



Radiofrequency electromagnetic radiation exposure effects on amygdala morphology, place preference behavior and brain caspase-3 activity in rats

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ABSTRACT

The purpose of the study was to evaluate the changes in amygdala morphology and emotional behaviors, upon exposure to chronic RF-EMR in adolescent rats. Four weeks old male albino Wistar rats were exposed to 900 MHz (power density: 146.60 $\mu\text{W}/\text{cm}^2$) from a mobile phone in silent-mode for 28 days. Amygdala morphology was studied using cresyl violet, TUNEL and Golgi-Cox staining. Place preference behavior was studied using light/dark chamber test and following this brain caspase-3 activity was determined. Number of healthy neurons was decreased in the basolateral amygdala and cortical amygdala but not in the central amygdala after RF-EMR exposure. It also induced apoptosis in the amygdala. RF-EMR exposure altered dendritic arborization pattern in basolateral amygdala but not in the central amygdala. Altered place preference and hyperactivity-like behavior was evident after RF-EMR exposure, but brain caspase-3 activity did not change. RF-EMR exposure perturbed normal cellular architecture of amygdala and this was associated with altered place preference.

1. Introduction

As a result of technological revolution, cell phones, which emit and receive radiofrequency electromagnetic radiation (RF-EMR) have become ubiquitous with an estimated 6.9 billion subscriptions globally (WHO, 2011). Majority of the children and adolescents carry at least a phone and uses it extensively. Recent reports suggest that, children as young as 13 years have been treated for addiction to mobile phones and this depicts the extent of impact of this technology amongst children (Lee et al., 2017). RF-EMR effects on various biological system is one of the major health concerns over the globe. As a consequence, researchers have extensively studied the effects of various frequencies of RF-EMR (such as 50 MHz, 1800 MHz, 2.5 GHz etc.) including the most widely used 900 MHz band that may cause changes on nerve cells in different brain regions.

RF-EMR is a form of energy and there are considerable reports suggesting that, RF-EMR specific types may affect biological tissues, particularly the brain (Barthélémy et al., 2016; Wang and Guo, 2016; Zhang et al., 2017; Kim et al., 2017). RF-EMR exposure for 50 min could

increase the brain glucose metabolism and this increase was in the parts of brain nearest to the antenna. However, the brain metabolism on the whole was almost same between on and off modes (Volkow et al., 2011). According to another report, three months of RF-EMR exposure induced increased MDA levels and reduction in antioxidant parameters in the rat hippocampus and cerebellum. Additionally, neuronal degeneration was found in the hippocampus and cerebellum. These changes were associated with significant over expression of cyclooxygenase-2 apoptotic gene and fragmentation of DNA (Hussein et al., 2016). RF-EMR exposure for 2 h/day for a month induced impaired spatial memory performance and lead to morphological changes in the hippocampal CA1 region such as degeneration of mitochondria, decreased synapses, and shorter postsynaptic densities in the rats (Li et al., 2012). As per another report, RF-EMR emitted from mobile phone affects the auditory potential in human subjects (Singh, 2015). In another study, it was found that, RF-EMR exposure for a month induced altered spatial learning and memory (Narayanan et al., 2009) and affected surviving cell count in the hippocampal CA3 region along with changes in dendritic arborization pattern in exposed group (Narayanan et al.,

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2015). In a report, it has been documented that, one month of exposure to RF-EMR had induced significant behavioral changes in rats as evident from elevated plus maze (EPM) test (Kumar et al., 2009). Additionally, RF-EMR exposure for a month lead to changes in oxidant and antioxidant status in hippocampus, amygdala, prefrontal cortex and cerebellum (Narayanan et al., 2014) and induced altered passive avoidance behavior and morphological changes in the hippocampus (Narayanan et al., 2010). As a result of substantial number of publications demonstrating the impact of RF-EMR on biological systems, researchers have opined that, there is an immediate need to evaluate the health implications of RF radiation (in the range 30 kHz–300 GHz) particularly on neurological disease, physiological addiction, cognition, sleep, and behavioral problems in addition to its role in cancer (Hardell, 2018).

Most of these reports have focused on RF-EMR induced changes in the cortex and hippocampus. Reports on the effect of RF-EMR on amygdala morphology, particularly nerve cell damage and surviving cells have not been reported so far. The amygdala is a collection of nuclear groups and is located deep in the temporal lobe (Johnston, 1923). The amygdaloid complex in rat consists of thirteen regions. As per 'Price's' nomenclature, amygdala nuclei are categorized into three major groups such as, the basolateral group, the cortical group and the centromedial group (Price et al., 1987). The different nuclei can be very well distinguished on the basis of cytoarchitecture and are referred as amygdaloid complex (Price et al., 1987; Amaral et al., 1992). This complex regulates memory, attention, emotions etc. (Davis, 1992; Kapp, 1992). However, the most studied and the best understood function of amygdala is its contribution to the detection of emotional events and the production of appropriate responses (emotional processing).

Researchers have used various tools to measure the emotional process in rats. The emotional processes are evaluated in rats by putting them into a situation of conflict. Rodents prefer darker areas over lighter areas but when presented to a novel environment they have a tendency to explore (Crawley and Goodwin, 1980). Although there are several tools used to measure the emotionality, one of the best studied and validated tools is light dark chamber (LDB) test. The advantage of LDB test is that it is being fast and easier to use. Moreover, prior training of animals is not required in this test.

Evidences suggest that, caspase-3 is vital for processes particularly associated with cell death or dismantling of certain cells and also the formation of apoptotic bodies (Hyman and Yuan, 2012). In addition to this, caspase-3 is also involved in cellular remodeling processes and these functions are independent of apoptosis. They include, dendritic pruning, metaplasticity and long term depression. One of the recent reports suggests that, in relation to the whole organism, caspase-3 deficiency in mice disturbs specific aspects of cognition and behavior, particularly attention and inhibitory control that results in a behavioral phenotype similar to attention deficit/hyper activity disorder (ADHD) (Lo et al., 2016).

Although the effect of RF-EMR on various brain regions has been studied, evidences on its effect on amygdala morphology and related behaviors is scanty. Hence, in the current study we investigated the effects of RF-EMR on morphology of amygdala nuclei, activity of caspase-3, and its possible impact on place preference behavior in rats.

