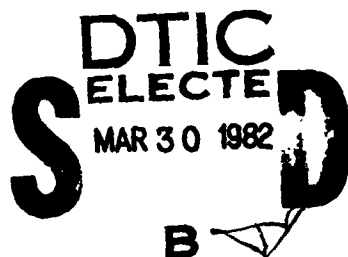


ADA 112737



Bioelectromagnetics 2:247-257 (1981)

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Effect of Nonionizing Radiation on the Purkinje Cells of the Rat Cerebellum

E. N. Albert, M. F. Sherif, N. J. Papadopoulos, F. J. Slaby, and J. Monahan

Department of Anatomy, The George Washington University Medical Center, Washington, DC, and Bureau of Radiological Health, Rockville, Maryland

In one experiment, Sprague Dawley rats (16-21 days of gestation) and their offspring were exposed to 100-MHz (CW) electromagnetic radiation at 46 mW/cm² (SAR 2.77 mW/g) for 4 h/day for 97 days. In another experiment, the pregnant rats were irradiated daily from 17 to 21 days of gestation with 2450-MHz (CW) microwaves at 10 mW/cm² (SAR 2 mW/g) for 21 h/day. In a third experiment, 6-day-old rat pups were irradiated 7 h/day for five days with 2450-MHz radiation at 10 mW/cm². Equal numbers of animals were sham irradiated in each group. Quantitative studies of Purkinje cells showed a significant and irreversible decrease in rats irradiated during fetal or fetal and early postnatal life. In animals exposed postnatally, and euthanized immediately after irradiation, significant decrease in the relative number of Purkinje cells was apparent. However, restoration apparently occurred after forty days of recovery.

Key words: nervous system, rat cerebellum, nonionizing radiation, Purkinje cells, microwaves, radiofrequency

INTRODUCTION

Effects of radiofrequency electromagnetic (RFEM) radiation on fetal development have recently been reported. These include occurrence of hemorrhage, resorption, exencephaly, and fetal death in conceptuses of CF-1 mice exposed during gestation to 2450-MHz microwaves at 123 mW/cm² [Rugh et al, 1974; Rugh et al, 1975; Rugh and McManaway, 1976]. Shore et al [1977] reported no effect on the litter size of the rats, but noted decreases in body and brain mass in animals exposed to 2450-MHz microwaves at 10 mW/cm² for five days during gestation. Berman et al [1978] exposed CD-1 mice to 2450-MHz radiation for 100 min daily at power densities that ranged from 3.4 to 28 mW/cm². They observed a decrease in mean of body mass and an increased incidence of exencephaly in fetuses expos-

Received for review November 6, 1980; revision received July 13, 1981.

Address reprint requests to Dr. E. N. Albert, Professor of Anatomy, Department of Anatomy, The George Washington University Medical Center, 2300 I St., NW Washington, DC 20037.

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ed at the highest power density. Thus, the current embryonic and fetal effect studies have examined gross teratology: body mass, litter size, and uterine resorptions, but none have involved investigation of subclinical or subtle changes in the central nervous system that might be due to exposure to RFEM fields at low intensities.

To identify possible effects of low-level RFEM energy on developing rat brains, we chose to examine the relative number of cerebellar Purkinje cells. The rationale for choosing the cerebellum and its Purkinje cells include the following considerations:

1. Development of the mammalian brain is dependent upon migration of nerve cells and involves patterns that are well understood for the cerebellum [Altman and Anderson, 1972; Das, 1977; Yu 1977].
2. Purkinje cells can be readily identified for qualitative and quantitative analyses.
3. Purkinje cells provide a relay station for all afferent input to the cerebellum as well as the major output from the cerebellum either directly or indirectly through the other cerebellar neurons [Jansen and Brodal, 1954; Palay and Chan-Palay, 1974; Jacobson, 1978]. Thus, a study of the cerebellum and its Purkinje cells provides a good experimental model for effects of RFEM radiation on the rat brain.

