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Research Paper

Age-dependent changes in the medial prefrontal cortex and medial amygdala structure, and elevated plus-maze performance in the healthy male Wistar rats

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ABSTRACT

Aging affects different parts of the brain structure and function. These changes are associated with several age-related emotional alterations like anxiety that is regulated by the amygdala and medial prefrontal cortex (mPFC). Thus, this study aimed to explore the effects of aging on the morphology changes in these regions. Twenty male Wistar rats were assigned to young and old groups. The anxiety level was evaluated by elevated plus-maze. Then, their brains were removed, fixed, cut, and stained with Cresyl Violet or Golgi-Cox. In addition to the estimation of stereological parameters, dendrite complexity, and spatial distribution of the neurons in the mPFC and amygdala were evaluated. Aging increased the medial amygdala volume and its total number of neurons, but it did not have a significant effect on these parameters in the mPFC. Furthermore, the size of the neurons in the mPFC increased, whereas the total length of the dendrite and its complexity significantly decreased with aging in this structure and increased in the amygdala. Although aging did not significantly change the dendritic spine density in both regions, old rats showed a more mature spine in the mPFC and more anxiety-like behavior. In conclusion, the increase of anxiety in the old individuals could be attributed to structural changes in the morphology of the dendrite and neuron and its spatial distribution in the mPFC and amygdala. The findings of this study partly support this hypothesis.

1. Introduction

Aging is considered a common physiological process that leads to the occurrence of several changes in the brain's structure and function (Dickstein et al., 2013; Rosenzweig and Barnes, 2003). However, these changes could be observed at all levels that range from molecules to morphology and function; also, they are associated with multiple etiologies (Peters, 2006). It should be noted that there are various types of changes that occurred in the neuronal morphology of the cerebral cortex during the normal aging process due to the neuronal type, region, and cortical layer (Duan et al., 2003).

It has been reported that the brain's volume or weight, reduce by a 5% aging ratio per decade after the age of 40, and specifically after 70 (Peters, 2006). However, the results showed that there was not a similar extent to the brain's changes within all the brain regions. For instance, it was reported that the frontal lobe was considered as one of the most affected areas, while the occipital cortex is associated with minimum changes (Peters, 2006).

It was assumed that the reduction of the volume of the brain areas is almost dependent upon cell loss and particularly, it occurs due to cell death. Stranahan et al. carried out an investigation and observed a significant age-related reduction in the number of principal neurons in the dorsal prefrontal cortex (PFC); however, they did not observe any change in the total number of neurons in the ventral PFC (Stranahan et al., 2012). Also, it was discussed by Avino et al. that the number of neurons within the amygdala nuclei of 52 human brains increased from childhood to adulthood (Avino et al., 2018).

This brain volume decrease could also be related to changes in the dendritic length and arbors. The dendrites are the key integrators of synaptic information in the neurons and play vital roles in neuronal

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plasticity. (Arikkath, 2012). However, there are controversies regarding various brain regions achieved through several investigations. As an example, Dickstein et al. observed that the dendritic complexity in the neurons of the frontal and temporal cortex decreases with aging, while it increases in the hippocampal pyramidal cell (Dickstein et al., 2013).

Dendrite spines are considered as dynamic structures that undergo remodeling over time. The properties of neural circuits and functions are mostly affected by changes occurring in the spine's morphology and density (Ethell and Pasquale, 2005). For example, the pyramidal cells in the second and third layers of the prefrontal cortex in aged animals lose the small, thin, and stubby spines; however, they do not lose the larger mushroom spines compared to young animals (Dumitriu et al., 2010). It was also found by Bloss et al. that there are progressive age-related reductions in spine density including 20 % loss among middle-aged rats, and 30 % loss among aged rats; furthermore, the majority of thin and stubby spines were susceptible to the aging reduction, while mushroom spines were mostly resistant (Bloss et al., 2011).

The information processing and function are highly dependent upon the neuronal size and morphology; however, the first factor is compatible with the neuron survival, and like other cells, the neurons might be reversibly/permanently atrophied or hypertrophied during the adult's life history. Changes in the sizes of the soma or dendritic complexity might be started during their middle-age (Finch, 1993). It is reported that aged rats showed atrophy in the forebrain cholinergic neurons (Martinez-Serrano et al., 1995), while the vasopressin neurons of the hypothalamus preoptic and paraventricular nuclei were hypertrophied among human being after 80 years of age (Vogels et al., 1990).

In addition to alterations in the volume, neuronal morphology, and dendritic complexity, the spatial distribution of the neurons can also be considered as an important factor for connectivity and functions of various brain areas. For example, it was reported that the spatial arrangement of coronary arteries was regular in healthy hearts, and it has been changed into a random pattern in the hearts with failures (Okabe et al., 2009).

It has also been found that emotional changes are another important aging aspect (Boguszewski and Zagrodzka, 2002) and it is well documented that the process of aging among human beings and animals is associated with several age-related behavioral alterations and decline in cognitive performance(Anderton, 2002; Pedersen et al., 2001). For instance, increase in anxiety with aging is reported by several investigations during recent years; these reports are on the basis of the evaluation of the rats' performances through considering the behavioral models of anxiety such as the elevated plus-maze (Boguszewski and Zagrodzka, 2002; Darwish et al., 2001; Miyagawa et al., 1998). Also, some investigations have connected behavioral impairments of aged animals to changes in the structure of specific brain regions (Gage et al., 1984).

There are two regions associated with a particular interest including medial PFC (mPFC) and amygdala, which are highly interconnected and play significant roles in regulating various emotional responses such as anxiety(Ernst and Fudge, 2010). Furthermore, both of these structures undergo considerable structural and functional changes during the development period (Caballero et al., 2016; Koss et al., 2014). Amygdala is associated with three sub-nuclei groups: the basolateral, cortical, and centro medial amygdala complex(Sah et al., 2003). The medial nucleus of the amygdala)MeA(nucleus was selected for the present study because the following traits have been determined for this part: the strong connection with PFC (Ko, 2017), its central role to generate innate emotional responses to chemo-signal (LeDoux, 2012), and high susceptibility to the aging compared to other amygdala nuclei (Herzog and Kemper, 1980).

The mPFC that consists of the anterior cingulate, prelimbic and infralimbic subregions in the rats, is located on the medial side of the hemisphere anterior and dorsal to the genu of the corpus callosum (Heidbreder and Groenewegen, 2003) and plays a critical role in the emotional processing (Papez, 1937). It is also known that changes in the structure and function of the prefrontal cortex might lead to several performance changes that occur as a result of aging (Raz et al., 2007). Moreover, the mentioned cortical area has reciprocal connections with the amygdala; also, they can commitantly be activated with the amygdala during the emotional stimuli representation(Ernst and Fudge, 2010).

The impacts of aging on some brain regions, have almost been recognized; however, the type of mechanism that might have age-associated changes in the mPFC and MeA is not completely known. It should be noted that elucidating these changes might lead to improvement of drug treatments that can restore or protect the neural circuits, or mediate the cognition and successful aging. Therefore, we study the impacts of aging on changes in the volume, cell number, neuronal size, dendrite arborization, spine density, and the spatial distribution of neurons in these areas. Also, the assessment of characterized anxiety-related behaviors of young and aged rats was carried out through the elevated plus-maze.

