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Research report

Effects of radiofrequency exposure on the GABAergic system in the rat cerebellum: clues from semi-quantitative immunohistochemistry

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Abstract

The widespread use of cellular phones raises the problem of interaction of electromagnetic fields with the central nervous system (CNS). In order to measure these effects on neurotransmitter content in the CNS, we developed a protocol of neurotransmitter detection based on immunohistochemistry and image analysis. Gamma-vinyl-GABA (GVG), an inhibitor of the GABA-transaminase was injected in rats to increase GABA concentration in the CNS. The cellular GABA contents were then revealed by immunohistochemistry and semi-quantified by image analysis thanks to three parameters: optical density (O.D.), staining area, and number of positive cells. The increase in cerebellar GABA content induced by GVG 1200 mg/kg was reflected in these three parameters in the molecular and the granular layers. Therefore, control of immunohistochemistry parameters, together with appropriate image analysis, allowed both the location and the detection of variations in cellular neurotransmitter content. This protocol was used to investigate the effects of exposure to 900 MHz radiofrequencies on cerebellar GABA content. Both pulsed emission with a specific absorption rate (SAR) of 4 W/kg and continuous emission with high SAR (32 W/kg) were tested. We observed a selective diminution of the stained processes area in the Purkinje cell layer after exposure to pulsed radiofrequency and, in addition, a decrease in O.D. in the three cell layers after exposure to continuous waves. Whether this effect is, at least partly, due to a local heating of the tissues is not known. Overall, it appears that high energetic radiofrequency exposure induces a diminution in cellular GABA content in the cerebellum. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: GABA

Keywords: Radiofrequency; Mobile phone; Neurotransmitter; GABA; Immunohistochemistry; Image analysis

1. Introduction

The interaction of electromagnetic fields (EMF) with living organisms presents an increasing source of interest nowadays. The extensive worldwide use of mobile phones raises the question of their possible biological effects, especially on the central nervous system (CNS), because of their use in close vicinity to the brain. To answer this question, it was necessary to study the effects of exposure to radiofrequencies (RF) emitted by mobile phones on neurotransmitter (NT) levels in the CNS.

The detection of variations of NT concentrations has long been a challenge to determine the effects of different factors on the CNS. On the one hand, the quantification of NT has been accomplished by a variety of methods [4,41,55,68] and often required chromatographic separation by high performance liquid chromatography (HPLC) [51,53,54,67,77]. These methods are very useful to determine NT concentrations in tissue homogenates but they do not give any precise information on NT location within a particular brain region. On the other hand, immunohistochemistry (IHC) has been extensively used to locate

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substances in brain structures [7,10,28,58]. Over the last decade, an increasing number of studies using IHC as a quantitative approach has emerged [9,19,30,49,59,74]. This technique is very attractive since it allows the semi-quantification of NT content and gives some crucial information on NT location, either at a macroscopic or a microscopic level [9].

Moreover, image analysis has been extensively used to detect and count cells stained for different markers: hormones [14,49], c-fos proteins [5] or NT in the CNS [7,58,74]. However, only a few studies used this method to quantify precisely some compound concentrations [30,59]. In fact, these latter studies used a standard calibration curve to assign a precise concentration of substance to an optical density (O.D.) value [30,59].

In the first part of this work, we tested the sensitivity of IHC and image analysis to semi-quantify cellular γ-aminobutyric acid (GABA) content in a pharmacological model in which altered GABA concentrations had been measured precisely elsewhere by HPLC [44]. GABA concentrations in the CNS are finely regulated by two main enzymes: one governing its synthesis (glutamate decarboxylase or GAD, EC 4.1.1.15) and the other one responsible for its catabolism (GABA-transaminase or GABA-T, EC 2.6.1.19) [15]. Thus, it is possible to change GABA concentrations in the CNS by using chemical compounds affecting the activity of these enzymes. Gamma-vinyl-GABA (GVG or vigabatrin) is a selective irreversible inhibitor of GABA-T in vitro and in vivo [29,61]. It induces a dramatic increase in GABA concentration in the CNS by reducing GABA-T activity by 80% [26], whatever its administration route [29,56]. GVG was shown to significantly enhance the GABA levels (measured by HPLC) in different brain areas, mainly in the hippocampus, the frontal cortex and the cerebellum [8,44]. In our experiment, the effect of GVG on cerebellar GABA content was semi-quantified by IHC and image analysis. We focused this study on the cerebellum because it is one of the richest GABA brain structures and GVG has been shown to enhance cerebellar GABA concentration up to 300% above control by HPLC technique [44].

After developing a suitable IHC and image analysis technique, this method was used for a first investigation of the effects of 900 MHz RF exposure on the GABA concentration in the cerebellum. To date, many studies have dealt with the putative effects of exposures to RF radiation on human health (for review, see Refs. [18,27,62]). Moreover, the effects of EMF on biological systems have been extensively investigated over the last 20 years [40,42,50,52]. Particular attention has been given to the effects of microwave irradiation on the CNS [1,6,21,37,71] especially on different NT systems, namely the cholinergic [13,38,39], the dopaminergic [17] or the glutamatergic and the GABAergic [35] systems. The present widespread use of cellular phones raises the question of the interaction of 900 MHz microwave irradiation with biological systems [18] and particularly with the CNS, since the RF are emitted very close to the human brain. In the last few years, several studies reported various effects of such EMF on the brain. For instance, pulsed EMF have been shown to influence human sleep [46,75], GSM (global system for mobile communication) radiotelephones have been implicated in the modification of the auditory brainstem response [31] and human brain activity [16], and a 915-MHz irradiation has been involved in the modification of the permeability of the blood brain barrier [64]. More recent investigations confirmed some of these previous results and showed that RF emitted by cellular telephones influenced human memory [33], EEG during awakening [22], sleep [23] or memory task [36] and cognitive functions [34,60]. However, no study has dealt with the effects of 900 MHz irradiation on NT themselves. This was the main goal of this study in which we submitted rats to 900 MHz RF exposure by means of a device delivering the same RF as those emitted by mobile phones. The effects of two different types of emission on the cerebellar GABA content are investigated in this study.

2. Materials and methods

2.1. Animals

Male Wistar rats at a body weight of about 180 g were purchased from IFFA CREDO (France). Following their arrival in the laboratory, the rats were kept under controlled environmental conditions (ambient temperature 22°C, 12-h light/dark cycle) and were accustomed to laboratory conditions 1 week before experiment. During this week, animals used for RF exposure were accustomed to their specific Plexiglas holders (Atelier Deco Volume, Talence, France) for 2 h a day. All the animals used in both experiments (GVG and RF exposure) weighed 220–250 g on the day of the experiment. Rats received food and tap water ad libitum. All experiments were performed in the morning to avoid the bias of circadian rhythms in endogenous transmitter systems. Animals were cared for and surgically handled in accordance with the European Communities Council Directive (24 November 1986, 86/609/EEC).

