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Effects of 900 MHz radiofrequency on corticosterone, emotional memory and neuroinflammation in middle-aged rats.

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ABSTRACT

The widespread use of mobile phones raises the question of the effects of electromagnetic fields (EMF, 900 MHz) on the brain. Previous studies reported increased levels of the glial fibrillary acidic protein (GFAP) in the rat’s brain after a single exposure to 900 MHz global system for mobile (GSM) signal, suggesting a potential inflammatory process. While this result was obtained in adult rats, no data is currently available in older animals. Since the transition from middle-age to senescence is highly dependent on environment and lifestyle, we studied the reactivity of middle-aged brains to EMF exposure. We assessed the effects of a single 15 min GSM exposure (900MHz; specific absorption rate (SAR) = 6 W/kg) on GFAP expression in young adults (6 week-old) and middle-aged rats (12 month-old). Brain interleukin (IL)-1β and IL-6, plasmatic levels of corticosterone (CORT), and emotional memory were also assessed.

Our data indicated that, in contrast to previously published work, acute GSM exposure did not induce astrocytes activation. Our results showed an IL-1β increase in the olfactory bulb and enhanced contextual emotional memory in GSM-exposed middle-aged rats, and increased plasmatic levels of CORT in GSM-exposed young adults.

Altogether, our data showed an age dependency of reactivity to GSM exposure in neuroimmunity, stress and behavioral parameters. Reproducing these effects and studying their mechanisms may allow a better understanding of mobile phone EMF effects on neurobiological parameters.

Keywords
Middle-age, Radiofrequency, Interleukin-1β, Interleukin-6, Corticosterone, Fear conditioning
1. Introduction

Over the last several decades, the rapid development of mobile phone technology has raised concerns about the cerebral effects of global system for mobile communications (GSM) 900 MHz microwaves. While older people represent an increasing share of cell phone users, aging-related vulnerability/the relationship between aging and vulnerability to electromagnetic fields (EMF) has rarely been assessed experimentally (Vecchio et al., 2010). The transition from adulthood to senescence seems closely dependent on life style and environmental factors, particularly during middle-age, its early onset. Indeed, middle-aged biological systems are particularly sensitive to toxicological factors, as shown by exacerbated behavioral impairments in middle-aged animals, when compared to younger animals, in response to lipopolysaccharide administration and traumatic brain injury (Hoane et al., 2004; Kohman et al., 2010). In addition, enrichment or self food-restriction diet during middle-age may postpone the appearance of age-related memory decline and neurochemical modifications (Frick et al., 2003; Kollen et al., 2010).

As a consequence, the onset of age-related deficits in middle-aged rodents shows great variability. These deficits include modified neurobiological parameters such as an increase in plasmatic levels of corticosterone (CORT), memory decline, elevation of interleukins (IL) levels, and astrocytes activation (Hayakawa et al., 2007; Lynch, 2010; Murray and Lynch, 1998). Cytokines, like the two immune mediators IL-1β and IL-6, are secreted by activated microglia and are known to trigger astrocytes activation (Woiciechowsky et al., 2004; Zhang et al., 2010). Astrocytes are non-neuronal central nervous system cells, implicated in neuronal synaptic plasticity (Vernadakis, 1996). Their activation, may lead to an astrogliosis phenomenon, characterized by an increased expression of glial fibrillary acidic protein (GFAP), the main intermediate filament of astrocytes (Eng et al., 1992).

Such biological responses are also elicited following exposure to toxicological or environmental factors. A transitory astroglial reactivity was observed 48 h to 72 h following an acute local exposure to GSM signal at high specific absorption rate (SAR) of 6 Watts/kg (W/kg) (Brillaud et al., 2007; Mausset-Bonnefont et al., 2004). This response was shown in several structures of the limbic system, a circuitry involved in both memory and emotional processes (Fanselow and Dong, 2010). GSM effects on IL expression have only been assessed in one in vitro study (Thorlin et al., 2006), while no in vivo animal studies have been published yet. GSM exposure was shown to affect plasmatic CORT levels (Mostafa et al., 2002) and to impair learning and memory (Fragopoulou et al., 2010; Narayanan et al., 2009).
Because common neurobiological parameters are affected by the ageing process and EMF exposures, we hypothesized a possible interaction between GSM exposure and middle-age-related vulnerability in these biological responses.

