

A Single Exposure to GSM-1800 MHz Signals in the Course of an Acute Neuroinflammatory Reaction can Alter Neuronal Responses and Microglial Morphology in the Rat Primary Auditory Cortex

Florian Occelli,^{d†} Julie Lameth,^{a†} Victor Adenis,^d Chloé Huetz,^d Philippe Lévêque,^b Thérèse M. Jay,^c Jean-Marc Edeline^{d*†} and Michel Mallat^{a*†}

^a Sorbonne Universités, UPMC Univ Paris 06, INSERM, CNRS, Institut du cerveau et de la moelle Epinière (ICM), Hôpital Pitié-Salpêtrière, boulevard de l'Hôpital, F-75013 Paris, France

^b Univ. Limoges, CNRS, XLIM, UMR 7252, F-87000 Limoges, France

^c INSERM, UMR_S 894, Physiopathologie des Maladies Psychiatriques, Centre de Psychiatrie et Neurosciences, Université Paris Descartes, Paris, France

^d Paris Saclay Institute of Neuroscience, Neuro-PSI, UMR 9197 CNRS & Université Paris-Sud, Orsay, France

Abstract—During mobile phone conversations, the temporal lobe neural networks involved in processing auditory information are exposed to electromagnetic fields (EMF) such as pulse-modulated GSM-1800 MHz radiofrequencies that convey wireless communications. The effects of these EMF on the brain affected by a pathological condition remain little investigated. In this study, rats injected with lipopolysaccharide (LPS) to induce neuroinflammation were exposed “head-only” to GSM-1800 MHz signals for two hours at a specific absorption rate (SAR) that reached an average value of 1.55 W/kg in the auditory cortex (ACx). Immunodetection of Iba1, a microglial marker, and electrophysiological recordings in the ACx three to six hours after global system for communication (GSM) exposure, or sham-exposure, showed that exposure to GSM-1800 MHz resulted in a growth of microglial processes and a reduction in spontaneous firing rate. More importantly, there was a significant reduction in evoked responses to artificial and natural stimuli and an increase in response duration. The response latency and the bandwidth of the frequency tuning were unchanged, but the GSM exposure led to a higher proportion of cortical sites exhibiting abnormally high acoustic thresholds. These modifications were not observed in rats exposed to GSM-1800 MHz without pretreatment with LPS. Together our data provide evidence that in neuroinflammatory conditions, acute exposure to GSM-1800 MHz can significantly affect microglia and neuronal activity underlying auditory perception. © 2018 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: auditory cortex, spectro-temporal receptive field, neuronal activity, microglia, lipopolysaccharide, electromagnetic field.

INTRODUCTION

Mobile phone communications are propagated by radiofrequencies and since the 1990s these communications use pulse-modulated signals among which is the global system for communication (GSM), which operates either in the 900-MHz or 1800-MHz bands. Potential health hazard of these electromagnetic fields (EMF) have motivated a broad spectrum of

laboratory studies that aim at determining biological responses to these EMFs. Many of these studies have focused on the CNS, given that the use of mobile phones often minimizes the distance between the brain and EMF source. In particular, the capacity of GSM signals to affect neuronal activity in humans has been approached by behavioral, electrophysiological or neuroimaging techniques. Consistent effects of EMF on cognitive performance could not systematically be established when assessing reaction times or accuracy (reviewed in (Valentini et al., 2010; Kwon and Hamalainen, 2011; Barth et al., 2012), but recent reports emphasize that this issue remains actively investigated (Vecchio et al., 2012; Byun et al., 2013; Guxens et al., 2016). Electroencephalographic analyses have revealed cortical responses induced by acute head exposure to GSM-EMF emitted by a mobile phone, albeit with marked differences depending on the study, which may originate

*Corresponding authors.

E-mail addresses: jean-marc.edeline@u-psud.fr (J.-M. Edeline), michel.mallat@upmc.fr (M. Mallat).

† Equal contributions.

Abbreviations: ACx, auditory cortex; BF, best frequency; EEG, electroencephalogram; EMF, electromagnetic fields; FRA, frequency response area; GSM, global system for communication; LPS, lipopolysaccharide; MUA, multi-unit activity; PBS, phosphate-buffered saline; SAR, specific absorption rate; SPL, sound pressure-level; STRFs, spectro-temporal receptive fields.

from variation in methodological design (Kwon and Hamalainen, 2011). The most consistently observed modifications are associated with the alpha band in the resting electroencephalogram (EEG). Several groups found an increase in EEG power in the alpha band (Croft et al., 2002, 2008; Curcio et al., 2005; Regel et al., 2007), whereas others reported reduced power (Maby et al., 2006; Perentos et al., 2013; Ghosn et al., 2015). Inter-hemispheric coherence of frontal alpha rhythms was found to be enhanced or reduced in elderly or young adults, respectively (Vecchio et al., 2007, 2010). Furthermore, it was observed that GSM-EMF can transiently increase cortical excitability assessed by recording motor-evoked potentials during transcranial magnetic stimulation of the motor cortex (Ferreri et al., 2006). Stimulating effects of EMF on cortical activity were also detected by measuring glucose metabolism with positron emission tomography (Volkow et al., 2011).

Besides studies involving human volunteers, the effects of GSM-related EMF on neuronal activity have been investigated in different animal species, *in vivo* or *ex vivo* in CNS slices or in neural cell cultures. These preclinical models are compatible with extended analyses at cellular or molecular levels, and they limit individual variability which is thought to affect the results from human studies (Loughran et al., 2012). However, the occurrence and amplitude of the potential EMF effects remain strongly dependent on experimental settings. Key parameters include the duration and number of exposures to EMF, as well as the specific absorption rate (SAR), which measures the rate at which EMF energy is absorbed by a unit mass of tissue (W/kg). EMF-induced changes in neuronal excitability were observed when performing electrophysiological recording of rat or avian neurons during EMF exposures. These changes involved increases or decreases in firing rate or burst rate (Tattersall et al., 2001; Beason and Semm, 2002; Moretti et al., 2013). Reductions in the threshold for firing action potentials or in neuronal accommodation were also detected in snail neurons recorded shortly after EMF exposure (Partsvania et al., 2011, 2013). In addition, it was found that exposure to GSM-900 MHz during the entire gestation period (6 h per day) decreased neuronal excitability of Purkinje cells in rat offspring (Haghani et al., 2013).

While most reports aimed at specifying the effect of EMF on the healthy CNS, recent studies have addressed how GSM-associated EMF may impact neuronal activity in the CNS already affected by a pathological process. Both EEG and transcranial magnetic stimulation-based investigations provided evidence that EMF-induced enhancement of excitability was prevented in human cerebral hemispheres affected by an epileptic focus (Tombini et al., 2013; Curcio et al., 2015). However, immunodetection of *cfos* and behavioral assessment showed that GSM-EMF can promote neuronal activity and seizures in rats treated with a subconvulsive dose of picrotoxin, (Lopez-Martin et al., 2006; Carballo-Quintas et al., 2011). Furthermore, improved cognitive performance was observed in mouse models

of Alzheimer's disease, as a consequence of repeated exposures to EMF (Arendash et al., 2012; Jeong et al., 2015).

It is now well established that most, if not all, CNS pathologies generate neuroinflammation, marked by reactive microglia, the immune resident CNS cells (Salter and Stevens, 2017; Wolf et al., 2017). Reactive microglia can affect the activity, or the wiring, of neuronal networks owing to a variety of mechanism, including microglial cell productions of cytokines, neuronal growth factors, reactive oxygen species, and glutamatergic agonist or phagocytic elimination of synapses (Cheret et al., 2008; Heneka et al., 2014; Mesci et al., 2015; Salter and Stevens, 2017).

