C. R. Physique 11 (2010) 592–601

Contents lists available at ScienceDirect

Comptes Rendus Physique

www.sciencedirect.com

Interactions between radiofrequencies signals and living organisms

In situ detection of gliosis and apoptosis in the brains of young rats exposed in utero to a Wi-Fi signal

Détection in situ de l'apoptose et de la gliose dans le cerveau de jeunes rats exposés in utero à un signal Wi-Fi

Saliha Aït-Aïssa [a,b,*], Bernard Billaudel [a], Florence Poulletier De Gannes [a,b], Annabelle Hurtier [a], Emmanuelle Haro [a], Murielle Taxile [a], Gilles Ruffie [c], Axel Athane [b], Bernard Veyret [a,b], Isabelle Lagroye [a,b]

[a] *Bordeaux University, IMS Laboratory UMR 5218 CNRS, Bioelectronics group, IPB - ENSCBP, 16, avenue Pey-Berland, 33607 Pessac cedex, France*
[b] *Bioelectromagnetics laboratory, École pratique des hautes études, ENSCBP, 33607 Pessac cedex, France*
[c] *Bordeaux University, IMS Lab., IMS Transfert-A2M, ENSCBP, 33607 Pessac cedex, France*

ARTICLE INFO

Article history:
Available online 3 January 2011

Keywords:
Wi-Fi signal
Young animals
Brain
In utero exposure
Apoptosis
Gliosis

Mots-clés :
Signal Wi-Fi
Jeunes animaux
Cerveau
Exposition in utero
Apoptose
Gliose

ABSTRACT

Pregnant rats were daily whole-body exposed or sham-exposed to a Wi-Fi signal in a free-running reverberation chamber at 0, 0.08, 0.4, and 4 W/kg for 2 h during the last 2 weeks of gestation (5 days/week). Following this in utero exposure, the pups were divided into two groups and 1 group continued exposure for 5 weeks after birth. Several brain areas were examined for gliosis and apoptotic cells. Comparison among sham and exposed groups revealed no significant differences, suggesting that in utero and post-natal exposure to Wi-Fi did not damage the brains of the young rats.

RÉSUMÉ

Des rates gestantes ont été exposées corps-entier à un signal Wi-Fi, sans contrainte de mobilité, dans une chambre réverbérante à des niveaux d'exposition de 0 ; 0,08 ; 0,4 et 4 W/kg et ce, durant les deux dernières semaines de gestation. Suite à cette exposition in utero quotidienne (2 h, 5 jours/semaine), chaque portée obtenue a été divisée en deux groupes dont un, poursuivant l'exposition jusqu'à 5 semaines après la naissance. La détection de gliose et de cellules apoptotiques a été réalisée au niveau de différentes régions du cerveau des jeunes rats. Aucune altération n'a été observée suite à l'exposition Wi-Fi in utero et post-natale.

1. Introduction

There is worldwide concern about the potentially greater sensitivity of children to environmental agents (e.g., heavy metals, chemicals, ionizing radiation, electromagnetic fields, etc.). In the case of radiofrequency (RF) fields, the current

* Corresponding author.
E-mail address: saliha.ait@ims-bordeaux.fr (S. Aït-Aïssa).

1631-0705/$ – see front matter
doi:10.1016/j.crhy.2010.10.005

generation of children is the first to be exposed to fields emitted by mobile phones and other wireless communication devices throughout their entire lives. The brain and central nervous system (CNS), considered to be sensitive targets for the effects of these new signals, have already been the subject of various experimental studies, but very few of these have been conducted in utero and post-natal. Following the 2006 World Health Organization (WHO) recommendations, studies on developing organisms are now ongoing, notably focusing on potential CNS injuries.

Brain injuries, particularly neurodegenerative disorders, are characterized by a progressive degeneration of neuronal cells in specific locations in the CNS [1]. One common feature of these diseases is that neuronal damage involves apoptosis, as well as atrophy, gliosis, and necrosis.

Astrogliosis represents a remarkably homotypic response of astrocytes to all types of CNS injuries, including injuries to the developing brain [2]. In vivo and in vitro studies have indicated that astroglial cells are activated following CNS injury, triggering an increase in glial fibrillary acidic protein (GFAP) expression [3,4]. GFAP expression is one of the earliest indicators of retinal damage. Indeed, GFAP expression in Müller cells appears to be a secondary response to loss of retinal neurons [5,6]. Otherwise, GFAP-rich, astrocytic amyloid plaques appear early in Alzheimer's disease (AD) [7] and recent molecular genetic studies have shown GFAP involvement leading to Alexander disease, a fatal neurodegenerative childhood disorder resulting from leukodystrophy [8,9]. GFAP increase, associated with reactive gliosis, a potent marker of cell response to neurotoxic insult, is also being investigated in the case of RF fields.

A genomic study of the adult rat brain using in situ hybridization histochemistry revealed that exposure to GSM-900 (0.3 and 1.5 W/kg) and 900 MHz continuous wave (7.5 W/kg) signals did not increase GFAP messenger RNA [10]. Acute local exposure to pulsed GSM-900 microwaves (15 min, 6 W/kg) induced an increase in immunoreactive GFAP in the brains of adult male rats [11]. However, data are still scarce, particularly for immature organisms. One study using electron microscopy reported an increase in glial cell population in 35-day-old male Wistar rats exposed at 2.45 GHz (2 h/day, 35 days, 0.11 W/kg) and compared to the control group, but GFAP expression was not evaluated [12].

Programmed cell death is involved during embryonic and foetal development, especially in the organization of the central nervous system. Cellular loss through apoptosis is reported to account to up to 50% during synaptogenesis [13].

Many of the toxic effects elicited by exposure to environmental stressors are reported to be mediated by the regulation of apoptosis [14]. Changes in the apoptotic process occur in most of the neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases, and amyotrophic lateral sclerosis [14–17]. Apoptosis has also been considered as an endpoint in investigations into potential induction of brain damage by RF. Numerous in vitro studies using human cell lines have produced contrasting results [18–22]. Three in vivo studies of animal brain tissue reported no effect of RF on apoptosis [23–25]. Most studies using animal primary cell cultures [26] and cell lines [27–29] found no effect of exposure on apoptosis. Nevertheless apoptosis was reported in rat primary neuronal cultures exposed to a CW 900 MHz RF field (2 W/kg, 24 h) [30]. Two studies using primary cultures prepared from immature animals (retinal cells of newborn mice and cortical neuron cell cultures from 24 h old rats) suggested an alteration in the apoptotic process [31,32]. However, once again, very few studies have investigated the potential apoptotic effects of RF exposure in utero or on immature organisms.

The main objective of our work was to assess whether exposure to a Wi-Fi signal had an impact on the CNS of young rats exposed in utero and during early life. Changes in astrocytic and apoptotic responses were detected in selected brain regions, notably those implied in post-natal brain development. Free-moving pregnant females were whole-body exposed in a reverberation chamber at several specific absorption rates (SAR), up to 4 W/kg. The presence of apoptotic neurons was assessed in the young rat brains and GFAP expression was used as a marker for astrocyte activation.