2. Materials and methods

2.1. Animals

Twenty male Wistar rats were purchased from the Institute of Animal Science Laboratory of Tehran (Medzist, Tehran, Iran). These animals were assigned to the young (2−3-month-old, n = 10) and old (18−20-month-old, n = 10) groups (Yang et al., 2018). They were kept under the standard 12–12 h light-dark cycle at room temperature (25 ± 2 °C) with normal humidity. Food and water were available ad libitum. All procedures in this experiment were performed by the Care and Use of Laboratory Animals (National Academy Press, 1996, Washington, USA) and approved by the Ethics Committee of Shiraz University of Medical Sciences (SUMS, Shiraz, Iran, Ethic code: IR.SUMS.REC.1397.77).

2.2. Evaluation of anxiety using elevated plus-maze

The Elevated Plus-Maze (EPM) was initially applied as a tool to test anxiety in rats (Pellow, 1985). EPM consists of two open arms (50 × 10 cm) surrounded by a 5-cm-high edge and two closed arms (50 × 10 cm) with 40 cm walls, which extended from the common central platform (5 × 5 cm). The entire apparatus is 50 cm above the floor. The EPM was stable on the floor and did not shake with locomotor activity of the rat. At first, the rats were placed on the middle platform that faced the open arm. Then, the behavior of the rat was recorded for 5 min using a camera (Sony, China). Finally, the animal behaviors were scored based on the recorded video for each animal.

After placing all of the 4 limbs of each rat in the arm, it was assumed that the rat had entered the arm. It was assumed that the investigated rat exited the arm when the animal was placed at least two front limbs on the external side of the arm. Percentages of the time spent on the open arms and their related entries are considered as reliable measures of anxiety (Serafim et al., 2012; Sudakov et al., 2013). Finally, the process of cleaning the maze was carried out after each animal test. It should be noted that all of the behavioral tests were carried out one day before the animals' sacrifice, from 8: 00 a.m. till 2:00 p.m. in a room with indirect lighting; we ensured that all four arms were similarly illuminated without shadows.

2.3. Tissue preparation

The animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). They were then transcardially perfused by phosphate-buffered saline (PBS) followed by 200–300 ml of 4% paraformaldehyde in PBS (1X, pH 7.4) The right brain hemispheres were rapidly removed, weighed and post-fixed in the same fixative overnight. The brain was then transferred to a 30 % sucrose solution in PBS 4 °C for 48−72 hrs, and the samples were kept at −80 °C.

Fifty μm-thick coronal sections of the brains were cut by cryostat (Leica, CM1860, Germany) and stored in a cryoprotectant solution at −20 °C until use for Cresyl Violet staining. An average of ten sections per animal were selected using uniform systematic random sampling and stained with Cresyl Violet for estimating the volume and neuron or non-neuron counting, and spatial distribution of the neurons in the mPFC (AP = 4.20 to -1.50) or MeA(-1.44 to -3.60) regions based on the rat brain atlas (Boyce and Gundersen, 2018; Paxinos and Watson, 2006).

2.4. Estimation of the volume

The live image of each Cresyl Violet stained brain section was transferred to a monitor through a stereomicroscope (Nikon, SM

Z745 T, Japan). The volume of the mPFC or amygdala (V) was estimated using point counting based on the Cavalier's principle by a stereological software (Stereo Lite, SUMS, Shiraz, Iran). For this purpose, the sum of the area of the favored structure "ΣA (sections)" (mPFC or amygdala) was multiplied by the distance between the sampled sections, considered as "d" (Kristiansen and Nyengaard, 2012). The following formula was used for volume estimating:

$$V_{(mPFC\ or\ MeA)} = \Sigma A_{(\text{sections})} \times d$$

2.5. Estimation of the numerical density and total number of neuron or non-neuron

Numerical density and the total number of cells (neuron or non-neuron) in the mPFC and MeA were estimated, using the optical disector method (Boyce and Gundersen, 2018). This technique eliminates bias in counting as a result of cell size or shape. In brief, cells were counted as they came into focus while scanning through the section. For each section, 8–10 unbiased counting frames were sampled in a systematically random fashion and examined under an Eclipse microscope (E200, Nikon, Tokyo, Japan), with a high-numerical-aperture (NA = 1.4)× 60 oil-immersion objective. Images were transferred to a computer using a high-resolution camera (BX51, Japan) connected to an electronic microcator with digital readout (MT12, Heidenhain, Traunreut, Germany) for measuring the movements in Z-direction with 0.5 μm precision. The number of cells was counted using computer-generated counting frames (StereoLite, SUMS, Shiraz, Iran). The mean density of the cells in each region was estimated as follows:

$$N_v = \frac{\sum Q^-}{\sum P \times a(f) \times h}$$

Where "∑Q-" is the total number of counted cells (neuron or non-neuron) within the sampling volume, "∑P" is the number of disector, "a/f" is the area of the frame (a/f = 0.000576 mm^2), and "h" is the height of the disector (0.020 mm). The total number of cells was estimated by multiplying the numerical density (Nv) and the volume of area (V). The brain cells were divided into two types: neurons and "others" which were termed non-neurons. The basic morphology of a normal neuron in Cresyl Violet staining consists of a large cell body or soma with neurites (dendrites and axon) emerging from the cell body, Nissl substance in the soma (and dendrites), the identifiable nucleus that is invariably pale or euchromatic with discrete nucleolus (Abusaad et al., 1999; Benes et al., 2001). Also, the nuclei of the neurons are round or ovoid; the nuclear envelope frequently has indentations and folding, which are not usually visible in non–neuron cells like astrocytes at the light microscope. Besides, large neurons virtually lack heterochromatin exception for 1–4 small granules attached to their large and well-defined nucleolus. A narrow rim of cytoplasm circling the entire nucleus is a useful feature to distinguish the small neurons from astrocytes (García-Cabezas et al., 2016).

2.6. Golgi - Cox staining procedure (modified briefly)

The Golgi-Cox stain solution was prepared (Zaqout and Kaindl, 2016), and the container bottle was covered with aluminum foil and stored for one month. The left brain hemispheres were removed and fixed in 4% paraformaldehyde for two weeks. They were then placed in a Golgi stain bottle in a dark place for two weeks, and the tissue was transferred into 30 % sucrose in PBS at 4 °C for ten days. After sinking, they were cut (100 μm) at -17 to −19 °C using a cryostat (Leica, CM1860, Germany). The brain sections were put in small baskets, rinsed in distilled water (5 min), and placed in the 20 % Ammonia solution (10 min); after rinsing in distilled water (5 min), they were dehydrated in graded ethanol and cleared by xylene (10 min). Finally, the sections were transferred on the gelatin-coated slide and left to dry for four days at room temperature (Smitha and Roopa, 2012).

2.7. Estimation of the cell diameter

Cell diameter of the neurons in the layer V of mPFC, which project to and regulate subcortical output a system like the amygdala(Gabbott et al., 2005), and also neurons of the MeA region were measured using a stereomicroscope and stereological software (Stereo Lite, SUMS, Shiraz, Iran). The live image of each brain section was evaluated according to the rat brain atlas in these regions. The horizontal and vertical diameters of the soma were measured and then averaged to give a mean cell diameter. At least twenty neurons in each area per group were analyzed (Bayram-Weston et al., 2016).