MATERIALS AND METHODS

Irradiation

Thirty-two Sprague Dawley rats formed the basis of the study. They were divided into the groups shown in Table 1. In each group, one half of the animals were irradiated, and the other half were sham irradiated.

Experiment No. 1

Pregnant Sprague Dawley rats with accurately timed pregnancies were transported from George Washington University (GWU) to Bureau of Radiological Health (BRH) for exposure. Animals were exposed in an anechoic chamber to 2450-MHz (CW) radiation at an average of 10 mW/cm² for 21 h/day for five days. The exposure commenced on the 17th day of gestation and ended at the conclusion of the 21st day. Two separate anechoic chambers were utilized in this study to provide either an exposure or a sham exposure. During exposure, animals

TABLE 1. Study Groups

Experiment No. 1	Immediate sacrifice	6 animals
	Recovery	6 animals
Experiment No. 2	Immediate sacrifice	6 animals
	Recovery	6 animals
Experiment No. 3	Immediate sacrifice	None
	Recovery	8 animals

were housed in Plexiglas containers with internal dimensions of $19 \times 11.4 \times 7.6$ cm. The tops and bottoms of these containers consisted of removable polystyrene grids to permit ventilation. Each container was positioned reproducibly on a Plexiglas rack 208 cm below a truncated horn antenna. Sham-exposed animals were similarly housed in a separate anechoic chamber.

In an effort to define the exposure of individual subjects, a series of three field-intensity measurements were made within each of the Plexiglas containers. The measurements were made with and without nonexperimental subjects in the remaining containers. All measurements were made with a nonperturbing, three-dimensional probe (Collins S/N57) connected to a transmitter (Collins S/N17) and an EIT digital receiver (EIT, Sterling, VA, S/N 1004).

It was determined that exposures varied both as a function of position within the container and also with time due to field perturbations produced by the movements of other animals. Consequently, although the average field intensity was 10 mW/cm^2 , an animal might have received as little as 4 mW/cm^2 or as much as 30 mW/cm^2 at any instant. Utilizing the Radiofrequency Radiation Dosimetry Handbook [Durney et al, 1977], specific absorption rates (SAR) for these pregnant rats were computed to range from 0.5 to 6 W/kg. The average SAR was approximately 2 W/kg for each of the animals.

During the 24-h cycle, the pregnant animals were removed twice daily from the Plexiglas cages of both anechoic chambers (irradiation and sham irradiation) to normal rat cages outside the chambers. This occurred once in the morning for 1.5 h, and once in the evening for 1.5 h. During these periods, the animals were given food and water ad libitum and the Plexiglas cages in the chambers were cleaned. After each 1.5-h period, the animals were replaced in the clean Plexiglas cages and again irradiated or sham irradiated. After the fifth day of irradiation or sham irradiation (day 21 of gestation), the animals were brought back to GWU where the dams delivered their pups within twelve hours. One half of the litters were used shortly after delivery (within 8 h). The remaining half of the litters were used 40 days after cessation of irradiation. Three control and three experimental animals were used in each case.

Experiment No. 2

In this experiment, 6-day-old pups along with the dams were transported from GWU to BRH. Matching sets of litter mates without their mothers were placed in well-ventilated Plexiglas cages in anechoic chambers for 3.5 h (9:00 AM–12:30 PM). One chamber was energized so that the pups were irradiated with 2450-MHz (CW) microwaves at 10 mW/cm^2 , while the other chamber was used for sham irradiation. After 3.5 h of irradiation, the pups were reunited with their mothers outside the anechoic chambers for 1.5 h for feeding. At 2 PM, control and experimental pups were again placed in their respective chambers and irradiated for an additional 3.5 h (2:00 PM–5:30 PM). The pups were then returned to the cages with their mothers until the next morning. Thus, the young rats were irradiated 7 h/day for five days. This procedure was repeated daily for five days. On the sixth day, the rats with their mothers were brought back to GWU. Six pups (three irradiated and three sham irradiated) were euthanized immediately on their arrival to GWU and another six pups were euthanized 40 days after cessation of irradiation.

