



# Dentate gyrus neurogenesis across different ages in male rats: an immunohistochemical approach

Ayman M. Ghallab<sup>1,2</sup> · Zeinab M. Alazouny<sup>1</sup> · Mai A. Samak<sup>1</sup> · Haidy G. Abdel Malek<sup>1</sup>

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## Abstract

Dentate gyrus is a fundamental sub-region of the hippocampus which is directly engaged in higher memory and cognitive functions. This study was performed to describe the histological and immunohistochemical changes in dentate gyrus in experimental animals during postnatal development. Forty four male albino rats were classified into four equal groups: new born group aged one day, adult group aged 3–6 months, early senile group aged 18–20 months and late senile group aged 30–31 months. Specimens of hippocampus were processed and prepared for routine hematoxylin and eosin stains and immunohistochemical expressions of calretinin, glial fibrillary acidic protein and Ki67 (Kiel 67). Morphometric data were statistically analyzed. The present results showed significant reduction in thickness of granule cell layer of late senile group. Interestingly, the mean number of immature neurons was significantly increased in early senile group, while it was significantly reduced in late senile group. The number of mature granule cells showed marked reduction in both early and late senile groups. Furthermore, the number of astrocytes and optical density of glial fibrillary acidic protein revealed significant age-dependent increase. Measurable difference in number of calretinin positive interneurons was detected between adult and senile groups. However, mean number of Ki67 immune positive nuclei expressed significant age-dependent reduction. This study concluded that interneurons play a substantial role in modulating dentate gyrus neurogenesis which occurs throughout life and steadily decreases during aging. It is recommended to focus on the different stimuli and factors that potentiate neurogenesis to prevent or treat cognitive deficiencies associated with aging.

**Keywords** Dentate gyrus · Calretinin · Aging · Neurogenesis, immunohistochemistry

## Abbreviations

Ki67	Kiel 67
DG	dentate gyrus
GFAP	glial fibrillary acidic protein
PBS	phosphate buffered saline
LSD	least significant difference
GCL	granule cell layer
SGZ	subgranular zone
GABA	gamma aminobutyric acid
H&E	hematoxylin and eosin

## Introduction

Hippocampal neurogenesis is believed to play an important role in modulating and maintaining learning, memory, and potentially other cognitive functions (Zhao et al. 2008). The proliferative capacity of adult neurogenesis in all mammals decreases with age however the timing of this and the extent to which it occurs in the human brain remains the subject of significant debate (Capilla-Gonzalez et al. 2015). The hippocampal formation is defined as the complex of six structures; hippocampus proprius, dentate gyrus (DG), presubiculum, parasubiculum, subiculum proprium and area entorhinal cortex (EC) (Andersen et al. 2000). Dentate gyrus is a trilaminar cortical structure with a characteristic U or V shape. It is a deep region within hippocampus and is surrounded by cornu ammonis. It has two blades: suprapyramidal which is the granule cell layer of DG laid between cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1) and the portion opposite this is the

✉ Ayman M. Ghallab  
aymanghallab@yahoo.com

<sup>1</sup> Department of Histology and Cell Biology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

<sup>2</sup> British University in Egypt (BUE), Cairo, Egypt

infrapyramidal blade. The region bridging the two blades (at the apex of V or U) is the crest (Andersen et al. 2007).

Neurogenesis in the adult DG of the hippocampus occurs constitutively throughout postnatal life, and the rate of neurogenesis within the DG can be modified under different physiological and pathophysiological conditions. Adult neurogenesis includes the process in which the division of a precursor cell occurs and the multi-step process (proliferation, differentiation, migration, targeting, and synaptic integration) which ends with the formation of a postmitotic functionally integrated new neuron (von Bohlen Und Halbach 2007). The progenitor cells lied in the subgranular zone of the DG and give rise to young neurons that can be integrated into existing neuronal circuits (Dokter and von Bohlen und Halbach 2012). Aging process is usually accompanied by cognitive decline and structural alterations in brain areas such as cortex and hippocampus which leads to morbidity and mortality (Bertoldi et al. 2017). Moreover, aging is the greatest risk factor for Alzheimer's disease which characterized by progressive memory loss and cognitive decline. Hippocampal circuitry is essentially vulnerable to aging and neurodegenerative conditions (Chohan et al. 2011). Dentate gyrus has represented a paramount focus of scientific interest during past few decades. However, there was a paucity of researches about its cytological contributions in aging process. Therefore, this study was conducted to through more light on the histological and immunohistochemical changes in the DG in male albino rats during postnatal development and aging and correlates these changes to interneuronal modulating functions.

## Materials and methods

### Experimental animals

Forty four male Wistar albino rats were divided according to age into four equal groups (eleven rats each). Group I (new born group): rats aged 1 day old weighing 10–50 g. Group II (adult group): rats aged 3–6 months weighing 150–200 g. Group III (early senile group): rats aged 18–20 months weighing 250–350 g. Group IV (late senile group): rats aged 30–31 months weighing 200–300 g (Buckland et al. 2013).