2.8. Evaluation of the dendritic complexity and length

Sholl analysis of Image J software (Java. NIH, USA) was applied to the Gogi-Cox stained sections to evaluate the dendritic complexity and dendrite length. Sholl analysis is a method of quantitative analysis of dendritic arbors, first described by Sholl in 1953. This method can be performed on both two and three-dimensional neuronal representations and gives an objective representation of the dendritic tree density and complexity.

The microscopic images for analysis were captured using a 100× oil-immersion objective lens. Thirty individual neurons in the layer V of mPFC or MeA regions in each group, which did not have truncated branches and were unobscured by neighboring neurons and glia, were selected for reconstruction based on the previously described method (Garrett and Wellman, 2009; Kutzing et al., 2010). To direct the analysis of a neuron of mPFC and MeA in the microscopic images, we imported each figure to "Image J" program. After setting the scale of the image, the beginning (or center) of the analysis was marked by clicking on the nuclei. In the next step, the analysis was done using the implementation of "Analyze" → "sholl" → "sholl analysis". Finally, the result for each cell analysis was obtained in a separate file (Sholl, 1953).

2.9. Estimation of the dendritic spine density and morphology

For quantification of dendritic spine density and morphology, the microscopic images of the Gogi-Cox stained sections were captured using a 100× oil-immersion objective lens of the interested region; then, we sampled 30 pyramidal neurons in the layer V of mPFC or neurons of the MeA per group. Dendritic spines are classified based on their shape into filopodium, thin, stubby, mushroom, and branched types. The filopodia and thin spines were considered immature while the stubby, mushroom and branched spines were mature ones (Smitha and Roopa, 2012). For each neuron, the spine density was estimated for 10 μm length located on the distal end of the 2nd or 3rd order branch of the one dendrite which was unobscured by the neighboring neurons and glia and with high resolution (Gillani et al., 2010; Shih et al., 2013).

2.10. Evaluation of the spatial distribution of the neurons

The Voronoi diagram of a set of points is Voronoi tessellation associated with the distribution of cell nuclei. Each polygon contains the region of space with an accumulation of cells close together. Therefore, the polygon area indicates the space that a cell occupies.

The mPFC or MeA neurons were mapped using the Image J Voronoi Plugin (Java. NIH, USA), which consisted of drawing a polygon area around each soma of the neurons. The area and the number of closest Voronoi polygons to each other were obtained. To draw the Voronoi polygon diagram, we analyzed the brain sections using the video-microscopy system; mPFC and MeA microscopic images were gained with an objective lens 40 × . The favored parameter was the nucleus of the neuron. Each figure was imported to "ImageJ" program. After setting the scale of the image, the neurons were marked by clicking on the nuclei. In the next step, the polygons were designed using the implementation of "Plugins" → "Analyze" → "Voronoi". Black and white images gained from the tessellated areas. Finally, areas of the polygons were measured by running "Analyze" → "Measure" system (Kamali Dolatabadi et al., 2019; Sarkala et al., 2019). The variability of polygon areas was easily analyzed by their variance. The coefficient of variation or CV (standard deviation of the polygon areas/mean×100) provides an index for the spatial distribution of the neurons: CV of 33%–64% is associated with a random distribution of the neurons; CVs less than 33 % have a regular pattern, and those more than 64 % are considered as a clustered distribution (Moroni et al., 2008). This CV is a classification and not a statistical comparison.

2.11. Statistical analysis

All data obtained at the end of the study were analyzed using software Prism 6 for Windows (GraphPad Software Inc., USA). First, the Kolmogorov-Smirnov test was used to determine the normal distribution of data. Data with normal distribution were analyzed by unpaired *t*-test. Second, if data did not have a normal distribution, it was analyzed using Mann-Whitney test. Data are reported as the mean ± SD. In all tests, the statistical significance level was considered $P < 0.05$. We performed a blind analysis of our data.

3. Results

3.1. Behavioral function: elevated plus-maze

Results of elevated plus-maze showed that compared to the young rats, both percentage of entrance (Mann-Whitney test, $p = 0.01$, $n = 10$) and time spent on open arms (*t*-test, $p < 0.0001$, $t = 7.15$, $df = 14$, $n = 10$) decreased in the older rats (Fig. 1). These two parameters were considered reliable measures of anxiety. Also, there was no significant age-related difference in the total arm entrance (*t*-test, $p = 0.08$, $t = 1.84$, $df = 15$, $n = 10$) between investigated groups.

3.2. The volume of area

The volume of mPFC and MeA was assessed by the stereological method. Results (Fig. 2) showed that there were no significant differences between the groups' mPFC volume (*t*-test, $p = 0.3$, $t = 1.04$, $df = 14$, $n = 6$); however, the MeA volume in old groups significantly increased compared to the young group (*t*-test, $p = 0.0008$, $t = 4.97$,$df = 9$, $n = 6$).

3.3. The total number of neuron and non-neuron cells

The data obtained through stereological methods showed no considerable differences in the total number of neuronal and non-neuronal cells between the young and old rats within the mPFC region (*t*-test, $p = 0.8$, $t = 0.2$, $df = 10$, $n = 6$). However, the total number of neurons in the MeA region significantly increased in the old rats (*t*-test, $p = 0.001$, $t = 4.37$, $df = 11$, $n = 6$), while the number of non-neuronal cells did not show any age-related difference (Fig. 3).

3.4. Cell diameter

Data showed that the cell diameter of the soma in the mPFC significantly increased in the old animals in comparison with young ones (*t*-test, $p = 0.008$, $t = 2.795$, $df = 35$, $n = 20$ neurons/group) but, there was no difference in the cell size of the amygdala between the groups (Fig. 4).

3.5. Dendritic length and complexity

The number of dendritic intersections with software lines was assessed by Sholl analysis in the MeA neurons and mPFC pyramidal cells (Fig. 5). The dendritic complexity was evaluated in four different concentric regions of the dendritic tree from the center of the soma; ≤50, 50–100, 100–150, and 150−200 μm.

The number of intersections in the distal part of the dendrites in the mPFC (further than 100 μm from neuronal soma) significantly did not show the change between young and old animals. However, its proximal dendrite regions (≤100 μm far from the neuronal soma) showed a significant decrease in the old rats when compared with young ones (Mann-Whitney, $p < 0.0001$, $n = 30$ neurons/group, Fig. 6A). Also, the total dendritic length in the mPFC neurons in old rats significantly decreased when compared to young ones (Mann-Whitney, $p < 0.0001$, $n = 30$ neurons/group, Fig. 6C). As in the mPFC, there was no difference between the number of intersections in the distal parts of the dendrites in the MeA neurons, but interestingly, the intersection number in its

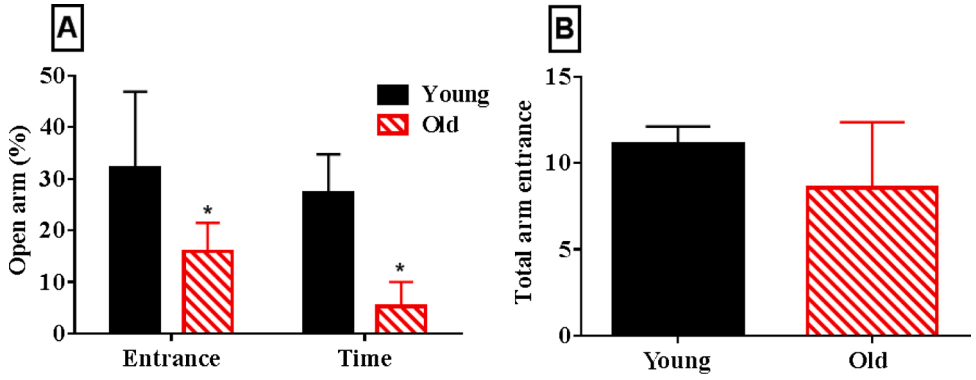

Fig. 1. A) Mean ± Standard deviation of the percentage of the entrance ($p = 0.01$) and time spent on open arms ($p < 0.0001$) decreased in young rats in comparison with old animals. B) Mean ± Standard deviation of the total arm entrance did not show a significant difference between the young and old rats.

