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Radiofrequency (4g) Exposure Effects on Dentate Gyrus structure and Function in Wistar rats: Pre- vs. Postnatal Impact

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ABSTRACT

The study investigates 4G radiofrequency-modulated electromagnetic fields (RF-EMF) impact on prenatal/postnatal neurogenesis in Wistar rats' dentate gyrus, crucial for memory and learning, amid concerns over gadget-related RF exposure. Thirty-five pregnant female Wistar rats were randomly assigned into seven groups (n=5/group): A-G. Group A was the control, while the rest were exposed to varying durations of electromagnetic fields. At birth, four pups/group were euthanized; the rest were exposed until postnatal day 35. Neurobehavioral tests (Y-maze, Open field), histological (Haematoxylin & Eosin), Immunohistochemical (Ionized calcium-binding adaptor molecule 1, caspase-3 assays), neurotransmitters (glutamate, serotonin, dopamine, Gamma Amino Butyric Acid), and Enzymes (Cytochrome-c-oxidase assay) were evaluated in dentate gyrus pre and postnatally. Radiofrequency radiation had mixed effects on dentate gyrus functions, impacting behavior both positively and negatively. Neurotransmitter and enzyme assays indicated mild negative effects, while histological and immunohistochemical analyses revealed aberrations in both pre and postnatal exposure groups. Altogether, 4G RF induced mild alterations in the overall structure of the dentate gyrus which could interfere with neurogenesis and neural plasticity.

***Keywords*:** Radiofrequency, neurogenesis, dentate gyrus, radiation.

Introduction

Neurogenesis is the process by which neural progenitor cells differentiate into mature neurons;[1] it begins embryonically, continues postnatally, and even throughout adulthood specifically in the dentate gyrus and the subventricular zone of the lateral ventricle.[2] Hippocampal neurogenesis plays a very important role in learning and memory.[3] It has been proposed that hippocampal-mediated learning could be a result of neuronal regeneration in the dentate gyrus.[4] The dentate gyrus is known to be the major structure for information transfer to the hippocampus.[5] It has been well established in previous literature that exposure to any environmental stressor at a critical brain developmental window can result in neurobiological and behavioural changes.[6]

Radiofrequency (RF) denotes the conveyance of energy via radio waves, encompassing any frequency within the electromagnetic radiation spectrum's oscillation rate. RF, encompassing radio waves and microwaves, constitutes a nonionizing form of radiation, lacking the energy necessary to disrupt chemical bonds or displace electrons.[7] The sources of RF could be natural (ultraviolet rays, earth, and ionosphere) or artificial (telecommunication devices, ovens, microwaves, and the rest). Although nonionizing radiation has a lower frequency and is generally considered safe, some types have enough energy to produce deleterious effects in living tissues.[8]

Recent studies show that EMF exposure at different frequencies can have adverse effects on health.[9] The brain is considered more exposed to RF because, during most conversations with mobile phones, it is usually placed close to the head.[10] Aslan *et al* .,[11] reported that exposure to Electromagnetic frequency (EMF) can lead to a variety of neurological conditions such as a decreased number of pyramidal and Purkinje cells in the Cornus ammonis. Low frequency of RF-EMF can affect spatial memory and place learning tasks in rodents when tested using water and radial arm maze.[12] Prenatal and early postnatal are the most sensitive and high-risk periods to be exposed to EMF.[13] The proper formation and differentiation of stem cells (neural) at the embryological development stage can be disrupted by exposure to EMF.[14] It can also influence neural function, regulation of neurotransmitter systems, neural survival, synaptic plasticity, and memory and learning.[15] In a report by Mausset et al,[16] EMFs prenatal exposure could distort granule cell neurogenesis at the dentate gyrus (DG) which could then lead to disturbance in postnatal behavior and cognitive functions in line with the hippocampus. Hence this study investigated the biochemical, histomorphological, immunohistochemical, and behavioural changes caused by 4G RF on the pre and postnatal developmental neurogenesis of the DG of Wistar rats.

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Materials and Methods

Experimental Animals:
Rats weighing 80 g - 100 g were utilized in this study, comprising 7 male and 35 female animals. The female rats were separated into seven groups, each group containing 5 individuals. A single male rat was placed in each cage for mating purposes, and pregnancy was validated by a vaginal plug, that was tagged on day 0 of gestation.