2.2. Drug treatment

GVG was administered intraperitoneally at different dosages: 1, 100 and 1200 mg/kg. Control rats were injected with 0.9% saline. Groups of six rats per dose were used in this experiment. All drugs were freshly dissolved and administered at a volume of 3 ml/kg.

2.3. RF experiment

For each experiment, two lots of 12 rats were used: one

exposed to RF emission and the other non-exposed (sham). All these rats were maintained in specific Plexiglas holders and the exposed rats were submitted to RF emission thanks to individual loop antennas (PIOM, Ecole Nationale Supérieure de Chimie Physique de Bordeaux, Pessac, France) fixed on the Plexiglas above their heads (Fig. 1). Four animals could be exposed at the same time. The antennas were connected to a 900 MHz RF power amplifier (Type RFS 90064, RFPA, Artigues-près-Bordeaux, France) which allowed the control of (1) the type of RF emission: pulsed to simulate the GSM emission (i.e. pulse emission during 576 μs every 4.6 ms), or continuous waves (CW), (2) the specific absorption rate (SAR) corresponding to the energetic flux received by the tissues (in W/kg). For the GSM type emission, the SAR used was 4 W/kg, whereas for the continuous emission, the SAR was fixed to 32 W/kg (corresponding to the maximal power delivered by the device). The duration of RF exposure was fixed at 2 h. Exposed and sham animals were placed in separate anechoic chambers during the 2-h experiment. The 2-h exposure to the higher SAR (32 W/kg) was well supported by the rats.

2.4. Determination of the SAR values

The power amplifier was calibrated in the PIOM laboratory (Ecole Nationale Supérieure de Chimie Physique de Bordeaux, Pessac, France). The SAR, expressed in W/kg, was calculated by the following relation: $SAR = c\Delta T/\Delta t$, with c: calorific capacity, ΔT: temperature variation, and Δt: time variation. This relation allowed to determine the SAR value by measuring the temperature variation thanks to a Vitek probe. This probe was placed in a rat phantom (middle of the brain) and measured experimentally the variation of temperature when the power amplifier was switched from off to on position.

The measure of the global temperature variation in the brain during RF exposure (4 W/kg) has been performed thanks to the same Vitek probe placed in the middle of the brain of a rat phantom. After 1-h exposure, the temperature

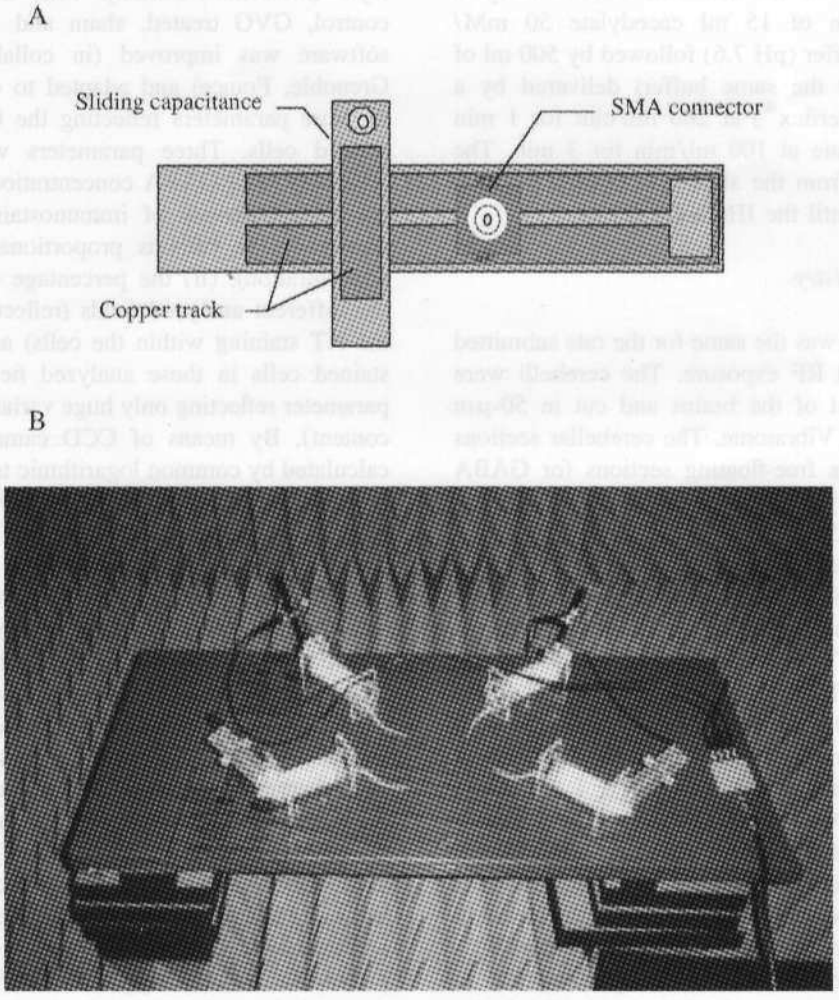

Fig. 1. Radiofrequency exposure system. (A) Individual loop antenna. (B) View of the experimental system used during a simultaneous exposure of four rats. Rats are maintained in Plexiglas holders, with individual loop antennas placed above their heads. Each antenna is connected to a 900-MHz power amplifier through a four-way power divider.

reached a plateau corresponding to a temperature increase in the brain of approximately 0.5°C.

A mathematical model of the SAR values was also performed, with the FDTD (finite difference time domain) mesh method by using the software XFDTD (Remcom, USA) and a loop antenna as source of emission. The SAR was then calculated by the relation $SAR = \sigma E^2/\rho$, where σ is the brain electrical conductivity (0.62 S/m), E is the electric field measured and ρ is the brain volume mass (1046 kg/m^3). Modelling showed that the SAR averaged on the whole brain was equal to the SAR measured experimentally at the position of the probe.

The SAR value was established at 4 W/kg per Watt emitted by the power amplifier (CW) in the whole brain (B. Veyret, personal communication).

2.5. Sacrifice and dissection of the brains

The animals were deeply anaesthetized with sodium pentobarbital 6% (i.p.), either 4 h after the injection of GVG (GVG experiment) or immediately after RF exposure (RF experiment). Tissue fixation was achieved by a transcardially perfusion of 15 ml cacodylate 50 mM/ sodium bisulfite 1% buffer (pH 7.6) followed by 500 ml of 5% glutaraldehyde (in the same buffer) delivered by a peristaltic pump (Masterflex®) at 200 ml/min for 1 min followed by a lower rate at 100 ml/min for 3 min. The brains were removed from the skull and postfixed in the same fixative at 4°C until the IHC experiment.