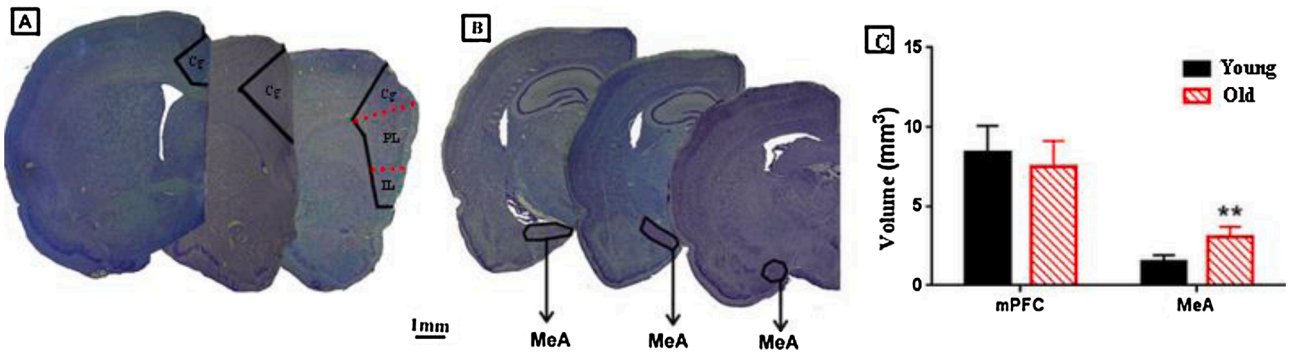

Fig. 2. Representative photograph of Cresyl Violet stained sections of (A) the cingulate (Cg), prelimbic (PL), and infralimbic (IL) parts of the medial prefrontal cortex (mPFC) and (B) medial nucleus of the amygdala (MeA); C) Mean ± Standard deviation of volume (mm^3) of the mPFC and MeA ($p = 0.0008$) in the young and old rats.

proximal 50 μm of the dendrite (in contrast to mPFC neurons) in old animals showed a significant increase in comparison with young animals (Mann-Whitney, $p < 0.0001$, $n = 30$ neurons/group, Fig. 6B). However, the total dendritic length of this region (in contrast to mPFC neurons) significantly increased in old animals when compared to the young one (Mann-Whitney, $p < 0.0001$, $n = 30$ neurons/group, Fig. 6D).

3.6. The dendritic spine density

The densities of all types of dendritic spines in the mPFC and MeA neurons were not significantly different between young and old rats (Tables 1,2). However, old animals had more mature dendritic spines (79 %) than young ones (60 %) in the mPFC (Table 1). Fig. 7 shows dendritic spine classification (A) and the representative dendrite of the pyramidal neurons in layer V of the mPFC (B).

3.7. The spatial distribution of the neurons

Voronoi tessellation of the mPFC and MeA neurons in the young and old groups is shown in Fig. 8. The data showed that 80 % of the polygons areas of the neurons in the mPFC region in the old rats were located in the range of 100−300 μm^2, while in the young animals, just 60 % of the polygons areas were located in this range. Furthermore, the old rats showed fewer neurons in the range of ≥400 μm^2 than young animals (Fig. 8A). Based on the coefficient of variation (CV) classification, the mean CV of polygon areas in both groups located in a random range (33 %–64 %), although old animals tended to have more regular distribution (34.5 %) than young rats (44.5 %) (Fig. 8B).

Regarding the MeA, about 70–80 % of the polygons areas of the neurons were located in the range of 100−300 μm^2 in both groups, however, polygons areas in the range ≤100 μm^2 in old animals are three times less than young animals and more neurons in the old rats are located in the range 300−400 μm^2 than young animals (Fig. 8C). The mean CV of polygon areas in both groups was located in a random range (33 %–64 %) and as in the mPFC, old rats had more regular distribution (39.5 %) than young ones (47.5 %) (Fig. 8D). Fig. 8(E–G) shows the representative spatial distribution of the neurons in the MeA.

4. Discussion

Aging leads to anatomical and physiological changes in various organs, especially the brain. These changes might have negative or positive effects on the emotional behaviors of older people. Therefore, it is supposed that age-related structural changes in various brain regions include mPFC and MeA, which are involved in anxiety mechanisms and might be associated with alterations in anxiety behaviors among aged rats. We have estimated changes in the volumes, cells' numbers and sizes, dendrite arborization and length, spine density, and spatial distribution of the neurons in both regions (mPFC, MeA) in young and old rats to achieve our goal, and EPM was used to evaluate the changes of anxiety behavior.

Behavioral evaluation by EPM in the present study showed that percentages of the entrance and the time spent on the open arm in older rats considerably decreased compared to the young rats. These two parameters have been reported as reliable criteria to measure anxiety in recent rodent studies (Walf and Frye, 2007). According to our EPM results, it seems that the anxiety level among old rats has increased. In line with this study, Imhof et al. observed a decrease in the number of entries and time spent on open arms (below 50 %) in the older Wistar rats than the younger ones (Imhof et al., 1993). Frussa-Filho et al. that have also used elevated plus-maze (and other anxiety model apertures) in two groups of 5-month and 20−24-month male Wistar rats showed an increase in anxiety behavior with aging (Frussa-Filho et al., 1991). However, Shoji and Miyakawa that have applied EPM on 2 to 25-month C57BL/6 J mice, did not report any significant impacts of aging on the percentages of the entries and time spent on open arms and anxiety (Shoji and Miyakawa, 2019). It should emphasize that various methodological differences include age, strain, or sex and number of the samples, and housing or testing conditions might lead to obtaining inconsistent findings in the EPM (Andrade et al., 2003; Nagy and Glaser, 1970).

Although aging leads to the reduction of mPFC volume, it increased this parameter in the amygdala (MeA). This finding is almost in agreement with previous studies. For example, Rubinow and Juraska showed that the volume of the basolateral nucleus of the amygdala increased between periods of adulthood and old age (Rubinow and Juraska, 2009). Several investigations studied human beings; for instance, Grieve et al. (2002) concluded that the frontal cortex's volume decreased, whereas it was preserved in the amygdala with aging (Grieve et al., 2005). Although the volume of a brain region gives rather unspecific information about the function of that region, it may indicate that structural changes of functional relevance take place in that specific area and thus, encourages more detailed studies of selected areas of interest. Changes in the volume of the area could be attributed to an alteration in the size or number of neurons (or glia) or both of them, as well as the dendritic length and its arborization.