The animals were obtained from the breeding animal house, Faculty of Medicine, Zagazig University. They were housed in a room illuminated for 12 h daily by daylight at room temperature, fed standard balanced diet and allowed water ad libitum (Suckow et al. 2006).

Experimental procedures: Upon reaching target age experimental animals (in accordance with guidelines of Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, Zagazig University) were anaesthetized using ether inhalation. When they became completely anesthetized, the

vault of each animal cranium was rapidly removed and the cerebral cortex was exposed. Brains were carefully dissected out, divided via coronal sections into two cerebral hemispheres according to rat brain atlas (Paxinos et al. 2012) then fixed in 10% neutral formal saline. Samples were divided into two sets to be processed for both histological and immunohistochemical studies (Seidler et al. 2002).

### Histological study

Specimens were dehydrated, embedded in paraffin wax, cut into 5  $\mu$ m sections with a rotary microtome (Leica RM 2025; Nassloch, Germany). Sections were encompassing the entire anterior–posterior axis of the hippocampus (10–12 sections) (Wolf et al. 2002). At least two different series of sections were examined in each specimen (hippocampus). Sections were mounted on glass slides then stained with hematoxylin and eosin (Bancroft and Gamble 2008).

### Immunohistochemical study

Immunohistochemical study was carried out applying the peroxidase labeled streptavidin Biotin technique for detection of glial fibrillary acidic protein (GFAP) as a marker for astrocytes (Larsson et al. 2004), calretinin as a marker for identification of interneurons (Gulyás et al. 1996; Huusko et al. 2015; Pelkey et al. 2017) and Kiel 67 (Ki67) for proliferating cells (Kim et al. 2009). Selected Paraffin sections (from –2.5 to –4.5 from Bregma) (Paxinos et al. 2012) were deparaffinized, rehydrated and washed in phosphate buffered saline (PBS) for 3–5 min.; peroxidase activity was quenched using 3% H<sub>2</sub>O<sub>2</sub> for 5 min and the sections were then rinsed with PBS for 15 min. Sections were blocked with 1.5% normal goat serum in PBS and were then incubated for 45 min at room temperature with the primary antibody. Sections were subsequently incubated with a second- stage biotinylated antibody (biotin-conjugated goat anti- rabbit immunoglobulin G (IgG), 1:200, 1 h, at room temperature). After rinsing in PBS, the reaction products were visualized by immersing the sections into the chromogen diaminobenzidine. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. For the negative control, the same steps were followed, but the primary antibody was replaced with PBS (Kiernan 2015; Polak and Van Noorden 2003). The primary antibodies used in the study were: (1) Monoclonal mouse anti-glial fibrillary acidic protein (anti-GFAP) delivered from Sigma Laboratories Ltd. (Catalog number MA5–12023), (2) Calretinin rabbit polyclonal antibody of Immunoglobulin G (IgG) type. Anti-calretinin antibody delivered from GeneTex Company (GeneTex, Inc. North America). (Catalog number GTX103261) and (3) Ki67 rabbit monoclonal antibody delivered from Dako Laboratories (UK) (Catalog number 275-

R17). Universal kits used the avidin biotin peroxidase system produced by Novacastra Laboratories Ltd. (UK).

**Morphometric study**

Quantitative morphometric measurements were achieved by using the Leica Qwin 500 Image analyzer (England) computer system at Image Analysis Unit, Department of Histology, Faculty of Medicine, Zagazig University. The thickness of the dentate granule cell layer, the number of dentate mature granule cells, immature cells and astrocytes, Optical density of GFAP were quantitatively assessed by the image analysis of three different high power fields (X400). The quantification occurred at level of granule cell layer of DG. Cells were quantified from coronal sections according to rat brain atlas (Paxinos et al. 2012). Counting the numbers of calretinin positive cells and Ki67 positive cells were quantitatively assessed by the image analysis of three different high power fields (X200). The numbers of immune positive cells were counted as a proportion of the total number of cells. The thickness of dentate granule cell layer was calculated and calibration was performed before each measurement by using the semiautomatic morphometric software program (Digitizer, version 2). The thickness of the granule cell layer was measured in micrometers. All measurements were tabulated in excel sheet where the mean values of each measurement in each case are calculated.