Thus, the aim of the present study was to assess GFAP levels in the cortex, striatum and hippocampus of young adult (6 week-old) and middle-aged male rats (12 month-old), 48 h after the same exposure parameters used in Brillaud’s study: single, 15 min, 900 MHZ GSM exposure (brain average SAR = 0 and 6 W/kg). We also evaluated pro-inflammatory cytokines (IL-1β and IL-6) expression in several brain structures (cortex, sub-cortex nuclei, olfactory bulb and cerebellum) and plasmatic levels of CORT. Emotional memory was assessed in the fear conditioning paradigm.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (6 week-old and 12 month-old) were purchased from Charles Rivers (France). Animals/The animals were kept in a controlled environment (12-h light/dark cycle, room temperature: 22°C) and received food and tap water ad libitum. Rats were housed two per cage. The cage’s environment was enriched with plastic cylinders identical in size, shape and appearance to GSM exposure rockets (Brillaud et al., 2007). Rats were handled and weighed daily for one week before the experiments began. The protocols were approved by the French State Council guidelines for the care and use of laboratory animals (Decree n° 87-849, October 19, 1987).

2.2. Experimental groups

- Age groups: For both ages, rats were randomly assigned to 2 independent exposure groups at SAR values of 0 or 6 W/kg (n = 8-9/group). On the first day of the experiment, the rats were subjected to the fear conditioning training. 48 h later, they were consecutively exposed to a single GSM signal for 15 min, and tested for sound memory. Contextual memory was tested 48 h after GSM exposure and was immediately followed by anesthesia, blood collection and sacrifice (Figure 1). Exposure doses and ages were counterbalanced over three independent experimental sessions to perform exposures and tests between 9:00 am and 12:00 pm.

- “Time schedule” control groups: Six week-old rats were randomly assigned to 12 independent groups (n = 6/group). Rats were sacrificed 30 min, 5h or 24h following (a) daily
handling (cage control (CC) group) (b) a single, 15 min, 6 W/kg GSM-exposure (c) a single, 15 min, sham (0W/kg GSM)-exposure, or (d) a single 15 min sham (0W/kg GSM)-exposure followed by a fear conditioning session (FC group). Treatments and exposure doses were counterbalanced over three independent experimental sessions to perform exposures and tests between 9:00 am and 12:00 pm.

2.3. Radiofrequency exposure system

Exposure set-up was performed as previously described (Dubreuil et al., 2002). A radio frequency power source (900–64 type, Radio Frequency Power Amplifier, France) emitting a 900 MHz EMF (1/8 duty factor) pulse modulated at 217 Hz was connected to a four-output divider. Each output was connected to a loop antenna allowing local exposure of four animal's heads simultaneously in an anechoic chamber. During exposure, each animal was placed in a Plexiglas rocket capped with a truncated cone on which an individual loop antenna was fixed. The rocket body was lined with air holes to facilitate breathing and minimize the rise in body temperature.

2.4. Fear Conditioning

2.4.1 Apparatus

Fear conditioning is a Pavlovian conditioning, in which a neutral conditioned stimulus is paired with an aversive unconditioned stimulus (in the present study, a sound and a foot shock, respectively). The measured responses were freezing and high activity. Freezing was defined as the complete immobilization of the animal except for respiratory movement for a minimum of 1 sec. High activity was used to evaluate locomotor activity and was defined as animal’s movement generating a pressure higher than 15 arbitrary unit (corresponding to 3 fold/times the pressure generated by the animal’s weight). Conditioning and tests occurred in two standard fear conditioning boxes purchased from Bioseb (France) and controlled by specific software (Panlab, Barcelona). As age differences were observed during conditioning on the last 60 sec of freezing (MaxFreezing) (figure 5b), the freezing response to the sound and the contextual tests was expressed as a percentage of MaxFreezing/as MaxFreezing percentage for each animal.

2.4.2 Protocol
The conditioning session occurred in environment 1 of the fear conditioning boxes. It began with a habituation period with no stimulus (no sound or foot shock, 3 min), followed by three conditioning cycles. One cycle included four consecutive steps: sound presentation (2 kHz; 80 db; 20 sec), no stimulus (10 sec), foot shock (0.25 mA; 1 sec), no stimulus (40 sec). To test contextual memory, animals were replaced for 2 min in environment 1. To perform the sound memory test, animals were introduced in boxes with a modified environment, defined as environment 2 (different box size, walls color, floor texture, scent and cleaning disinfectant) for 2 min and were exposed to the sound presentation (2 min).