The current study did not illustrate any considerable changes in the total number of the neuron or non- neuron cells in the mPFC region between the young and old rats. However, the total number of neurons in the older rats significantly increased in the MeA region. Consequently, this might partly be one reason for the volume increase in this area. In line with this study, Stranahan et al. also did not observe a considerable change in the total number of neurons in the ventral part of PFC (Stranahan et al., 2012). In the amygdala region, in agreement with the present results, the stereological study in 52 human brains also showed that the number of neurons in the amygdala of typically developing humans increased from youth to adulthood (Avino et al., 2018). Rubinow and Juraska also showed that the volume of the basolateral region

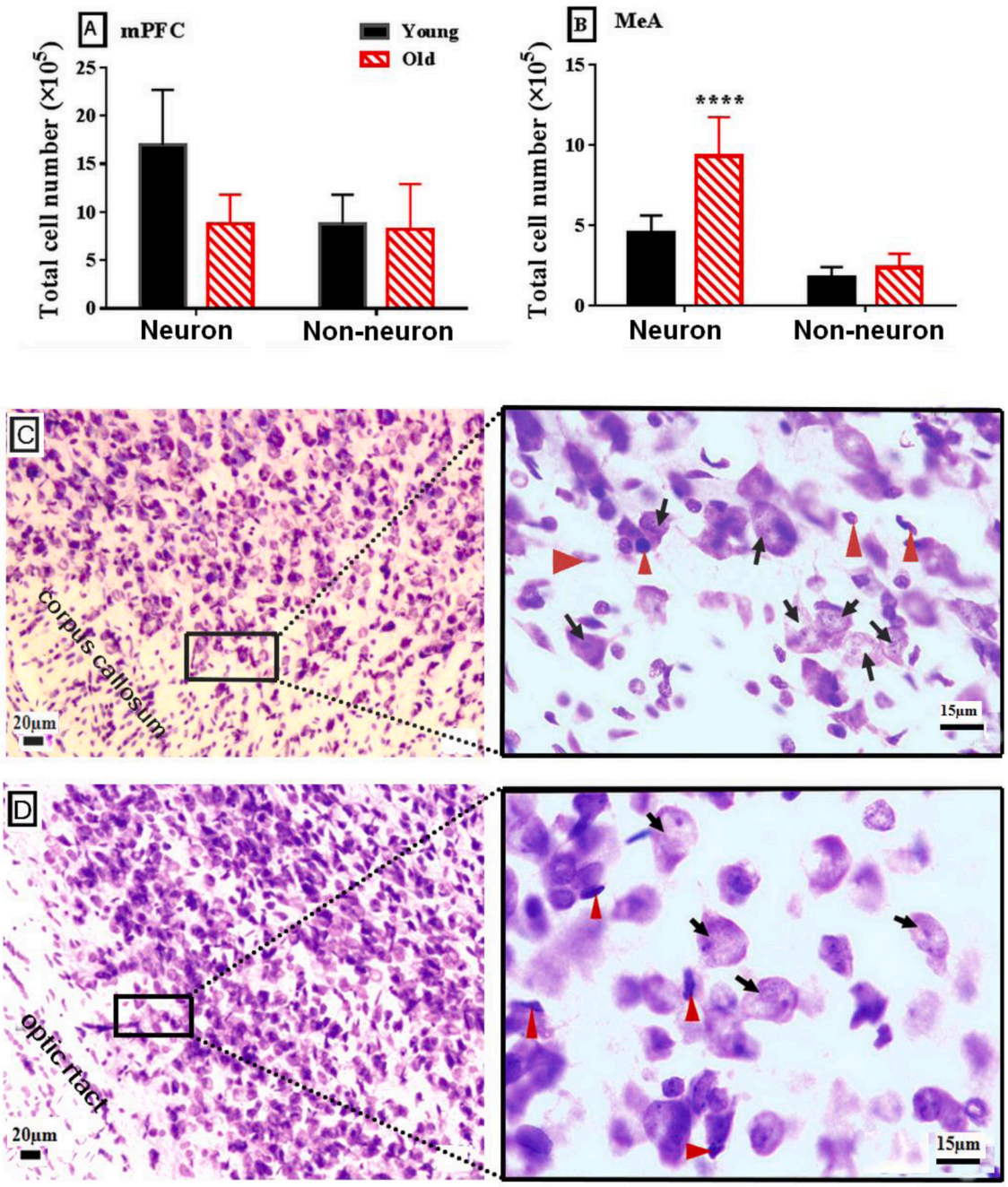

Fig. 3. Mean ± Standard deviation of the total number of neuron and non–neuron cells in the medial prefrontal cortex (mPFC, A) and medial nucleus of the amygdala (MeA, B) in the young and old male rats. Representative photograph of the Cresyl Violet stained section shows the neurons (black arrows) and non-neurons (red arrowhead) in the mPFC (C) and MeA (D) regions. **** Significant difference ($p < 0.001$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

of the amygdala and its glia number increased with aging; however, the number of neurons remained stable (Rubinow and Juraska, 2009).

The stereological assessment of the cell body size of the pyramidal neurons within layer V of mPFC showed significantly larger neurons among old rats than younger ones. In agreement with this finding, Finch (1993) also concluded that rhesus monkeys and human beings generally show hypertrophy in the forebrain neurons with aging. He described that the neuron hypertrophy of normal humans was progressive up to the age of 60 years throughout this structure. He also described neuron hypertrophy for rats, in various age groups (Finch, 1993). It has been reported that physiologic hypertrophy is usually adaptive and leads to functional improvement (King, 2006). More studies are necessary to elucidate the mechanisms of hypertrophy in the mentioned cell.

Regarding MeA, the size of the neurons was not different between young and old rats. To the best of our knowledge, Berdel et al. (1997) have just carried out an investigation that was in line with the current study and reported no changes in the size of neurons within the amygdala during postnatal periods (Berdel et al., 1996).

Sholl analysis was applied for the pyramidal neurons of layer V in the mPFC and neuron of the MeA region to determine the dendrite complexity and total dendritic length. Our results showed that aging led to the reduction of both total dendritic length and dendrite complexity in its proximal parts (≤50 µm distance from the neuronal soma) of the mPFC region (Fig. 6A, C); In contrast, these parameters increased in the

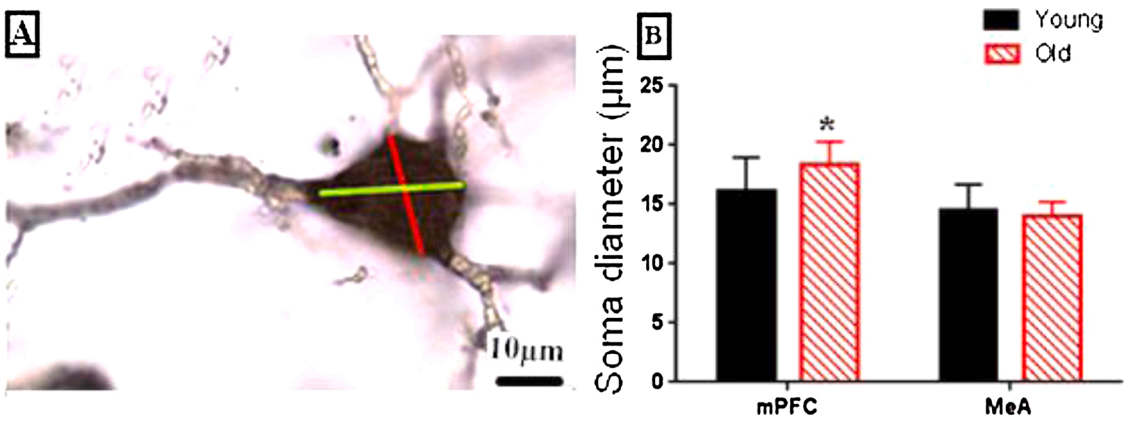

Fig. 4. A) Representative Golgi- Cox stained neurons of the medial prefrontal cortex (mPFC) and size estimation method. B) Mean ± Standard deviation of the average diameter of the pyramidal neurons (µm) in the layer V of mPFC ($p = 0.008$) and neurons of the medial nucleus of the amygdala (MeA) in the experimental groups.