Lipopolysaccharide (LPS) injection in rodents has been widely used to model neuroinflammation and the sickness syndrome triggered by acute infections (Dantzer, 2001; Dantzer et al., 2008; Hoogland et al., 2015). These pathological states are highly frequent in human populations and they are thought to favor exaggerated and deleterious inflammatory responses in microglia during aging or in brains affected by neurodegenerative diseases (Perry and Holmes, 2014; Norden et al., 2015). Investigating the effect of EMF in LPS-treated rats, we recently found that a 2-h head-only exposure to GSM-1800 MHz can transiently modify cortical neuroinflammation and key components of excitatory neurotransmission, as revealed by EMF-induced reductions in the level of interleukin-1 β gene expression, increased length or branching of microglial processes and changes in phosphorylation of AMPA receptors (Lameth et al., 2017). These effects were observed 24 h following EMF exposure in the dorso-medial part of the cerebral cortex where the local SAR averaged 2.9 W/kg (Lameth et al., 2017). Importantly, our study indicated that the neuroinflammatory state was necessary to promote CNS cell response to GSM-1800 MHz, because, in healthy rats, the very same exposure to GSM-1800 MHz had no effect on AMPA receptor phosphorylation, IL-1 β gene expression (Lameth et al., 2017) or on markers commonly used to detect glial cell reactions (Watilliaux et al., 2011).

In the present study, we used the same GSM-exposure settings applied to LPS-injected rats to further investigate the impact of EMF on neuronal activity under a neuroinflammatory condition. Our aim was to determine whether a single head-only GSM-EMF exposure can alter electrophysiological responses of cortical neurons to sensory stimuli, a few hours after termination of the EMF exposures. We focused on the auditory cortex, which, in humans, is close to the GSM source, and is necessarily activated during mobile phone communications. We show here that when this cortical region is affected by neuroinflammation, a 2-h GSM exposure leads to changes in both microglial cell morphologies and in neuronal response to artificial and natural stimuli. Our dosimetric analyses indicate that these cell responses can be obtained with local SAR values of less than 2 W/kg. These values are far below those previously reported to have no behavioral effects in normal rats (up to 3.5 W/kg) (Dubreuil et al., 2002,

2003), but they remain higher than those estimated in the brain of mobile phone users (Wiert et al., 2008; Ghanmi et al., 2014).

EXPERIMENTAL PROCEDURES

Subjects

Data were collected in the cerebral cortex of 29 adult male Wistar rats obtained from Janvier Laboratories at 55 days of age. Rats were housed in a humidity (50–55%) and temperature (22–24 °C)-controlled facility on a 12 h/12 h light/dark cycle (light on at 7:30 A.M.) with free access to food and water. All experiments were conducted in accordance with the guidelines established by the European Communities Council Directive (2010/63/EU Council Directive Decree), which are similar to those described in the *Guidelines for the Use of Animals in Neuroscience Research of the Society of Neuroscience*. The protocol was approved by the ethics committee Paris-Sud and Centre (CEEA N°59, project 2014-25, national agreement 03729.02) using the procedures 32-2011 and 34-2012 validated by this committee.

The animals were habituated to the colony rooms for at least one week before LPS treatment and exposure (or sham exposure) to GSM-EMF. Twenty-two rats were injected intraperitoneally (i.p.) with *E. coli* LPS (250 µg/kg, serotype 0127:B8, SIGMA) diluted in sterile endotoxin-free isotonic saline 24 h before GSM or sham exposure ($n = 11$ per group).

Exposure to GSM-1800 MHz

As in a previous study (Lameth et al., 2017), exposure of rats to GSM was carried out 24 h after LPS injections (11 animals) or without LPS treatment (four animals). The animals were lightly anesthetized with Ketamine/Xylazine (Ketamine 80 mg/kg, i.p.; Xylazine 10 mg/kg, i.p.) before exposure to prevent movements and ensure reproducible position of the animal's heads below the loop antenna emitting the GSM signal. Half of the rats from the same cages served as control (14 sham-exposed animals, among which 11 rats pretreated with LPS): they were placed below the loop antenna with the energy of the GSM signal sets to zero. The weight of the exposed and sham-exposed animals were similar (exposed: 300 ± 10 g; sham-exposed 314 ± 13 g; unpaired t -test $t(23) = 0.53$, $p = 0.31$). All the anesthetized animals were placed on a metal-free heating pad to maintain their body temperature around 37 °C throughout the experiment. As in previous experiments, the exposure duration was set at 2 h. After exposure, the animals were placed on another heating pad in the surgery room.

EMF exposure system

The exposure system was identical to that used previously (Watilliaux et al., 2011) and has been described in detail elsewhere (Leveque et al., 2004). Briefly, a radiofrequency generator emitting a GSM electromagnetic field (RFP, 1/8 duty factor) pulsed at 217 Hz (pulse emission every 4.6 ms for 546 µs) was connected to a four-output divider, allowing simultaneous

exposure of four animals. Each output was connected to a loop antenna, (Sama-Sistemi srl; Roma) enabling local exposure of the animal's head. The loop antenna consisted of a printed circuit, with two metallic lines engraved in a dielectric epoxy resin substrate (dielectric constant $\epsilon_r = 4.6$). At one end, this device consisted of a 1-mm-wide line forming a loop placed close to the animals' head. As in previous studies (Leveque et al., 2004; Watilliaux et al., 2011; Lameth et al., 2017), SARs were determined numerically using a numerical rat model with the Finite Difference Time Domain (FDTD) method (Yee, 1966; Kunz and Luebbers, 1993; Taflov, 1995). They were also determined experimentally in homogeneous rat model using a Vitek or Luxtron probe for measurement of rises in temperature. In this case, SARs, expressed in W/kg, were calculated using the following equation: $SAR = C \Delta T / \Delta t$ with C being the calorific capacity in J/(kg·K), ΔT , the temperature change in °K and Δt , the time in seconds. Numerically determined SAR values were compared with experimental SAR values obtained using homogenous models, especially in the equivalent rat brain area. The difference between the numerical SAR determinations and the experimentally detected SAR values was less than 30%.

Fig. 1 shows the SAR distribution in the rat brain with a rat model, which matched that of the rats used in our study in terms of weight and size (see Lameth et al., 2017). The SAR values were the highest in cortical areas located directly below the loop antenna: they were 2.94 ± 0.24 (mean $\pm$ std) in the dorso-medial part of the cortex (Lameth et al., 2017). In contrast, in the auditory cortex (ACx) (Fig. 1 bottom middle panel), local SAR was reduced to 1.55 ± 0.71 W/kg. This value dropped to 0.68 ± 0.57 W/kg in the cortical region located ventrally to the ACx (bottom right), which is part of the piriform and the entorhinal cortex. As the weight of the exposed rats were homogenous, differences in tissues' thickness at the level of the head were probably negligible, so the actual SAR in ACx or other cortical regions was probably very similar from one exposed animal to another.