Experiment No. 3

Pregnant Sprague Dawley rats were exposed to 100-MHz-CW RFEM fields at 46 mW/cm² (SAR 2.77 mW/g) at the Environmental Protection Agency, Research Triangle Park, NC (EPA) during 8:00 AM–2:00 PM. Details of the exposure facility and dosimetry are published elsewhere [Smialowicz et al, 1979]. The pregnant dams, starting on the sixth day of gestation through the end of pregnancy, and then their offspring were exposed for 4 h daily for 97 days. Experimental and sham-irradiated offspring were delivered to GWU fourteen months after cessation of the irradiation and were euthanized immediately. Four sham-exposed and four irradiated animals were used in this experiment.

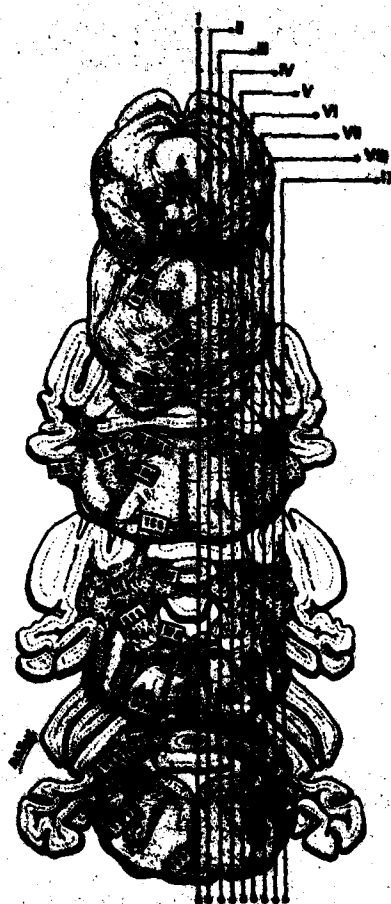
Histological Analysis

In all three experiments, the brain of each experimental animal was processed with its corresponding control. Animals were anesthetized by pentobarbitol sodium (50 mg/kg) and fixed by cardiac perfusion with 10% buffered formalin. The cerebella were removed, divided in the midsagittal plane, and embedded in paraffin. The blocks were serially sectioned at 10 μ and stained with hematoxylin-eosin, Luxol fast blue, and DeMyer's silver stain for axons [DeMyer, 1958]. Six to nine parasagittal planes were selected and used as constant levels for reference during matching of the cerebellar sections from experimental and control animals.

Four to six serial sections were studied at each plane. The determination of the parasagittal planes was based on the neuroanatomical cytoarchitecture of both the brainstem and the cerebellar nuclei and peduncles (Fig. 1). Sections of lateral parasagittal planes, beyond the middle of the nucleus dentatus and the lateral limit of both the nucleus cochlearis ventralis and the tractus spinalis trigeminalis were avoided. Beyond these lateral parasagittal planes, the cerebellum does not yield complete sections.

Each section was projected onto a sheet of paper. The outlines of the whole cerebellar parasagittal section, as well as those of the demarcation between the cerebellar cortex and the white core and the interface between the molecular and granular layers, were outlined. The areas bordered by these outlines were measured by a polar planimeter and expressed in mm². The mean value of the areas of corresponding sections from experimental and control animals, and the number of Purkinje cells at each plane were calculated. Parasagittal sections were preferred because these planes were perpendicular to the surface of the folia and presented the largest number of Purkinje cells in a single layer [Palkovits et al, 1971].