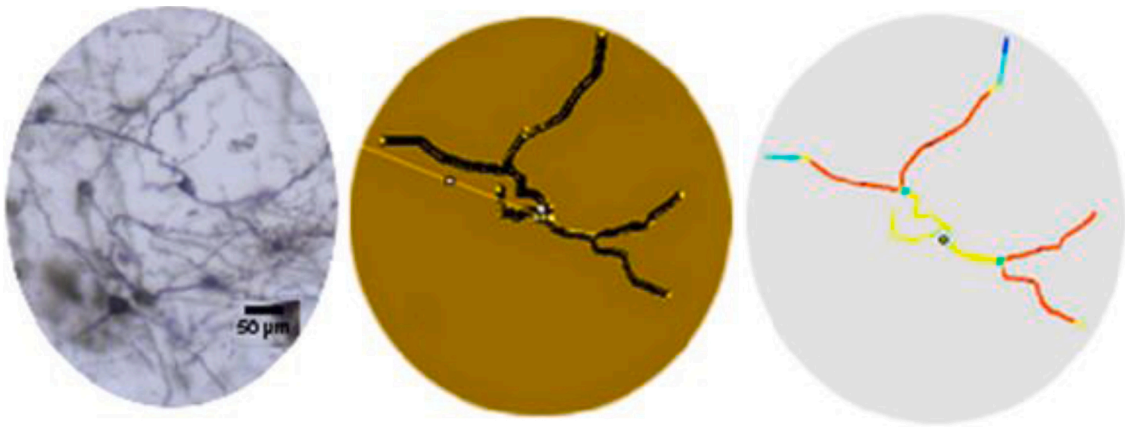

Fig. 5. Representative skeletonized neuron in the Sholl analysis with the Image J program: the number of intersections made by dendritic material every 10 µm away from the cell body was counted using concentric spheres; warmer hues (red) indicate higher and coldest hues (blue) the lowest number of the intersection. The average of total dendrites length was estimated by counting 4.57 ± 0.85 primary dendritic branches per neuron in the young and 4.36 ± 0.67 in the old animals in the mPFC and 2.78 ± 0.42 and 2.85 ± 0.66 primary dendritic branches per neuron in the MeA for the young and old rats, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

MeA with aging (Fig. 6B, D). In other words, the nueons of old rats within mPFC are associated with shorter dendritic length and probably fewer primary dendritic branches in comparison with young rats. Koss et al. also reported a reduction in dendritic materials of the mPFC neurons' basilar tree from 35 to 90-day females rats (Koss et al., 2014). In contrast, Markham et al. have observed an increase in the dendritic materials of the prefrontal cortex between young (20-day) and old (90-day) rats (Markham et al., 2013). This contradictory finding can be attributed to the difference in the age of animals or the selected layer of the neurons within the mPFC (layer III and 3rd month in their study and layer V and 18th month in the present study).

Considering MeA, old rats have longer dendritic length and more primary branches. Previous studies also showed an increase in dendritic arborization within the amygdala among old rats (Rubinow et al., 2009). These changes in dendritic morphology might represent more plasticity within the amygdala of aged individuals. In contrast, Zehr et al. have reported that the number of cell body-emanated dendrites become pruned during puberty in the terminal dendritic spines (Zehr et al., 2006). First, Zehr et al. have used Syrian hamsters in the puberty period, while Rubinow et al. (Rubinow et al., 2009) and the present study have studied much older rats. Second, their reports are regarding terminal dendrites and our findings are related to the proximal dendrites (Fig, 6B). It should be noted that, at least here, there is not sufficient evidence regarding the way aging changes the dendrite length or complexity.

Changes in the dendritic length of these two regions among aged animals might have resulted from alterations in the density or type of dendritic spines and,consequently, the synaptic plasticity. Our findings imply that aging did not significantly change the total number of all types of dendrite spines in the neurons of the amygdala and mPFC; however, old rats showed more mature spines in mPFC neurons. Bloss et al. (2011) have described a report that was highly in agreement with our findings; they claimed that the number of dendritic spines within mPFC remains stable during the aging procedure (Bloss et al., 2011). However, Markham et al. demonstrated a considerable reduction in the spine density of the dendrites among the rats' mPFC during the aging process (Markham and Juraska, 2002). As mentioned above, this difference might be due to differences in age and animal strain or layer of study in the PFC.

To the best of our knowledge, there was not any study regarding MeA and aging in the rats; however, in line with the present study, Rubinow and Juraska (2009) described an age-related increase in the dendritic material of the principal neurons in the rat basolateral amygdala (BLA) (Rubinow and Juraska, 2009). It is worth noting that the increase in both dendritic complexity and neurons' total number (not the size of soma) within MeA might be considered as the reason for increase in the volume of this structure in old animals. Regarding mPFC, our results showed that the mPFC in the old rats had larger neurons and more mature spines than young ones that may indicate compensatory action of the neurons