2. Material and methods

2.1. Animal exposure

Female Wistar rats ($n = 60$, Janvier, Le Genest-St-Isle, France) were received 3 days post coitum (with no guarantee of pregnancy), and acclimated for 4 days in the animal facility. They were randomly distributed into five groups: four RF-exposed groups and one cage-control group. Females were exposed randomly in a reverberating chamber at whole-body SAR levels of 0, 0.08, 0.4, or 4 W/kg (sham-exposure, public and professional exposure limits and ICNIRP critical level, respectively). Sham-exposed rats underwent the same procedures as exposed animals, except for the presence of the Wi-Fi signal. Exposure at all SAR levels was conducted with 12 dams per condition. The cage-control group (CC) was kept in the animal facility ($n = 12$) with minimum handling.

RF exposure of dams and their embryos lasted 2 weeks during gestation (2 h/day, 5 days/week from day 6 to 21 of gestation). After birth, three pups per litter were exposed with their mothers for an additional 5 weeks post-natal until weaning (G1 group $n = 3$ per litter), while the rest of the litter, exposed only in utero, was kept in the animal facility for 5 weeks (G2 group, $n = 3$–15 per litter). All biological tests were carried out blind on 5-week-old rats from the G1, G2 and cage control groups. Since G1 and G2 were not handled in the same way in terms of separation from the mothers, for each group the reference was the respective sham-exposed counterparts. The experimental protocols followed the recommended ethical procedures of French legislation and the CNRS ethics committee.

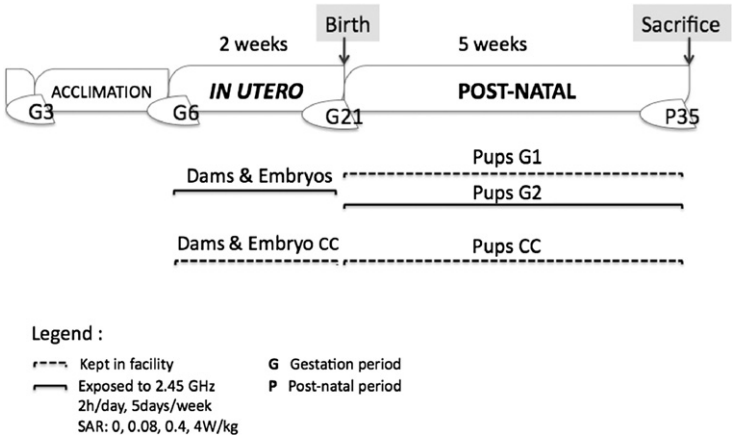


Fig. 1. Animal exposure protocol.

Fig. 1. Protocole d'exposition des animaux.

2.2. Exposure system

The free-running exposure system was designed as a cubic reverberation chamber ($150 \times 150 \times 150$ cm) with 6 dipole antennas, activated at random, and three paddles for mode stirring. The signal was Wi-Fi (2.45 GHz, IEEE 802.11 b/g 1999) based on the "dialog" between two PCs equipped with Wi-Fi cards (Netgear WG311v3 802.11g Wireless PCI adapter). Attenuation levels were set to yield SAR levels of 0, 0.08, 0.4, and 4.0 W/kg in the dams. A 70-W amplifier provided such levels without reaching saturation. The four animal cages were placed in a $40 \times 40 \times 40$ cm volume at the centre of the chamber. Calculations using numerical phantoms of the pups gave higher SAR levels in the pups compared to the mothers, peaking at 9 ± 3 W/kg at the end of the first week of life for the highest SAR level of 4 W/kg in dams, and decreasing later to reach the SAR level of the mothers [33].

Calculations take into account the relative positions of the pups during exposure, with respect to the mother and siblings, based on the webcam recordings for three typical configurations at different ages. It is noteworthy that exposure at 2450 MHz yields absorption that are more superficial than at the most commonly used GSM frequencies of 900 and 1800 MHz. Moreover, the averaged dielectric properties of adult rats are $\sigma = 1.7$ S/m and $\varepsilon = 40$ at 2450 MHz. Peyman et al. (2001) have demonstrated that the dielectric properties decrease with the age of the rats [34]. The homogeneous dielectric properties of the pup model were extrapolated based on this data.

2.3. Brain cryosections

Five-week-old rats ($n = 12$/condition, one per litter) were anesthetized using isoflurane (Baxter, Maurepas, France), perfused, and fixed via the heart with 4% paraformaldehyde (unless otherwise mentioned, chemicals come from Sigma–Aldrich, St Quentin Falavier, France).

At that time, brains were taken for analysis, coded and kept in fixative solution overnight at 4 °C, cryo-preserved in 20% sucrose for 48 h at 4 °C, frozen in isopentane, and stored at −80 °C. Ten-micrometer brain cryosections were prepared from the Z1 (Interaural 8.20 mm, Bregma −0.80 mm) and Z2 (Interaural 5.2 mm, Bregma −3.80 mm) zones in each sample (Cryostat Leica CM1900). Ten regions were observed for immunoreactivity, including the hippocampus (DG and CA1, CA2, CA3), cortex (retrosplenial cortex M1, primary auditory cortex M3, and motor cortex F1), striatum (Caudate Putamen CPu), Amygdala (M4), and preoptic area (F4) (Fig. 1). Dried cryosections were kept at −20 °C prior to processing for the TUNEL assay or −80 °C for GFAP staining by immunohistochemistry.

2.4. GFAP staining by immunohistochemistry

Glial fibrillary acidic protein levels were assessed using immunohistochemistry. Dried slides were processed using an autostainer (Dako Autostainer, DakoCytomation, Trappes, France) at 22 °C. Slides were thawed at room temperature during 1 h, and then washed in PBS-Triton-X100 0.3% for 30 min. Endogenous peroxidases were inhibited with 0.03% H_2O_2 and non-specific sites saturated with 10% horse serum (GIBCO™ Invitrogen, Cergy Pontoise, France) in Phosphate Buffer Saline (PBS). Slides were then washed in 0.3% PBS-Tween and incubated for 1 h with the primary purified antibody (C-19) (Santa Cruz Biotechnology, California, USA) (1:100) raised in goat (DakoCytomation, Trappes, France).