2.6. Immunohistochemistry

The protocol of IHC was the same for the rats submitted to GVG treatment and RF exposure. The cerebelli were separated from the rest of the brains and cut in 50-μm sagittal sections with a Vibratome. The cerebellar sections were then processed as free-floating sections for GABA immunodetection. They were pretreated with trypsine 0.05%–EDTA 0.02% (5 min, Gibco). After washing (2×5 min) in Tris-buffered saline (Tris 0.05 M, NaCl 9‰, pH 7.6 — TBS), the sections were incubated with a rabbit polyclonal antibody against GABA (Geffard, France) diluted at 1:5000 in TBS containing goat non-specific serum (NSS) 1% and Triton 0.1% for 48 h at 4°C. After washing in TBS (2×10 min), sections were incubated with a goat anti-rabbit immunoglobulin G (Tebu) diluted at 1:200 in TBS (containing goat NSS 1%) for 1 h at room temperature, washed 2×5 min in TBS and incubated with a rabbit peroxidase–antiperoxidase complex (DAKO) diluted at 1:200 in TBS (containing goat NSS 1%) for 1 h at room temperature. After the last washes (2×5 min) in TBS, immunoreactivity was revealed with 0.037% 3-3′-diaminobenzidine hydrochloride (DAB, Sigma) diluted in TBS (pH 7.6) in the presence of 0.02% H_2O_2. Particular care was taken to ensure a comparable coloration procedure between control and experimental cerebellar sections: (i) the duration of the incubation in the DAB-H_2O_2 solution was strictly controlled and fixed at 8 min for all the sections, (ii) the control and experimental cerebella were always processed in parallel. The reaction was terminated by rinsing several times in TBS. The sections were then mounted on glass slides in 0.06 M phosphate buffer, dried for 24 h at ambient temperature and coverslipped with DePeX mounting medium (Gurr®). They were then dried for at least 3 days before examination under the microscope and further image analysis.

2.7. Image analysis

An upright optical microscope (Leica DMLB) coupled with a Color Camera Tri CCD (Donpisha) and image analysis software IPS 4.06 — Unilog (Alcatel, Grenoble, France) were used. A neutral filter was inserted to highlight the brown peroxidase staining.

The analysis was performed on live-acquired images of 768×576 pixels (corresponding to a tissue surface of 0.043 mm^2, objective 20, ocular 2.5) in each cerebellar cell layer (molecular, Purkinje cells and granular layers) of control, GVG treated, sham and exposed animals. The software was improved (in collaboration with Unilog, Grenoble, France) and adapted to our aim which was to measure parameters reflecting the GABA quantity in the stained cells. Three parameters were chosen for their reliability with GABA concentration in neuronal cells: (i) the optical density of immunostained cells (staining intensity of the cells is proportional to the local GABA concentration), (ii) the percentage of stained cell area in the different analyzed fields (reflecting the distribution of the NT staining within the cells) and (iii) the number of stained cells in these analyzed fields (the less sensitive parameter reflecting only huge variations in cellular GABA content). By means of CCD camera, O.D. values were calculated by common logarithmic transform of the ratio of incident to transmitted light. The whole O.D. range (0–+2) was divided into 256 digitized values (0–255, corresponding to 8 bits) to highlight variations in staining intensity. All the parameters described above were measured for the cells detected as positive ones. The cells were considered positive if their O.D. values were higher than a defined threshold. This threshold was chosen as the O.D. value above which only cell bodies (and not processes) were detectable in the molecular and granular layers. It was determined thanks to live-acquired images to fit perfectly with the detection of the entire cells. It was therefore very important to readjust the threshold value for each section to obtain the optimal conditions to detect only the cell bodies of the stained cells and discard some artifacts which would induce a misevaluation of the parameters. Interestingly, in the Purkinje cell layer, the accumulation of GABA in basket terminals was also detected with these parameters.

Six sections per rat and three fields per section (corre-

sponding to the three cell layers in the cerebellum) were analyzed. Each analyzed field was chosen randomly in each cerebellar layer of each section and delimited by the experimenter through interaction with the software. For each analyzed field, the mean O.D. (M.O.D.) value was obtained by averaging O.D. values of all stained profiles in this field. The background O.D. of each section was systematically subtracted from this M.O.D. In this way, a single M.O.D. value was obtained for each layer of each section. As the analysis was carried out on six sections per rat, six M.O.D. values were obtained for each cell layer in each animal. The percentage of the staining area in the analyzed field was calculated as the ratio between the stained cell area and the total area of the analyzed field in one layer. Thus, six values of percentage of stained cell area were obtained for each cell layer in each rat. The number of stained cells in the analyzed field of each layer was obtained by counting individual positive cells. Six values of stained cell number were then calculated for each cell layer in each rat. In the Purkinje cell layer, this number was not calculated as we detected there only the basket terminals.

2.8. Drug

GVG was generously supplied by Marion Merrell Bourgoin S.A. (Bourgoin-Jallieu, France).

2.9. Statistical analysis

A Mann–Whitney test was used to compare the effects of GVG treatment or RF exposure on each parameter. The comparisons were performed on 36 values per group (six sections per rat, six rats per group) and 72 values per group (six sections per rat, 12 rats per group), respectively, for GVG and RF experiments.

Differences were considered significant when $P<0.05$ (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

3. Results

3.1. Validation of the IHC as a semi-quantitative approach

The first part of this work was devoted to determining the sensitivity of IHC and image analysis in the detection of cerebellar GABA content changes after GVG treatment. Fig. 2 illustrates the difference of the cell staining between cerebellar sections of control, GVG 1, 100 and 1200 mg/kg treated rats. Note the difference of staining of the cell bodies between control and treated animals in the molecular and granular layers (Fig. 2A–D), especially for the highest dose of GVG (Fig. 2A and D). The difference of staining between control and GVG 1200 mg/kg is highlighted when observed at higher magnification (Fig. 2E–F). We then decided to focus our study on the highest dose of GVG, i.e. 1200 mg/kg.

We first analyzed the O.D. values of stained cells, reflecting the local GABA concentrations. In the molecular layer, GVG 1200 mg/kg induced a 132% increase in the M.O.D. of stained cells compared with control (Fig. 3A, $P<0.001$). In the Purkinje cell and the granular layers, we did not observe such an effect on the M.O.D. (Fig. 3A). Interestingly, a lower dose of GVG (100 mg/kg) was as efficient as the highest dose (1200 mg/kg) in inducing an increase in the M.O.D., whereas 1 mg/kg had no effect on this parameter (data not shown).

The second parameter measured was the area of staining, indicating the distribution of GABA in the cells. It appeared that this parameter was also differently affected in each cerebellar cell layer after treatment with GVG (Fig. 3B). GVG 1200 mg/kg induced a significant increase (137%) in the area of stained cells in the molecular layer (Fig. 3B, $P<0.001$), and to a smaller extent (130%), in the granular layer (Fig. 3B, $P<0.05$) compared to controls. Lower doses of GVG (1 and 100 mg/kg) had no effect on this parameter (data not shown). In the Purkinje cell layer, no statistical significant difference could be observed even at the highest GVG dose (Fig. 3B).