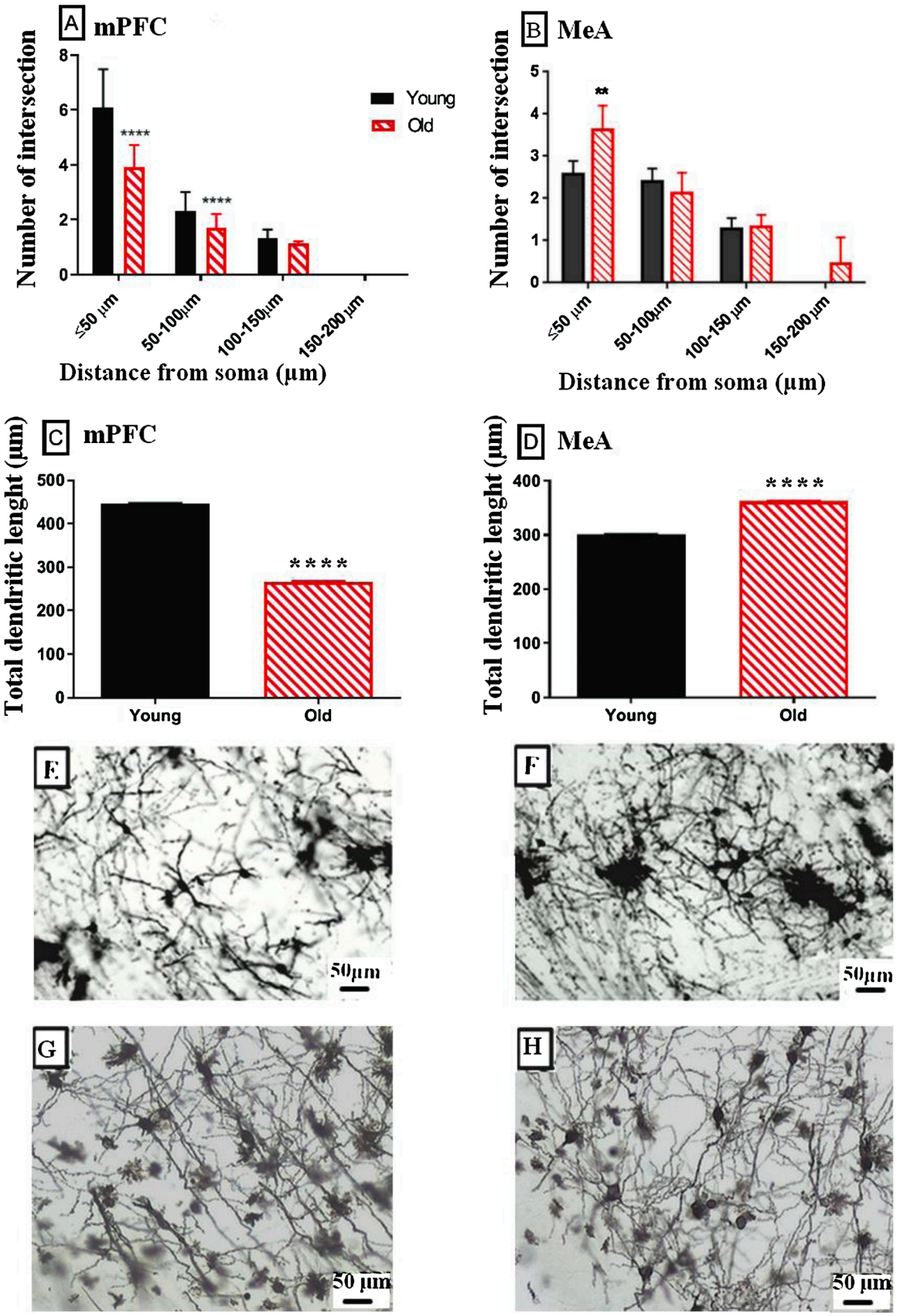

Fig. 6. Intersection number (Mean ± Standard deviation) in different dendrite distances from the soma (≤50, 50-100, 100-150, and 150-200 µm) in the medial prefrontal cortex (mPFC, $p < 0.0001$, A) and the medial nucleus of the amygdala (MeA, $p < 0.0001$, B). Mean ± Standard deviation of total dendritic length (µm) in the neurons of mPFC (C, $p < 0.0001$) and MeA (D, $p < 0.0001$). Representative Golgi-Cox-stained photographs show dendrite arborization of the neurons in the MeA area (E, young; F, old) and the mPFC (G, young; H, old) of male rats.

Table 1
Mean ± Standard deviation of dendritic spine density (spine/10 μm on the distal end of 2nd or 3rd order branches of the one dendrite) in the 30 neurons within the medial prefrontal cortex in the young and old male rats.

Type of spine	Young	Old
Mushroom	0.46 ± 0.91	0.75 ± 1.35
Stubby	3.93 ± 1.83	5.16 ± 2.29
Branched	0.0	0.0
Thin	2.46 ± 2.99	1.25 ± 2.29
Filopodia	0.46 ± 1.06	0.25 ± 0.45
Total	7.33 ± 2.38	7.417 ± 1.50
Mature (%)	60	79

Table 2
Mean ± Standard deviation of dendritic spine density (spine/10 μm on the distal end of 2nd or 3rd order branch of the one dendrite) in the 30 neurons within the medial nucleus of the amygdala in the young and old male rats.

Type of spine	Young	Old
Mushroom	2.53 ± 1.61	2.23 ± 1.48
Stubby	4.84 ± 3.26	5.61 ± 2.43
Branched	0.0	0.0
Thin	3.84 ± 1.99	4.38 ± 2.75
Filopodia	1.76 ± 1.83	1.16 ± 0.83
Total	13 ± 6.75	13.46 ± 4.15
Mature (%)	56	61

for cognitive decline and better function (Markham and Juraska, 2002), and support for more connection in this area. Old rats' amygdala might receive more projections from mPFC, which is due to more dendritic branches in order to control their emotions; however, this hypothesis has to be clarified by future studies.

Although changes in the size and number of neurons or their dendrites and spines, and the volume of these areas have significant impacts, the spatial distribution of the neurons might also play a considerable role in their connectivity and functions. Therefore, the spatial arrangement of mPFC and MeA neurons in young and old animals was carried out for the first time in the current study by using the Voronoi tessellation test. Our Voronoi tessellation findings showed that neurons within both mPFC and MeA areas were randomly distributed among old and young male rats. It was also indicated that the neuronal distribution of both areas among aged animals tended to change into the regular arrangement. However, we could not find any study that has evaluated the spatial arrangement of the neurons within the two mentioned regions under healthy or pathologic conditions.

Sarkala et al. reported that there was a regular spatial arrangement of CA1 hippocampal pyramidal neurons among healthy rats; however, it changed into a random pattern after the brain ischemia (Sarkala et al., 2019). Although, it is not clear how the changes of the neurons' spatial distribution within mPFC and amygdala regions affect the connectivity and function, the distribution pattern of cell bodies and neuritis can lead to the mechanical forces and indirectly reflects the functional changes in these regions (Friedl and Gilmour, 2009).

Finally, we evaluated the correlation of anxiety (open arm entrance and time percentage) with the structural parameters: volume, cell number, CV of Voronoi tessellation and dendritic length. Pearson results showed that there was a positive correlation between the anxiety level in EPM and neuronal cell number ($p = 0.04$, $r = 0.81$) and CV of Voronoi tessellation ($p = 0.05$, $r = 0.9$) in the MeA area.

In summary, it is evidenced by several studies that aging is associated with deleterious effects on the structure and functions of different brain areas including mPFC and MeA. First, the majority of studies and reports have focused on one brain region, while there are various brain regions involved in one function that should be simultaneously investigated. Changes in one region's parameters might not be necessarily associated with similar alterations within another area (such as data regarding mPFC and MeA in the current study). Second, it seems that old individuals might represent more anxiety behaviors than young ones; however, this parameter could be due to various structural changes