2. Materials and methods

2.1. Animals and maintenance

Four week old male albino Wistar rats (50–60 g) were acquired from Central Animal Research Facility of our university. The rats were taken from six different litters and were kept in plastic cages which measured 41 cm × 28 cm × 14 cm. Each of the cage housed three rats. Water and food was given to them *ad libitum* and a 12:12 h L:D environment was maintained in an air-conditioned room. Institutional animal ethics

committee approval pertaining to the current research was obtained.

2.2. Experimental groups and study design

Rats were grouped into; A: Control, B: Sham exposed and C: RF-EMR exposed. The animals of group A were housed in the home cage throughout the entire experimental period (28 days). Animals of group B were exposed to a cell phone in switch-off mode (1 h/day). Animals of group C were exposed to 900 MHz RF-EMR from a mobile phone (1 h/day, in silent mode, without any ring tone) for 28 days. The study was carried on in two separate stages. In stage-I; the morphology of amygdala was studied. 36 rats ($n = 12/\text{group}$) were used in this stage and they were divided into various groups as stated earlier. On 29th experimental day, 6 animals from each group were killed, brain was removed and processed for cresyl violet staining, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and the other animals were used for modified Golgi-Cox staining. In stage-II; place preference behavior was studied. Thirty six rats ($n = 12/\text{group}$) were used in this stage, and they were divided into different groups as cited above. To study their place preference, on 29th experimental day, all rats were tested on light-dark chamber and then euthanized. The brain was shelled out to study caspase-3 activity in the amygdala, hippocampus, and prefrontal cortex.

2.3. RF-EMR exposure and power measurements

The mode of RF-EMR exposure and the dose were used in the current study was in accordance with previous publications (Narayanan et al., 2009, 2013). Animals (3/cage) of the RF-EMR group were subjected to 900 MHz radiation through a cell phone with a maximum power level output 2 W/kg (SAR; 1.15 W/kg). The cell phone was placed in a small wire-mesh cage with a wooden bottom, in the center of the home cage. This was particularly done to prevent rats being in contact with the phone. Rats were exposed to radiation for 7 days per week (1 h/day) for 28 days. To expose rats with 900 MHz radiation, the phone which was placed in the cage was activated repeatedly by giving unattended calls (50 calls/h). This was performed with the help of an auto dialer unit (indigenously made to befit our needs) which can dial four phone at a time. All these phones were acquired from the same manufacturers with the same SAR specifications. Measurement of power density was performed in the cage by SPECTRAN HF-2025E with MCS real-time spectrum analyzer software, Aaronia AG, (Germany). Peak power density was noted to be 146.60 $\mu\text{W}/\text{cm}^2$ at 3 cm distance from the cell phone when the phone was ringing (Narayanan et al., 2013).

2.4. Cresyl violet (CV) staining and quantification of surviving neurons

CV stain was made as per the previous published reports (Narayanan et al., 2015). The sections were embedded with paraffin and then stained with cresyl violet as reported in the earlier research (Narayanan et al., 2015; Bancroft and Stevens, 1990). The slides were coded by an independent person before quantitative analysis. The method reported earlier (Narayanan et al., 2015) was followed to count viable cells in different amygdala nuclei (basolateral amygdala, central amygdala, and cortical nuclei). Briefly, viable neuron count was done using a light microscope (Magnus MLX, Microscope) which was equipped with an ocular micrometer and a stage micrometer (Erma, Tokyo, Japan). Hundred divisions in the micrometer measured 250 μm on the stage. Ten brain sections of each animal were counted for the number of viable neurons in 250 μm^2 area. Decoding of the slides was done only after the statistical comparison of the groups was completed.

2.5. Golgi-Cox staining and dendritic quantification

After the experimental period animals were euthanized. The brain

was then shelled out and was subjected to impregnation in freshly prepared Golgi-Cox solution for two weeks. After two weeks, they were processed for Golgi-Cox staining. This procedure was performed, by following earlier published protocol (Narayanan et al., 2014). Central and basolateral amygdalaoid neurons (10/rat) were traced under 400 × magnification through a Camera Lucida, obtained from LABKRON (India). The following criterion was used for the selection of neurons for tracing. a) Neurons should belong to the basolateral and central nuclei of amygdala. b) Selected neurons should have got stained completely and entire dendritic profile must be distinguishable. c) Any branch should not have truncation within 100 µm distance from the cell body. d) Selected neurons must be well separated from nearby neurons. Quantification of dendritic branching points and intersections was performed using concentric circles method (Sholl, 1953). Number of branching points were noted up to a distance of 100 µm from the cell body and dendritic intersections were counted up to a distance of 140 µm from cell body.

2.6. TUNEL staining and quantification of apoptotic cells in the amygdala

The paraffin embedded brain sections were submerged in xylene for 10 min, and then in ethanol solutions (2 min each in 100%, 95%, 70%, 50%, 30%). Following this they were immersed in 1X PBS for 2 min and then for about 2 min in distilled water. The slides were then heated at 95 °C for 10 min in 0.1 M citrate buffer for epitope retrieval. These sections were stained to detect DNA strand breaks using APO-BrdU™ TUNEL Assay Kit with Alexa Fluor® 488 Anti-BrdU (Molecular Probes®, Invitrogen, USA) as per the manufactures instructions. Briefly, slides were first rinsed 3 times with the rinse buffer provided with the kit. Following this DNA labeling solution (Reaction buffer + TdT enzyme + Brd UTP) was prepared as per the manufacturer's protocol. The sections were overlaid with the DNA labeling solution and incubated at 37 °C for 60 min in a humid chamber. Then the slides were rinsed twice with rinse buffer. Antibody staining solution (Alexa Fluor 488 dye + Anti BrdUAb + Rinse Buffer) was prepared as per the manufacturer's instructions. The sections were overlaid with the Antibody staining solution and incubated at room temperature for 30 min in a dark humid chamber. Propidium iodide (provided with the kit) was poured on top of all sections and incubated at room temperature for 30 min in a dark humid chamber. The sections were later fixed permanently with DPX. TUNEL stained sections were first analyzed qualitatively using a fluorescence microscope. Photomicrographs were captured at 100 × magnification using a fluorescence microscope (Olympus BX 51 attached with Andor's EMCCD camera) and processed for quantification. TUNEL positive cells were counted manually (500 µm² area) from these images of successive amygdala sections. Three rat brains were examined per group, and the mean number of apoptotic cells was represented.