Surgical procedures for electrophysiological recordings

At the end of exposure, the animal was supplemented with additional doses of Ketamine (20 mg/kg, i.p.) and Xylazine (4 mg/kg, i.p.) until no reflex movement could be observed after pinching the hind paw. A local anesthetic (Xylocaine 2%) was liberally injected subcutaneously in the skin above the skull and on the temporal muscles. After placing the animal in a stereotaxic frame, a craniotomy was performed above the left temporal cortex. The opening was 9 mm wide starting at the intersection point between parietal and temporal bones and 5 mm high as in our previous studies (Manunta and Edeline, 1997, 2004; Manunta and Edeline, 1998; Cotillon and Edeline, 2000; Cotillon-Williams and Edeline, 2003). The dura above the ACx was carefully removed under binocular control without damaging the blood vessels. And the end of the surgery, a pedestal in dental acrylic cement was built to allow atraumatic fixation of the animal's head during the

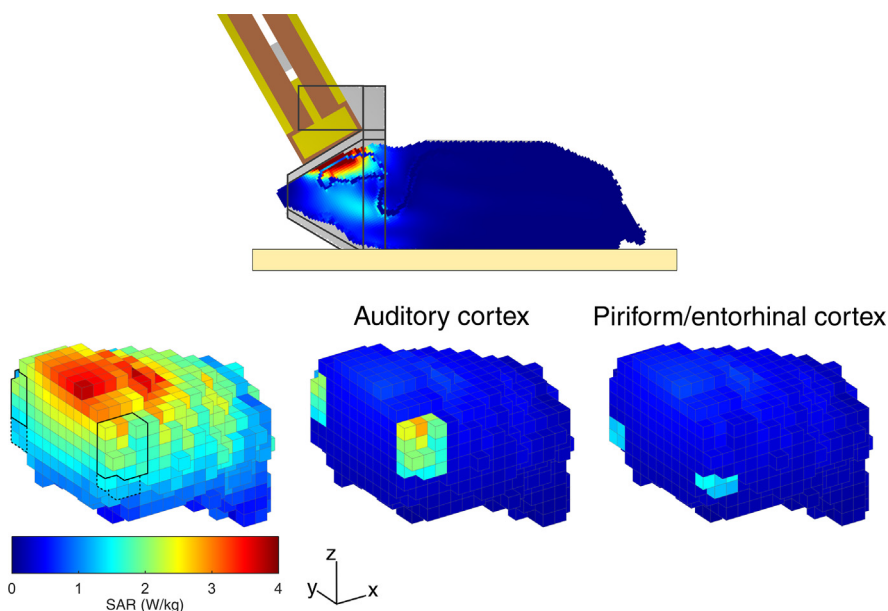


Fig. 1. Dosimetric analysis of specific absorption rates (SARs) in the rat brain during exposure to GSM-1800 MHz. The heterogeneous model of phantom rat and loop antenna described by [Leveque et al. \(2004\)](#) was used to evaluate the local SAR in the brain with a 1-mm³ (adult brain) cubic mesh. (Top) Global view of the rat modeled (adult rat) in the exposure setup with the loop antenna above its head and the metal-free heat pad (yellow) under the body. (Bottom) Distribution of SAR values in the adult brain at 1-mm³ spatial resolution. The areas delimited by the thick black contours on the left correspond to the primary auditory cortex in which microglia and neuronal activity were analyzed; the area delimited by the dashed line contains the piriform and entorhinal where the SAR value was lower (right panel). The color-coded scale of SAR values shown in (bottom) applies to all numerical simulations shown in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

recording session. The stereotaxic frame supporting the animal was placed in a sound-attenuating chamber (IAC, model AC1).

Recording procedures

Data were from multiunit recordings in the primary ACx (area AI) of 16 rats including 10 animals pretreated with LPS. Extracellular recordings were from arrays of 16 tungsten electrodes (TDT, ϕ : 33 μ m, < 1 M Ω) composed of two rows of eight electrodes separated by 1000 μ m (350 μ m between electrodes of the same row). A silver wire (ϕ : 300 μ m), used as ground, was inserted between the temporal bone and the dura mater on the contralateral side. The estimated location of the primary ACx was 5 mm posterior to Bregma and 7 mm, 3 mm ventral to the superior suture of the temporal bone (corresponding area A1 in Paxinos & Watson 2005). The raw signal was amplified 10,000 times (TDT Medusa) then processed by a multichannel data acquisition system (RX5, TDT). The signal collected from each electrode was filtered (610–10000 Hz) to extract multi-unit activity (MUA). The trigger level was carefully set for each electrode (by a co-author blind to the exposed or sham-exposed status) to select the largest action potentials from the signal. On-line and off-line examination of the waveforms suggests that the MUA collected here was made of action potentials generated by 3–6 neurons at the vicinity of the

electrode. At the beginning of each experiment, we set the position of the electrode array in such a way that the two rows of eight electrodes sampled neurons responding from low to high frequency when progressing in the rostro-caudal direction.

Acoustic stimuli

Acoustic stimuli were generated in Matlab, transferred to a RP2.1-based sound delivery system (TDT) and sent to a Fostex speaker (FE87E). The speaker was placed at 2 cm from the rat's right ear, a distance at which the speaker produced a flat spectrum (± 3 dB) between 140 Hz and 36 kHz. Calibration of the speaker was made using noise and pure tones recorded by a Bruel & Kjaer microphone 4133 coupled to a preamplifier B&K 2169 and a digital recorder Marantz PMD671. Spectro-temporal receptive fields (STRFs) were determined using 97 gamma-tone frequencies, covering eight (0.14–36 kHz) octaves presented at 75-dB SPL at 4.15 Hz in a random order. The frequency response area (FRA) was determined using the same set of tones and presented from 75-dB to 5-dB SPL in random

order at 2 Hz. Each tone was presented eight times at each intensity.

Responses to natural stimuli were also evaluated. In pilot studies ([Occelli et al., 2016](#)), we observed that, regardless of the neurons' best frequency (BF), rat vocalizations rarely elicit robust responses in AI, whereas heterospecific ones (such as song birds or guinea pig vocalizations) often trigger robust and reliable responses across the entire tonotopic map. Therefore, we tested here cortical responses to guinea pig's vocalizations (a whistle call used in ([Gaucher et al., 2013a](#))) concatenated in a 1 s stimuli, presented 25 times. This vocalization has its fundamental frequency between 2.5 and 5 kHz and harmonics up to 20 kHz (see [Fig. 2](#) in [Gaucher et al., 2013a](#), and [Fig. 2](#) in [Gaucher et al., 2013b](#)).

Inserting an array of 16 electrodes in the cortical tissue almost systematically induces deformation of the cortex. We gave at least a 20-minute recovery period to allow the cortex to return to its initial shape, then the array was slowly lowered. Spectro-Temporal Receptive Fields (STRFs) were used to assess the quality of our recordings and to adjust the electrodes depth. The recording depth was 300–700 μ m, which corresponds to layer III/IV and the upper part of layer V according to ([Roger and Arnault, 1989](#)). When a clear tuning was obtained for at least 12 of the 16 electrodes, and that the stability of the recordings was satisfied, the protocol started by presenting the acoustic stimuli in the following