We chose to count Purkinje cells in complete cerebellar sections to compensate for the reported differences in their population densities between the anterior and posterior lobes [Armstrong and Schild, 1970], as well as the local variations in each folium due to the arbor vitae appearance of folding of the cerebellar cortex [Braitenberg and Atwood, 1958; Fox and Barnard, 1957; Armstrong and Schild, 1970; Palkovits et al, 1971]. Only Purkinje cells present displaying a visible nucleolus were counted (Figs. 2 and 3). Purkinje cells in the molecular and granular layers were not counted. All cell counts were performed double blind by two individuals. Density of Purkinje cells expressed as Purkinje cells per mm² of the cerebellar cortical area in each section, and then the numbers were averaged for all sections and planes per animal and groups of animals. The mean relative num-



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Fig. 1. Graphic representation of the nine parasagittal planes in a series of transverse sections of mid-brain, pons, and upper medulla of the rat brainstem. The neuronal structures through which parasagittal planes pass are shown.



List of Abbreviations for Figures 1-3. BP = Basis pedunculi; DBC = Decussation of branchium conjunctivum; GL = Granular layer; ML = Molecular layer; NCV = Ventral cochlear nucleus; ND = Dentate nucleus; NE = Nucleus emboliformis; NF = Fastigial nucleus; NG = Globose nucleus; NMV = Motor nucleus of the trigeminal nerve; N MES V = Mesencephalic nucleus of the trigeminal nerve; NOS = Superior olivary nucleus; NP = Pontine nuclei; NR = Red nucleus; N S V = Sensory nucleus of trigeminal nerve; NVS = Superior vestibular nucleus; P = Inter peduncular nucleus; SN = Substantia nigra; TCS = Corticospinal tract; T SP V = Spinal tract of the trigeminal nerve; N III = Nucleus of oculomotor nerve; IV N = Nucleus of trochlear nerve; VI N = Nucleus of abducent nerve; VII N = Nucleus of facial nerve; VII G = Genu of the facial nerve.



Fig. 2. Representative microphotograph of a parasagittal section of rat cerebellum. The arrows point to the interface (Purkinje cell line) between the molecular and granular layers ($\times 40$). (Legend same as in Fig. 1.)

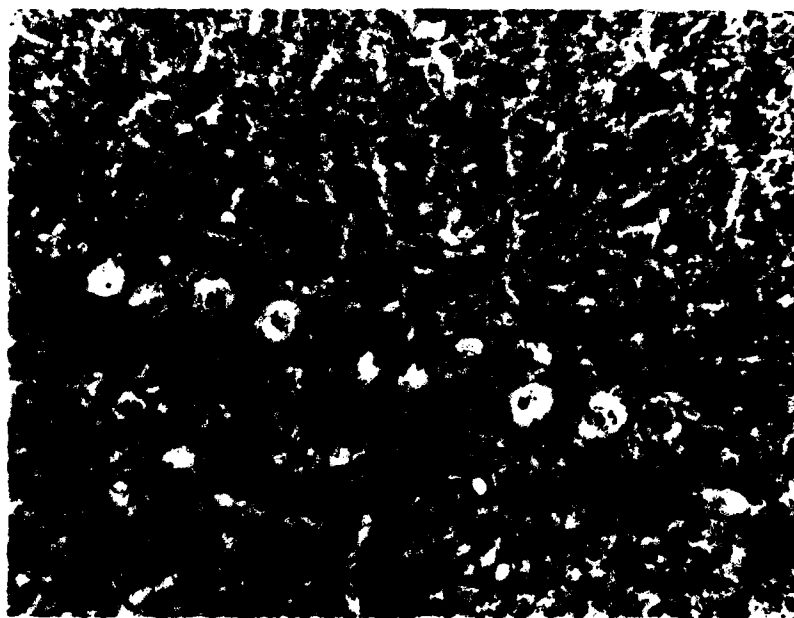


Fig. 3. Representative microphotograph of the rat cerebellar cortex showing the Purkinje cells arranged in a row at the interface between molecular and granular layers. The arrow points to Purkinje cells without visible nucleoli. Such cells were not counted. (Legend same as in Fig. 1.)