2.7. Analysis of place preference behavior

Light dark box test was done to evaluate the place preference behavior in rats. The LDB was made indigenously and modified to befit our needs. It was made out of Plexiglas as this material does not retain odors after washing or cleaning. It consists of light and dark boxes with same dimensions (35 cm × 35 cm × 35 cm). An opening measuring 8 × 8 cm connects the two chambers. A video camera (Sony) was fixed on a tripod stand right above the LDB to video record the activities of rat in the apparatus. This set-up remained constant during the entire experiment. The video camera was connected to a PC (installed with Panlab SMART version 2.5 Image analysis system, Barcelona, Spain). The PC was kept in a separate room adjacent to the experiment room where LDB test was conducted. This was particularly done to avoid the experimenter presence induced change in rat behavior which would otherwise be a confounding factor. To specifically control the lighting conditions in the experiment room, with no interference from the

uncontrolled sources of light, the test was done in an interior room (hermetic to exterior light as possible) between 07:00 pm to 11:00 pm. The lighting in the experiment room was controlled to obtain 5 lx in the dark chamber. However, the illumination (using 60 W incandescent light bulb) is set in a manner to obtain 600 lx in the light box. Each rat was kept in the dark box facing the wall and the start button was pressed immediately. The LDB and PC were kept in two different rooms. Once the animal was kept in dark box, the experimenter immediately moved to the other room (to avoid any change in animal behavior as a result of presence of the experimenter). Each rat was subjected to a single test (5 min duration), and during this period each rat's behavior was carefully analyzed and recorded. The path taken by each animal was tracked manually by a trained individual. After each rat was tested, the apparatus was thoroughly cleaned by 0.1% acetic acid solution to eliminate odors left by previous animal. During data acquisition, through the computer keyboard by pressing the corresponding numerical key, various events were recorded. In the current study, the following parameters were recorded and evaluated: percentage time animal spent in dark & bright boxes, latency to emerge to bright box, number of entries into bright & dark boxes, distance travelled in bright and dark boxes, total distance travelled, and number of defecation boli.

2.8. Caspase-3 activity

The Caspase-3/CCPP32 Colorimetric Assay Kit (Biovision, USA) was used for assaying caspase-3 activity in various brain regions. Freshly dissected tissues were homogenized in cell lysis buffer provided by the manufacturers on ice. The tissue homogenate was centrifuged at 4 °C and the supernatant was transferred to a microcentrifuge tube and immediately processed for assay. Protein concentration in the lysate was estimated by Lowry's method (Lowry et al., 1951). 100 µl of lysate supernatant was mixed with 50 µl of 2X reaction buffer containing 10 mM DTT (Dithiothreitol). This was mixed with, 5 µl of 4 mM DEVD-pNA (Ac-Asp-Glu-Val-Asp *para*-nitroanilide) substrate and incubated at 37 °C for 2 h. The optical density was read at 405 nm using a microtiter plate reader. For the quantification of Caspase 3 activity, five different solutions of pNA were prepared in cell lysis buffer. These were read at 405 nm using a spectrophotometric microtitre plate reader and calibration curve was made. From this calibration curve the slope was calculated and it was found to be 0.025. The Caspase-3 activity was calculated by using the following formula: $\Delta\text{ODU} \times 1/\text{curve slope}$; ie, $\Delta\text{ODU} \times 40$. The activity was expressed as U/µg of tissue protein. One unit (U) of Caspase-3 activity is defined as the amount of enzyme, which produces 1 nmol of pNA in 2 h at 37 °C.

2.9. Statistical analysis

All data was presented as Mean ± SE. Significant difference between groups were tested by one way ANOVA, and Tukey's test. P value < .05 was taken as statistically significant at 5% level of significance. GraphPad Prism statistical package (version 5.1) was used for data analysis.

3. Results

3.1. Degree of neuronal survival in the basolateral and central amygdala nuclei

General morphology of cresyl violet stained basolateral and cortical nuclei exhibited cell loss in RF-EMR exposed group to some extent. This was indicated by darkly stained neurons, interposed with normal cells. Qualitative examination of BLA, CeA, CoA of both control (Fig. 1A1–C1) and sham exposed (Fig. 1A2–C2) groups under higher magnification revealed plenty of large healthy cells with abundant Nissl body. Though many neurons in the BLA and CoA of RF-EMR exposed group had similar characteristics as controls, many karyopyknotic