![Week 1](image1.png)

**Week 1**

**Handling**

**Week 2: Fear conditioning test**

**Day 1** 9:00 am - 12:00 pm

7 min

**Conditioning**

**Day 2** 9:00 am - 12:00 pm

4 min

**Single GSM exposure 0 or 6 W/kg for 15 min**

**Day 3** 9:00 am - 12:00 pm

**Sound test**

**Day 4** 9:00 am - 12:00 pm

2 min

**Contextual test**

**Day 5** 9:00 am - 12:00 pm

**Blood collection and Sacrifice**

**Figure 1**

Experiment design. On day 1, rats were subjected to the conditioning session of the fear conditioning test. 48 h after, they were tested for the sound memory immediately after GSM exposure. 48h later, rats were tested for the contextual memory. Then, blood was collected and rats were sacrificed. Exposure and behavior tests were conducted between 9:00 and 12:00 pm.

2.5. Plasma and brain sample preparation

Plasmatic CORT was assessed on sacrifice day to avoid the stress induced by blood collection during behavioral tests. Rats were anesthetized with isoflurane. Blood was collected from *venae cavae* in EDTA and centrifuged (10000 rpm/10 min/4°C). Plasma was stored at -80°C until assayed. The rats were subjected to an intra-cardiac perfusion with 0.1% phosphate buffered saline (PBS). The brains were quickly removed and the two hemispheres were separated and randomly assigned to Enzyme Linked Immuno Sorbant Assay (ELISA) or immunohistochemistry samples preparation. For immunohistochemistry, the brain hemispheres were fixed/placed in 4 % paraformaldehyde (PFA) for 4 days, incubated in 30% sucrose solution, frozen in isopentane at -50°C, and stored at -80°C. Using a cryostat
microtome, 25 μm sagittal brain slices were obtained and stored at -20°C in cryoprotectant solution until processing as free-floating sections. For ELISA, the olfactory bulb, cortex, subcortex nuclei and cerebellum of the other hemispheres were dissected on ice and stored at -80°C.

2.6. Plasmatic corticosterone

A CORT competitive assay was performed with a commercially available kit (assay designs, correlate-EIA CORT, R&D) according to the manufacturer's instructions. Briefly, polyclonal anti-CORT antibody and alkaline phosphatase-labeled CORT were added to the pre-coated wells. After 2h incubation at room temperature, the excess reagents were washed away and substrate was added. After 1h incubation, the enzymatic reaction was stopped with trisodium phosphate solution. CORT concentrations (ng/ml) were inversely proportional to the intensity of the yellow color read at 405 nm.

2.7. GFAP Immunohistochemistry

Brain slices were incubated in 30% H₂O₂ for 20 min before addition of normal goat serum blocking solution. Sections were incubated overnight at 4°C with the primary antibody anti-GFAP (rabbit antibody, Abcam ab7260), then with the secondary biotinylated antibody (anti-rabbit IgG, ABC kit Abcam ab8627). Sections were incubated with an Avidine Biotine Solution (ABC kit Abcam ab 8627), and staining revealed with 3,3’- diaminobenzidine tetrahydrochloride (Sigma tabs), 30% H₂O₂. Sections were washed in PBS to stop the reaction.

GFAP image analysis was performed using Visilog 6.8 imaging software (Noesis, France), by measuring the percentage of stained surface over a manually defined area (optical density), excluding the interface of adjacent tissues (10 × objectives). GFAP was assessed in the cortex, striatum and hippocampus, the structures where an effect of GSM exposure was previously described (Brillaud et al., 2007; Mausset-Bonnefont et al., 2004).

2.8. IL Enzyme Linked Immuno Sorbant Assay

Structures were homogenized by sonication in an ice-cold Tris/HCl/EDTA buffer containing a 4% protease inhibitor (Roche) and 1% phosphatase inhibitor (Sigma) cocktail. Samples were centrifuged (9800 rpm/15 min/4°C) and the supernatant was collected. A Lowry test was used to measure total protein content in each sample. IL-1β and IL-6 were quantified using commercialized sandwich ELISA kits (Rat IL-1β /IL-1F2 and Rat IL-6, DuoSet ELISA Development system, R&D systems) according to the manufacturer’s
instructions. Raw data were presented for controls and sham groups, while GSM effects were presented as percentages of sham group.