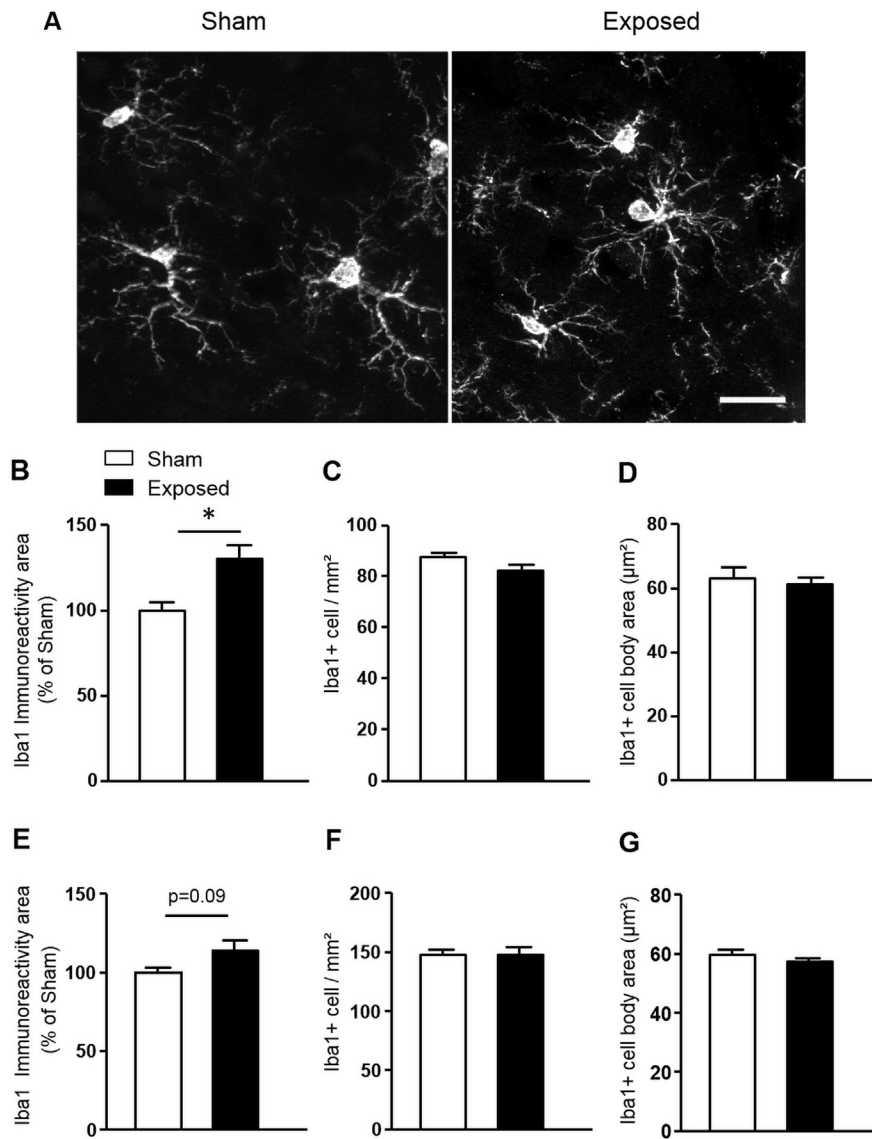


Fig. 2. Effect of GSM-1800 MHz on microglia in, or ventral to, the auditory cortex in LPS-injected rats. (A) Representative stacked views of microglia stained with anti-Iba1 antibody in coronal sections of the auditory cortex from LPS-injected rats that were perfused 3–4 h after sham or GSM exposure (exposed). Scale bar = 20 μ m. (B–D) Morphometric assessment of microglia in the auditory cortex. (B) Spatial coverage of the microglial marker Iba1, 3–4 h after sham or GSM exposure (exposed). Data represents the area of anti-Iba1 staining normalized to mean values from sham-exposed animals. (C, D) Counts of anti-Iba1-stained microglial cell bodies (C) and area of microglial cell bodies (D) in exposed and sham-exposed animals. (E, F, G) Microglia analyses in the area located ventrally to the auditory cortex (piriform and entorhinal cortex): Iba1 spatial coverage (E) and counts (F) or area (G) of Iba1-positive cell bodies. All the data are mean $\pm$ SEM from six animals, * $p < 0.05$, Student's unpaired- t test.

order: (1) Gamma-tones to determine the STRF at 75-dB SPL (5 min), (2) Gamma-tones at intensities ranging from 75- to 5-dB SPL (at 2 Hz) to determine the Frequency Responses Area (FRA, lasting 12 min), (3) vocalizations (25 repetitions) at 75-dB SPL. Presentation of the entire set of stimuli lasted 30 min, with periods of 2 min of spontaneous activity collected before and after these stimuli. This set of acoustic stimuli was used with the electrode array positioned at 3–6 locations per animal in the primary ACx.

Data analysis

The STRFs derived from MUA were obtained by constructing post-stimulus time histograms (PSTHs) for each frequency with 1-ms time bins. All spikes falling in the averaging time window (starting at stimulus onset and lasting 100 ms) were counted. Thus, STRFs are matrices of 100 bins in abscissa (time) multiplied by 97 or 129 bins in ordinate (frequency). For visualization, all STRFs were smoothed with a uniform 5×5 bin window, but the actual values of response latency and bandwidth were from raw data.

From the STRF obtained at 75-dB SPL, the BF was defined as the frequency (in kHz) where the highest firing rate was recorded. At each intensity, peaks of significant response were automatically identified using the following procedure: A positive peak in the MUA-based STRF was defined as a contour of firing rate above the average level of the baseline activity (estimated from the ten first milliseconds of STRFs at all intensity levels) plus six times the standard deviation of the baseline (spontaneous) activity. For a given cortical recording, four measures were extracted from the peak of the STRF.

First, the “response strength” was the total number of spikes falling in the significant peaks. Second, the “response duration” was the time difference between the first and last spike of the significant peaks. Third, the “bandwidth” was defined as the sum of all peaks width in octaves. Forth, the response latency was measured as the time at which the firing rate was 6 times above spontaneous activity (i.e. the latency of the significant contour).

The response to the natural stimulus (guinea pig whistle) was quantified by the firing rate (number of action potentials) emitted during the 345 ms of presentation of that stimulus. We also computed the spike-timing reliability coefficient (CorrCoef), which quantifies the trial-to-trial reliability of the response. This index was computed for each vocalization: it corresponds to the normalized covariance between each pair of spike trains recorded at presentation of this vocalization and was calculated as follows:

$$\text{CorrCoef} = \frac{1}{N(N-1)} \sum_{i=1}^{N-1} \sum_{j=i+1}^N \frac{\sigma x_i x_j}{\sigma x_i \sigma x_j}$$

where N is the number of trials and $\sigma x_i x_j$ is the normalized covariance at zero lag between spike trains x_i and x_j where i and j are the trial numbers. Spike trains x_i and x_j were previously convolved with a 10-ms-wide Gaussian window. Based upon computer simulations, we have previously shown that this CorrCoef index is not a function of the neurons' firing rate (see for details [Gaucher et al., 2013a](#); [Aushana et al., 2018](#)).

Immunohistochemistry

Twelve LPS-treated rats, submitted to identical GSM or sham exposure protocols ($n = 6$ per group), were used for immunohistochemistry in the cerebral cortex. Three to four hours after exposure (or sham exposure), adult rats were deeply anesthetized with pentobarbital and perfused with 0.1 M phosphate-buffered saline (PBS) followed by paraformaldehyde 4% in 0.1 M PBS, pH 7.4. Brains were post-fixed overnight in paraformaldehyde 4%, then cryoprotected by immersion in PBS containing 25% (w/v) sucrose and frozen in -70°C isopentane. Immunostaining was performed on coronal sections (20- μm -thick) cut on a cryostat (Microm, Heidelberg, Germany) and mounted on gelatin-coated Superfrost glass slides (Menzel-glaser, Freiburg, Germany). Sections were blocked in PBS containing 0.1% Triton-X100 and 10% goat serum (30 min at room temperature) and then incubated overnight at 4°C with rabbit polyclonal anti-Iba1 (1:400, Wako Chemicals). Bound antibodies were detected by applying biotinylated goat anti-rabbit IgG (GE Healthcare, Velizy Villacoublay, France) for 2 h at room temperature, followed by Alexa Fluor 488-conjugated streptavidin (Life technology, Saint Aubin, France) for 30 min at room temperature. Sections were counterstained with Hoechst 3342 dye (2 $\mu\text{g}/\text{ml}$) and mounted in fluoromount G (Clinisciences). Control staining, performed by omitting primary antibodies, was negative.

Image analysis

Quantitative image analyses were performed as described previously ([Cheret et al., 2008](#)). Images were captured using a Zeiss AxioImager Z1 microscope equipped with the apotome system. A $10\times$ NA 0.50 Fluor objective (Zeiss microscope) was used to acquire images with equal exposure times. For each animal and each cortical region assessed, immunostained microglial cells were analyzed in microscopic fields ($6 \times 10^5 \mu\text{m}^2$ area) acquired in 12 sections spanning areas of the auditory, piriform or dorsolateral entorhinal cortex and distributed over a cortical region extending in the rostro-caudal axis between 4 and 6.4 mm caudal to the bregma. Image analysis was performed blind relative to the experimental groups. The microglial cell bodies, confirmed by nuclei counterstaining, with Hoechst dye were counted manually. The area stained by anti-Iba1 and that of Iba1-stained cell bodies were determined following assignment

of a threshold to eliminate background immunofluorescence, using Image J software.