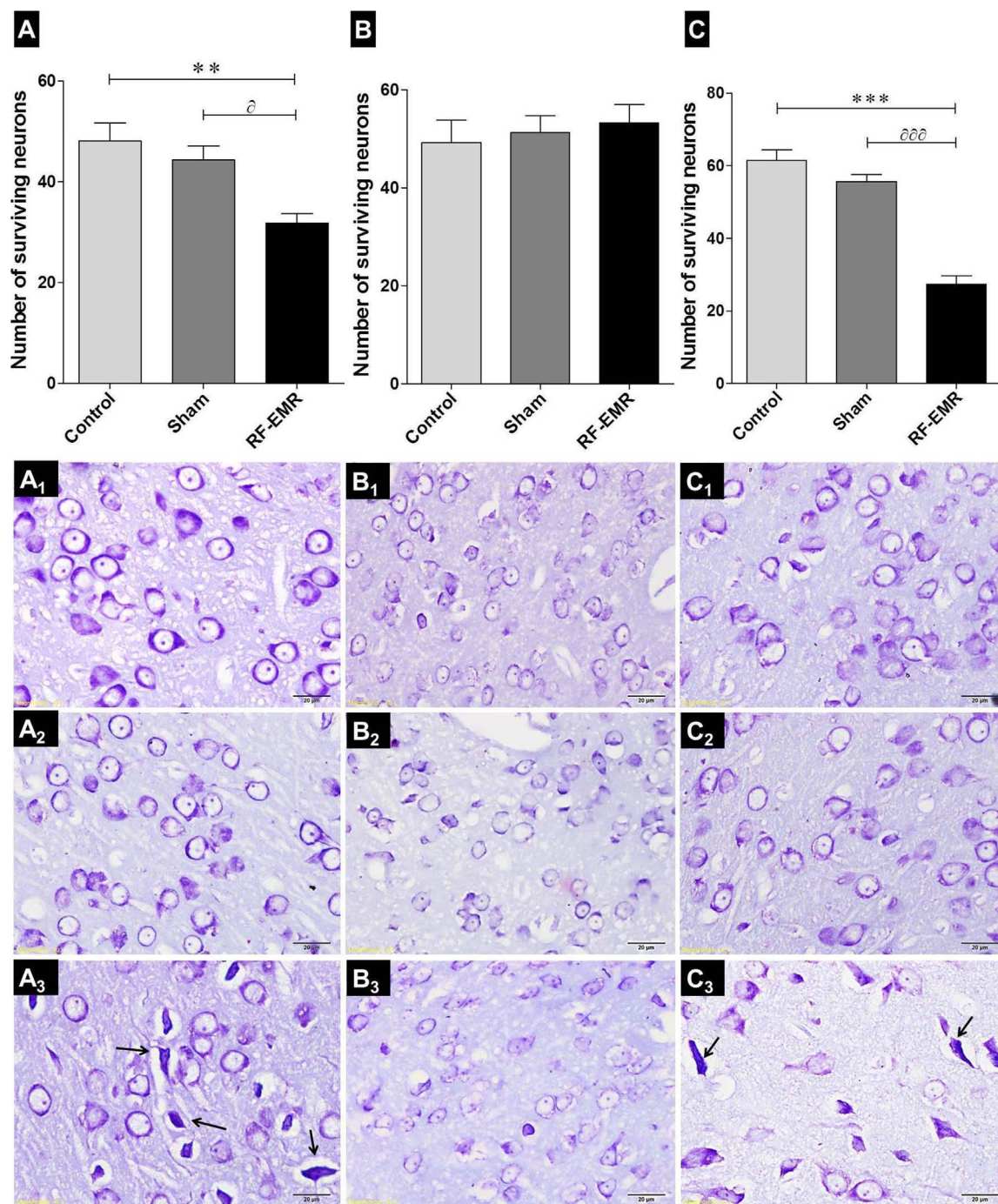


Fig. 1. Surviving neuron counts in BLA (A), CeA (B) and CoA (C) and photomicrographs of CV stained BLA (A1–A3), CeA (B1–B3), CoA (C1–C3) from various groups. (Control- A1, B1, C1; Sham- A2, B2, C2; RF-EMR- A3, B3, C3), Scale bar 20 μ m & magnification 400 $\times$ in all the panels. ($\partial p < .05$, $**p < .01$, $***\partial\partial\partial p < .001$).

neurons (indicated by arrows) were found between the normal neurons, indicating possible nerve cell injury (Fig. 1A3, C3). This change was evident in cortical nuclei compared to BLA (Fig. 1A3, C3). However, the darkly stained neurons were not found to be high in CeA of RF-EMR group compared to other groups studied (Fig. 1B3). Analysis of healthy/surviving cell count revealed that, the mean number of healthy cells was decreased in the BLA and CoA of RF-EMR group compared to control and sham exposed rats (Fig. 1A & C). However, it was not found to be decreased in the CeA of RF-EMR exposed group compared to other groups studied (Fig. 1B).

3.2. Dendritic morphology of basolateral and central amygdala neurons

3.2.1. BLA total dendritic processes

The number of dendritic processes was found to be 5.283 ± 0.116 in control, 5.374 ± 0.106 in sham exposed and 5.224 ± 0.233 in the RF-EMR group. When compared with the controls, the total number of dendritic process from soma was not significantly different in the test group.

3.2.2. BLA dendritic intersections

In comparison to controls, the dendritic intersections, which is a measure of dendritic length was reduced in test group and it was evident from 20 μ m length concentric circle onwards. In RF-EMR group,