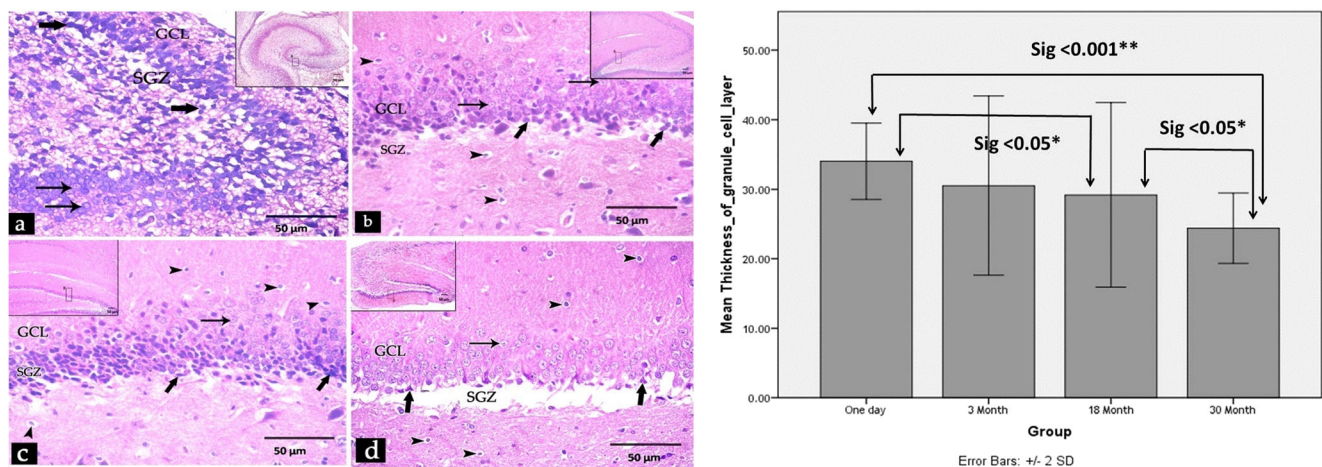
Statistical analysis of the results was expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare normally distributed variables in more than two groups. Post-hoc least significant difference (LSD) test was used according to homogeneity of variances.

The tests were two sided.  $P < 0.01$  was considered statistically significant and  $p \geq 0.05$  was considered statistically non-significant. All data were analyzed using Statistical Package for Social Science (SPSS) 18.0 for windows (SPSS Inc., Chicago, IL, USA) & MedCalc 13 for windows (MedCalc Software bvba).

**Results**

**Histological and morphometric results (Fig. 1)**

Hematoxylin and eosin (H&E) stained sections of new born group (I) revealed dentate gyrus (DG) of the hippocampus consisted of molecular layer, granule cell layer (GCL) and polymorphic cell layer. The granule cell layer (GCL) contained many compactly arranged mature granule cells with rounded pale vesicular nuclei. Many neurons with oval dark nuclei and paucity of cytoplasm were detected in subgranular zone (SGZ) (Fig. 1a). Sections of adult group (II) stained with H&E showed the dentate gyrus GCL with many compactly arranged granule cells. Few glial cells with darkly stained nuclei surrounded by lightly stained space were also detected (Fig. 1b). Sections of early senile group (III) stained with H&E showed the dentate gyrus GCL with granule cells with rounded pale vesicular nuclei and many cells with dark nuclei and scanty cytoplasm were seen in SGZ (Fig. 1c). Sections of late senile group (IV) stained with H&E revealed thin dentate gyrus GCL that contained few granule cells, edematous SGZ and numerous glial cells with darkly stained nuclei were also observed (Fig. 1d).



**Fig. 1** A higher magnification of the boxed area in inset showing GCL (arrow) containing many mature granule cells with rounded pale vesicular nuclei (new born and adult). Multiple neurons (thick arrows) with oval dark nuclei and paucity of cytoplasm in SGZ (a & b). Few granule cells in senile and late senile groups (c & d). Numerous neurons with oval dark

nuclei and scanty cytoplasm (c) are also noticed, however, few ones are observed in late senile group (d). Glial cells with darkly stained nuclei surrounded by lightly stained space (arrow head) are few in adult (b) and numerous in senile groups (c & d) [H&E, Scale Bar = 50 µm] and the thickness of granule cell layer in the different studied groups

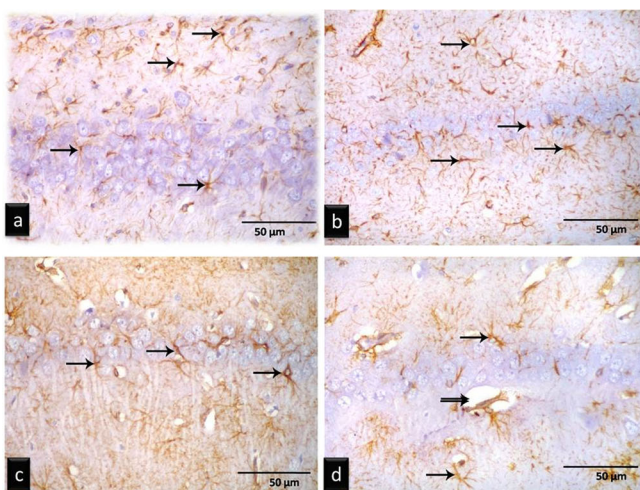
**Morphometric results** The mean values of the granule cell layer thickness were  $24.3982 \pm 2.54132 \mu\text{m}$  in the new born group,  $29.1889 \pm 6.64713 \mu\text{m}$  in the adult group,  $34.0255 \pm 2.73913 \mu\text{m}$  in the early senile group and  $30.5227 \pm 6.44952 \mu\text{m}$  in the late senile group. Statistical analysis of these results revealed that there was statistically significant decrease in the thickness of granule cell layer (GCL) of the late senile group (IV) compared to the new born group (I) as  $P$  value  $<0.001$ . On the other hand, results showed no significant difference between group (I) and group (II) and non-significant difference between group (II) and group (III). Post-hoc test comparisons using LSD (least significant difference) test indicated that the mean of GCL thickness in new born and adult groups was significantly different from late senile group. However, the mean of GCL thickness in early senile group was significantly different from the mean of new born and late senile groups.