Thereafter, the sections were incubated with 1:200 biotinylated anti-goat antibody for 30 min, and then with avidin-biotin-peroxidase, according to the supplier's recommendations (Universal LSAB2™ kit, DakoCytomation, Trappes, France). Finally, the presence of GFAP was revealed as a brown colour using peroxidase diaminobenzidine substrate supplemented with nickel (Vector SK-4100, Abcys U.S., France). Brain sections from ischemic rats were used as positive controls (2 vessel

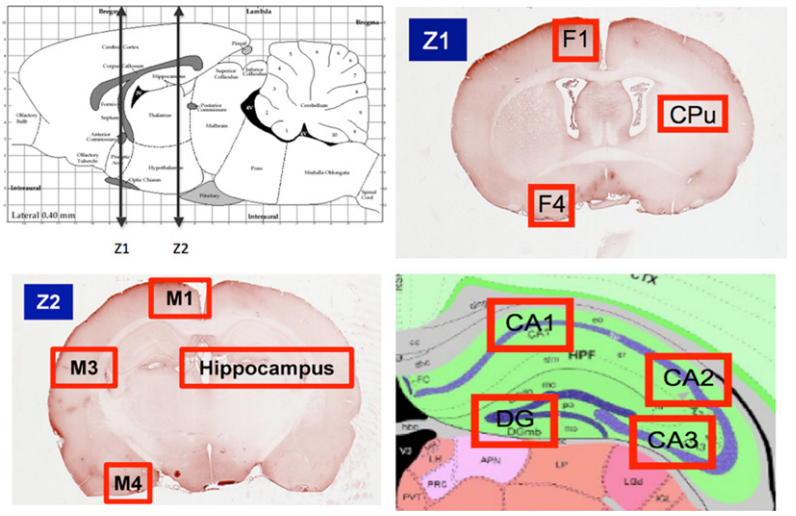


Fig. 2. Brain areas tested for apoptosis and astrogliosis immunoreactivity. Z1: interaural 8.20 mm, bregma −0.80 mm. Z2: interaural 5.2 mm, bregma −3.80 mm. DG: dentate gyrus; CA1-3: cornus ammonis, fields of hippocampus; M1: retrosplenial cortex; M3: primary auditive cortex; M4: amygdala; F1: motor cortex; F4: preoptic area; CPu: caudate putamen.

Fig. 2. Régions du cerveau observées pour la mise en évidence d'apoptose et d'astrogliose par immunoréactivité. Z1 : interaural 8,20 mm, bregma −0,80 mm. Z2 : interaural 5,2 mm, bregma −3,80 mm. DG : gyrus denté ; CA1-3 : cornus ammonis, domaines de l'hippocampe ; M1 : cortex retrosplénial ; M3 : cortex auditif primaire ; M4 : amygdala ; F1 : cortex moteur ; F4 : zone pré optique ; CPu : putamen caudé.

occlusion ischemia; 2-h reperfusion 120 min) (Fig. 2). Brain structures of interest were identified under the microscope (×400) (ZEISS Axiovert 50, Olympus digital camera, Fisher-Bioblock, Illkirch, France) and the images analyzed using Aphelion software, version 3.2 (ADCIS S.A. & Amerinex Applied Imaging, Caen, France).

2.5. Apoptosis detection

Apoptosis was detected in situ via DNA strand breaks on brain slices, using the TUNEL assay with an in situ cell-death detection kit, specifically set up for neuronal applications (NeuroTACS II kit, Trevigen, USA).

Cryosections fixed on Superfrost slides were permeabilized-using Neuropore™ (Trevigen, Gentaur, Paris, France) according to the manufacturer's instructions. Slides were immersed in quenching solution (Methanol + 30% H_2O_2) then in 1*X* terminal deoxynucleotidyl transferase (TdT) labelling buffer. Samples were covered with 50 µl labelling reaction mixture (1*X* TdT labelling buffer + TdT dNTP mix + 50*X* Mn^{2+} + TdT enzyme) and incubated in a humidity chamber at 37 °C for 1 h. The labelling reaction was stopped in 1*X* TdT stop buffer. Samples were covered with 50 µl streptavidine-horseradish peroxidase solution and incubated at room temperature for 10 min. Slides immersed in diaminobenzidine solution were washed twice in deionized water before blue counterstaining (1 min). Slides were washed by dipping in the following solutions in turn: tap water, ammonium water, tap water, 70% ethanol (twice), 95% ethanol (twice), 100% (twice), and xylene (twice). Slides were mounted using DPX histology mountant and dried overnight. Positive controls were generated using TACS-nuclease that produced DNA breaks in every cell (Fig. 2). Morphology and immunoreactive coloration associated with apoptotic neurons were observed under the microscope (ZEISS Axiovert 50). Each brain area in each sample was photographed (Olympus digital camera, Tokyo, Japan). The total number of apoptotic cells per section was determined by direct visual counting (×200 magnification) as intact (well-defined, blue–violet stained, round) or irreversibly damaged (densely brown-stained, polygonal shaped) neurons. Groups for each subdivision were compared with the equivalent region in the sham-exposed brains and expressed as positive or negative apoptosis observations.

2.6. Statistical analysis

At each SAR level, 12 female rats were exposed and the statistical power was such that the detection of a 35% difference with the sham-exposed group was possible at $p < 0.05$. In the statistical analysis, only one pup per litter was considered, so that it becomes an independent sample, with a total of 9 to 12 pups from different mothers per condition. The non-parametric Kruskal–Wallis Exact test (STATXACT® Software) was used to compare GFAP immunoreactive areas among the four SAR groups, followed by the Mann–Whitney test to determine the inter-group statistical significance. Using this analysis, G1 and G2 pups were compared to their respective sham-exposed counterparts but no comparison was made between both groups. After decoding, sham-exposed groups were compared to cage-control group and the analysis showed no difference for the parameters of interest (data not shown).