Experimental Design/Procedure:
The female Wistar rats were divided into seven groups (n=5) " table 1". Mating with male rats induced pregnancy, confirmed by the presence of a vaginal plug marking day 0 of gestation. Most pregnancies lasted 21 days, a typical period for Wistar rats. Customized cages with controlled exposure to radio-frequency radiation were provided, with each group assigned one mobile phone and Wi-Fi modem. The distance between the cage base and the devices was measured for standardization. At birth, four offspring per group were randomly chosen and sacrificed to assess the immediate effects of gestational radiation exposure. The remaining offspring were observed until Post-natal day 35 to evaluate longer-term impacts on development and health.
The experimental procedures were performed with approval from the Ethical Committee for Health Research at Babcock University (BUHREC) with the registered number 762/19.

Table 1: The Animal Grouping and Treatment

GROUPS	NO OF ANIMALS (ADULT FEMALES)	NO OF ANIMALS (ADULT MALES)	NO OF ANIMALS (PUPS)	DURATION
A	5	1	4	No radiation
B	5	1	4	12 hours every day
C	5	1	4	12 hours/3 days in a week (Monday, Wednesday, and Sunday)
D	5	1	4	6 hours per day
E	5	1	4	6 hours/3 days in a week (Monday, Wednesday, and Sunday)
F	5	1	4	24 hours everyday
G	5	1	4	24 hours/ 3 days in a week

The groupings are consistent with the initial groupings as stated in the methodology. However, neonates in group F were lost

Neurobehavioral evaluation
The neurobehavioral evaluation was carried out on PND 30 using the open field test and Y-maze. The open field test is used to test for explorative behaviour in rodents, while the Y-maze spontaneous alternation parameter is used to check for short-term and spatial memory in rodents.[17]
The Open Field Test (OFT) serves as a widely used assessment tool for evaluating exploratory behaviour and overall activity levels in rodents, enabling measurement of both qualitative and quantitative aspects. Essentially, the OFT consists of a spacious enclosed cuboid container with barriers preventing escape, measuring 72cm x 72cm x 36cm (Length x Width x Height respectively). One side of the wall is made up of plexiglass for rat visibility, red lines were used to divide the floor into sixteen 18 x 18cm ^{2} and a central square (18 x 18cm) was drawn in the middle of the open field using blue marker. The fundamental and prevalent outcome of interest, movement, can be affected by various factors such as motor function, exploratory tendencies, freezing responses indicative of fear, illness, and the specific time within the circadian rhythm, among numerous other variables. Each animal was placed in the centre square and allowed to explore for five minutes, in this time the animals were recorded using a monitoring camera; outcomes such as defecation, grooming, rearing, center square entry, and number of lines crossed were evaluated[18]. It is important to note that after each animal's time, the apparatus is cleaned using cotton wool and ethanol to avoid olfactory and visual cues.[18]
The Y-maze test is sensitive to hippocampal damage and gene manipulations (Creative Biolabs). Assessment can be conducted by permitting mice to explore all three arms of the maze, each situated at a 120° angle to the others, driven by rodents' innate curiosity to investigate new territories.[17] The maze was sanitized with 70% ethanol to remove smell and dirt and a video camera was placed above it for recording. After the rat was placed at the center of the maze, it was allowed to explore the three arms freely for three minutes. To compute the percentage of alteration, the count of arm entries and triads is documented. An entry is registered when all four limbs are within the arm. This study was done for all the animals in each group and it was done twice for each of them. After each animal exploration, the maze was cleaned with 70% ethanol to remove all dirt and smell.[19]

Sacrifice and histological analysis
On Postnatal Day 35, the animals were euthanized via cervical dislocation. Subsequently, the skull was meticulously opened to facilitate the retrieval of brain tissues. The hippocampal formation was identified and extracted, then preserved in 10% formal saline to initiate histological procedures for Haematoxylin & Eosin, Cresyl violet, Luxol fast Blue, and Immunohistochemical assessment.

Biochemical assay
Brain tissues intended for biochemical assays underwent homogenization in ice-cold 0.1M phosphate buffer at a ratio of four volumes to facilitate further assessment of neurotransmitter levels and enzyme activities.

Glutamate
Glutamate level was conducted following the method described by Adelodun *et al*.,[20] The dentate gyrus was homogenized by adding 2 ml of sucrose and a pestle for grinding, after which the homogenate was poured into clean sample bottles and was kept in a centrifuge at 4000G for 15 minutes. Supernatants were moved to fresh sample bottles for quantifying glutamate levels using ELISA kits. A microplate reader at 450nm was used, with samples adjusted to 50µL with assay buffer.