### Immunohistochemical and morphometric results

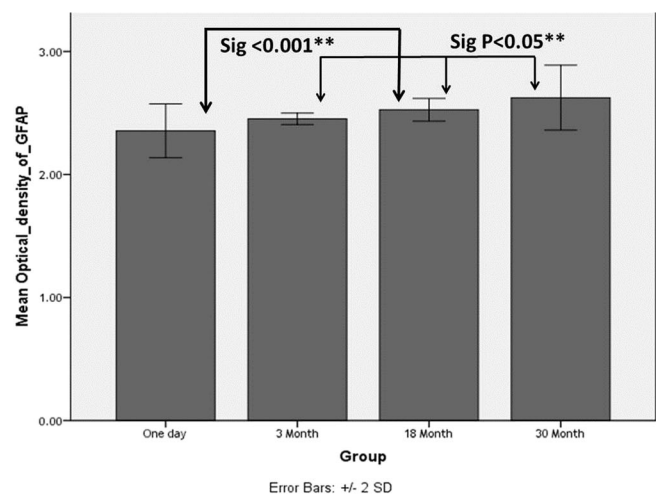
**Immunohistochemical and morphometric results for GFAP (Fig. 2)** Immuno peroxidase technique for GFAP showed astrocytes with positive reaction. Astrocytes appeared as small bodies with few short thin processes in new born group (I) (Fig. 2a). They appeared larger in size with multiple thin processes in adult group (II) (Fig. 2b). In early senile group (III) multiple strong positive astrocytes appeared larger in size with many thick processes (Fig. 2c). Similar findings were detected in late senile group (IV) with dilated perivascular space (Fig. 2d). Morphometric results: mean values of the optical density of GFAP were  $2.4511 \pm 0.00935$  in the new born group;  $2.4400 \pm 0.01549$  in the adult group;  $2.4745 \pm$

$0.01864$  in the early senile group and  $2.4601 \pm 0.00806$  in the late senile group. Statistical analysis of these results revealed that there was statistically significant increase in the mean of optical density of GFAP towards the late senile group (IV) compared to the new born group (I) as  $P$  value  $<0.001$ . While there was no statistically significant difference between new born group (I) and late senile group (IV). Post-hoc test comparisons using LSD test indicated that the mean number of optical density of GFAP of new born group was statistically significant different from early senile group. Also the adult group its mean was statistically significant different from older age groups. While early senile group was significantly different from all age groups.

**Immunohistochemical and morphometric results for calretinin (Fig. 3)** Immunohistochemical staining for calretinin showed strong positive immune reaction in numerous interneurons of newborn group (I) (Fig. 3a). Strong positive immune reaction was expressed in many interneurons with large nerve cell body and prominent dendrite in adult group (II) (Fig. 3b). However, few immuno-positive interneurons were noticed in early senile group (III) (Fig. 3c). Few positive immune reactions for calretinin appeared in interneurons in late senile group (IV). Some of interneurons appeared with cytoplasmic reaction, while others appeared with cytoplasmic and nuclear reaction (Fig. 3d). Morphometric results: mean number of immune positive interneurons was  $7.1618 \pm 1.75149$  in the new born group;  $2.6764 \pm 0.61331$  in the adult group;  $1.9169 \pm 0.23464$  in the early senile group and  $1.1645 \pm 0.10053$  in the late senile group. Statistical analysis of these results revealed that there was statistically significant decrease in the mean of the number of immune positive cells of



**Fig. 2** Immuno-reactivity of GFAP (arrow heads) showing astrocytes with small bodies and few short thin processes in new born group (a). Astrocytes appear larger in size with multiple thin processes in adult group (b). Multiple astrocytes appear larger in size with multiple thick



processes in early & late senile groups (c & d). [Immuno peroxidase technique for GFAP, Scale Bar =  $50 \mu\text{m}$ ] and mean values of Optical density of GFAP for astrocytes

calretinin as a proportion of the total number of cells in adult, early senile and late senile groups (II, III, IV) compared to the new born group (I) as  $P$  value  $<0.001$ . However, non-significant difference was detected between early and late senile groups. Post-hoc test comparisons using LSD test indicated that the mean of the number of immune positive cells of calretinin as a proportion of the total number of cells in each age group was statistically significant different from other age group one.