Statistical analysis

Differences between groups of animals were analyzed using non-parametric Chi-square test or the parametric unpaired Student t -test. Unpaired t test was applied checking the assumption of equal variances and Gaussian distributions with F -test and the Kolmogorov and Smirnov (K-S) tests using Prism software. For the electrophysiological data, all statistical analyses were performed as if each recording was independent, but cluster analysis was also performed by taking the position of the electrode array as cluster. All the statistical effects reported in this paper still hold when cluster-based analysis was performed.

RESULTS

Microglial morphology in or ventrally to the ACx

Adults rats were injected with LPS 24 h before the 2 h head-only exposure to GSM-1800 MHz or the sham exposure. To determine whether GSM-EMF impacted LPS-responsive immune cells in the ACx, we analyzed cortical sections stained with anti-Iba1 that selectively reveals microglia ([Kettenmann et al., 2011](#)). [Fig. 2A](#) illustrates microglial morphologies in sections of ACx fixed 3–4 h after sham or GSM exposure. LPS-stimulated microglia showed a bush-like cell morphology marked by highly ramified tortuous processes in both sham and GSM-exposed animals. However, microglial processes appeared more developed in GSM-exposed animals. Consistently, quantitative image analyses showed that the total area of Iba1 immunoreactivity in optic fields of ACx was significantly increased by 30% in RF-exposed rats compared with sham-exposed animals ([Fig. 2B](#); unpaired t -test $t(10) = 3.24$; $p < 0.01$). In contrast, GSM exposure had no significant effect on the number ([Fig. 2C](#); unpaired t -test $t(10) = 1.79$; $p = 0.10$) or the area ([Fig. 2D](#); unpaired t -test $t(10) = 0.43$; $p = 0.67$) of microglial cell bodies revealed by Iba1 staining. Together, these data indicate that in animals exposed to GSM-1800 MHz for 2 h, there was an enlargement of the spatial domain occupied by microglial processes in the ACx, detectable 3–4 h after the end of the exposure. The mean SAR value in the ACx was 1.55 W/kg ([Fig. 1](#)). To determine the microglial response at a lower SAR value, Iba1 immunostaining was also analyzed in a cortical region located ventrally to the ACx, where the average SAR was reduced to 0.68 W/kg (see [Fig. 1](#), bottom right panel). In this cortical region, the total area of Iba1 immunoreactivity tended to increase in GSM-exposed animals ([Fig. 2E](#), 14% increase in the mean value) but the difference between GSM- and sham-exposed animals did not reach significance (unpaired t -test $t(10) = 1.87$; $p = 0.09$). Also, GSM exposure had no significant effect on the number ([Fig. 2F](#); unpaired t -test $t(10) = 0.03$; $p = 0.97$) or on the area of microglial cell body ([Fig. 2G](#); unpaired t -test $t(10) = 1.16$; $p = 0.27$).

Responses of ACx neurons to pure tones and natural sounds

Initially, 352 recordings were collected in sham-exposed animals and 416 recordings were collected in exposed animals. The electrophysiological results presented below are from recordings displaying significant STRFs (i.e. evoked responses 6 standard deviations above spontaneous firing rate): 293 Multiunit (MUA) recordings obtained in 5 sham-exposed rats and 350 MUA recordings obtained in 5 GSM-exposed rats. Both in exposed and sham-exposed animals electrophysiological recordings were performed between 3 h and 6 h after the end of the exposure. For each animal, the insertion of the electrode array in the ACx and the adjustment of the trigger level for each electrode was made under blind conditions to the status of the animal (exposed or sham-exposed).

In all animals (exposed or sham-exposed animal), responses to pure tones were observed between 4.0 and 6.0 mm posterior to Bregma, i.e. at the classical location for the rat primary ACx (Manunta and Edeline, 1998; Polley et al., 2007). On each animal, we observed a clear gradient of best frequencies (BF) of about five octaves in 2 mm with the high frequencies (up to 36 kHz here) located rostrally. Fig. 3 shows two examples of tonotopic gradients observed in a sham-exposed (Fig. 3A) or exposed animal (Fig. 3B). The range of BF observed at 75 dB in the two groups of animals (Fig. 3C) were from 1.1 kHz to 36 kHz and their distribution's did not differ between groups (Chi-square = 0.49; ns).

As shown in Fig. 4, analyzing the different parameters derived from spectro-temporal receptive fields (STRFs) collected at 75 dB revealed striking differences between exposed and sham-exposed animals. First, the response strength (i.e. the total number of action potentials falling in the significant contours of STRFs) was weaker in exposed rats compared with sham-exposed controls (unpaired *t*-test $t(641) = 4.95$; $p < 0.0001$; Fig. 4A). An even more pronounced effect was observed when comparing the firing rate obtained at the BF: the BF responses were much lower in exposed rats compared with sham-exposed animals (unpaired *t*-test $t(641) = 7.12$; $p < 0.0001$, Fig. 4B). In addition, the response duration was significantly longer in exposed rats compared with sham-exposed animals (unpaired *t*-test $t(641) = 6.24$; $p < 0.0001$; Fig. 4C). In contrast, both the frequency bandwidth (Fig. 4D) and the response latency (Fig. 4E) did not differ between the two groups ($t(641) = 0.49$, $p = 0.62$ and $t(641) = 1.13$, $p = 0.14$, respectively). The effects observed on the responses strength tested with pure tones were confirmed when analyzing the response to a natural stimulus. The responses of ACx neurons to the guinea pig whistle were significantly weaker in exposed rats compared with their sham-exposed counterparts ($t(641) = 5.96$, $p < 0.0001$; Fig. 4F) and the spike timing reliability of neuronal responses (quantified by the CorrCoef Index) was significantly lower in exposed than

in sham-exposed animals (0.19 vs. 0.26 unpaired *t*-test $t(641) = 5.64$; $p < 0.0001$). In addition, quantifying the spontaneous firing rate of cortical neurons during 2-min periods of silence also revealed a marked decrease in firing rate between exposed and sham-exposed animals (unpaired *t*-test, $t(641) = 3.29$, $p < 0.002$).

To determine if the GSM-induced decreased responsiveness required LPS treatment, MUA recordings were collected in exposed and sham-exposed animals in the absence of LPS injection. In these conditions, we obtained 213 recordings in four exposed animals and 178 recordings in three sham-exposed animals. There were no differences between exposed and sham-exposed animals in terms of (i) responses strength (104 vs. 105 s/s, unpaired *t*-test $t(336) = 0.98$; $p = 0.78$), (ii) firing rate at the BF (266 vs. 249 s/s unpaired *t*-test $t(336) = 1.12$, $p = 0.16$), (iii) response latency (12.65 vs. 13.06 ms, unpaired *t*-test $t(336) = 0.52$; $p = 0.54$) and (iv) frequency bandwidth of the STRFs (3.85 vs. 3.76 octave, unpaired *t*-test $t(336) = 0.78$; $p = 0.11$). Under these conditions, there was also no difference between the neuronal responses obtained in exposed and sham-exposed animals at presentation of the guinea pig whistle (37.8 vs. 39.2 s/s, unpaired *t*-test $t(336) = 0.98$; $p = 0.26$).