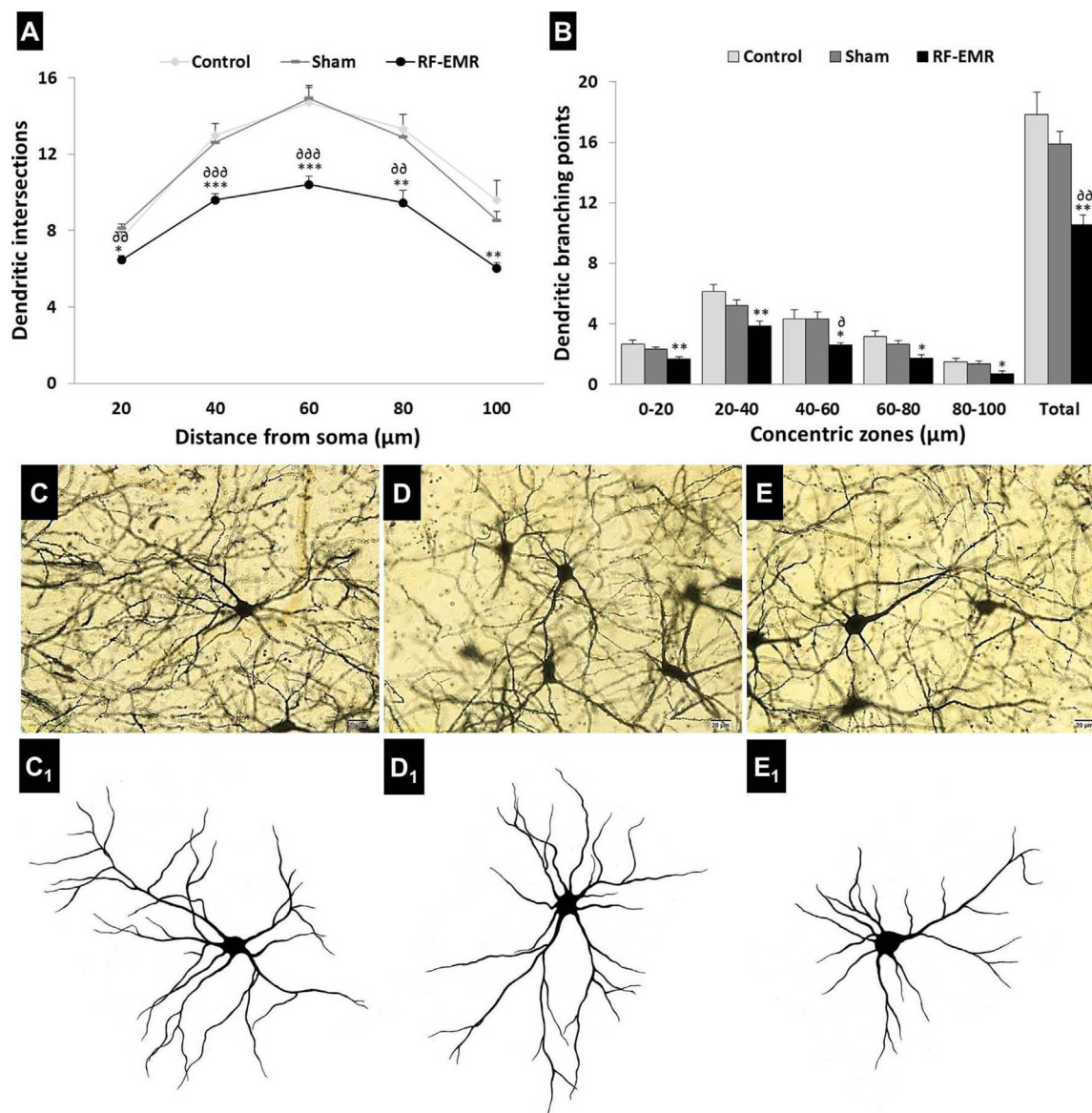


Fig. 2. RF-EMR effects on BLA pyramidal neuron dendritic intersections (A), branching points (B). Photomicrographs and camera lucida tracings of Golgi-Cox stained basolateral amygdala pyramidal neurons from control (C & C1), sham exposed (D & D1) and RF-EMR exposed (E & E1) rats. Scale bar 20 μm, magnification 200 × in panel C, D & E. Scale bar 20 μm in panel C1, D1 & E1. Camera lucida tracings were done at 400 × magnification. (*, $p < .05$; **, $p < .01$; ***, $p < .001$).

compared to controls, the mean dendritic intersections were decreased at 20 μm concentric circle length (Fig. 2A). Additionally, at concentric circles 40 μm and 60 μm length dendritic intersections were significantly decreased in RF-EMR group (Fig. 2A, E & E1). The mean number of dendritic intersections was reduced to ~10 in RF-EMR exposed group, but this was ~14 in other groups. This trend was even evident at 80 μm and 100 μm length. At 80 μm length dendritic intersections found to be reduced in test group compared to control and sham groups, but at 100 μm length this difference was only significant with control group (Fig. 2A).

3.2.3. BLA dendritic branching points at different concentric zones

Though generally a reduction in dendritic branching was seen in RF-EMR group, this was considerably evident between 20–40 μm and 40–60 μm concentric circles (CC) studied (Fig. 2B, E, E1). In other concentric circles studied, the decrease was significant with only control but not so with the sham exposed group (Fig. 2B).

3.2.4. BLA total dendritic branching points

In RF-EMR group when compared to controls, the total number of branching points was significantly decreased. When the branching points were ~15 in controls it was reduced to ~10 in RF-EMR group (Fig. 2B). The above results indicate a deleterious effect of RF-EMR on the dendritic morphology of BLA pyramidal neurons (Fig. 2E, E1).

3.2.5. CeA dendritic intersections

When compared to controls, the dendritic intersections were slightly increased in RF-EMR exposed rats but not with sham exposed rats (Fig. 3A, E, & E1). This decrease was found only at concentric circles 60 μm and 80 μm but in all other CC's (20, 40, 100, 120 & 140 μm) this was observed to be similar across groups.

3.2.6. CeA dendritic branching points

Branching points were similar across groups at 0–20 and 20–40 concentric zones. When compared to control, in RF-EMR group the dendritic branching points at 40–60 concentric zone was slightly higher. However, this difference was not evident when RF-EMR vs sham