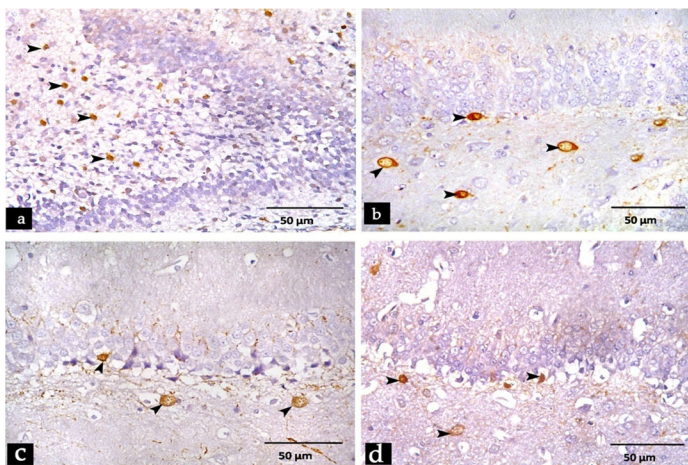
**Immunohistochemical and morphometric results for Ki67 (Fig. 4)** Immunohistochemical staining for Ki67 showed positive immune reaction in numerous nuclei of DG of new born group (I) (Fig. 4a). Adult group (II) showed positive immune reaction for Ki67 in many nuclei of DG (Fig. 4b). Early senile group (III) showed positive immune reaction for Ki67 in few nuclei of DG (Fig. 4c). Late senile group (IV) showed positive immune reaction for Ki67 in few nuclei of DG (Fig. 4d). Morphometric results: mean number of immune positive nuclei for Ki67 was  $57.5100 \pm 8.14101$  in the new born group;  $69.5455 \pm 10.87533$  in the adult group;  $74.0909 \pm 6.84769$  in the early senile group and  $18.1818 \pm 3.60051$  in the late senile group. Statistical analysis of these results revealed that there was statistically significant decrease in the mean of the number of immune positive nuclei of Ki67 as a proportion of the total number of cells in the late senile group (IV) compared to the new born group (I) as  $P$  value  $<0.001$ . Post-hoc test comparisons using LSD test indicated that the mean of the number of immune positive nuclei of Ki67 as a proportion of the total number of cells in the new born and adult group was statistically significant different from older age groups (early and late senile). While the mean number of immuno-positive nuclei count of Ki67 was statistically significant different from all other age groups.

**Mean number of dentate granule cells, immature cells and astrocytes (Fig. 5 & Table 1)**

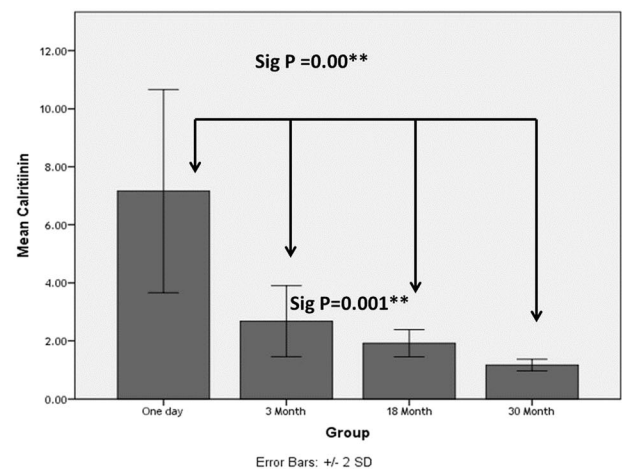
There was statistically significant decrease in the mean number of mature granule cells from the new born group (I) towards the adult and late senile groups (II&IV) as  $P$  value  $<0.001$ . There was statistically significant decrease in the mean number of immature granule cells from the new born group (I) towards the late senile group (IV) as ( $P$  value  $<0.001$ ). Also, there was statistically significant increase between the adult group (II) and early senile group (III) ( $P$  value  $<0.001$ ). Statistically significant increase in the mean number of astrocytes cell count was reported the new born group (I) towards the adult, early senile and late senile groups (II, III, IV) ( $P$  value  $<0.001$ ).