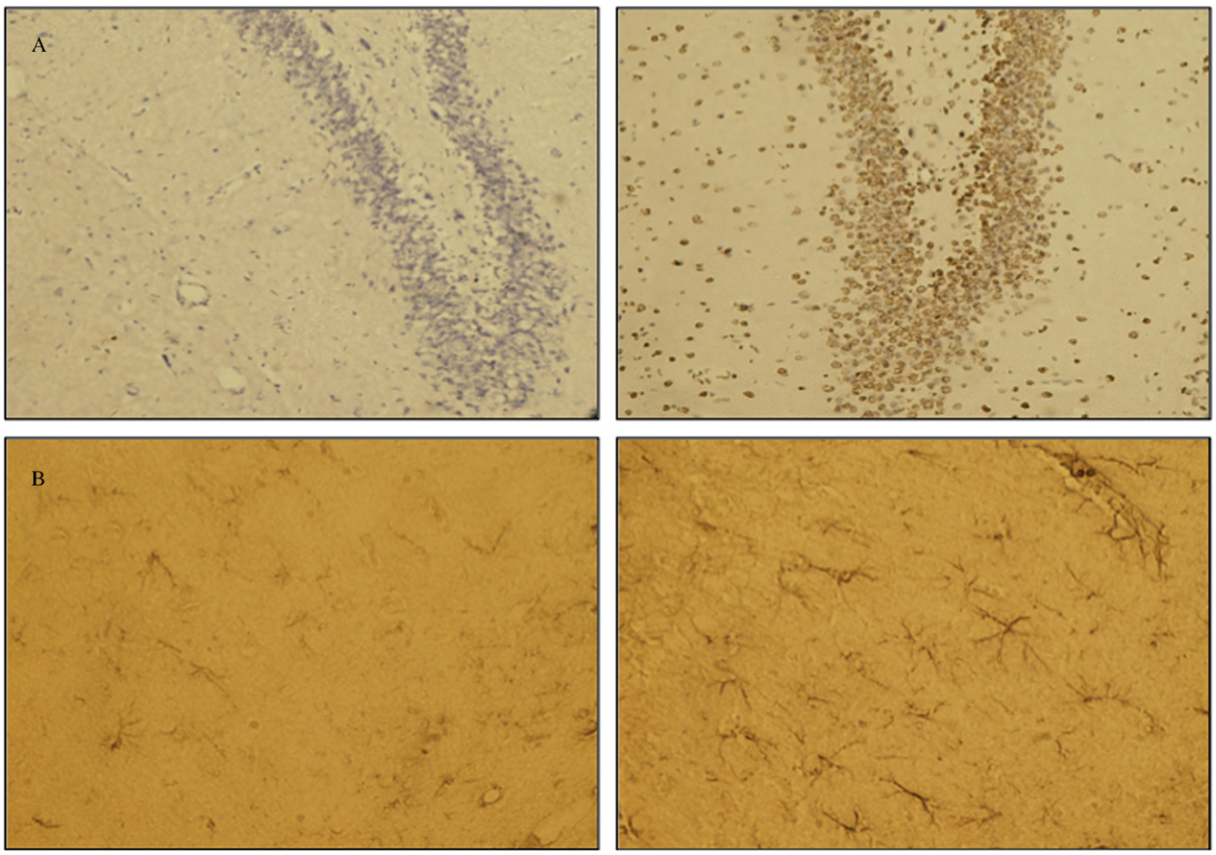


Fig. 3. Cage control and positive controls for in situ GFAP and apoptosis staining. (A) DG dentate gyrus Z2, TUNEL test for brain of a sham (left) and of a nuclease-positive control rat (right) (×200). (B) CA2, immunohistochemical staining of GFAP for sham (left) and ischemia positive control (right) (×400).

Fig. 3. Marquage GFAP et détection de l'apoptose in situ dans les cerveaux de rats contrôles cages et contrôles positifs. (A) DG Dentate gyrus Z2, test TUNEL sur rats contrôle cage (à gauche) et contrôle positif avec adjonction de nucléase (à droite) (×200). (B) CA2, marquage immuno-histochimique de la GFAP sur le cerveau d'un rat contrôle cage (à gauche) et contrôle positif Ischémie (à droite) (×400).

3. Results

We previously reported that in utero Wi-Fi exposure had no effect on gestational outcome [35]. There was no statistically significant difference among groups in maternal body-weight gain from day 6 to 21 of gestation. Out of a total of 60 female rats, 52 completed pregnancy, with a distribution of 9–12 dams per group. The percentage of pregnant rats was similar in all groups ($\geqslant$75%).

The dams gave birth to 3–15 pups per litter. There were no statistical differences among the number of pups per litter or the pups' body-weight in all groups. Daily observation of both dams and pups did not reveal any clinical signs of suffering.

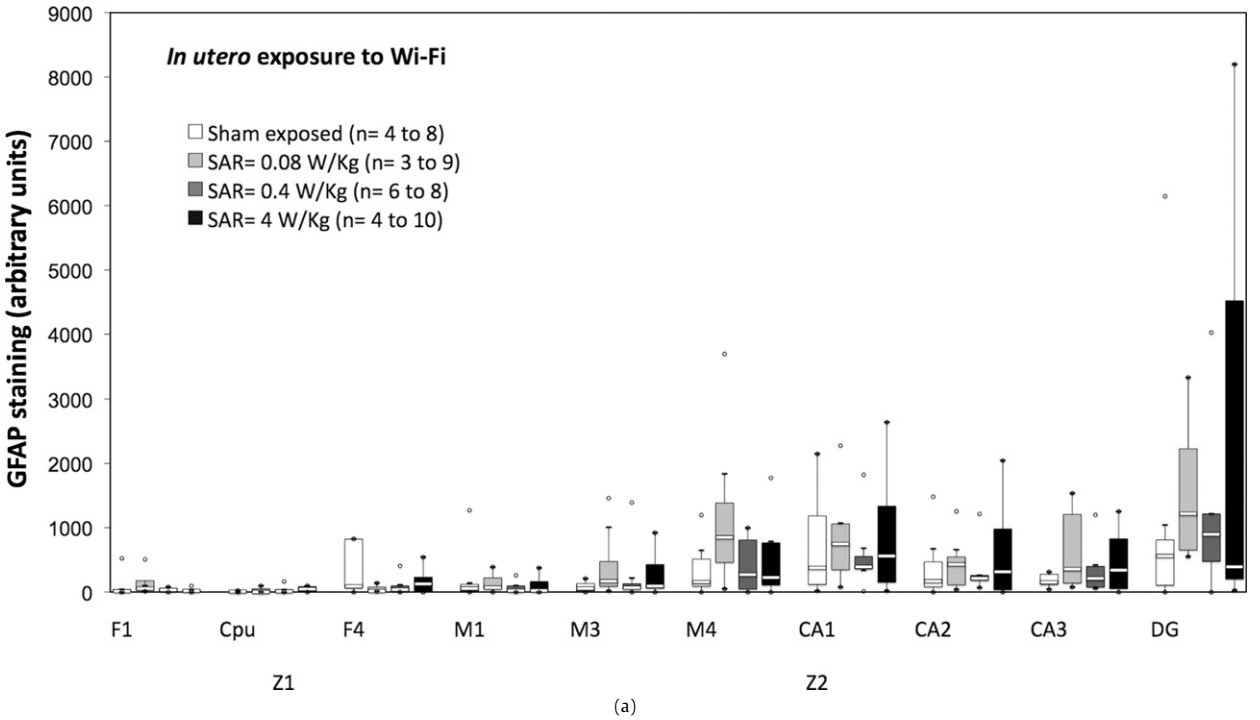
3.1. In situ GFAP detection

Immunocytochemistry revealed the presence of brown immunoreactive areas, indicative of GFAP expression, particularly in the cerebral cortex and hippocampus. Two VO-ischemia controls exhibited an increase in GFAP positive cells, whereas no change was observed in the sham group (Fig. 3B). Slice labelling quantification, illustrated in Tukey's diagram (Fig. 4), showed no statistically significant differences in immunoreactive areas in the brains between Wi-Fi-exposed and sham-exposed groups ($p > 0.15$ in all case).