TABLE 2. Effect of 2.45-GHz Radiation on the Relative Number of Purkinje Cells of Rats Irradiated in Utero And Sacrificed 40 Days After Delivery*

	Control	Experimental	Percentage change in relative number of Purkinje cells
Mean number of Purkinje cells per mm ² of cortical area	48.47 SEM $\pm$ 2.26	35.87 SEM $\pm$ 1.71	25.83% P < 0.01
Ratio of cortical to total area	0.77	0.79	
Ratio of molecular to cortical area	0.59	0.59	

*All values of the table are the mean values of all areas in parasagittal sections at different planes studied in each group.

bers of Purkinje cells were compared accordingly for each group of control and experimental animals.

Cerebella of 32 albino rats were processed according to the described methods yielding a total of approximately 1,434 sections for study (matching, projection, planimetry). This study represents a total count of approximately 420,000 Purkinje cells.

RESULTS

The effect of irradiation on the relative number of rat cerebellar Purkinje cells are summarized in Tables 2, 3, and 4. All values referred in the tables are the mean values of the section and planes obtained from the cerebella of each group of animals.

Experiment No. 1 (Table 2)

a. Rats irradiated in utero from day 17 through 21 and euthanized on day 1 of postnatal life. The cerebella of these animals were not mature enough to clearly display the Purkinje cell layer. As a result many of the Purkinje cells could not be positively identified by light microscopy. Therefore, we chose not to include data from this group (six animals) on the Purkinje cells numbers.

b. Rats irradiated in utero (day 17 through 21) and euthanized 40 days after delivery. In this and the remaining group of animals, the cerebellar maturation had reached a point where a clear layer of Purkinje cells could be positively identified at the molecular-granular interface. The statistical analysis of Purkinje cell counts showed that there was significantly fewer (25.83%) cells in the experimental animals than in the control animals.

Experiment No. 2 (Table 3)

a. Rats irradiated postnatally (6-10 days) and euthanized immediately after irradiation. In this group, experimental animals showed a significant decrease in the relative number of Purkinje cells (24.37%) in contrast to control animals.

TABLE 3. Effect of 2.45-GHz Radiation on the Relative Number of Purkinje Cells of Rats Irradiated Between Six and Ten Days After Delivery*

	Control	Experimental	Percentage of change in relative number of Purkinje cells
Animals euthanized immediately after irradiation			
Mean number of Purkinje cells per mm ² of cortical area	115.68 SEM $\pm$ 5.30	87.46 SEM $\pm$ 4.62	24.37% P < 0.01
Ratio of cortical to total area	0.76	0.76	
Ratio of molecular to cortical area	0.49	0.50	
Animals euthanized 40 days after irradiation			
Mean number of Purkinje cells per mm ² of cortical area	33.98 SEM $\pm$ 1.05	31.61 SEM $\pm$ 1.22	7.01% P < 0.1
Ratio of cortical to total area	0.77	0.78	
Ratio of molecular to cortical area	0.60	0.61	

*All values are the mean values of all areas in parasagittal sections at different planes studied in each group.

TABLE 4. Effect of 100-MHz Radiation on the Relative Number of Purkinje Cells of Rats Irradiated in Utero and for 90 Days After Delivery and Sacrificed 14 Months After Irradiation*

	Control	Experimental	Percentage of change in relative number of Purkinje cells
Mean number of Purkinje cells per mm ² of cortical area	28.33 SEM $\pm$ 1.21	24.74 SEM $\pm$ 0.71	12.67% P < 0.05
Ratio of cortical to total area	0.68	0.69	
Ratio of molecular to cortical area	0.62	0.62	

*All values are the mean values of all areas in parasagittal sections at different planes studied in each group.

b. Rats irradiated postnatally (6–10 days) and euthanized 40 days after cessation of irradiation. There was a nonsignificant decrease of 7.01% in the relative number of Purkinje cells in the irradiated animals.

Experiment No. 3 (Table 4)

a. Rats irradiated in utero and for 97 days postnatally and euthanized 14 months after irradiation. The relative number of Purkinje cells was significantly fewer (12.67%) in experimental animals than in control animals.