In the LPS-treated animals, the quantification of cortical thresholds obtained was based upon responses obtained when testing the frequency response area (FRA, see Methods). This quantification was performed in two different ways. First, for each animal and each position of the electrode array, we plotted the minimal threshold (in dB SPL) obtained for each frequency. These minimal thresholds were averaged across positions for a given animal, then across animals. Second, we averaged all the thresholds values derived from all the positions of the electrode arrays across all animals. These averaged values of thresholds were the mean thresholds obtained for each frequency, and were not minimal thresholds. In both cases, at a given intensity and at a given frequency, a response was considered to be significant when the firing rate was 6 standard deviations above the spontaneous firing rate. As shown in Fig. 5A, the minimal thresholds recorded in the exposed and sham-exposed animals were quite similar across the range of tested frequencies (1.1–36 kHz). In contrast, for a large range of frequencies (from 2.2 to 11 kHz), the average thresholds were higher in exposed animals compared with sham-exposed animals (Fig. 5B; unpaired *t*-test $t(10) = 3.45$, $p < 0.0001$). This was confirmed by analyzing the proportion of recordings exhibiting low (<25 dB), mid (25–50 dB) and high acoustic thresholds (>50 dB). As shown in Fig. 5C, in the low (<2.2 kHz) and high frequencies (>11 kHz), the distribution of low, mid and high thresholds did not differ between exposed and sham-exposed animals (in both cases, Chi-square = 0.74, ns). In contrast, in the middle frequencies (2.2–11 kHz), the two distributions significantly differed (Chi-square = 5.67, $p < 0.0001$): there was a large decrease in the number of low thresholds and a twofold increase in the number of high

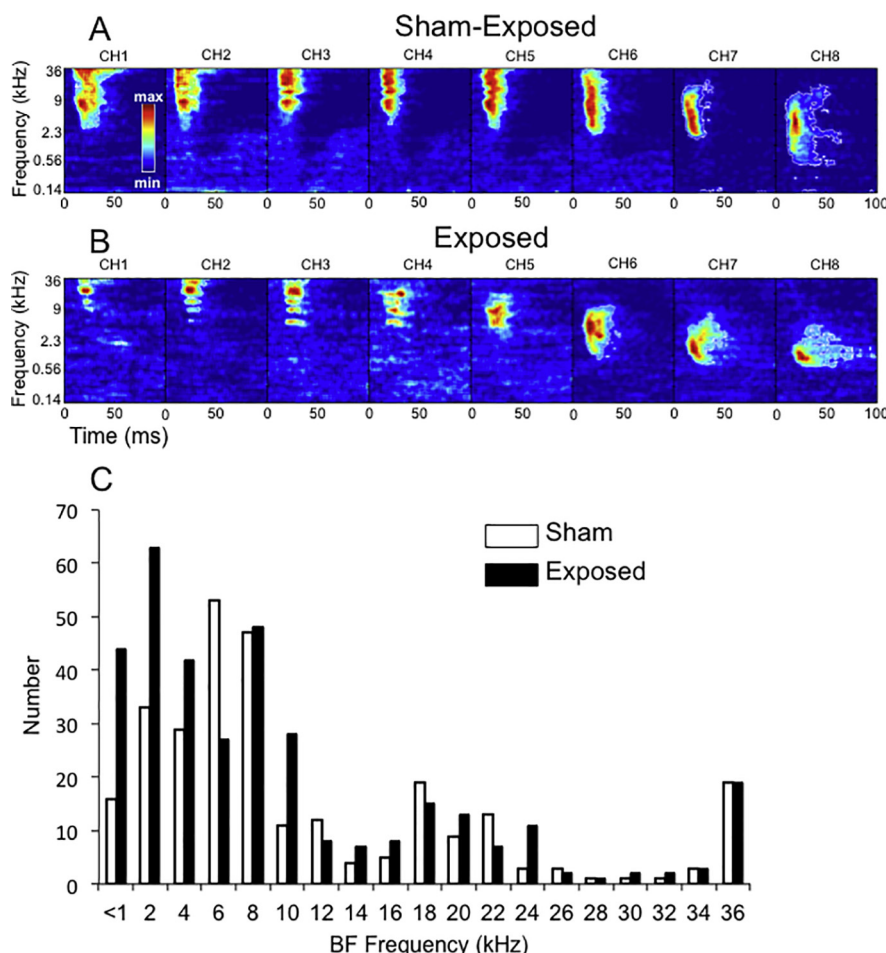


Fig. 3. Examples of spectro-temporal receptive fields in sham-exposed and exposed rats. Representative spectro-temporal receptive fields (STRF) simultaneously recorded from one of the two electrode arrays positioned in the primary auditory cortex. For each of the eight electrodes (CH1–CH8), the color code represents the firing rate (red being the higher) from 0 to 100 ms after the beginning of the stimulus (abscissa) and from 0.14 to 36 kHz (ordinates). The best frequency (BF) was defined as the frequency eliciting the largest number of action potentials. Both in A and in B, there was a progressive shift of the BF values from high to low frequency when progressing from the rostral to caudal direction. Note that there was a tendency for responses of longer duration in the exposed animals. (C) Distribution of the BF values in the Sham-exposed group (Sham) and the in exposed group (Exposed). There was no statistical difference between the two distributions (Chi-square = 0.49, ns). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

thresholds in the exposed animals. Altogether, these analyses indicate that despite the similarities in minimal thresholds, exposed rats differ from sham-exposed animals by enlarged proportions of ACx neurons displaying high thresholds during the 3–6 h following exposure to GSM-1800 MHz.

DISCUSSION

In the present study, anesthetized rats pretreated with LPS were exposed for 2 h to GSM-1800 MHz signals to further specify the effect of GSM-EMF on reactive glial cells and on sensory-evoked responses in the CNS affected by a neuroinflammatory process. Focusing on the ACx, we found that the GSM exposure led to changes in microglial cell morphology together with

significant alterations in neuronal responses to both pure tones and communication sounds.

Using exactly the same GSM exposure settings applied to healthy or LPS-injected rats, we previously found that CNS cell responses to GSM-EMF were only promoted under neuroinflammatory conditions (Lameth et al., 2017). In particular, comparing the cortex of LPS-injected rats fixed 24 h after GSM or sham exposures revealed a GSM-triggered increase in the spatial domain occupied by microglial processes. The microglial response was observed 24 h after exposure in the dorso-medial cortex where the local SAR reached a value of 2.94 W/kg, but it was no longer detectable 3 days after GSM exposure (Lameth et al., 2017). Similar to an EMF-emitting mobile phone (Wiat et al., 2008), our setup, based on a loop antenna generates EMF that are unevenly absorbed within the brain (Fig. 1). The SAR values within a given CNS region critically depend on its distance from the loop antenna. In the current study, we have extended our observations of the microglial response with respect to the post-exposure time, the level of EMF absorption and the activity of neuronal networks. Our results indicate the GSM-triggered extension of microglial processes already occurred as soon as 3 h after GSM exposure in the ACx where the average SAR value was 1.55 W/kg. Our results also suggest that the threshold SAR for this early microglial response, is higher than 0.68 W/kg, because this value corresponds to the average SAR in a cortical region where the spatial domain covered by microglial processes showed a trend

toward an increase but was not significantly modified.