The last parameter taken into account was the number of stained cells which is affected only if the GABA content varies dramatically (see Section 2). In both molecular and granular layers, GVG 1200 mg/kg induced a strong increase (148 and 152%, respectively) in this parameter compared with controls (Fig. 3C, $P<0.001$). Lower doses of GVG induced also an increase in the number of positive cells (data not shown).

As a summary, our approach allowed the detection of an increase in the cerebellar GABA content induced by GVG in both molecular and granular layers.

3.2. Effects of 900 MHz RF exposure on cerebellar GABA content

This approach was consequently used as a sensitive method to determine the effects of 900 MHz RF exposure on the cellular GABA content in the cerebellum. We observed different results depending on the type and power of RF emission. After exposure to low power RF (GSM, SAR 4 W/kg), the M.O.D. of stained cells were not statistically different in RF and sham exposed rats, whatever the cell layer ($P>0.05$, Fig. 4A–C left side). Interestingly, there was a 16% diminution in the area of stained processes in the Purkinje cell layer in the RF exposed rats compared to sham ones ($P<0.01$, Fig. 5B left side; Table 1).

In the rats exposed to higher RF power (continuous waves or CW, SAR 32 W/kg), the decrease in the stained processes area in the Purkinje cell layer was also detected (17% decrease, $P<0.01$, Fig. 5B right side; Table 1). Moreover, a 13% decrease in the area of stained cells was

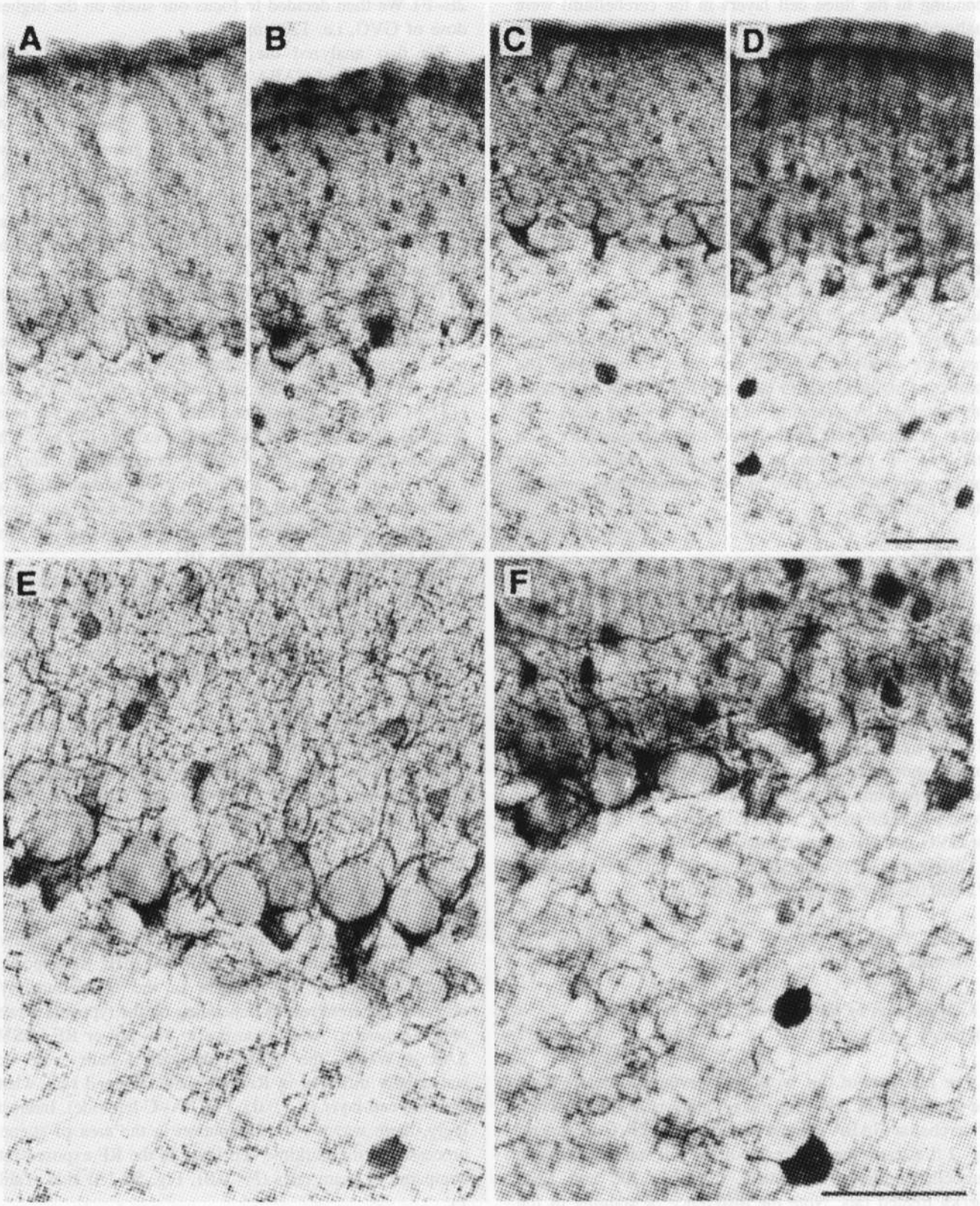

Fig. 2. Effect of GVG treatment on the staining of GABAergic cells in the rat cerebellum. Each photograph contains the molecular layer (upper part), the Purkinje cell layer (middle part) and the granular layer (lower part). (A–D) Views at low magnification (×250), A: control, B: GVG 1 mg/kg, C: GVG 100 mg/kg, D: GVG 1200 mg/kg. (E–F) Views at high magnification (×500), E: control, F: GVG 1200 mg/kg. Scale bars: 50 μm.