**Discussion**

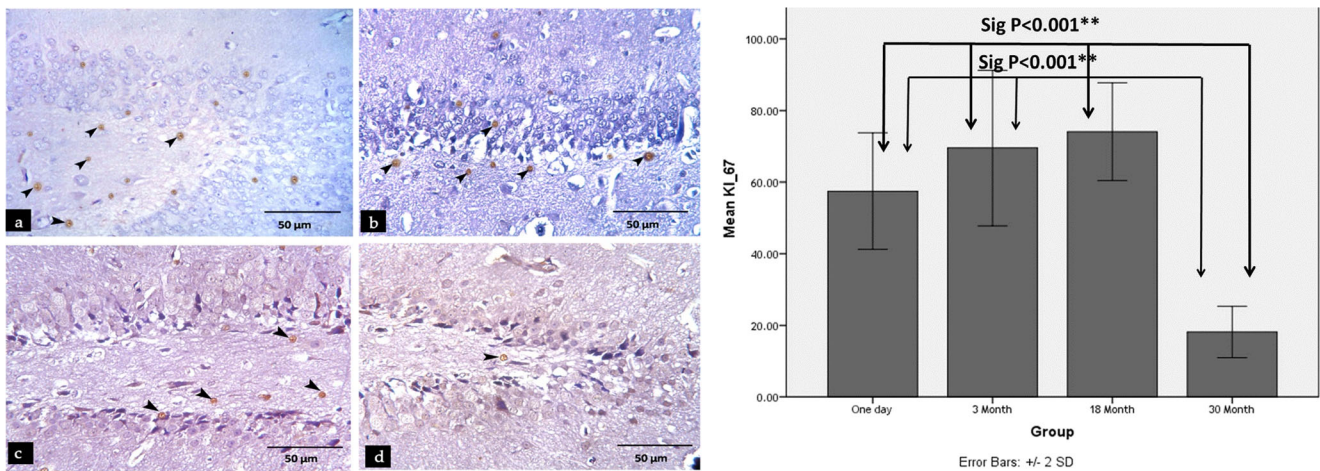
Neurogenesis in the postnatal human brain occurs in two neurogenic niches; the subventricular zone (SVZ) in the wall of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus. The extent to which this physiological process continues into adulthood is an area of ongoing research (Dennis et al. 2016). Subgranular zone of the hippocampus contains the microenvironment that is permissive for neuronal development to occur. This microenvironment is called the neurogenic “niche.” The niche comprises the precursor cells themselves, their immediate progeny and immature neurons, other glial cells and endothelia, very likely immune cells, microglia, and macrophages, and an extracellular matrix (Mercier et al. 2002). This study was conducted to through more light on dentate gyrus neuro-cytomorphological and



**Fig. 3** Immuno-reactivity of calretinin (arrow heads) is strong positive in (a) newborn group and (b) adult group, few positive in (c) early senile group and (d) late senile group. [Immuno peroxidase technique for



calretinin, Scale Bar = 50 µm] and mean number of immuno-positive interneurons for calretinin in the different studied groups



**Fig. 4** Immuno-expression of Ki67 (arrow heads) is positive immune reaction in numerous nuclei of DG in (a) new born group and (b) adult group, is positive immune reaction in few nuclei of DG in (c) Early senile

group and (d) Late senile group. [Immunoperoxidase technique for Ki67, Scale Bar = 50  $\mu$ m] and mean number of immuno-positive nuclei for Ki67 count in the different studied groups

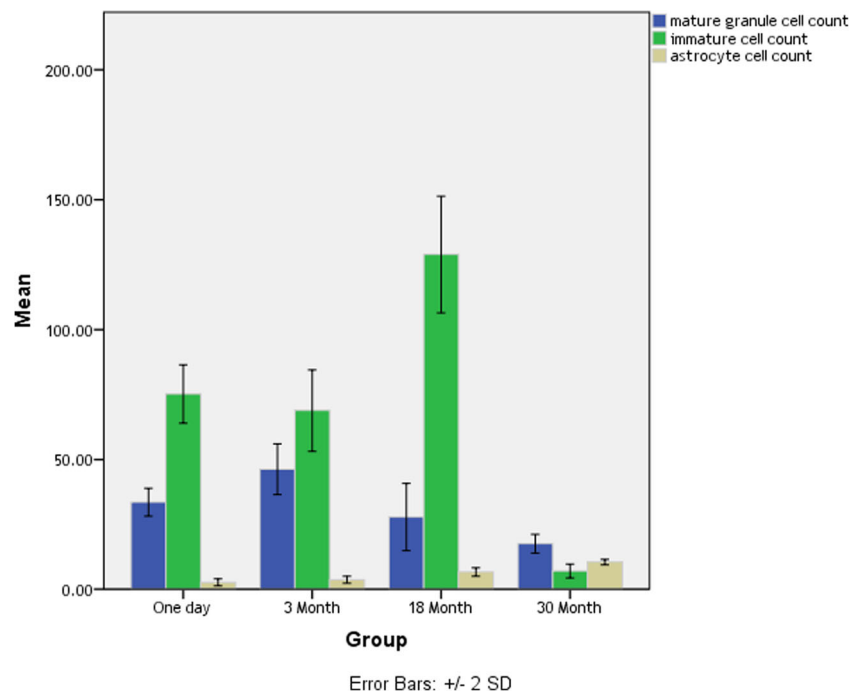
immunohistochemical alterations during postnatal development and to evaluate correlated interneuronal modulating functions.