Microglia are highly plastic cells that undergo permanent change in their shape owing to the cycle of extensions and retractions of their cell processes (Tay et al., 2017). Continuous cell process remodeling was also observed by live imaging of reactive microglia under proinflammatory conditions triggered by LPS injection (Kondo et al., 2011; Gyoneva et al., 2014). Therefore, it is most likely that the enlargement of the microglial domain observed in GSM-exposed animals resulted from a shift in the balance between extensions and retractions of microglial processes. The mechanisms responsible for this shift remain to be specified. EMF energy absorbed by microglial cells could possibly affect the activity of microglial components that control the growth or ramification

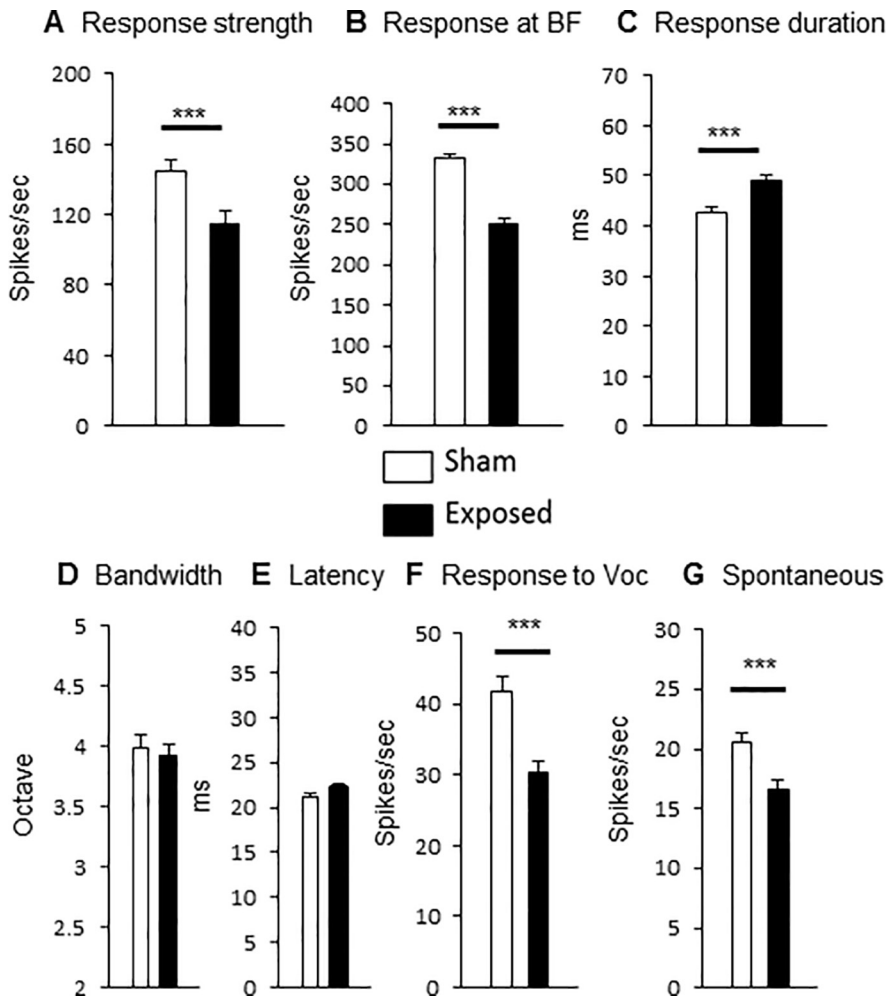


Fig. 4. Quantification of the responses to artificial and natural stimuli in the two groups of animals. (A–E) Quantification of the receptive field parameters. The response strength (total number of action potential in the significant contours of the STRFs) (A) and the responses at the best frequency (B) were significantly lower in exposed rats compared with sham-exposed animals. The duration of tone-evoked response was significantly longer (C) in the exposed animals, whereas there was no difference in the bandwidth (D) and latency (E) of the responses between the two groups. The response to the natural stimuli (guinea pig vocalization) was also lower in the exposed animals (F) and so was the spontaneous activity (G). All graphs are mean $\pm$ SEM based on 350 multiunit recordings in the exposed group and 293 multiunit recordings in the sham-exposed group.

of microglial cell processes. These components include purine-related ectoenzymes or receptors, nucleoside transporters, integrin-binding proteins and the two-pore domain K^+ channel THIK-1 (Matyash et al., 2017; Meller et al., 2017; Madry et al., 2018). Alternatively, GSM exposure could affect extrinsic signals that regulate the motility of microglial cell processes. In support of this possibility, we here found that the changes in microglial cell morphology were concomitant with GSM-induced changes in neuronal activity, whereas accumulating evidence indicates that the level of neuronal firing, or neurotransmitter release, strongly impacts the motility of microglial cell processes (Fontainhas et al., 2011; Li et al., 2012; Dissing-Olesen et al., 2014; Eyo et al., 2014).

Three to six hours after GSM or sham exposure, neuronal responsiveness was assessed in the primary ACx by testing the responses to pure tones and

communication sounds. A clear tonotopic gradient was observed in all exposed and sham-exposed animals, indicating that the exposure did not induce map disorganization. The fact that the response latency and the bandwidth of spectral tuning were similar in the two groups of animals is an indication that, on average, the sampling of neuronal activity was performed in both groups in the primary ACx (and not in a secondary area) and was probably from the same cortical layers.

Several mechanisms can potentially contribute to the weaker responses observed in the cortex of exposed animals when tested with pure tones and vocalizations. First, these weaker responses can result from an intrinsically reduced excitability of cortical cells following exposure to the GSM 1800 MHz. Several *in vitro* studies, directly quantifying neuronal properties, have pointed out that GSM exposure alters neuronal excitability. For example, cultures of cortical neurons exposed for 3 min to GSM-1800 MHz (at a SAR of 3.2 W/kg) displayed a 30% lower firing rate and bursting rate (Moretti et al., 2013), an effect that reversed after exposure. Also, exposure to GSM-900 MHz during the entire gestation period (6h per day) decreased neuronal excitability of Purkinje cells of rat offspring (Haghani et al., 2013). In mollusk ganglia, exposure to 1800 MHz at SAR level of 8.2 W/kg reduced the threshold for firing action potentials and reduced neuronal accommodation (Partsvania et al., 2013). Arguing in favor of a decrease in intrinsic

excitability, we also detected that, in absence of any acoustic stimulation, spontaneous firing rate was lower in exposed than in sham-exposed animal.

However, we cannot discard the possibility that GSM exposure could also affect synaptic transmission either from thalamo-cortical synapses or from cortico-cortical synapses. It is now largely documented that in ACx, the breadth of spectral tuning is not exclusively determined by afferent thalamic projections and that intracortical connections endow additional spectral input to a cortical site (Kaur et al., 2004; Metherate et al., 2005; Happel et al., 2010). In our experiment, the fact that cortical STRFs display similar bandwidths in exposed and sham-exposed animals can be viewed as an indirect indication that the impact of the GSM-EMF was not on the cortico-cortical connections. This also suggests that

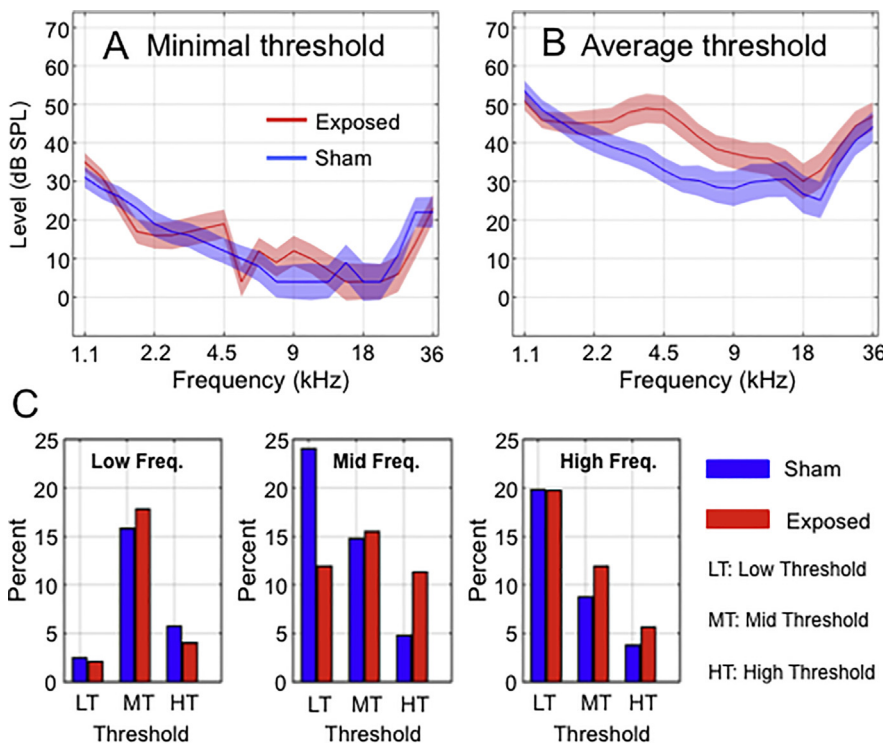


Fig. 5. Quantification of minimal and average threshold from the frequency response areas (FRA). (A) Quantification of the minimal thresholds based on the frequency response areas (FRA). For each position of the electrode array, the lowest threshold for a given frequency was considered and these values were averaged across electrode arrays then across animals. (B) Quantification of the average thresholds based on the frequency response areas (FRA). For each position of the electrode array, all the thresholds were considered (not only the minimal one). (C) Distributions of the Low (LT), Middle (MT) and High (HT) thresholds for three ranges of frequencies. In low frequencies (< 2.25 kHz), the distribution did not differ between exposed and sham-exposed animals. In mid frequencies (2.25–11 kHz) the distributions significantly differ with a lower proportion of low threshold (< 25 dB) and a higher proportion of high thresholds (> 50 dB) in the exposed animals. The two distributions did not differ in the high frequency range (above 11 kHz).