2.9. Statistics

Statistical analyses were performed using the SPSS 15 software (Inc., Chicago, IL, USA). Values were given as mean ± standard deviation of mean (SEM) per group. Raw data were analyzed after logarithmic transformation and, percentages, after square root transformation. Levene’s test was used for variance homogeneity. Analyses were performed using three-way analysis of variance/variance analysis (ANOVA) (for age × exposure dose × time and age × exposure values × structure interaction, with repeated measures on time and structure) and two-way ANOVA (for time × age, time × exposure values, age × exposure values and structure × exposure values interaction, with repeated measure on time and structure). The main effects were analyzed using Bonferroni’s or Dunnett’s post-hoc corrected t-test. Time and structures were treated as within subject factors while exposure doses were treated as between subject factors. Effects were considered significant when p < 0.05. Grubb’s test was used for outlier’s detection.

3. Results

3.1 Absence of age or GSM exposure effect on GFAP staining

Figure 2 shows GFAP staining in the cortex, striatum and hippocampus of 6 week-old and 12 month-old rats following GSM exposure. ANOVA analysis showed significant structure effect (F_{2,24} = 97, p < 0.001) and structure × age interaction (F_{2,24} = 5, p = 0.02). However, no age effect (F_{1,12} = 1.9, p = 0.2), treatment effect (F_{1,12} = 0.02 p = 0.9), age × treatment (F_{1,12} = 0.9, p = 0.4), structure × treatment (F_{2,24} = 0.4, p = 0.6) or structure × age × treatment interaction (F_{2,24} = 2.2, p = 0.1) was obtained. Bonferroni’s post-hoc corrected t-test showed higher GFAP staining in the hippocampus compared to the other structures (p < 0.05) (Figure 2b).
Figure 2

GFAP immunostaining in the cortex (Cx), the striatum (Str) and the hippocampus (Hp) of 6 week- and 12 month-old rats exposed to GSM signal for 15 min at a SAR value of 0 or 6 W/kg. (a) Objective 10×; scale bar, 200µm (b) Higher magnification in the cortex, objective 40×; scale bar, 50µm. (c) Absence of GSM exposure or age effect on GFAP staining in three cerebral structures. 6 week- and 12 month-old rats were subjected to GSM exposure (0 and 6 W/kg) and sacrificed two days later to assess GFAP immunostaining. *** p < 0.001 significantly higher than Cx and Str in sham young adults. * p < 0.05 significantly higher than Cx in sham middle aged rats. +++ p < 0.001 significantly higher than Cx and Str in correspondly exposed groups. N = 5-6/group.

3.2 Effect of age and GSM exposure on cerebral IL-6 and IL-1β
No age differences were obtained regarding basal IL levels in the sham exposed groups (p > 0.94) (Table a). Figure 3 (a and b) shows IL-6 and IL-1β following GSM exposure in the cortex, the sub-cortical nuclei, the olfactory bulb and the cerebellum, in 6 week- and 12 month-old rats following GSM exposure.

**Figure 3**

GSM effect on (a) IL-6 and (b) IL-1β in the cortex (Cx), the sub-cortical nuclei (S-Cx), the olfactory bulb (OB) and the cerebellum (Crb) in 6 week- and 12 month-old rats. Rats were subjected to GSM exposure (0 and 6 W/kg) and sacrificed two days later to quantify IL. * p < 0.05 significantly increased compared to sham groups of both ages. ## p < 0.01 significantly increased compared to 6 week-old exposed group. N = 8-9/group. (c) IL levels were not modified in the brain of 6 week-old rats sacrificed 30 min, 5h and 24h after daily handling (CC), GSM sham-exposure followed by fear conditioning session (FC), GSM sham-exposure (0 W/kg) or GSM-exposure (6 W/kg).

ANOVA analyses performed on the IL-6 and IL-1β measurements showed, respectively, the absence of structure effect (F3,96 = 0.3, p = 0.8; F3,96 = 0.8, p = 0.5), treatment
effect ($F_{1,32} = 2.2, p = 0.1; F_{1,32} = 0.7, p = 0.4$), structure × age interaction ($F_{3,96} = 0.14, p = 0.9; F_{3,96} = 1.8, p = 0.1$), structure × treatment interaction ($F_{3,96} = 1.4, p = 0.2; F_{3,96} = 0.8, p = 0.5$), or structure × age × treatment interaction ($F_{3,96} = 0.04, p = 0.9; F_{3,96} = 1.6, p = 0.2$).

For IL-6, no age ($F_{1,32} = 0.2, p = 0.9$) or age × treatment interaction ($F_{1,32} = 0.03, p = 0.9$) was observed.