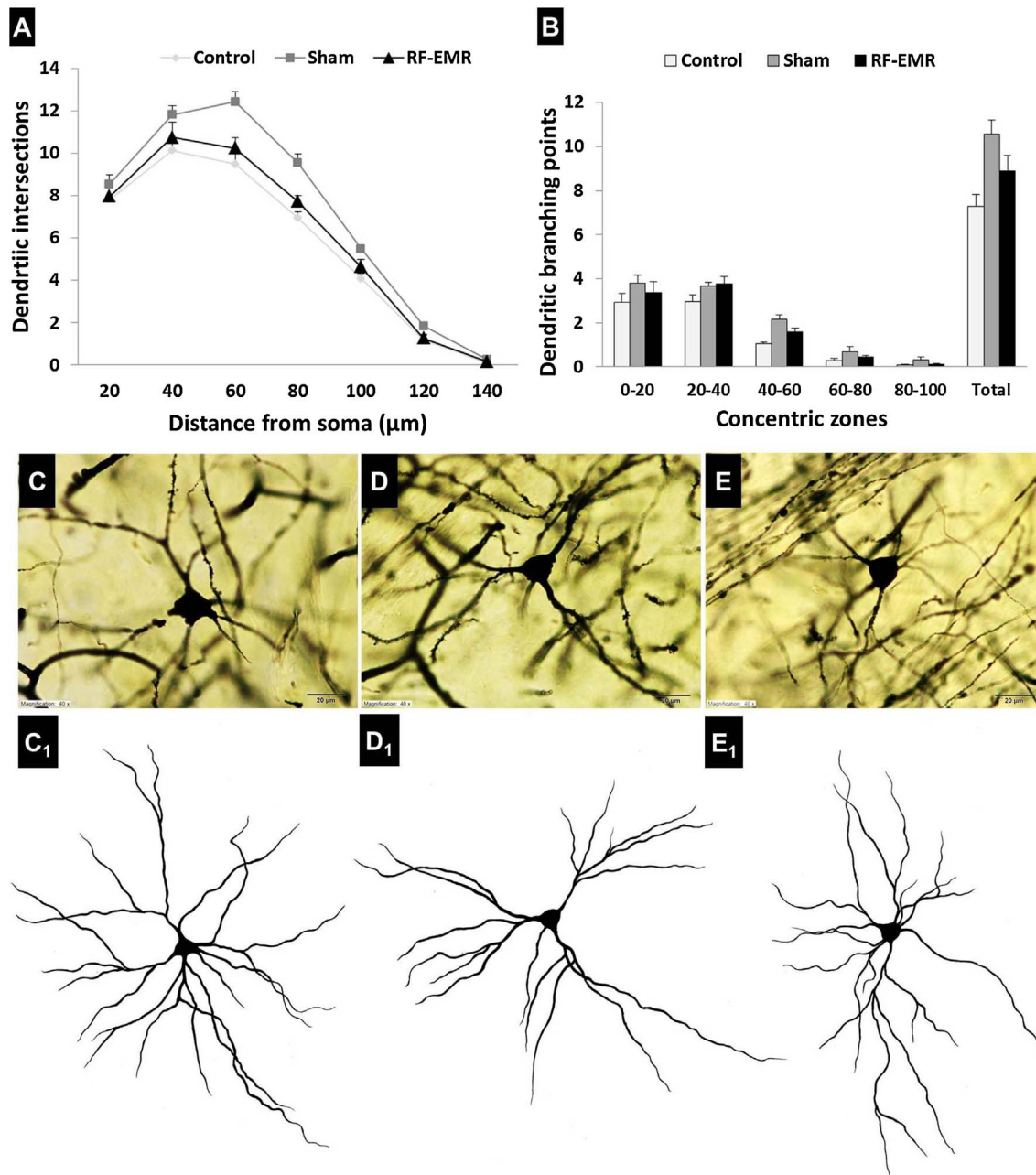


Fig. 3. RF-EMR effects on dendritic intersections (A) branching points (B) in neurons of CeA. Photomicrographs and camera lucida tracings of Golgi-Cox stained CeA neurons from control (C & C1), sham exposed (D & D1) and RF-EMR exposed (E & E1) rats. Scale bar 20 μm , magnification 400 $\times$ in panel C, D & E. Scale bar 20 μm in panel C1, D1 & E1. Camera lucida tracings were done at 400 $\times$ magnification. ($\partial p < .05$; $\partial\partial p < .01$).

group comparison was performed. Branching points were similar in all three groups at 60–80 and 80–100 concentric zones (Fig. 3B). In RF-EMR group the total branching points were found to be elevated (Fig. 3B, E & E1) compared to control.

3.3. Analysis of apoptosis in the amygdala

Morphological evaluation of TUNEL stained brain sections from RF-EMR exposed group revealed several TUNEL positive cells (apoptotic cells; Fig. 4C1) in the amygdala compared to other groups (Fig. 4A1 and B1). One-way analysis of variance test followed by Tukey's test revealed a significant difference in the mean number of TUNEL positive cells/500 μm^2 in RF-EMR group compared to other groups (Fig. 4C1 & D).

3.4. Analysis of place preference behavior

The entrance latency to emerge to light box was not found to be statistically different in different groups (Fig. 5A). However, percentage of time elapsed in the light box was significantly elevated in the test group when compared with control and sham groups (Fig. 5B). Percentage of distance travelled in the bright box was elevated in RF-EMR exposed rats but this was not significantly different ($p = .07$; Fig. 5C). When compared to control/sham rats, total crossings across chambers were significantly increased in RF-EMR rats (Fig. 5D). This indicates the impulsive nature (hyperactivity like) behavior of rats (Fig. 5I). Total distance travelled and fecal boli were increased in RF-EMR exposed rats (Fig. 5E, F). The above results demonstrate an altered place preference and hyper-activity like behavior with RF-EMR exposed group (Fig. 5I).

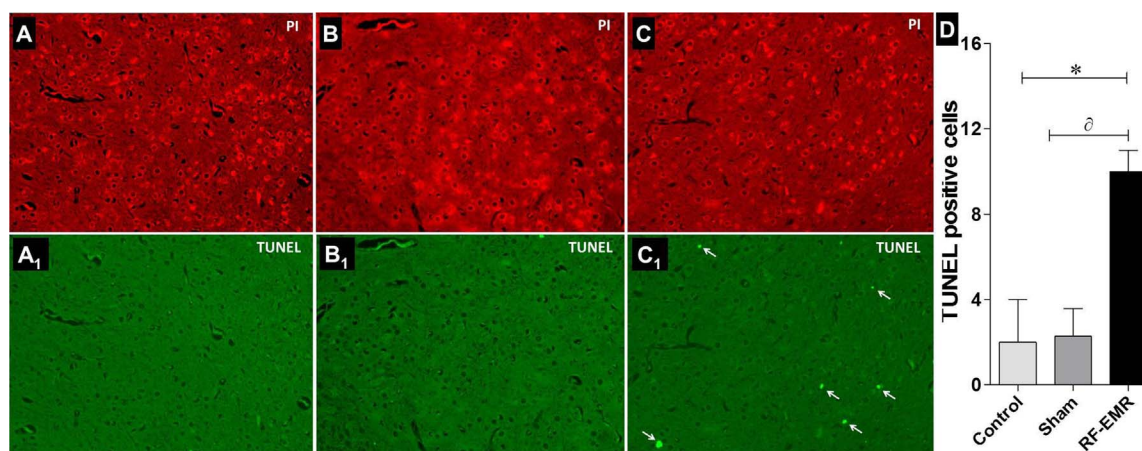


Fig. 4. Representative photomicrograph of propidium iodide (A–C) and TUNEL (A1–C1) stained amygdala from control (A & A1) sham (B & B1), and RF-EMR groups (C & C1). Quantification of TUNEL positive cells in the amygdala of various groups (D). Scale bar- 50 μ m, Magnification 100 $\times$.

3.5. Analysis of caspase-3 activity

3.5.1. Caspase-3 activity in the amygdala

In the amygdala, the caspase-3 activity was not found to be considerably altered in test group when compared to other groups. It was observed to be comparable in all these groups as the statistical analysis showed no difference in the means of various groups studied (Fig. 6A).

3.5.2. Caspase-3 activity in the hippocampus

When compared to control and sham rats, the caspase-3 activity was not observed to be significantly different in RF-EMR group. Caspase-3 activity mean values in the hippocampus of control and sham exposed rats were 1.056 ± 0.403 U/ μ g of tissue protein and 1.116 ± 0.409 U/ μ g of tissue protein respectively. However, it was slightly decreased (0.788 ± 0.180 U/ μ g of tissue protein) in RF-EMR group but it was not significantly different when compared to other groups (Fig. 6B).

3.5.3. Caspase-3 activity in the prefrontal cortex

Caspase-3 activity in the prefrontal cortex was also not found to be changed due to RF-EMR exposure. The mean values were found to be 0.225 ± 0.079 , 0.248 ± 0.070 , 0.235 ± 0.083 in control, sham and test group respectively and these were comparable (Fig. 6C).