connections from other cortical areas exposed at SARs higher than those measured in the ACx (Fig. 1) are probably not responsible for the altered responses reported here.

Here, the lowest values of acoustic threshold remained similar in exposed and sham-exposed animals. However, there was a larger proportion of cortical recordings showing high values of threshold in exposed vs. sham-exposed animals. Given that it has been proposed that cortical acoustic thresholds are mainly controlled by the strength of thalamo-cortical synapses (Kaur et al., 2004; Happel et al., 2010), it can be suspected that thalamo-cortical transmission is partially reduced by the exposure, either at the pre-synaptic (decrease in glutamate release) or at the post-synaptic level (decrease in the number of receptors or in their affinity).

That the duration of cortical responses was longer in exposed animals is a quite interesting observation. In all species, the phasic “onset” response lasting a few tens of milliseconds is one of the prominent characteristics of the primary ACx. This phasic response is controlled by the feedforward inhibition, which silences pyramidal cells after their initial response to thalamic inputs (Wehr and

Zador, 2003; Tan et al., 2004). Partially reducing the contribution of feed-forward inhibition by pharmacological agents can lead to longer response duration (Gaucher et al., 2013a). The longer response duration observed in the ACx of aged animals were also interpreted as a sign of reduced intracortical inhibition (Ling et al., 2005; Turner et al., 2005; Gourevitch and Edeline, 2011; Occelli et al., 2016). Thus, one possibility is that the GSM-EMF had more of an impact on intracortical interneurons than pyramidal cells. This possibility should be tested by future *in vitro* studies.

Based on culture conditions optimized for survival of neurons *in vitro* in the absence of glial growth (Brewer et al., 1993), the study by Moretti and colleagues (2013) strongly suggests that exposure to GSM-1800 MHz can reduce neuronal firing irrespective of any effect of the EMF on glial cells. However this neuronal response was observed during the GSM exposure and ended with the exposure (Moretti et al., 2013). In our study, the GSM-triggered alterations in neuronal firing were observed *in vivo*, from 3 to 6 h after the end of the GSM exposure, concomitantly to GSM-triggered extension of microglial processes. In light of accumulating evidence that neuronal firing or neurotransmission is

modulated by cell-to-cell contact between reactive microglia and neurons (Hoshiko et al., 2012; Bechade et al., 2013; Kato et al., 2016; Szalay et al., 2016; Cantaut-Belarif et al., 2017), it seems possible that the extension of microglial processes induced by GSM-EMF, in turn contributed to the down regulation of neuronal firing observed in GSM-exposed animals. In our previous study focused on the dorso-medial part of the cortex, we showed that GSM-induced extension of microglial cell processes, downregulation of Interleukin-1 β gene or post-translational modification of AMPA receptors did not persist 72 h after the GSM exposure (Lameth et al., 2017). Considering the lower SAR value in the ACx compared with that in the dorso-medial area (1.55 vs 2.94 W/kg), it seems reasonable to expect that the modifications in microglial cell processes and in neuronal activity reported here were transient. The actual longevity of the effects described here, and their behavioral consequences, remain a question for future studies.

By analyzing neuronal responses in ACx, our study assesses whether acute exposure to GSM-1800 MHz can affect the cortical processing of auditory information. In humans, investigations have shown that 10- to 30-min exposures to EMF emitted by GSM mobile phones

had no consistent effect on the auditory processing assessed at the cochlear (Ozturan et al., 2002; Parazzini et al., 2005; Uloziene et al., 2005; Paglialonga et al., 2007; Bamiou et al., 2008) or brainstem level (Arai et al., 2003; Oysu et al., 2005; Sievert et al., 2005; Stefanics et al., 2007; Kwon et al., 2010). Furthermore it was reported that in healthy rats, repeated head exposure to GSM-900 or 1800 MHz at a local ear SAR of 2 W/kg did not affect the function of cochlear outer hair cells (Galloni et al., 2005a,b). Similarly, when the ears of guinea pigs were exposed for two months to GSM-900 MHz (5days/week, 1 h/day, SAR between 1 and 4 W/kg) there was no detectable change in the amplitude of distortion-product otoacoustic emissions (DPOAE) and in thresholds of auditory brainstem responses (Aran et al., 2004).

Interestingly, it was recently reported that cultured spiral ganglion neurons treated with LPS undergo autophagy and enhanced production of reactive oxygen species in response to a GSM-1800 MHz exposure at a SAR of 4 W/kg. These cells responses were not observed in the absence of LPS treatment (Zuo et al., 2015). In our *in vivo* analysis, we provide evidence that under LPS-triggered proinflammatory conditions, acute head exposure to GSM-1800 MHz can interfere with auditory processing as indicated by weaker responses and higher thresholds observed from ACx neurons. These alterations were promoted in the context of the LPS-triggered immune activation because they were not observed in rats exposed to GSM in the absence of LPS pretreatment.

Our result may be considered within the context of the public exposure limit of 2 W/kg set for human head exposure. From dosimetric analyses carried out in different brain structures using mobile phone models (Wiat et al., 2008; Ghanmi et al., 2014), it can be assumed that SAR values at the level of the human ACx should remain below 0.5 W/kg, owing to the distance of the EMF source and the dielectric properties of the tissues surrounding the brain. This upper limit is clearly below the average SAR value (1.55 W/kg) present in the rat ACx in our experimental setting. We did not find a statistically significant microglial cell response in cortical regions where the average SAR was 0.68 W/kg, but it remains possible that under LPS-triggered inflammatory conditions, CNS cells could react to SAR value of less than 1 W/kg.

The high prevalence of neuroinflammation in human populations and the worldwide use of wireless communication require additional investigations that aim at defining the effect of GSM-EMF on the CNS under proinflammatory conditions. We show that in rats undergoing acute neuroinflammation, a single exposure to GSM-1800 MHz induces both growth of cell process in proinflammatory effector cells such as microglia, and modifications in spontaneous and evoked activities of ACx neurons. These changes in neuronal activity are indicative of functional alterations in auditory processing and they do not occur in healthy rats submitted to a same GSM exposure. These results extend our knowledge of the CNS cell responses to GSM-EMF in neuroinflammatory states. In light of rising evidence that microglia-to-neuron contacts modulate the activity of

neuronal networks and conversely, that neuronal activity influence the motility of microglial processes, it remains to determine if there are causal relationships between the microglial response to GSM-EMF and the changes in firing rate of ACx neurons. On the one hand, they can be two independent alterations occurring in these two cell types with no causality between them. On the other hand, they can be linked, one of these alterations being partly responsible for the other. Future investigations are also needed to determine whether human neuropathologies may promote neural cell responses to GSM exposures below the limits set for the general public.

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