For IL-1β, a significant age effect ($F_{1,32} = 4.4, p = 0.04$) and age × treatment interaction ($F_{1,32} = 4.6, p = 0.04$) were detected. ANOVA analysis performed on separate structures’ data showed a significant age effect ($F_{1,32} = 5.8, p = 0.02$) and age × treatment interaction ($F_{1,32} = 5.2, p = 0.03$) for IL-1β only in the olfactory bulb. Bonferroni’s post-hoc corrected t-test showed significantly increased IL-1β in the olfactory bulb of GSM-exposed 12 month-old rats compared to 6 week- and 12 month-old sham rats ($p = 0.04$) and to GSM-exposed 6 week-old rats ($p = 0.01$).

Cerebral IL levels were not modified by a 6 W/kg-exposure as compared to a sham GSM-exposure, and by a sham GSM-exposure or fear conditioning procedure (FC) as compared to the CC group, after 30 min, 5h and 24h ($p > 0.9$) (Figure 3c).

3.3 Corticosterone response to GSM is age-dependent

Figure 4 shows plasmatic levels of CORT in GSM- and sham-exposed 6 week- and 12 month-old groups. ANOVA analyses showed a significant dose effect ($F_{1,20} = 4.5, p = 0.04$), but no age effect ($F_{1,20} = 0.02, p = 0.8$) or dose × age interaction ($F_{1,20} = 1.9, p = 0.2$). Bonferroni’s post-hoc corrected t-test showed that GSM exposure significantly increased CORT in the 6 week-old group ($p < 0.05$), while it had no effect in middle-aged rats.

The CORT level was significantly increased 30 min, but not 5 or 24 h after the fear conditioning procedure compared to sham- and 6 W/kg GSM-exposed group ($p = 0.001; p = 0.006$) and the CC group ($p = 0.03$). However, the CORT level was not modified by 6 W/kg as compared to sham GSM-exposure, and by sham-GSM-exposure compared to the CC group, after 30 min, 5h and 24h ($p > 0.9$) (Figure 4b).
Figure 4

(a) Plasmatic level of corticosterone in 6 week- and 12 month-old sham and GSM-exposed rats. Rats were subjected to GSM exposure (0 and 6 W/kg) and blood was collected two days after.* p < 0.05 CORT was significantly increased by GSM exposure in the 6 week-old rats. N = 6/group. (b) CORT levels were significantly increased in the plasma of rats subjected to 0 W/kg sham-exposure followed by a fear conditioning session (FC) compared to cage control group (CC) \(^a\) p < 0.05, sham-exposed group (0 W/kg) \(^b\) p < 0.001 and GSM-exposed group (6 W/kg) \(^c\) p < 0.01 at 30 min but not 5 or 24h after procedures.

<table>
<thead>
<tr>
<th>Time</th>
<th>CC (0 W/kg)</th>
<th>FC (0 W/kg)</th>
<th>0 W/kg</th>
<th>6 W/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>30mn</td>
<td>190 ± 123</td>
<td>360 ± 137(^{a,b,c})</td>
<td>111 ± 102</td>
<td>161 ± 83</td>
</tr>
<tr>
<td>5h</td>
<td>284 ± 100</td>
<td>283 ± 85</td>
<td>246 ± 95</td>
<td>226 ± 72</td>
</tr>
<tr>
<td>24h</td>
<td>91 ± 79</td>
<td>86 ± 57</td>
<td>107 ± 110</td>
<td>129 ± 85</td>
</tr>
</tbody>
</table>

3.4 Age and GSM effect on emotional memory

Figure 5 (a and b) shows the percentage of time spent in high activity and freezing during the conditioning session. ANOVA analyses performed on the data summarized in figure 5a showed a significant time effect (\(F_{12,384}= 88, p < 0.001\)), age effect (\(F_{1,32}= 12, p = 0.002\)) but no time \(\times\) age interaction (\(F_{12,384}= 1.32, p = 0.2\)). T-test showed a significant increase of high activity in the 12 month-old group as compared to the 6 week-old rats at T90 sec to T210 sec (\(p < 0.001\)). ANOVA analyses performed on the data summarized in figure 5b showed a significant time effect (\(F_{12,384}= 134, p < 0.001\)) but no time \(\times\) age interaction (\(F_{12,384}= 1.3, p = 2.3\)) or age effect (\(F_{1,32}= 1.7, p = 0.2\)). ANOVA performed on MaxFreezing responding showed a significant age effect (\(F_{1,32}= 8.2 p = 0.007\)). T-test showed that the percentage of time spent freezing was significantly higher in the 6 week-old group as compared to the 12 month-old rats at T360 sec (\(p = 0.04\)) and T390 sec (\(p = 0.03\)).
Figure 5