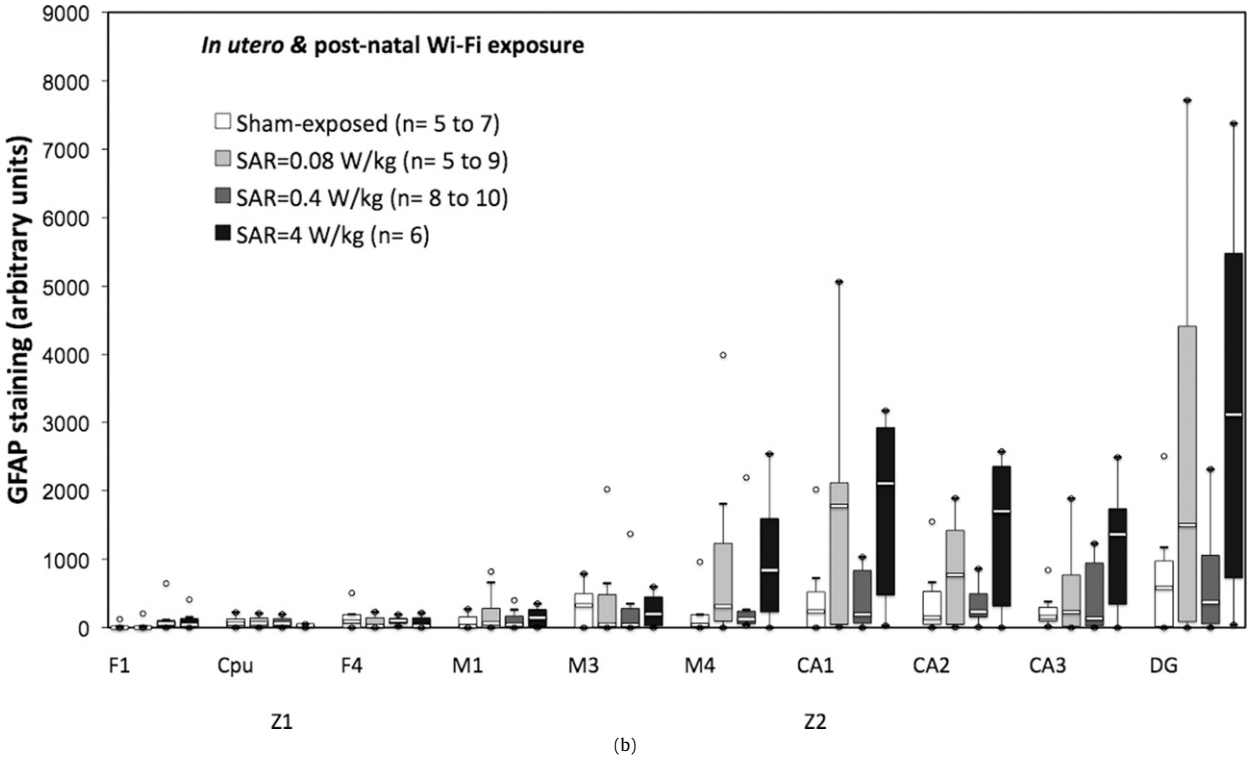
This observation was true in the superficial part of the brain (cortex) as well as in deeper structures (striatum, hippocampus) for groups exposed in utero only or in utero and during early life. These results suggest that Wi-Fi exposure does not induce persistent astroglia activation in the immature rat brain.

3.2. Apoptosis TUNEL assay

Apoptosis was detected in situ using the TUNEL assay on tissue sections from the brains of exposed rats (Fig. 5). Standard microscopic examination revealed neither morphological changes nor cell death in any region of interest in either Wi-Fi-



(a)



(b)

Fig. 4. GFAP staining levels in selected brain areas of young rats exposed: (a) only in utero to Wi-Fi signal; (b) in utero and post-natal to Wi-Fi signal. Tukey's diagram showing box and whisker plots of the distribution of GFAP staining intensity exhibiting in the different SAR-level groups of rats and in the different brain areas. The central box shows the 25th and 75th percentiles, with the median indicated by a white line inside the box. The whiskers extend to the minimum and maximum values, excluding outliers, which are plotted individually outside this range.

Fig. 4. Intensité de marquage GFAP par région dans les cerveaux de jeunes rats exposés : (a) uniquement in utero au signal Wi-Fi ; (b) in utero et en post-natal au signal Wi-Fi. Le diagramme de Tukey permet d'illustrer la distribution de l'intensité de coloration du GFAP pour les différents niveaux de DAS et dans les différentes zones du cerveau de rats. La zone centrale montre les 25ème et 75ème centiles, la médiane est indiquée par une ligne blanche à l'intérieur de la boîte. Les « moustaches » s'étendent aux valeurs minimales et maximales, les valeurs aberrantes à l'exclusion sont représentées individuellement en dehors de cette plage.

or sham-exposed rat brain sections. Only positive-control tissue sections treated with nuclease showed a strong signal in almost all cells (Fig. 3A). In these positive sections, cells containing fragmented nuclear chromatin, characteristic of apoptosis, exhibited the brown staining typically associated with cell condensation. Conversely, the cage-control rat brains showed no staining. Taken together, the lack of staining and apoptotic cells in the various brain areas studied, in superficial as well as deeper structures, suggested that early life exposure to the Wi-Fi signal did not alter neuronal apoptotic processes.

4. Discussion

Deployment of new wireless communication technologies has raised concerns about the health effects of RF signals, such as Wi-Fi, particularly on young subjects, in view of the potentially greater sensitivity of developing brains. Few studies have addressed the effects of RF exposure on immature animals. In our experiments, we investigated the possible influence of prenatal 2.45 GHz Wi-Fi exposure on the brain integrity of young rats. For this purpose, we used young rats exposed in utero in a reverberating chamber specially designed for homogeneous whole-body, free-moving animal exposure. This exposure system was particularly advantageous to avoid restraining stress for pregnant rats and their pups [33]. The young rats were sacrificed 5 weeks after birth, corresponding to sexual maturity, after exposure either in utero only or in utero plus 5 weeks of early life.

We focused on two brain development processes, potentially leading to pathological processes following brain injuries: GFAP expression and apoptosis. Neurons in most regions of the brain are formed during gestation and the process of neurogenesis is almost complete before birth.

Nevertheless, for some structures, such as the DG in the hippocampus and the subventricular zone of the lateral ventricle, neurogenesis begins during gestation and continues during the early postnatal period then, at a slower rate, into adulthood [36]. GFAP expression and apoptotic cells were, therefore, detected in selected regions of the young rat brain.

In our experiment there was no change in astroglia activation on 5-weeks old rats, evaluated via GFAP immunostaining, in response to RF exposure. Detection of GFAP via immunostaining is possible as soon as the late gestation period and GFAP mRNA expression has been detected on foetal day 15 with an increase up to day 21 [37]. Between birth and day 20, anti-GFAP staining showed an evolution to the adult pattern (mature shaped astrocytes) [38]. Proliferation and differentiation of astrocytes in rats occur mainly during postnatal brain development during which inflammation of the CNS is characterized by reactive astrogliosis, resulting in astrocyte proliferation or astrocytic hypertrophy [39]. GFAP expression has been proposed as a specific marker for assessing astrocyte response to injury, particularly in mature brain [4,40]. Nevertheless, immature astrocytes have also been reported to express GFAP, notably following early differentiation induced by neurodevelopmental toxicants [41].