The present work revealed that new born group (I) dentate gyrus H&E stained sections consisted of molecular layer, granule cell layer and the polymorphic layer forming upper blade and lower blade. It occupied the area between Cornu ammonis 3 (CA3) and the Para-hippocampal gyrus. It had a toothed shape and the hippocampal sulcus appeared as a deep groove in adult (II), early senile (III) and late senile (IV) groups. However, in late senile group (IV) the subgranular zone appeared bullous. These findings were in agreement with Amaral et al. (2007) & Witter (2007). Basically in agreement with Cummings et al. (2005) & Seri et al. (2001) the present results revealed that granule cell layer of DG contained compactly arranged mature granule cells with rounded pale vesicular nuclei. The present work noticed that SGZ contained oval shaped neurons with darkly stained nuclei and paucity of cytoplasm; Kempermann et al. (2004) described them as an early stage of immature neurons. Llorens-Martín et al. (2016) confirmed that these maturational stages are identified on the basis not only of the expression of specific molecular markers but also of their unique morphological features. Chohan et al. (2011) proved that immature granule cells are unique neuro-precursor cells that maintain mitotic activity in adulthood; they ultimately differentiate into mature granule neurons with rounded pale vesicular nuclei and pale acidophilic cytoplasm. Statistically significant decrease in GCL thickness was evident in late senile group (IV) compared to the new born group (I), this was in line with Wisse et al. (2014). Varela-Nallar et al. (2010) explained this with age dependent loss of afferents from the entorhinal cortex, reduced rates of precursor cells

proliferation and reactive oxygen species (ROS) theory of aging. This research investigated differential cell count of GCL that confirmed this interpretation. Interestingly, the present observation showed that early senile group (III) revealed significant increase in the number of immature neurons in the SGZ. Subsequently, this explains the non-significant difference in GCL thickness between adult and early senile groups. Li et al. (2008) clarified that compensatory increase of immature cell proliferation rate accompany neurodegenerative disorders as Alzheimer's disease. However, the newly generated neurons in dentate gyrus do not differentiate to mature neurons.

Astrocytes are critical for neuronal survival in central nervous system; as they regulate energy metabolism and maintain blood-brain barrier (Dringen et al. 2000; Zonta et al. 2003). Examination of H&E stained sections of early and late senile groups revealed observable increase of astrocytes, the present statistical data confirmed these findings. Astrocytes appeared with small rounded nuclei surrounded by lightly stained cytoplasm in agreement with Vinters and Kleinschmidt-DeMasters (2018). Borlongan et al. (2000) described this result as reactive gliosis or astrogliosis which is characterized by compensatory accelerated synthesis of GFAP. Rodríguez-Arellano et al. (2016) added that reactive astrogliosis is associated with dystrophic neurite plaques. Glial fibrillary acidic protein (GFAP) is the principal intermediate filament in mature astrocytes. It is thought to be important in modulating astrocyte motility and shape by providing structural stability to astrocytic processes (von Bohlen Und Halbach 2007). Immunohistochemical stained sections revealed increase of GFAP optical density in astrocytes in late senile group (IV) compared to new born group (I). In addition, increase in num-

**Fig. 5** Quantification of dentate gyrus cells counts in the different studied groups



ber and size of astrocytes in their GFAP-immunoreactivity with astrocytic foot processes edema around the blood vessels with dilated perivascular spaces were observed in late senile group (IV). These findings were in agreement with Larsson et al. (2004) and Xiaoli et al. (2006).

Using Ki67 as a marker for proliferating cells proved significant reliability as it is expressed during mitosis and has a short half-life. Kim et al. (2009) stated that Ki67 is a good marker for detecting proliferating cells during neurogenesis of the adult brain. In the current study, examination of immunohistochemical stained sections for Ki67 revealed that positive nuclei were abundant in the new born group, decreased in the adult one, reached low values in the early senile group then almost disappeared in late senile group. The present study confirmed this finding as statistically significant decrease in number of immune positive nuclei of Ki67 in the late senile group was reported compared to the new born group. This work observed that Ki67 immuno-reactive cells were dispersed in almost all of the layers of dentate gyrus with more concentration in the SGZ. These observations were in agreement with Ambrogini et al. (2004); Liu et al. (2000) who proved that granular cells neurogenesis is pronounced within the first three postnatal weeks and continues into adulthood at a reduced rate. They added that, after proliferation of neural precursor cells within the SGZ, newborn cells migrate toward the GCL. The recent work of Boldrini et al. (2018) proved that ongoing hippocampal neurogenesis is mandatory to sustain higher memory and cognitive functions. Ki67 positive nuclei observed in this study were similar to the nuclei of granule

cells in size and shape. This suggests that these nuclei may be destined to become granule cells. Skilling et al. (2017) stated that neurogenesis related circuits modifications could steer granule cells integration into dentate gyrus-neocortex networks.