Percentage of time spent (a) in high activity and (b) freezing during the conditioning phase in 6 week- and 12 month-old rats. Age differences were observed in the MaxFreezing (last 60 sec). * p < 0.05, ** p < 0.01, *** p < 0.001 significantly increased compared to the other age group. N = 16-18/group.
Figure 6a shows time spent freezing during the first 30 sec in environments 1 and 2 in “conditioned rats”. ANOVA analyses showed a significant age effect ($F_{1,62} = 8, p = 0.006$), environment effect ($F_{1,62} = 5, p = 0.03$) but no age × environment interaction ($F_{1,62} = 4, p = 0.06$). No difference in freezing was shown between 6 week-old and 12 month-old rats in environment 1 ($p > 0.05$). The 12 month-old rats spent significantly less time freezing in environment 2 (modified environment) compared to environment 1 (know environment) ($p = 0.004$), while 6 week-old rats showed no responding differences between the two environments.

Figure 6

Freezing in response to (a) environment 1 versus environment 2, (b) sound, and (c) context. (a) ** $p < 0.01$ significantly decreased compared to environment 1. (b) Significant age × exposure × time interaction was obtained between groups (c) responding of GSM exposed 12 month-old rats was significantly increased compared to: * $p < 0.05$, ** $p < 0.01$ GSM exposed 6 week-old rats and + $p < 0.05$ sham exposed 6 week-old rats. N=8-9/group.

Figure 6 (b and c) shows the percentage of time spent freezing in response to the sound and the context. ANOVA analyses performed on the data summarized in figure 6b
showed a significant time effect ($F_{3,90} = 5, p = 0.03$) and age × exposure × time interaction ($F_{3,90} = 3.2, p = 0.03$). However, no exposure effects ($F_{1,30} = 0.74, p = 0.39$), time × exposure ($F_{3,90} = 0.28, p = 0.8$), age × time ($F_{3,90} = 1.2, p = 0.3$) or age × exposure ($F_{1,30} = 0.94, p = 0.87$) was obtained. ANOVA analyses performed separately on data for the 6 week- and 12 month-old groups showed no time effect or time × treatment interaction ($p > 0.4$). No significant treatment effect was found in the 6 week-old group ($F_{1,15} = 0.8, p = 0.4$) or the 12 month-old group ($F_{1,15} = 0.17, p = 0.67$). ANOVA analyses performed separately on data for the sham- and GSM-exposed groups showed no time × age interaction ($F_{3,42} = 2.6, p = 0.06$ and $F_{3,48} = 0.8, p = 0.5$, respectively) or age effect ($F_{1,14} = 1.3, p = 0.27$ and $F_{1,16} = 0.5, p = 0.42$) in both groups. A significant time effect ($F_{3,48} = 6.3, p = 0.001$) was shown only in GSM exposed groups. No significant group effect was shown for each time point ($p > 0.7$).

ANOVA analyses performed on the data summarized in figure 6c showed a significant time effect ($F_{3,90} = 11, p < 0.001$), age effect ($F_{1,30} = 10, p = 0.003$) and age × treatment interaction ($F_{1,30} = 3.4, p = 0.04$). ANOVA analyses performed separately on the 6 week- and 12 month-old groups showed a significant time effect ($F_{3,48} = 8.7, p < 0.001$ and $F_{3,42} = 3.1, p = 0.03$, respectively) in both groups. No exposure effect or time × exposure interaction was obtained in both ages groups ($p > 0.6$). ANOVA analyses performed separately on sham- and GSM-exposed groups’ data showed a significant age effect in the GSM-exposed group ($F_{1,16} = 11.7, p = 0.004$) and significant time effect in both groups ($p < 0.001$). For each time point, a significant group effect was shown ($p < 0.05$). Dunnert’s post-hoc corrected t-test showed a significant increase in freezing time in middle-aged GSM-exposed rats compared to GSM-exposed young adults ($p = 0.03$, T30 sec and $p = 0.002$, T60 sec) and to sham-young adults ($p = 0.04$, T30 sec).
4. Discussion

The aim of this study was to assess the potential vulnerability of neuro-immunity, stress and emotional systems of middle-aged animals in response to an acute GSM exposure. Our first data indicated that, conversely to earlier published work, acute GSM exposure did not induce astrocytes activation. Our second result suggested that some biological responses of GSM-exposed rats were age-dependent: we found an increase in olfactory bulb IL-1β and fear memory in GSM-exposed middle-aged rats, and increased plasmatic CORT levels in GSM-exposed young adults.