Glial alteration has been reported for instance in foetal alcohol syndrome [42]. A study using an acute ethanol administration during the prenatal period reported disrupted generation of specific glial cells (Bergmann glia cells) during early postnatal development [43]. GFAP expression has also been demonstrated in glial cells and enhanced GFAP expression has been observed in the acutely damaged brain and in numerous brain injuries and neurodegenerative diseases [5,7,8,44,45].

Our observations do not suggest any increase in GFAP levels, or alterations in its distribution, in Wi-Fi-exposed animals. This was true after both 2-week in utero and 7-week exposure. Our results are in line with those obtained by evaluating GFAP expression by immunohistochemistry following chronic exposure of C57BL mice to a CDMA signal (849 and 1763 MHz, 7.8 W/kg) [23]. This finding is also corroborated by a study that found no alternation in GFAP messenger RNA in adult rat brains (GSM-900, 1.5 and 3 W/kg; and 900 MHz continuous wave, 7.5 W/kg) [10]. Furthermore, no change in GFAP level was observed in a study using the same method, following 10 days' chronic GSM-900 exposure (45 min/day 5 days/week, 24 weeks, 1.5 W/kg) [46]. These authors also reported increased GFAP stained areas at higher brain-averaged SAR levels of 6 W/kg, and two other studies by the same group using a single, 15-min exposure (GSM-900, 6 W/kg) produced similar results [11,47]. Therefore, astroglia activation caused by local exposure at high SAR levels may be due to a thermal effect. However, primary cultures, enriched in astroglial cells, exposed to a GSM-900 signal at 3 W/kg or a continuous wave 900 MHz signal at 27 and 54 W/kg did not exhibit any change in total intracellular GFAP content [48]. Interestingly, these authors noticed a change in GFAP levels and cell morphology in the positive controls following a temperature increase of only 1 °C, thus emphasizing the need for well-designed temperature control in all experiments using microwaves on biological systems.

Our study is the first to evaluate in situ GFAP expression following in utero and neonatal Wi-Fi exposure. The results suggest that, under our experimental conditions, exposure to a 2.45 GHz Wi-Fi signal does not induce reactive and persistent gliosis.

Apoptosis in the brain, under normal physiological conditions, is believed to occur mainly during prenatal development, decline early after birth, and remain minimal in the adult brain. The axonal and dendritic growth and synaptogenesis processes may represent a "window of vulnerability" for the action of external agents. An increase in neuronal apoptosis accompanying the normal development of the brain is observed during exposure to these agents with cognitive consequences in the learning phase and in adulthood [49].

Moreover, studies have shown that exposure to ethanol during early development increases neuronal death in rodent brain [50,51]. Thus, prenatal exposure to ethanol caused changes in the expression of bcl-2, bax, and caspase 3 in the developing rat cerebral cortex and thalamus [52]. Restraining stress drastically inhibited hippocampus cell proliferation in

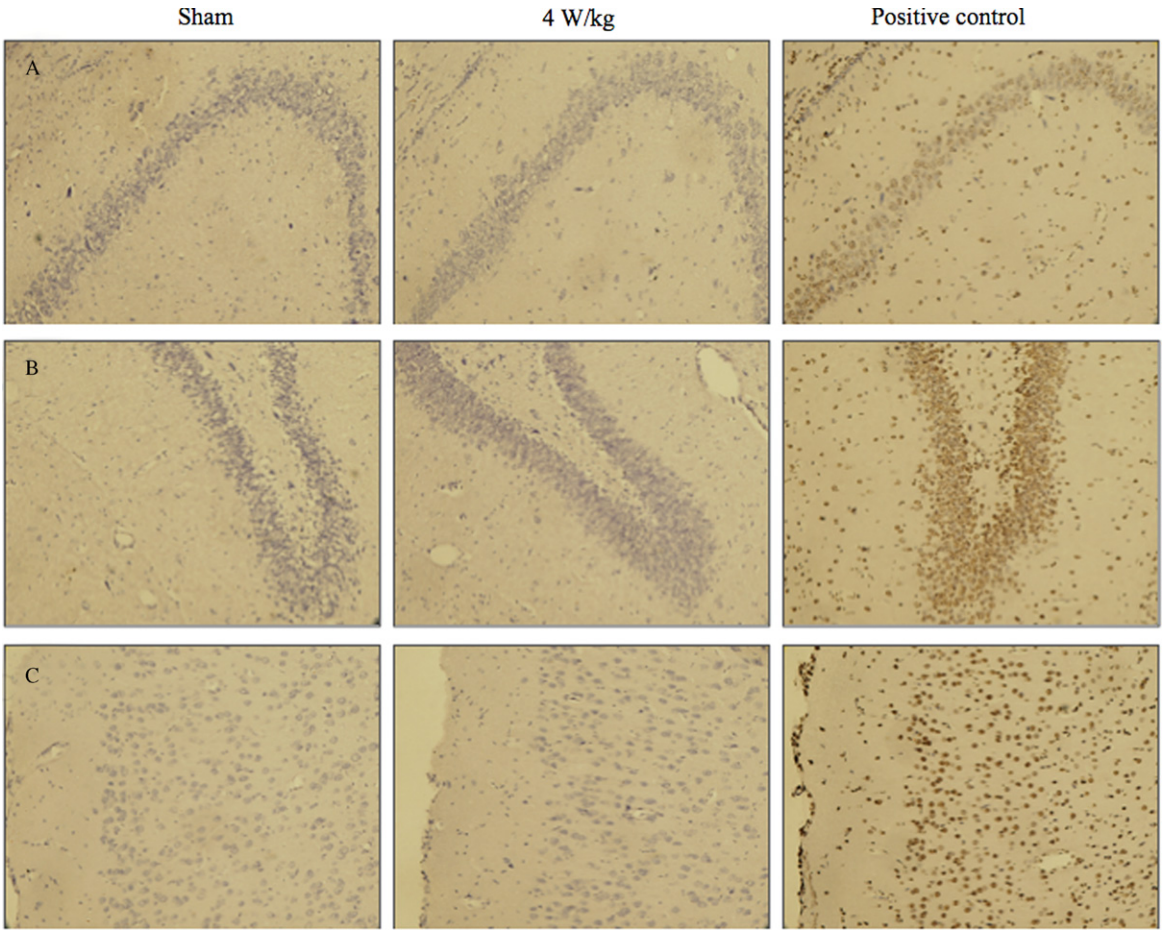


Fig. 5. Representative images of apoptotic neurons identification using the TUNEL assay. (A) CA2 cornu ammonis 2. (B) DG dentate gyrus. (C) F1 motor cortex. Sham: TUNEL negative, blue–violet stained. Wi-Fi signal at 4 W/kg: blue–violet stained similar to sham. Positive control (TACs nuclease): TUNEL positive, brown stained.