It is noteworthy that in all experiments, the ratios of cortical to total section, and molecular to granular areas remained unchanged between control and experimental animals. This is interpreted to mean that the radiation had no effect on the proportional thickness of the various layers of the cerebellar cortex.

DISCUSSION

Fetuses and newborn rats exposed to two frequencies of RFEM radiation (100 MHz and 2450 MHz) and at two power densities (46 mW/cm² and 10 mW/cm²) with similar calculated SAR values (2.77 mW/g and 2 mW/g) produced similar effects.

The principal changes involved a significant decrease in the mean number of cerebellar Purkinje cells. This decrease was observed when the rats were irradiated in utero (days 17–21 of gestation), and also when irradiation occurred during gestation (days 6–21) as well as postnatally. Under these two conditions, the decrease in Purkinje cells appears to be permanent, since it was observed in animals euthanized 40 days and 14 months after cessation of irradiation.

These findings raise questions about the effects of irradiation, such as: Does irradiation interfere with genesis and/or maturation of Purkinje cells from the neuroepithelium? Was the migratory pattern of these cells altered by irradiation so that they did not reach their final destination? Were the postmitotic Purkinje cells destroyed during early stages of formation?

Irradiation could affect the proliferative activity of the neuroepithelium, because the cerebellar rudiment and Purkinje cells arise during the second half of

gestation [Addison, 1911; Hamilton and Mossman, 1976]. This developmental stage coincides with the in utero irradiation period of our experiments. The radiation could also affect the migratory pattern of Purkinje cells and other microneurons. This could then prevent Purkinje cells from reaching the interface between molecular and granular areas and forming the normal synaptic contacts with the parallel fibers. During this period, unlike the Purkinje cells, the external granular layer (which gives rise to cerebellar microneurons) begins to exercise its proliferative and migratory activities including development of parallel fibers and their synaptic connections with the Purkinje dendrites. Normal proliferation, migration, and development of these microneurons is considered essential for normal maturation of Purkinje cells [Altman, 1972]. RFEM radiation appears to affect the proliferating external granule cells that give rise to the cerebellar microneurons. This will be the subject of a separate manuscript.

When rat pups were exposed postnatally (days 6-10) and euthanized immediately, we again observed a significant decrease in the mean relative number of Purkinje cells in experimental animals. Conversely, when animals from the same group were euthanized 40 days after irradiation, there was no significant difference in the relative number of Purkinje cells between control and experimental animals. This indicates that the decrease in Purkinje cells was reversible. The temporary decrease in the immediately euthanized animals can be explained by suggesting that the radiation affected the migration pattern of Purkinje cells, but not the proliferative activity of the external granular layer.

Observations of gross behavior of the animals, prior to euthanizing, did not reveal any abnormal functional or behavioral characteristics. Under light microscopy no degenerative changes were observed in Purkinje cells. These observations are consistent with our previous findings [Albert and DeSantis, 1975] in Chinese hamsters exposed to 2450-MHz (CW) microwaves at 25 and 20 mW/cm². Thus, it appears that even in the presence of 12-25% decrease in the relative number of Purkinje cells, no obvious functional clinical symptoms could be observed. This is in accord with the fact that an 18%-25% loss in the number of Purkinje cells of man and rat due to aging does not result in manifestations of neurological signs or symptoms [Hodge, 1894; Dolley, 1911; Ellis, 1920; Tetsuo, 1928]. On the contrary, in clinical conditions, such as olivopontocerebellar atrophy, parenchymatous cerebellar degeneration or ataxia telangiectasia, there is considerable decrease in the number of Purkinje cells, along with other accompanying neuropathological lesions.

Further studies on the mechanism of interactions between RFEM radiation and Purkinje cells and other cerebellar neurons are in progress.

ACKNOWLEDGMENTS

Supported by EPA contract #68-02-3257 and the Department of Navy #N00014-78-C-0527.

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