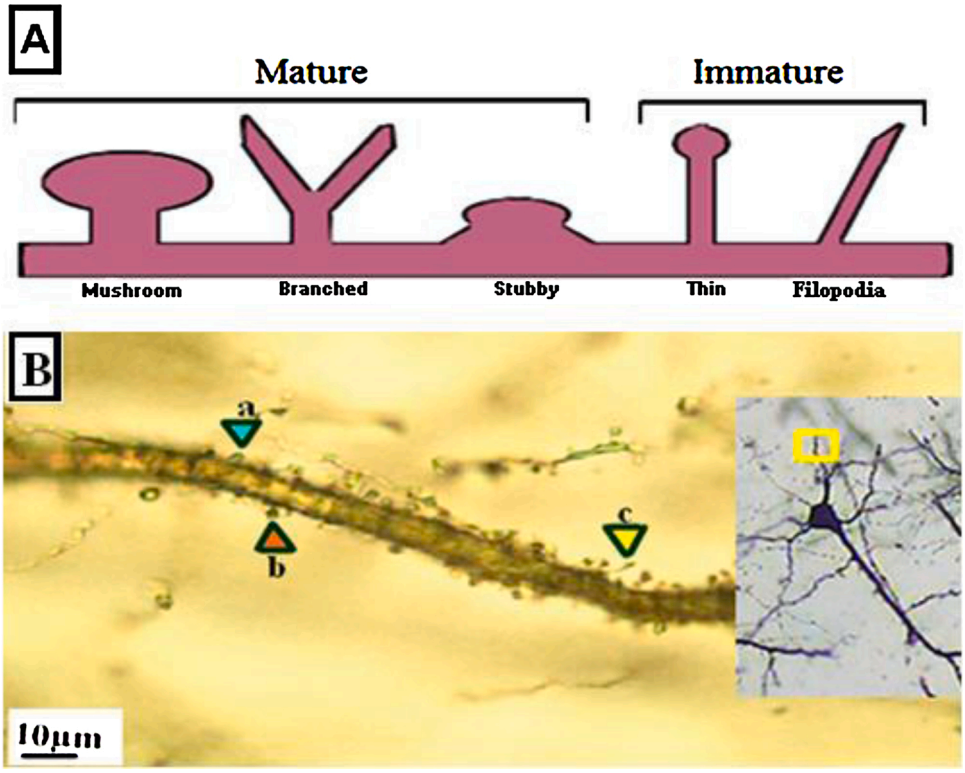

Fig. 7. A) Classification of dendritic spines (Mature and Immature). B) Representative photograph of mushroom (b), stubby (a), and thin (c) dendritic spines on the distal end of the 2nd or 3rd order branch of dendrite on the medial prefrontal cortex.

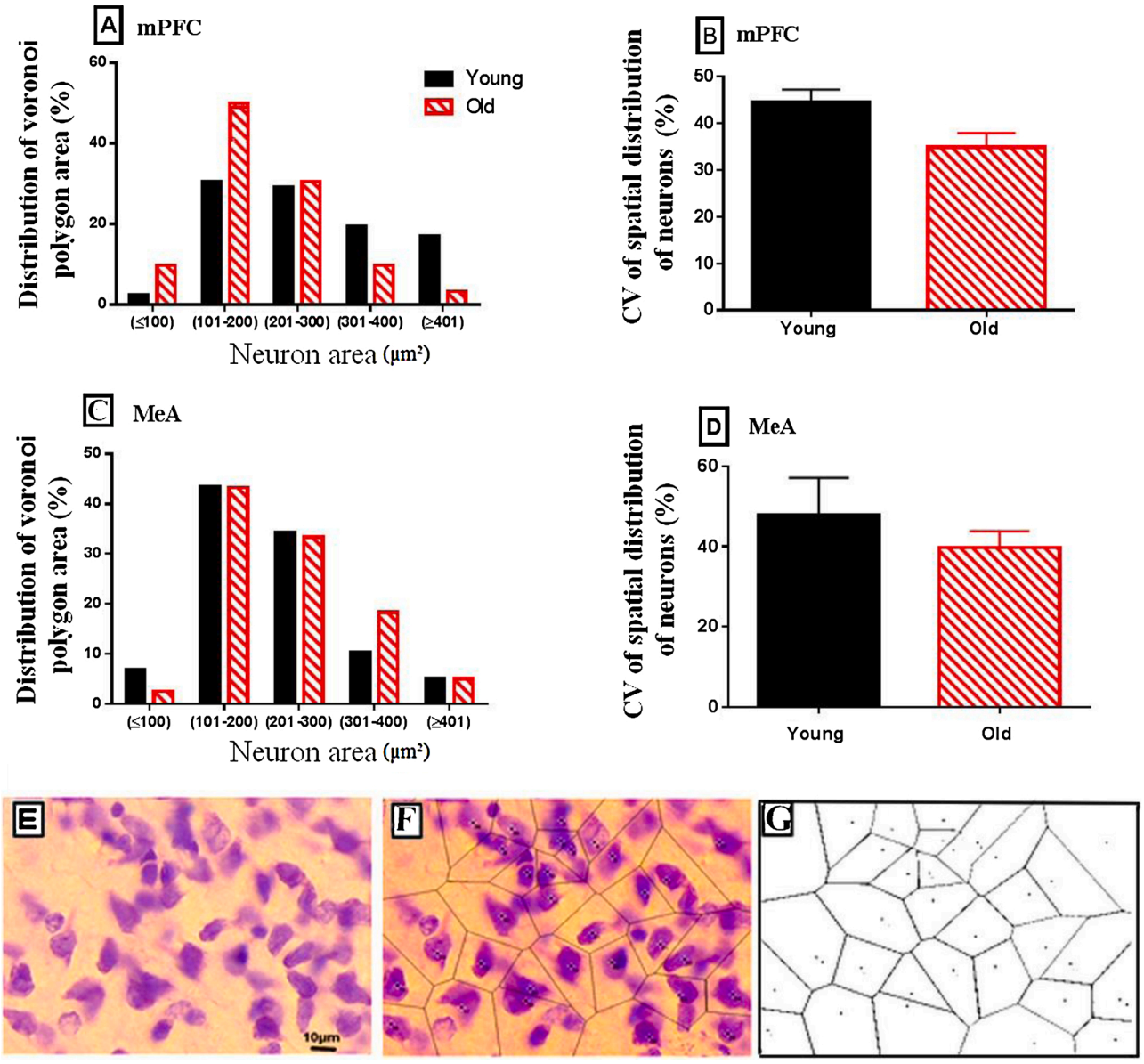

Fig. 8. Mean ± Standard deviation of Voronoi polygon area (%) and Coefficient of Variation (CV) within the medial prefrontal cortex (mPFC, A, B) and medial nucleus of the amygdala (MeA, C, D). Representative photograph, polygon area, and schematic of Voronoi tessellation of the neuron in the MeA (E, F, and G, respectively).

including the size, number, or dendrite complexity of the neurons within these two regions. It is proposed in the current study that the structural changes in mPFC and MeA regions among the mentioned rats could be related to this behavioral change; however, more investigations have to be carried out to clarify this hypothesis.

Ethics statement

All procedures in this experiment were performed by the Care and Use of Laboratory Animals (National Academy Press, 1996, Washington, USA) and approved by the Ethics Committee of Shiraz University of Medical Sciences (SUMS, Shiraz, Iran, Ethic code: IR.SUMS. REC.1397.77).

CRediT authorship contribution statement

N. Sotoudeh: Conceptualization, Formal analysis, Investigation, Methodology, Software, Writing - original draft. **M.R. Namavar:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing. **A. Zarifkar:** Conceptualization, Project administration, Resources, Writing - review & editing. **A.R. Heidarzadegan:** Investigation, Methodology.

Conflicts of interest

The authors declare that they do not have any known competing financial interest or personal relationshipthat could have appeared to influence the work reported in this paper.

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