Fig. 5. Images représentatives du test TUNEL pour l'identification des neurones apoptotiques. (A) CA2 cornu ammonis 2. (B) DG dentate gyrus. (C) F1 cortex moteur. Sham : Test TUNEL négatif, coloration violette. Signal Wi-Fi à 4 W/kg : coloration violette similaire au sham. Contrôle positif (TACs nucléase) : Test TUNEL positif, coloration brune.

pregnant rats and during early neonatal development and significantly increased caspase-3 activity within the hippocampus [53].

Caspase activity following RF exposure has been investigated as a marker of apoptosis in numerous studies using animal and human cells, but few have demonstrated any effect [19,21,25–28,30]. In a series of studies, primary cortical neuron cultures from embryonic rats exposed to CW and GSM 900 MHz signals showed no statistically significant difference in caspase activity. However, caspase-independent apoptosis was reported under specific exposure conditions and out of physiological temperature (CW, 2 W/kg, 39 °C) [26,30]. Experiments using Annexin V as an apoptosis marker mostly produced negative results, notably on murine neuroblastoma cell lines exposed to GSM-900, 24 h, 2 W/kg [29] and human Molt4 cells exposed to different American RF signals at SAR up to 3 W/kg [18].

Only one study reported positive results on retinal ganglion cells from newborn mice exposed to 2.45 GHz signals (6–18 h, no dosimetry), but the authors did not exclude the possibility of a thermal effect [32]. Several genes related to apoptosis expression have also been studied, but only in vitro studies reported possible effects, notably on the Bcl2 gene [20,24,31]. One study reported Bax gene up-regulation in cultured mouse neurons and astrocytes following exposure to a genuine mobile phone (1900 MHz, 2 h, no dosimetry) [31]. A study on cell proliferation and apoptosis in the brains of C57BL mice exposed to a CDMA signal (849 and 1763 MHz, 7.8 W/kg) reported no effect due to RF [23].

Our results are in agreement with a previous study using the TUNEL assay on adult rats exposed head-only to GSM-900 (2 h, 0, 0.14, and 2.0 W/kg), where apoptosis was not observed [54]. To assess whether in utero exposure to Wi-Fi affected the cell death process in young rat brains, we investigated in situ apoptosis on tissue sections taken from exposed rats, using the TUNEL assay, a potent tool for apoptosis detection, as it facilitates analysis of topographical patterns of apoptosis, characteristic of different types of neuronal injury [1]. The various brain areas studied, in superficial as well as deeper structures, did not exhibit staining of DNA breaks, indicative of apoptosis or apoptotic cells, in any of the sham or exposed

groups (Fig. 5). In vivo exposure to the Wi-Fi signal, including the gestation period, did not, apparently, induce post-natal apoptosis. Only one other study has investigated the effects of RF exposure on neuronal death in young rats [39]. This behavioral and morphological study reported no degenerative changes or dying neurons following whole-body GSM-900 exposure (22 h/day, 5 weeks, 0.3 or 3.0 W/kg).

In conclusion, under our experimental conditions, whole-body in utero exposure with and without extended postnatal exposure to a Wi-Fi signal at SAR levels up to 4 W/kg for the dams and 12 W/kg for the pups, did not trigger persistent astroglia activation or induce apoptosis in the brains of young rats. These observations suggest that prenatal exposure to Wi-Fi has no deleterious effects on the integrity of the developing rat brain.

Acknowledgements

This work was supported by the French "Fondation santé et radiofréquences" (Grant 2006-6) and France Telecom R&D (Grant No. 46142804). The authors would like to thank Dr. Joe Wiart for his cooperation and advice, particularly on the exposure system.