4.1. Middle-aged model

Human middle-age (40 to 65 years old) is characterized by the occurrence of early signs of ageing, such as mood disorders and declarative memory decrease (Byers et al., 2010). During this period, the onset of senescence is highly dependent on age, sex, species and strains (Frick et al., 2003; Hebda-Bauer et al., 2007). Since environmental factors and lifestyle also play a key role in the progression of ageing, middle-aged models potentially display measurable reactivity to environmental challenges and may be a useful tool for toxicological investigations (Frick et al., 2003; Hebda-Bauer et al., 2007; Kohman et al., 2010).

In the present study, 12 month-old Sprague Dawley male rats were chosen to model middle-age. They displayed the same phenotype as young adults regarding CORT, GFAP and IL levels. Accordingly, no deficits were reported for CORT levels in 10 month-old Sprague Dawley and for IL in 12 month-old Fisher 344 rats and BALB/c mice (Gee et al., 2006; Kohman et al., 2010; Rasmussen et al., 1999). Deficits in Sprague Dawley and Wistar rats were reported later in the middle-age period (approximately 15 month-old), for micro and macroglia markers (CD11b, MHC II and GFAP) and IL levels, respectively (Bilbo, 2010; Hoane et al., 2004; Minogue et al., 2007).

Regarding behavioral performances, the assessment of age-related decline (as memory) is hampered in late ageing subjects, as it overlaps with senescence-linked disabilities (such as apathy, sleep disorders, modified locomotor activity) that lead to important inter-individual variability (Bilbo, 2010; Byers et al., 2010; Hodes and Shors, 2007; Moretti et al., 2011). Therefore, middle-aged models’ assets are displaying less intra-group variability, then, requiring fewer experimental subjects (Moyer and Brown, 2006), and mitigating ageing-related biases in behavioral approaches (such as differences in locomotor activity) (Pesic et al., 2010).
In the present study, on the contrary, we showed increased locomotor activity to novelty and decreased freezing during fear conditioning in middle-aged rats. To allow interpretation of behavior assessments, freezing during tests was expressed as percentage of freezing during conditioning, and the absence of differences in locomotor activity was checked during the test sessions using the high activity parameter. In our protocol, the contextual test was performed four days after conditioning, which did not allow robust contextual memory in young adults. On the contrary, middle-aged rats displayed contextual memory and tended to freeze more to/at the sound than young adults did. A previous study reported that 12 month-old Sprague Dawley rats showed more anxiety-related behavior than young rats, without memory impairment, suggesting that trait anxiety may have determined stronger fear memory in our study (Moyer and Brown, 2006). Based on anatomy, overlapping brain circuitries mediate memory, stress, fear, anxiety and nociception, involving the prefrontal cortex, hippocampus, amygdala and hypothalamus (Fendt and Fanselow, 1999; LeDoux, 2000). Thus, additional modalities, including sensory skills, ability to freeze, reactivity to novelty or sensorimotor functions may explain differences between young adults and middle aged rats (Hodes and Shors, 2007).

4.2. GSM exposure and neuro-immune system

An indication of injury progression may be given by pro-inflammatory factors, as IL are secreted within 1 to 8 hours by neurons and microglia and within 2 to 8 days by astrocytes following an acute excitotoxic brain injury (Pearson et al., 1999; Taupin et al., 1993).

According to our data, a 15 min acute 6 W/kg GSM-exposure does not seem to influence the neuro-immune and neuro-inflammatory axes in young adults, despite our primarily formulated hypothesis. Indeed, IL-1β and IL-6 were not modified from 30 min to 48 h post exposure and the GFAP response previously shown by Brillaud et al. (2007) and Mausset-Bonnefond et al. (2004) was not reproduced, while we used a similar protocol.

Our study was one of the first to assess these biomarkers, as only one study has been published and showed the absence of EMF-effects on IL secretion in primary astroglial and microglial cultured cells (Thorlin et al., 2006). Regarding the astroglial response to GSM, the literature is characterized by a high variability, as shown by negative (Fritze et al., 1997; Grafstrom et al., 2008) and positive effects (Ammari et al., 2010; Brillaud et al., 2007; Mausset-Bonnefont et al., 2004). Protocol differences may explain part of this variability, as, GFAP expression can be decreased by stress (Laping et al., 1994). In our protocol, the use of
fear conditioning in our protocol and the demonstration that it increases CORT after 30 min must be highlighted as a possible interacting parameter.