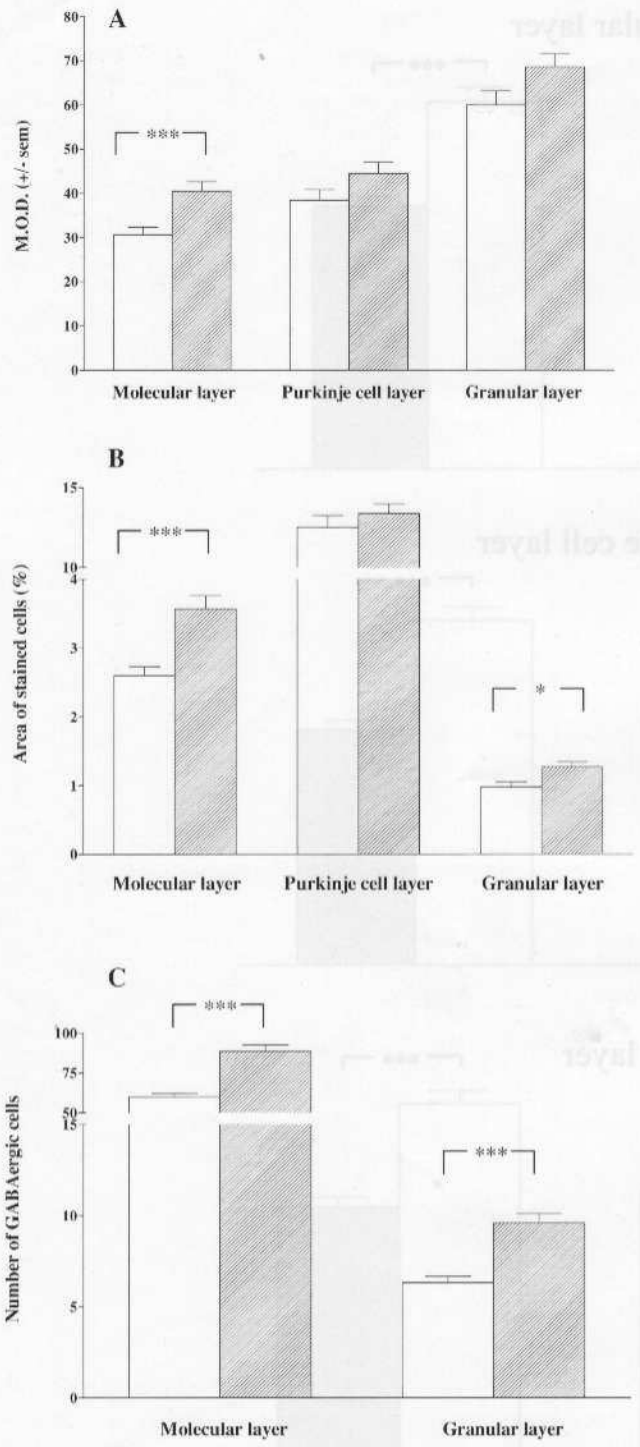

Fig. 3. Effect of GVG (1200 mg/kg) on the mean optical density (M.O.D.), the staining area and the number of GABAergic cells in the three cell layers of the rat cerebellum. (A) M.O.D. of stained cells; (B) staining area of GABAergic cells; (C) number of GABAergic cells. Empty bars: control; hatched bars: GVG 1200 mg/kg. Each column represents the mean±S.E.M. of 36 values (six rats, six values per rat). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

observed in the molecular layer ($P<0.05$, Fig. 5A right side; Table 1). This result was strengthened by the diminution of the M.O.D. in the exposed rats in all three cell layers: 28, 32 and 27% decrease in the molecular, Purkinje cell and granular layers, respectively ($P<0.001$, Fig. 4A–C right side; Table 1). The number of stained cells was affected neither by the GSM nor the CW type of exposure whatever the cell layer considered (Fig. 6). Altogether, these results suggest that RF exposure exerts an effect on the concentration and distribution of the GABA content and that this action is amplified for high energetic RF exposure. Interestingly, this effect appears at a lower dose in the Purkinje cell layer.

4. Discussion

In the first part of this study, we showed that IHC allowed the detection of variations in the cellular GABA content when coupled with appropriate image analysis. We chose to use this method (rather than HPLC) to obtain specific information on the precise cellular location of NT concentration variations inside a particular brain structure. Indeed, subtle differences concerning one specific cell or a given circuit can be overlooked by bulk analysis of NT content with HPLC. In order to calibrate our immunohistochemical approach, we used GVG (Vigabatrin), a potent selective inhibitor of GABA-T [29,44], to induce an increase in the GABA concentration in the cerebellum. Our technical approach needed, firstly to develop a reproducible IHC protocol and, secondly to define appropriate analysis parameters in order to reflect the intracellular GABA content in the immunostained cells. The reproducibility of IHC was achieved by (1) treating control and experimental sections in parallel and (2) strictly controlling the duration of the DAB incubation. Image analysis was performed by the measurement of different parameters. This procedure required the definition of an appropriate threshold for the cell detection. This threshold, defined as the O.D. value above which cell bodies were detected in their totality, had to be reset for each section since the staining of the cells could vary between the sections. It has been shown that the definition of a threshold could vary between different experimenters and mainly within observers themselves [26]. Therefore, special care was given to this step and the staining areas corresponding to unidentified 'phantoms' were systematically discarded from the analysis (see Section 2). To obtain comparable data between sections, it was also necessary to subtract the O.D. of the background of each section from the M.O.D. of the cells.

We chose to measure three parameters on the sections, each giving a different and complementary information on the cellular GABA content. Indeed, the O.D. values reflected the local GABA concentration inside each cell (the intensity of the staining is proportional to the quantity of NT [59]), the staining area rather indicated the distribution of GABA all over the cell, and the variation of