Glutamate levels were determined using the manufacturer's standard curve (ab83389, Abcam).

Dopamine

The assay utilized the competitive inhibition enzyme immunoassay technique, as documented by Atack*et al.*,[21] A microplate was coated with a monoclonal antibody tailored to dopamine. A competitive inhibition reaction ensued between biotin-labeled dopamine and unlabeled dopamine (both standards and samples) with the dopamine-specific antibody pre-coated on the microplate. Following incubation, any unbound conjugate was washed away. Avidin linked to Horseradish Peroxidase (HRP) was introduced into each well and allowed to incubate. Subsequently, upon addition of the substrate solution, the intensity of color developed was inversely correlated to the concentration of dopamine in the sample. Following the procedural steps, 50µL of stop solution was introduced, and absorbance was promptly read at 450 nm.

Serotonin

Serotonin levels were determined using the method described by Owolabi *et al.*,[22] In this procedure, 100 µL of acylated standards, controls, and samples were dispensed into appropriate wells of 5Ht/5-HIAA Microtiter Strips, followed by adding 25 µL of 5Ht antiserum to all wells. Plates were sealed with adhesive foil and incubated for 15 to 20 hours at 2 to 8 °C. The standard serotonin ELISA protocol was adhered to consistently. Absorbance was measured within 10 minutes using a microplate reader set at 450 nm, with a reference wavelength ranging from 620 nm to 650 nm.

Gamma Amino Butyric Acid (GABA)

Brain homogenates were prepared and centrifuged at 3000G for 20 minutes; the supernatant was collected and assayed by bringing the samples to a 50µL standard solution and adding the GABA antiserum to each well. Subsequently, the solution was thoroughly mixed, and 100 µL of stock solution was added. Absorbance was then at a wavelength of 450 nm was measure, with reference wavelengths set at 620 nm and 650 nm.

Cytochrome-C-oxidase

Following the homogenization of the brain tissues (dentate gyrus), enzymes (cytochrome c-oxidase were analyzed. ELISA analysis was conducted at 450 nm utilizing a microplate reader, according to the manufacturer's instructions provided.

Statistical analysis

Statistical analysis was done using Graph Pad Prism 8.4.2 software, where raw data was transformed into grouped data and analyzed via one-way analysis of variance (ANOVA). The outcomes were presented as mean ± error of the mean (SEM) in tabular format. Subsequently, the Student Newman-Keuls post hoc test was employed to compare means, identifying any significant differences, with a significance level set at $P<0.05$.

Results and Discussion

The dentate gyrus is recognized for its crucial involvement in learning and memory formation. Consequently, this research aimed to explore 4G radiofrequency radiation (RFR) effects on prenatal and postnatal neurogenesis within the dentate gyrus of Wistar rat models, employing biochemical, histological, and immunohistochemical methodologies.

Cytochrome-c-oxidase (CcO) activity in the brain is a specific indicator of oxidative metabolism.[23] Being the final enzyme in the electron transport chain, CcO is closely linked to mitochondrial dysfunction.[24] The present study (Figure 1) demonstrated increased CcO activity in both prenatal and postnatal dentate gyrus, consistent with Owolabi et al.'s findings[22] indicating a positive effect of radiofrequency exposure on prenatal and postnatal brain neurochemistry. However, this contrasts with Amari et al.'s findings,[25] where exposure to GSM signals led to decreased CcO activity in certain brain areas, including the dentate gyrus. Given the high energy demand during neurogenesis, the observed increase in CcO activity underscores its significance in brain development, as evidenced by studies in zebrafish showing neurodegeneration due to CcO deficiency.[26]

During early development, Gamma-Aminobutyric Acid (GABA) initially acts excitatory before transitioning to a different role alongside glutamate. GABA has implications in various neurogenesis processes, while glutamate, the primary excitatory neurotransmitter in the CNS, plays crucial roles in neurodevelopment, learning, and stress responses.[20] This study observed increased levels of both GABA (Figure 2) and glutamate (Figure 3) across experimental groups, consistent with Owolabi et al.'s findings[22] following radiofrequency exposure. However, abnormal increases in GABAergic signaling may impede development, while glutamatergic over-activity could hinder neurogenesis.[27]