For the first time, our study investigated the question of a GSM effect on GFAP and IL response in middle-aged rodents. We found that cerebral GFAP and IL-6 were not modified while IL-1β was increased only in the olfactory bulb, 48 h following GSM exposure. This response may be explained by the fact that this structure is anatomically small and protrudes near the GSM source. A lack of homogeneity in IL-1β secretion through brain regions may also depend on the patterns of neural activation in response to a stimulus (Minami et al., 1991). Similar middle aged-related IL hyper-reactivity was demonstrated in response to lipopolysaccharides (Kohman et al., 2010).

4.3. GSM exposure, stress and emotional memory

Fear is an apprehensive arousal response to an explicit threat and refers to the activation of the defensive behavioral system (Fendt and Fanselow, 1999). Fear conditioning is based on both a Pavlovian conditioning and the induction of fear in response to an aversive unconditioned stimulus. Both processes are complex and rely closely on CORT release.

The increased CORT levels found in GSM-exposed 6 week-old rats were not in accordance with the literature showing the absence of a CORT levels modification relative to EMF exposure in in vivo studies in adult Fischer 344 and Wistar rats (Prochnow et al., 2011; Stagg et al., 2001). However, previous studies showed middle-aged related differences in plasmatic CORT or corticotropin-releasing factor responses to different types of stress when compared to young animals (Nolan et al., 1991; Pisarska et al., 2000). Reports of increased CORT levels for 3 days post-stress exist (Gaillet et al., 1991; Ottenweller et al., 1994), however, in most cases, the plasmatic CORT response corresponds to a transitory peak occurring within the first 30 min post (stress) stimulus (Xu et al., 1997). Our experiments showed that the CORT release was significantly increased 30 min but not 5 or 24 hours following fear conditioning, while acute GSM exposure alone did not affect CORT in young adults. These data strongly suggest that acute GSM is not perceived as a stressor. While it is hazardous to speculate about a potential link between CORT increase and GSM exposure performed 48h before, one may hypothesize that higher CORT levels, observed 3 days post exposure may correspond to increased reactivity of the hypothalamic-pituitary-adrenal axis to sacrifice-induced stress that would be affected by GSM and fear conditioning interaction; however, this hypothesis warrants reproducing adequate experimental groups in a single experiment.
Our data appears to contradict the acute anxiogenic effect of CORT in the fear response (Mitra and Sapolsky, 2008) as increased CORT level was not linked to an increased fear response in GSM-exposed young adults. Indeed, this group rather displayed a tendency toward a decrease in response to the sound, which may be underlined by the fear-reducing effects of CORT, linked to its ability to inhibit the recall of previously acquired, emotionally arousing information (Roozendaal et al., 2009; Sartori et al., 2011). In the same test, exposed middle-aged rats displayed an increased freezing response. One may hypothesize that this increase to results from increased IL-1β in the olfactory bulb. Indeed, olfactory bulbectomy is a model of depression, and has been characterized in 19 month-old Sprague Dawley male rats by emotional and mood-related behavioral impairments, including a tendency toward an increased freezing response (Slotkin et al., 1999).

Performing tests of depression-like behavior and fear conditioning using a discrete olfactory conditioned stimulus may allow/make it possible to determine behavioral impacts of GSM-induced increased IL in the olfactory bulb (Pugh et al., 1999). Lastly, at both ages, GFAP and IL in the hippocampus and sub-cortical nuclei, including key structures implicated in the fear conditioning paradigm, were not subjected to GSM or age effects. More generally, current literature is characterized by discordant GSM effects on memory (positive effects: (Fragopoulou et al., 2010; Narayanan et al., 2009) ; negative results: (Ammari et al., 2008; Dubreuil et al., 2002)), which may be explained by the weakness or specificity of GSM action in particular protocols or types of memory processes.

5. Conclusion

The present study showed middle-age-related vulnerability to an acute GSM exposure. Biomarkers of such vulnerability were: enhanced emotional memory and impaired corticosterone response while IL showed only a weak and isolated increase in the olfactory bulb. Reproducing these effects and studying the underlying mechanisms may allow a better understanding of the interaction between mobile phone EMF and the neurobiological tissue through ageing.

References