References

[1] E.C. Hirsch, Animal models in neurodegenerative diseases, in: Neuropsychiatric Disorder: An Integrative Approach, 2007, pp. 87–90.
[2] A.R. Little, J.P. O'Callaghan, Astrogliosis in the adult and developing CNS: is there a role for proinflammatory cytokines? Neurotoxicology 22 (2001) 607–618.
[3] H. Franke, et al., The reaction of astrocytes and neurons in the hippocampus of adult rats during chronic ethanol treatment and correlations to behavioral impairments, Alcohol 14 (1997) 445–454.
[4] J.P. O'Callaghan, K. Sriram, Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity, Expert Opin. Drug Safety 4 (2005) 433–442.
[5] V. Sarthy, Focus on molecules: glial fibrillary acidic protein (GFAP), Exp. Eye Res. 84 (2007) 381–382.
[6] M.R. Verardo, et al., Abnormal reactivity of Müller cells after retinal detachment in mice deficient in GFAP and vimentin, Invest. Ophthalmol. Vis. Sci. 49 (2008) 3659–3665.
[7] R.G. Nagele, et al., Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease, Neurobiol. Aging 25 (2004) 663–674.
[8] R. Li, et al., GFAP mutations in Alexander disease, Int. J. Dev. Neurosci. 20 (2002) 259–268.
[9] M. Pekny, M. Pekna, Astrocyte intermediate filaments in CNS pathologies and regeneration, J. Pathol. 204 (2004) 428–437.
[10] K. Fritze, et al., Effect of global system for mobile communication microwave exposure on the genomic response of the rat brain, Neuroscience 81 (1997) 627–639.
[11] A.L. Mausset-Bonnefont, et al., Acute exposure to GSM 900-MHz electromagnetic fields induces glial reactivity and biochemical modifications in the rat brain, Neurobiol. Dis. 17 (2004) 445–454.
[12] R. Paulraj, J. Behari, Protein kinase C activity in developing rat brain cells exposed to 2.45 GHz radiation, Electromagn. Biol. Med. 25 (2006) 61–70.
[13] R.W. Oppenheim, Cell death during development of the nervous system, Annu. Rev. Neurosci. 14 (1991) 453–501.
[14] R. Franco, et al., Environmental toxicity, oxidative stress and apoptosis: ménage à trois, Mutat. Res. 674 (2009) 3–22.
[15] O. Ekshyyan, T.Y. Aw, Apoptosis: a key in neurodegenerative disorders, Curr. Neurovasc. Res. 1 (2004) 355–371.
[16] M. Okouchi, et al., Neuronal apoptosis in neurodegeneration, Antioxid. Redox Signal 9 (2007) 1059–1096.
[17] A.M. Gorman, Neuronal cell death in neurodegenerative diseases: recurring themes around protein handling, J. Cell Mol. Med. 12 (2008) 2263–2280.
[18] G.J. Hook, et al., Measurement of DNA damage and apoptosis in Molt-4 cells after in vitro exposure to radiofrequency radiation, Radiat. Res. 161 (2004) 193–200.
[19] P. Merola, et al., Proliferation and apoptosis in a neuroblastoma cell line exposed to 900 MHz modulated radiofrequency field, Bioelectromagnetics 27 (2006) 164–171.
[20] M. Buttiglione, et al., Radiofrequency radiation (900 MHz) induces Egr-1 gene expression and affects cell-cycle control in human neuroblastoma cells, J. Cell Physiol. 213 (2007) 759–767.
[21] A. Höytö, et al., Radiofrequency radiation does not significantly affect ornithine decarboxylase activity, proliferation, or caspase-3 activity of fibroblasts in different physiological conditions, Int. J. Radiat. Biol. 84 (2008) 727–733.
[22] Y. Cao, et al., 900-MHz microwave radiation enhances gamma-ray adverse effects on SHG44 cells, J. Toxicol. Environ. Health A 72 (2009) 727–732.
[23] T.H. Kim, et al., Local exposure of 849 MHz and 1763 MHz radiofrequency radiation to mouse heads does not induce cell death or cell proliferation in brain, Exp. Mol. Med. 40 (2008) 294–303.
[24] F. Yilmaz, et al., Whole-body exposure of radiation emitted from 900 MHz mobile phones does not seem to affect the levels of anti-apoptotic bcl-2 protein, Electromagn. Biol. Med. 27 (2008) 65–72.
[25] S. Dasdag, et al., Effect of mobile phone exposure on apoptotic glial cells and status of oxidative stress in rat brain, Electromagn. Biol. Med. 28 (2009) 342–354.
[26] V. Joubert, et al., No apoptosis is induced in rat cortical neurons exposed to GSM phone fields, Bioelectromagnetics 28 (2007) 115–121.
[27] V. Joubert, et al., Microwave exposure of neuronal cells in vitro: study of apoptosis, Int. J. Radiat. Biol. 82 (2006) 267–275.
[28] A. Höytö, et al., Proliferation, oxidative stress and cell death in cells exposed to 872 MHz radiofrequency radiation and oxidants, Radiat. Res. 170 (2008) 235–243.
[29] J. Moquet, et al., Exposure to low level GSM 935 MHz radiofrequency fields does not induce apoptosis in proliferating or differentiated murine neuroblastoma cells, Radiat. Prot. Dosimetry 131 (2008) 287–296.
[30] V. Joubert, et al., Apoptosis is induced by radiofrequency fields through the caspase-independent mitochondrial pathway in cortical neurons, Radiat. Res. 169 (2008) 38–45.
[31] R. Zhao, et al., Studying gene expression profile of rat neuron exposed to 1800 MHz radiofrequency electromagnetic fields with cDNA microassay, Toxicology 235 (2007) 167–175.
[32] X.R. Zhou, et al., The study of retinal ganglion cell apoptosis induced by different intensities of microwave irradiation, Ophthalmologica 222 (2008) 6–10.
[33] T. Wu, et al., Whole-body newborn and young rats' exposure assessment in a reverberating chamber operating at 2.4 GHz, Phys. Med. Biol. 55 (2010) 1619–1630.
[34] A. Peyman, A.A. Rezazadeh, C. Gabriel, Changes in the dielectric properties of rat tissue as a function of age at microwave frequencies, Phys. Med. Biol. 46 (6) (2001) 1617–1629.

[35] S. Aït-Aïssa, et al., In-utero and early-life exposure of rats to a Wi-Fi signal: gestational outcome and screening of immune markers in sera, Bioelectromagnetics, submitted for publication (manuscript number BEM-10-0132).
[36] R. Bartesaghi, S. Severi, S. Guidi, Effects of early environment on pyramidal neuron morphology in field CA1 of the Guinea-pig, Neuroscience 116 (2003) 715–732.
[37] M. Sancho-Tello, et al., Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures, Glia 15 (2) (1995) 157–166.
[38] S.K.R. Pixley, De Veilis, Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin, Develop. Brain Res. 15 (2) (1984) 201–209.
[39] M. Huleihel, H. Golan, M. Hallak, Intrauterine infection/inflammation during pregnancy and offspring brain damages: possible mechanisms involved, Reprod. Biol. Endocrinol. 2 (2004) 17.
[40] C. Zimmer, et al., Altered glial fibrillary acidic protein immunoreactivity in rat brain following chronic hypoxia, Neuroscience 40 (1991) 353–361.
[41] G. Krinke, Developmental neurotoxicity. The Laboratory Rat. The Handbook of Experimental Animals, Academic Press, 2000, 756 pages.
[42] M. Guizzetti, M. Catlin, L.G. Costa, Effects of ethanol on glial cell proliferation: relevance to the fetal alcohol syndrome, Fetal and Pediatric Pathology 18 (1998) 433–443.
[43] F.P. Perez, et al., Electromagnetic field therapy delays cellular senescence and death by enhancement of the heat shock response, Exp. Gerontol. 43 (2008) 307–316.
[44] F. Razavi-Encha, J.C. Larroche, D. Gaillard, Infantile familial encephalopathy with cerebral calcifications and leukodystrophy, Neuropediatrics 19 (1988) 72–79.
[45] Y. Kitamura, Y. Nomura, Stress proteins and glial functions: possible therapeutic targets for neurodegenerative disorders, Pharmacol. Ther. 97 (2003) 35–53.
[46] M. Ammari, et al., Effect of a chronic GSM 900 MHz exposure on glia in the rat brain, Biomedicine & Pharmacotherapy 62 (2008) 273–281.
[47] E. Brillaud, A. Piotrowski, R. de Seze, Effect of an acute 900 MHz GSM exposure on glia in the rat brain: a time-dependent study, Toxicology 238 (2007) 23–33.
[48] T. Thorlin, et al., Exposure of cultured astroglial and microglial brain cells to 900 MHz microwave radiation, Radiat. Res. 166 (2006) 409–421.
[49] D. Rice, S. Barone, Critical periods of vulnerability for the developing nervous system: evidence from humans and animal, Models Environ. Health Perspect. Suppl. 108 (2000) S3.
[50] C.R. Green, et al., Chronic prenatal ethanol exposure increases apoptosis in the hippocampus of the term fetal Guinea pig, Neurotoxicol. Teratol. 27 (2005) 871–881.
[51] P. Jaatinen, J. Rintala, Mechanisms of ethanol-induced degeneration in the developing, mature, and aging cerebellum, Cerebellum 7 (2008) 332–347.
[52] S.M. Mooney, M.W. Miller, Effects of prenatal exposure to ethanol on the expression of bcl-2, bax and caspase 3 in the developing rat cerebral cortex and thalamus, Brain Res. 911 (2001) 71–81.
[53] D.L. Van den Hove, et al., Prenatal stress and neonatal rat brain development, Neuroscience 137 (2006) 145–155.
[54] F. Poulletier de Gannes, et al., Effects of head-only exposure of rats to GSM-900 on blood-brain barrier permeability and neuronal degeneration, Radiat. Res. 172 (2009) 359–367.