Dopamine and serotonin, neurotransmitters regulating mood and implicated in anxiety and depression, also influence neuroplastic changes prenatally and postnatally within the hippocampal formation and cortex.[28] This study noted significant changes in dopamine (Figure 4) and serotonin (Figure 5) levels across all groups exposed to prenatal and postnatal radiofrequency, aligning with Ismail et al.'s[28] findings suggesting stress-related dopaminergic effects and anxiogenic serotonin effects. Increased serotonin levels may lead to learning impairments and spatial memory deficits.[29]

The Y-maze test (Figure 6) evaluated spatial memory, showing no significant differences across exposed groups compared to the control. However, spontaneous alternation decreased in moderate and low intermittent exposure groups, suggesting that radiofrequency exposure intensity may influence neurobehavioral alterations.[30] The open field test (Figure 7) revealed decreased exploratory behavior across exposed groups, consistent with previous reports.[31]

Histological assessment (Plate 1) revealed altered dentate gyrus structure following prenatal and postnatal radiofrequency exposure, with notable cell loss and condensed granule cells. These findings align with studies reporting hippocampal cell loss due to EMF exposure.[32] Additionally, immunohistochemical analysis showed mild to moderate microglial (Plate 2) activation in postnatal dentate gyrus, suggesting potential neuroinflammatory responses, but no acute inflammation at birth, consistent with previous studies.[33,34]

Expression of caspase-3 (Plate 3), a mediator of apoptosis, was mild in prenatal groups but varied in postnatal groups, possibly affecting synaptic pruning and plasticity. The dose-dependent effects observed indicate that prolonged exposure may induce more significant apoptotic potentials.[35]

Conclusion

Exposure to 4G radiofrequency radiation had a mild effect on the behavioural, biochemical, histoarchitectural, and immunohistochemical profile of the prenatal and postnatal dentate gyrus. However, the effects were in a time and intensity-dependent manner which suggests that consistent and long-term exposure could produce more deleterious effects which will directly or indirectly harm neurogenesis and neuroplasticity. The mechanism of action of 4G-RF on the blood-brain barrier permeability should be further explored.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Figure 1: Bar Chart showing pre-natal and postnatal Cytochrome C oxidase concentration across the groups. Values are mean ± SEM of data obtained. P values (p <0.05). *; Indicates statistical significance when compared to the control group (A), #; Indicates statistical significance when compared to the 12 hrs continuous group (B) $; Indicates statistical significance when compared to the 12 hrs intermittent group (C), @; Indicates statistical significance when compared to the 6 hrs continuous group (D).

Figure 2: Bar Chart showing pre-natal and postnatal GABA concentration across the groups. Values are mean ± SEM of data obtained. P values (p <0.05), *; Indicates statistical significance when compared to the control group (A) at p<0.05, #; Indicates statistical significance when compared to the 12 hrs continuous group (B) at p<0.05, $; Indicates statistical significance when compared to the 6hrs intermittent group (E) at p<0.05.

Figure 3: Bar Chart showing pre-natal and postnatal glutamate concentration across the groups. Values are mean ± SEM of data obtained. P values (p <0.05). *; Indicates statistical significance when compared to the control group (A) at p<0.05, #; Indicates statistical significance when compared to the 12 hrs continuous group (B) at p<0.05, $; Indicates statistical significance when compared to the 12 hrs intermittent group (C) at p<0.05, @; Indicates statistical significance when compared to 6 hrs intermittent group (E) at p<0.05; Indicates % statistical significance when compared to 24 hrs continuous group (F) at p<0.05

Figure 4: Bar Chart showing pre-natal and post-natal Dopamine concentration across the groups. Values are mean ± SEM of data obtained. P values (p <0.05). *; Indicates statistical significance when compared to the control group (A) at p<0.05, #; Indicates statistical significance when compared to the 12 hrs continuous group (B) at p<0.05, $; Indicates statistical significance when compared to the 12 hrs intermittent group (C) at p<0.05, @; Indicates statistical significance when compared to 6hrs continuous group (D) at p<0.05.

Figure 5: Bar chart showing levels of Serotonin in Pre and post-natal dentate gyrus.

Figure 6: Bar chart showing the Y-Maze parameters in the postnatal experimental groups.

