, including hormonal and genetic variances, which in turn impact responses to treatments[33, 39]. Previous studies have identified neuropathic pain-related and sex-specific abnormalities in alpha oscillations across the DPC that could underlie aberrant neuronal communication in nociceptive processing and modulation[40]. Our findings underscore the importance of considering

these sex-specific alterations induced by acute inflammatory pain in the brain.

4 Conclusion

The present study established a MEG methodology in a rat model of inflammatory pain. We reported increased alpha activities after acute inflammatory insult (CFA) or non-harmful injection (saline) in both female and male rats. Specifically, our results revealed that inflammatory insult could induce heightened alpha activity specifically in female rats, even in the absence of external painful stimuli, indicating the heightened perception of spontaneous pain in females during inflammation. These findings provide additional information to a deeper understanding of sex-specific pain responses and underscore the necessity of incorporating sex differences into pain management strategies.

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基于光泵磁力计脑磁图的炎症痛模型大鼠脑活动性别差异研究*

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摘要　目的　脑磁图（magnetoencephalography，MEG）是一种非侵入性的神经成像技术，能够检测和记录由大脑电活动产生的磁场。与基于超导量子干涉仪（superconducting quantum interference devices，SQUID）的MEG相比，基于光泵磁力计（OPM）的MEG具有灵敏度更高、空间分辨率更高和成本更低的优势。但目前大多数研究为临床研究，缺乏基于OPM技术开展的MEG动物实验研究。疼痛是一种不愉快的感官和情感体验。疼痛感知涉及大脑活动的复杂变化，并显示出性别差异。尽管临床上通过MEG发现了与疼痛相关的神经元活动，但其特点尚未阐明，关于疼痛相关大脑活动变化的基础研究仍然不足。本研究旨在用MEG技术在大鼠模型中研究炎症性疼痛（通过完全弗氏佐剂（Complete Freund's Adjuvant，CFA）诱导）对大脑活动的影响，分析疼痛感知期间的大脑活动变化，并探索疼痛相关MEG信号的性别差异。**方法**　本研究使用成年雄性和雌性Sprague-Dawley大鼠。通过在爪内注射CFA（100 μl，50%溶液，稀释于生理盐水）诱导炎症性疼痛，对照组注射生理盐水。采用von Frey纤维在基线及注射后1 h评估疼痛行为。进行MEG记录时，麻醉大鼠的头部放置OPM，并置于多层磁屏蔽内，以确保信号的清晰度。每只大鼠进行两个15 min的MEG记录，其中包括5 min的基线记录和10 min的机械刺激阶段。通过去除伪影和累积频谱图对自发脑活动进行时频分析，生成聚焦于4~30 Hz频率范围的频谱图。**结果**　注射生理盐水/CFA前后，雄性和雌性大鼠的机械刺激都能提高alpha波活动（4~30 Hz）。与注射前的状态相比，注射生理盐水/CFA会增强雌雄大鼠的平均alpha波活动。值得注意的是，雌性大鼠在注射CFA 1 h后表现出的平均alpha波活动高于静息状态下注射生理盐水后的平均alpha波活动。此外，通过von Frey行为学测试测量的疼痛阈值没有出现性别差异，但在注射CFA后的静息状态下，雌性大鼠比雄性大鼠表现出更高的平均alpha频谱功率。**结论**　与雄性大鼠相比，雌性大鼠对炎症性疼痛的感知能力更强。综上所述，CFA注射后大脑alpha波活动表现出性别差异，在炎症性疼痛期间处于静息状态的雌性大鼠大脑alpha波活动静息水平较雄性大鼠高。本研究提供了一种基于OPM的MEG记录用以研究麻醉动物脑活动的方法。此外，本研究的结果有助于深入理解疼痛相关的神经活动和疼痛性别差异。

关键词　脑磁图，疼痛，性别差异，alpha波活动，炎症

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