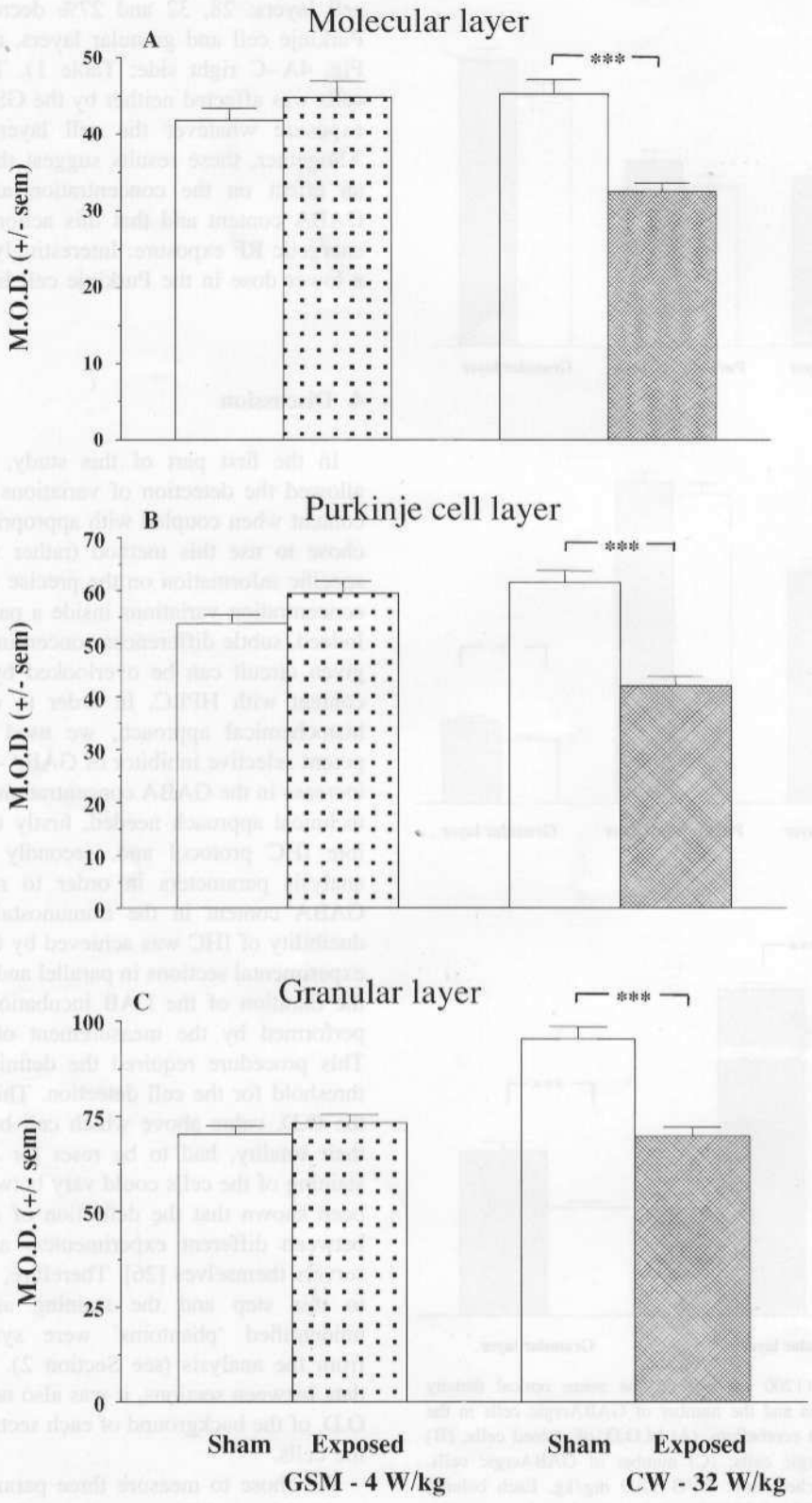

Fig. 4. Effect of radiofrequency exposure on the mean optical density (M.O.D.) of GABAergic cells in the rat cerebellum. Left side of the histogram: radiofrequency exposure to 4 W/kg GSM (pulsed emission); right side of the histogram: radiofrequency exposure to 32 W/kg CW (continuous emission). A: molecular layer; B: Purkinje cell layer; C: granular layer. Each column represents the mean±S.E.M. of 72 values (12 rats, six values per rat). *** $P<0.001$.

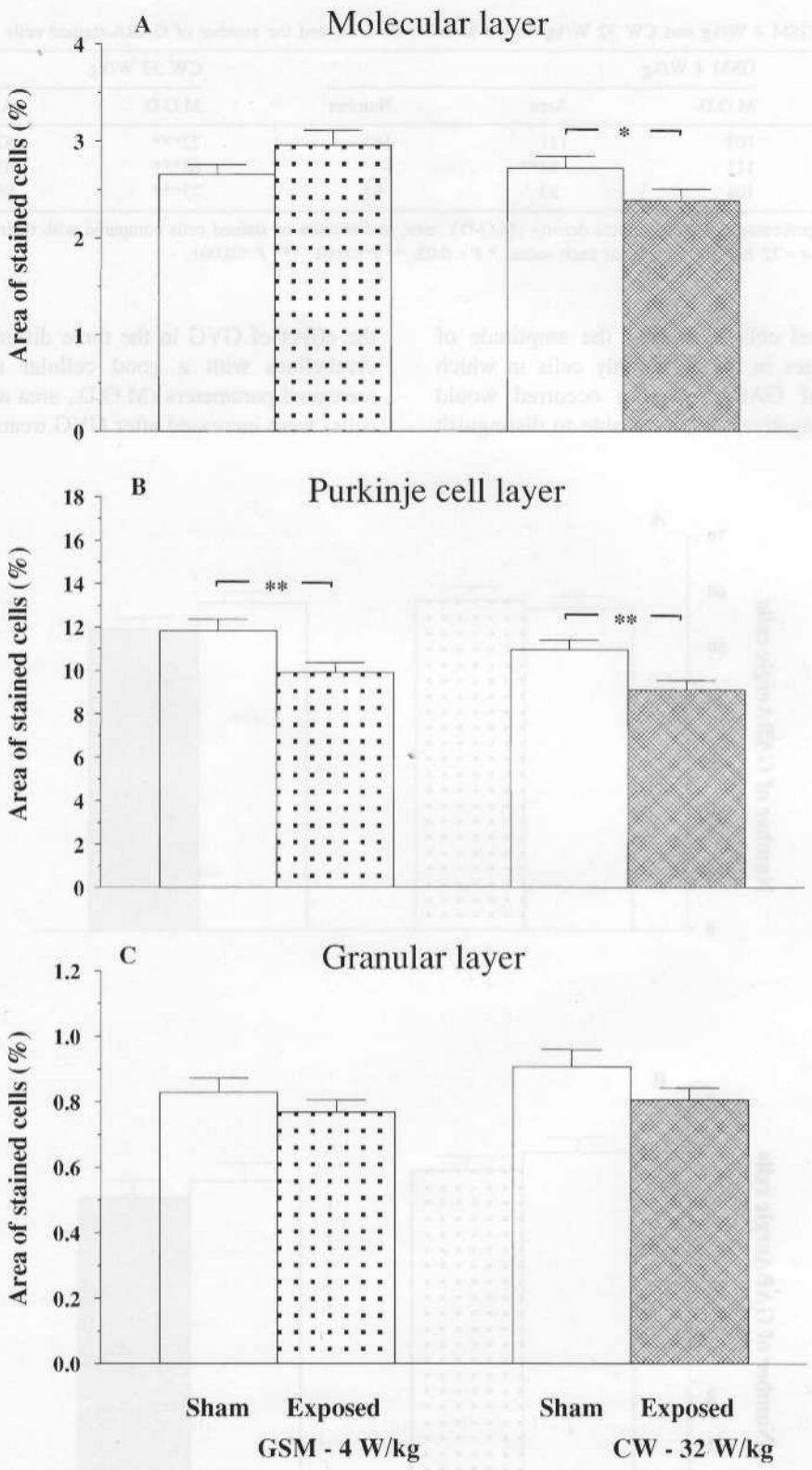

Fig. 5. Effect of radiofrequency exposure on the staining area of GABAergic cells in the rat cerebellum. Left side of the histogram: radiofrequency exposure to 4 W/kg GSM (pulsed emission); right side of the histogram: radiofrequency exposure to 32 W/kg CW (continuous emission). A: molecular layer; B: Purkinje cell layer; C: granular layer. Each column represents the mean±S.E.M. of 72 values (12 rats, six values per rat). * $P<0.05$, ** $P<0.01$.