Calretinin is a marker for specific non-pyramidal  $\gamma$ -aminobutyric acid (GABA)-ergic interneurons within hippocampus (Brandt et al. 2003). We observed that mean number of calretinin immuno-reactive cells revealed significant decrease in adult, early senile and late senile groups (II, III, and IV) compared to the new born group (I). These findings were reported by Andrews-Zwilling et al. (2010) who also stated that the population of CR-expressing cells within the granule cell layer of DG have not been characterized in sufficient detail. Takahashi et al. (2010) clarified that calretinin is a marker of various cell lines in the dentate gyrus: hilar mossy cells and interneurons of SGZ. However, calretinin-positive interneurons are expressed in all layers of hippocampal zones, including areas Cornu ammonis 1-Cornuammonis3 (CA1-CA3) and the DG (Brandt et al. 2003). Somogyi and Klausberger (2005) reported that axons of interneurons in the Cornu ammonis 1 (CA1) region of the hippocampus traverse the hippocampal sulcus and also innervate the dentate gyrus. de Guevara-Miranda et al. (2017) suggested that hippocampal interneurons are directly engaged in regulation of adult hippocampal neurogenesis based on their proximity to adult neural stem cells in the SGZ. Most hippocampal interneurons share a common characteristic they are GABAergic, producing and releasing this largely inhibitory

**Table 1** Comparison between mature granule, immature and astrocyte cells counts in different studied groups

Variable	Group I	Group II	Group III	Group IV	Significance
Mature granule cell count	33.5455 ± 2.69680	46.2727 ± 4.90083	27.8182 ± 6.47793	17.5455 ± 1.80907	0.00**
Immature cell count	75.1818 ± 5.61815	68.8182 ± 7.83349	128.9091 ± 11.22902	7.0000 ± 1.34164	0.00**
Astrocyte count	2.6364 ± .67420	3.6364 ± .67420	6.6364 ± .80904	10.4545 ± .52223	0.00**

\*\* : Significant ( $p < 0.001$ )

neurotransmitter (Maccaferri and Lacaille 2003). During early postnatal development, newly generated neurons follow a sequence of events through which they form their synaptic connections (Ben-Ari 2002). Initially the neurons are “silent”, meaning that they have no spontaneous postsynaptic currents to the commonly applied agents e.g. GABA. Zhao et al. (2006) reported that upon the formation of GABAergic synapses they become sensitive to depolarization by GABA, followed by the development of glutamatergic inputs, and lastly a switch to hyperpolarization by GABA, a sign of neuronal maturity. Adult-generated neurons in SGZ pass a similar progression steps to initiate synaptic connectivity with hippocampal neuronal circuit (Espósito et al. 2005). Ables et al. (2010) added that GABAergic interneurons regulate the slow proliferation of putative neural stem cells which are the source cells of adult neurogenesis. Furthermore, Deisseroth et al. (2004) highlighted that as adult-generated cells differentiate into mature dentate gyrus granule cells; they not only respond to GABA but also receive GABAergic inputs. This input is important as it depolarizes the maturing cells and elevates their intracellular  $Ca^{2+}$  levels via activation of voltage-gated calcium channels. GABAergic interneurons have the power to regulate the differentiation of adult-generated DG cells. However, with this complex integration of interneurons into the hippocampal circuitry and proposed interneuron regulation of synaptic activity in the hippocampus, interneurons have received little attention in regard to how they affect the neurogenic niche and the generation of new neurons in the adult brain (Houser 2007). The biological mechanisms responsible for brain neuronal aging are not yet fully investigated;  $Ca^{2+}$  is widely believed to have a role in the process. Schwaller (2014) stated that  $Ca^{2+}$  homeostasis control appears to be a fundamental property of healthy neurons, so the  $Ca^{2+}$  binding proteins such as calretinin are of considerable importance. Although calretinin emerges as a multi-functional protein it is also associated with development i.e., cell proliferation and differentiation. Masiulis et al. (2011) suggested that various signaling factors regulate interneuron GABAergic activity and thus adult-neurogenesis in an activity-dependent manner. Herrup (2010) confirmed the interplay between interneurons and hippocampal neurogenesis. This research can suggest that there is a bidirectional crosstalk between the age-induced decline in interneurons and the decline in adult neurogenesis. The present study concludes that

interneurons play a substantial role in modulating dentate gyrus neurogenesis which occurs throughout life and steadily decreases during aging. It is recommended to focus on the different stimuli and factors that potentiate neurogenesis to prevent or treat cognitive deficiencies associated with aging.

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### Compliance with ethical standards

**Conflict of interest** The authors declare no conflicts of interest.

**Ethical approval** All procedures performed in the present study involving experimental animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, Zagazig University, Egypt.

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