4. Discussion

The association between mobile phone RF-EMR and its biological effects has been a burning topic of discussion and research in recent years (Wang and Guo, 2016; Zhang et al., 2017; Kim et al., 2017; Hardell, 2018; Mokarram et al., 2017). Nonetheless, it is relatively under-explored as the speed at which this technology has been advanced has outpaced research capabilities. Results of the current study portrays that, the surviving nerve cell count was reduced in certain nuclei of amygdala (BLA & CoA) but not in certain other regions (CeA). Similarly there was a reduction in the dendritic arborization pattern in BLA pyramidal neurons but slightly increased dendritic arborization in CeA after RF-EMR exposure. All these suggest a morphological alteration in the amygdala due to RF-EMR exposure. The BLA may undergo structural reformation when exposed to various stressors such as immobilization, maternal stress, etc. (Vyas et al., 2002; Vyas et al., 2003; Mitra et al., 2005; Weinstock, 2008). In an earlier study, chronic immobilization stress had increased basolateral amygdala pyramidal neuron dendritic arborization and increased anxiety like behaviors (Vyas et al., 2002; Vyas et al., 2003; Mitra et al., 2005). However, in this study we found decreased arborization in BLA neurons but not much change in the CeA. This is corroborating with the argument that, 'dendritic remodeling of BLA by stress seems to be stressor specific'. In

other words, the response (eg; dendritic remodeling) of BLA to stress depends mainly on the type of stress applied.

When we critically evaluate the real time videotaped recordings obtained during the place preference behavior, we can see that, there is a lack of experience to evaluate unpredicted stimuli and select a suitable response in RF-EMR group compared to controls. They were behaving impulsively in the apparatus compared to controls. This could be due to the stress that induced by RF-EMR along with morphological changes that were undergoing in the amygdala. There could be an imbalance in the sensory processing of emotional stimuli due to morphological changes in the amygdala which could have triggered this behavioral change. A report indicates that, social isolation for 6 weeks in rats induced longer time of the room choice, more entries to light box, more rearing and this perhaps explained by their higher anxiety and a lack of experience to evaluate unpredicted stimuli and select a suitable response (Shuikin et al., 2003). It is also interesting to note that, decreased amygdala size is correlated with anxiety like behavior in humans with panic disorders (Massana et al., 2003; Hayano et al., 2009).

The crucial question to be answered is what lead to cell death or dendritic remodeling in the amygdala? Is it due to apoptosis? Or is this a result of necrosis? TUNEL staining and caspase-3 activity assays were performed to understand the role of activation of apoptotic pathway leading to nerve cell damage. Although we got a positive result in the TUNEL staining, we could not get an affirmative result in caspase-3 assay corroborating with the cellular changes in the amygdala. The caspase-3 assay was performed after the behavioral testing and this took 3 days to complete. By the time we performed the caspase-3 assay, its activity might have come down (if it could have got elevated) to the baseline. This could be one of the possible reasons for not observing a change in caspase-3 activity. Report suggests that, the activation of caspase is a transient phenomenon and to record a change in the activation of caspase-3, the assay have to be performed in 24–28 h' time after the experimental procedure. This opinion is corroborating with TUNEL staining results of the current study. As represented in the results, several TUNEL positive neurons were observed in the amygdala of RF-EMR exposed rats. Other researchers have also reported the presence of apoptotic cells (TUNEL positive cells) in certain brain regions (hippocampal CA1, CA3 and dentate gyrus) of mice exposed to 835 MHz RF-EMR chronically and the authors suggest that this might affect the cell viability in the hippocampus regions (Maskey et al., 2010). The possibility of other mechanisms such as autophagy or necrosis cannot be excluded in the amygdala, but at present we cannot categorically propose this as we do not have evidences to pinpoint these mechanisms. The possibility of the existence of a caspase independent pathway for apoptosis cannot be ruled out. Earlier reports indicate that,