Table 1
Effect of RF exposure to GSM 4 W/kg and CW 32 W/kg on the M.O.D., the area, and the number of GABA-stained cells in the rat cerebellum

	GSM 4 W/kg			CW 32 W/kg		
	M.O.D.	Area	Number	M.O.D.	Area	Number
Molecular layer	107	111	103	72***	87*	92
Purkinje cell layer	111	84**	–	68***	83**	–
Granular layer	104	93	95	73***	89	95

Each value represents the percentage of mean optical density (M.O.D.), area, and number of stained cells compared with their respective sham values in each cerebellar cell layer. $n=72$ fields (12 rats) for each value. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

the number of stained cells displayed the amplitude of GABA content changes in the cells (only cells in which dramatic variation of GABA quantity occurred would become positive or negative). We were able to distinguish the effect of GVG in the three different cell layers of the cerebellum with a good cellular resolution. All three measured parameters (M.O.D., area and number of stained cells) were increased after GVG treatment with the highest

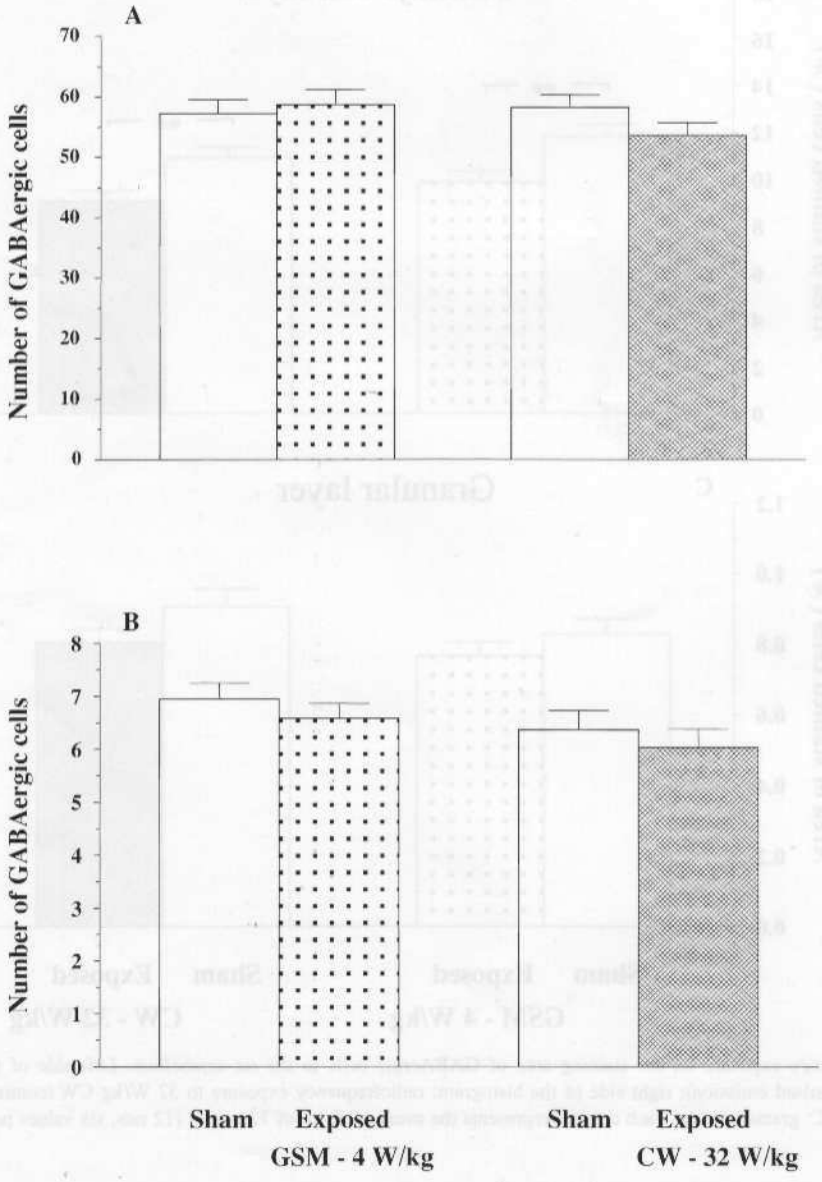

Fig. 6. Effect of radiofrequency exposure on the number of GABAergic cells in the rat cerebellum. Left side of the histogram: radiofrequency exposure to 4 W/kg GSM (pulsed emission); right side of the histogram: radiofrequency exposure to 32 W/kg CW (continuous emission). A: molecular layer; B: granular layer. Each column represents the mean±S.E.M. of 72 values (12 rats, six values per rat).

dose (1200 mg/kg), mainly in the molecular layer, whereas no obvious changes were observed in the Purkinje cell layer. This latter observation could be due to either the saturation of the parameters measured (M.O.D., staining area) in this particular cell layer or the fact that GVG acts essentially in the perikarya [65] and less so in the processes. Indeed, the staining pattern observed in the Purkinje cell layer corresponded to the basket cells processes and not to the cell bodies of Purkinje cells.

Therefore, the increase of cerebellar GABA content induced by GVG (1200 mg/kg) was highlighted at the levels of both intracellular GABA concentration and subcellular GABA distribution. Several studies have been published on the effects of GVG on the brain GABA content measured by HPLC [44,61] but these measurements concerned the global content of both intra and extracellular GABA in particular brain structures without any cellular resolution. The determination of the GABA concentration by HPLC showed that GVG (1200 mg/kg) induced an increase of 300% of the GABA content in the cerebellum [44]. With our technical approach, we were able to find increases in intracellular GABA content reaching 150% of the control values. We could explain the difference between our results and those of Löscher and Hörstermann [44] by (1) the difference of the cellular compartment measured: only the intracellular one in our investigation vs. both intra and extracellular in their work and (2) by the type of cells measured: only neuronal cells in our study vs. both neuronal and glial cells in theirs. It was shown that GVG was able to increase the GABA content in nerve terminals [43,66] but GABA-T is mainly located in nonsynaptosomal mitochondria, especially in glial cells [56,65,69]. Therefore, the effect of GVG measured on neuronal cells would constitute a small part of the global effect of GVG. We did not evaluate the GABA content in glial cells since they were too small to be taken into account. As it was shown that GABA-T presented a high activity in neuronal perikarya [65], the strong effect of GVG observed on the GABA content in neuronal perikarya could be induced by a direct action on the neuronal GABA-T.

In summary, IHC seems to be a powerful complementary method to locate, with a high spatial resolution, the effects of a treatment on NT contents inside particular cells. Biochemical techniques such as HPLC measure precisely the concentrations of extracellular and intracellular NT in homogenates from defined brain regions [51,53,54,67] but they cannot provide any information on variations in molecules concentrations among neurons with a cellular resolution. For example, HPLC could not allow the differentiation of the effects of GVG in the three cerebellar cell layers, whereas IHC and image analysis could achieve this goal. The usefulness of our approach is based on the appropriate definition of the parameters (optical density, area and number of stained cells) and the standardization of the technical conditions. Nevertheless, it is obvious that IHC is only a semi-quantitative method and that it presents some technical limits: (i) the detection of the positive cells depends on the threshold fixed by the experimenter, (ii) the measures between the different sections are variable (in order to avoid this variability, the parameters were measured on six sections per animal), (iii) the parameters reflect indirect GABA concentrations since they depend on the intensity of the staining, (iv) the duration of incubation with DAB during IHC process must be strictly controlled to avoid staining variations between sections.