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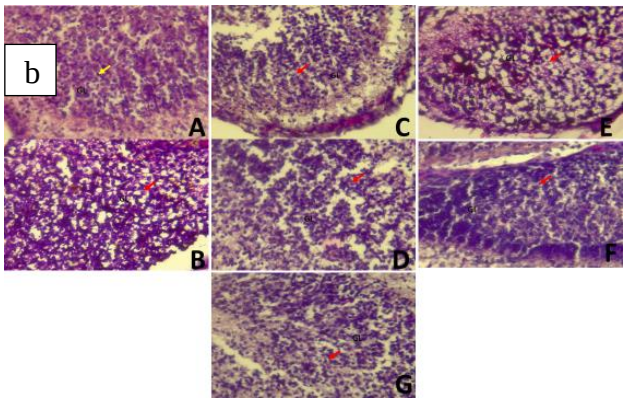
a

Figure 7: Bar chart showing the open field test parameters in postnatal experimental groups

Histological and immunohistochemical evaluation of the Prenatal and postnatal dentate gyrus



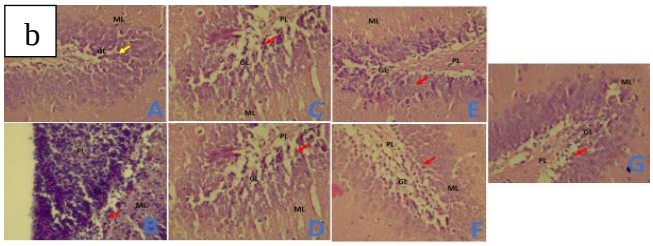
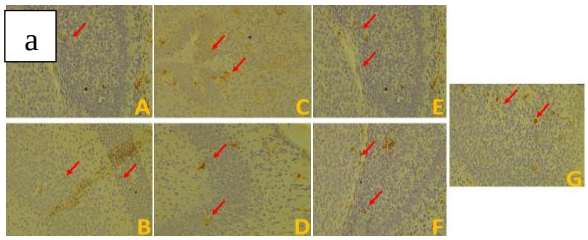


Plate 1a: Photomicrographs showing H&E staining of the dentate gyrus of the pre-natal hippocampal formation (X 400) (ML- molecular layer, GL- granule layer and PL- polymorphic layer)

Plate 1b: Photomicrographs showing H&E staining of the dentate gyrus of the post-natal hippocampal formation (X 400) (ML- molecular layer, GL- granule layer and PL- polymorphic layer)



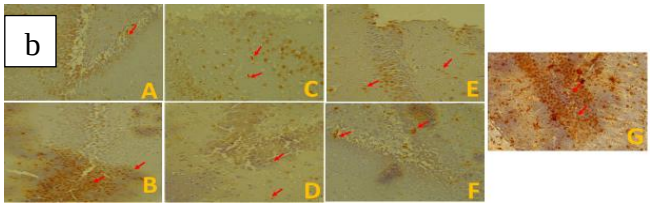


Plate 2a: Photomicrographs showing IBA-1 expression in the prenatal dentate gyrus X400 (Red arrows represents the mild to extensive microglial activation in groups B-G)

Plate 2b: Photomicrographs showing IBA-1 expression in the postnatal dentate gyrus X400

(Red arrows represents the mild to extensive microglial activation in groups B-G).

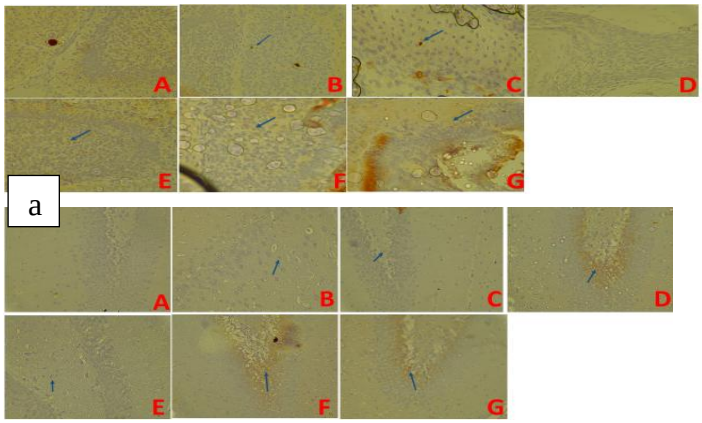


Plate 3a: Photomicrographs showing Caspase- 3 expression in the Prenatal Dentate gyrus

Blue arrows indicate mild expression of apoptotic proteins in groups B, C, E, F and G.

Plate 3b: Photomicrographs showing Caspase- 3 expression in the Postnatal Dentate gyrus

Blue arrows indicate mild to moderate expression of the apoptotic protein in groups B- G

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