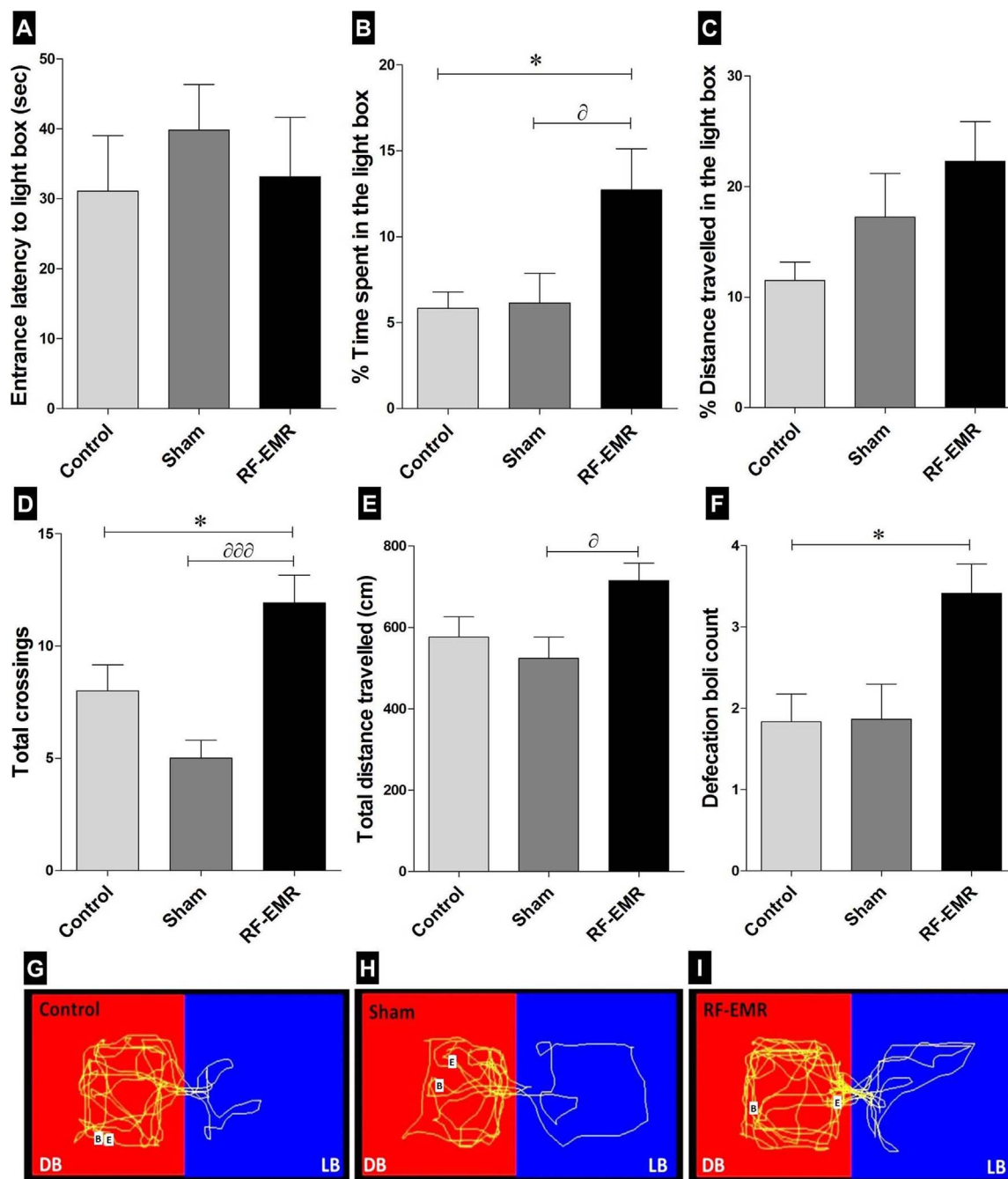


Fig. 5. RF-EMR effect on entrance latency to light box (A), percentage time elapsed in the light box (B), percentage distance travelled in the light box (C), total crossing (D), total distance travelled (E), and number of defecation boli (F). Real time videotaped records depicting animal trajectories during LDB test (G, H, I). (* ∂ $p < .05$, $\partial\partial\partial$ $p < .001$).

continuous wave (CW) 900 MHz Radiofrequency fields (RF) for 24 h induced apoptosis through a caspase-independent pathway that involves apoptosis inducing factor (AIF) in primary neuronal cultures of rats (Joubert et al., 2008). Since in the current study we have not evaluated this aspect (RF-EMR effect on cultured neurons), further research in this line is needed to get a comprehensive picture.

RF-EMR emitted from mobile phones can have two effects such as thermal and non-thermal. It is possible that, thermal effect is not the only effect of RF-EMR. Non thermal effects could have played a role in inducing a change in the amygdala morphology. Unfortunately, several exposure parameters, such as, frequency, modulation, power density, make it extremely difficult to compare and draw specific conclusions at non-thermal levels of RF-EMR exposure. Hence, disagreements persist in certain reports and careful interpretation is required while

extrapolating the data to human subjects. RF-EMR exposure induced oxidative stress in turn triggering cell death or altered morphology/neuronal connections leading to altered place preference behavior in rats could be a possibility. Reports suggest that, increased oxidative stress markers (increased MDA levels) were seen in the brain (Shehu et al., 2016) and particularly in the amygdala when rats were exposed to RF-EMR for a month (Narayanan et al., 2014). There are also reports indicating that, oxidative stress in the hypothalamus & amygdala may modify the fear circuits functioning and this can be one of the reasons for the development of anxiety (Gingrich 2005; Hovatta et al., 2005). In a recent work, authors report that, anxiety like behavior in mice is induced by oxidative stress and this can be reversed by Phosphodiesterase-2 (PDE2) inhibition (Masood et al., 2008). Amygdala also modulates plasticity (especially related to fear learning and memory

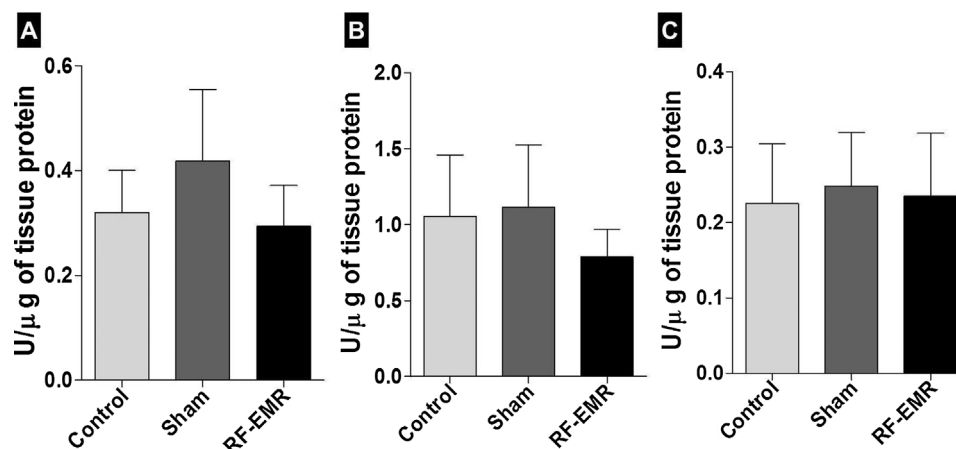


Fig. 6. RF-EMR effect on caspase-3 activity in the amygdala (A), hippocampus (B) and prefrontal cortex (C) of various groups.

formation) in certain other brain regions which are the areas for the memory storage (Cahill and McGaugh, 1998; Cahill et al., 1999; McGaugh 2002). These studies suggest that, while plastic changes take place in BLA during fear conditioning it also involves in modulating the plasticity in other brain regions. Which means that, if there is a change in amygdala morphology it could also change the functionality of other related brain regions (such as hippocampus) necessary for regulating anxiety or fear behavior.

Emotionality is controlled by the interplay of a number of neurotransmitters. Reports indicate a link between RF-EMR exposure and amino acid neurotransmitters imbalance in various brain regions (midbrain, cerebellum and medulla) of young and adult male albino rats (Noor et al., 2011). Another study evaluated the level of neurotransmitters in the brains of fetal rats chronically exposed to cellular phones and it was found that, both norepinephrine and dopamine were increased in group exposed to mobile phone for 10 min each and 3 times a day. However, both neurotransmitters were reduced in group exposed to mobile phone for 60 min each and 3 times a day (Jing et al., 2012). Possibly, neurotransmitter alteration after RF-EMR exposure could be another attribute for behavioral changes in RF-EMR exposed rats. Additionally it should be noted that, the neural correlates of emotion processing and its interaction with motor networks is highly complicated with the involvement of several neurotransmitters and brain regions. Therefore, only with present research results, we cannot categorically suggest a possible reason for the changes that are seen in place preference behavior and in amygdala. Further studies in this regard will give insight in to the possible neuronal basis for such a change in amygdala after RF-EMR exposure.

5. Conclusion

Chronic RF-EMR exposure perturbed normal cellular architecture of rat amygdala which was characterized by decreased surviving neuronal count in BLA, CoA and decreased dendritic arborization pattern in BLA. Apoptosis is found to be one of the probable attribute for the decreased surviving neuronal count in various regions studied. The reformed amygdala cellular architecture was associated with altered place preference behavior when rats were tested on LDB. Nonetheless, brain caspase-3 levels were not altered after chronic RF-EMR exposure. These results suggest an impact of chronic exposure to RF-EMR on amygdala cellular architecture and related behaviors in rats.

Conflict of interest

The authors declare that they have no conflict of interest.

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