Despite these technical limits, we showed that this method could be suitable to study and estimate the effects of extrinsic factors on NT concentrations in the CNS and to differentiate these effects in several cell layers with a good cellular resolution. We thus chose to use IHC and image analysis to investigate the effects of RF exposure on the cerebellar GABA content.

The results obtained with this technique demonstrated that RF exposure had an influence on the cellular GABA content in the cerebellum. This effect clearly depended on the type and power of RF emitted. The frequency of emission used in this study was 900 MHz, whatever the type and power of emission. The RF emitted with a pulsed mode (GSM type) and delivering a SAR of 4 W/kg induced a decrease in the staining area in the Purkinje cell layer. This indicated that RF exposure, even at the lowest dose tested (SAR 4 W/kg), could affect the extent of GABA content in the basket terminals, implying that RF exposure induced a redistribution of GABA in these processes. A similar decrease in the Purkinje cell layer was also observed, together with a slight decrease in the molecular layer, when the RF were emitted in a continuous mode and delivered a higher SAR (32 W/kg). This effect, observed even at the lowest RF power tested, contrasted with the absence of effect of GVG in this particular layer. Since the staining pattern observed in this layer was only due to the basket cell terminals, this result suggests that RF may act, at first, in the terminals, probably by increasing GABA release.

The decrease of staining pattern was even more evident at the M.O.D. level, since a strong diminution of this parameter occurred in the three cerebellar layers. This latter result indicated that exposure to very high doses of RF affected not only the distribution of GABA but also most likely the local concentration.

Whatever the considered cell layer, the number of positive cells remained constant between sham and exposed rats. Interestingly, cells remained detectable after RF exposure even if their GABA content had decreased. This provides a strong argument in favor of the adequacy of the detection parameters used since even a significant decrease in local GABA content maintained cells in the range of detection. The differential effect of GVG and RF exposure on the number of GABAergic cells could be explained by the nature of these two factors. Indeed, variations in this parameter reflected only very important changes in intracellular GABA concentrations. Therefore, it is not so

surprising that a very potent pharmacological drug like GVG was far more efficient in modifying this parameter than an environmental factor like RF exposure.

Altogether, these results indicated that exposure to 900 MHz RF inducing high SAR in the tissues (4 W/kg and above) interfered with cerebellar GABA content. Since variations of GABA content in the CNS have some physiological and behavioral consequences [12], the effect of RF exposure on the GABAergic system should be further investigated. It is worth noting that this effect was more important when the power of energy deposited in the tissue was higher. Whether the diminution of GABA content (induced by RF) was due to an increase in GABA release or degradation or a decrease in GABA synthesis is not known. However, the predominance of decrease in the basket terminals of the Purkinje cell layer suggests that the former is more likely.

Baxter et al. [3] showed that focused microwave irradiation to the head facilitated rapid diffusion of GABA from areas of high concentrations to adjacent areas of lower concentration. This means that microwave irradiation could increase GABA release in the cerebellum, which would explain the diminution of cellular GABA content observed after RF exposure.

A GABA uptake system could also participate in the decrease in the cerebellar GABA content observed in our experiment after 2 h of RF exposure. Indeed, it is known that the neuronal level of GABA depends on the functioning of a metabolic cycle between neurons and glia [57,63]. GABA released from the nerve terminals is further taken up into the nerve terminals themselves or into the glial cells by two separate high-affinity uptake systems [25]. RF exposure could thus influence these GABAergic uptake systems to induce a decrease in neuronal GABA uptake. Further experiments are needed to investigate the effects of RF exposure on these uptake systems.

Several studies have shown that other RF could provoke some changes in different NT systems. Indeed, Lai et al. [39] showed that a 45-min exposure to 2450 MHz pulsed RF induced a decrease in the choline uptake in the CNS whereas a 20-min exposure to the same RF induced rather an increase in the choline uptake [38]. It was also shown that microwave exposure to 2450 MHz inactivated enzyme systems. Indeed, microwave sacrifice is commonly used in order to inactivate enzymes responsible for post-mortem changes in NT concentrations [2,24,48,70]. Microwave irradiation induced an inactivation of both GAD and GABA-T enzymes, leading to a constant post-mortem GABA concentration [32]. Even if these irradiations were very intense and brief, it seems possible that a longer exposure to lower intensity irradiation could also affect different enzymes leading to a decrease in neuronal GABA content.

In this study, we investigated for the first time the effects of 900 MHz RF, i.e. the emission frequency of mobile phones, on the NT themselves. Even though the maximal SAR induced by the mobile phones emitting in GSM mode is 0.8 W/kg, we decided to expose the animals to higher SAR (a minimum of 5-fold the SAR induced by mobile phones) in order to test the adequacy of our experimental tools to measure a significant variation of GABA parameters. Our results indicated that this dose of RF emission interfered with the GABAergic system. However, the mechanism of RF action is not known: the effects could be driven either directly by a specific effect of the electromagnetic field or indirectly via local heating of the tissues. The literature reports different issues to this question: Mason et al. [47] showed that 5.02 GHz microwave radiation provoked a thermal stress inducing an increase in several amino acid concentrations in hypothalamic and caudate nuclei, whereas another study reported the non-thermal effect of 960 MHz RF on cell proliferation [73]. In our conditions (SAR 4 W/kg), the elevation of the brain temperature measured in a rat phantom was approximately 0.5°C after a 2-h exposure (see Section 2). As this measure did not take into account the thermal diffusion in the tissue, the blood circulation and the thermoregulation of the animal, a local heating may not be responsible for the observed effects. For the higher SAR (32 W/kg), a thermal effect could not be excluded but it is quite difficult to estimate since no measure of temperature has been performed precisely with this SAR. However, even if this SAR would induce an increase in the brain temperature, the thermoregulation should restore the temperature in a physiological range.

It is worth noting that RF emitted by mobile telephones induced very smooth variations of brain temperature. Indeed, it has recently been shown that 900 MHz RF with a SAR of 1.6 W/kg provoked a temperature rise in the brain of approximately 0.1°C [72,76]. Therefore, the possibility of a thermal effect after GSM exposure could be ruled out in those conditions.

As a conclusion, this study is a pioneer in the field of investigation of the effects of RF on cellular NT concentration in the CNS. Because of the widespread use of devices emitting RF, and especially mobile phones, this field of research is now open to test some eventual biological effects on the CNS. The possibility of therapeutic effects of RF on the brain could not be excluded, since they are used elsewhere as therapeutic tool in several medical approaches (for review, see Refs. [11,20,45]). Further investigations with complementary techniques will be necessary to understand the mechanism of RF interactions with NT and their receptors as well as their putative physiological implications.

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