

Investigating of Brain Development in Rat Fetuses, Newborns and Adults

Remzi Yiğiter*, Osman Nuri Keleş†, Atakan Yücel‡, Yıldırım Kalkan†, İsmail Can§, Mustafa Güleç‡, Nermin Yücel||, Ünsal Aydınöğlü‡, Bünyami Ünal†

ABSTRACT

The brain is a part of the central nervous system that has drawn the attention of humankind since antiquity. Although there are thousands of studies on the brain, its mysteries have not been fully understood. Neurogenesis has a critical role in the development of the brain. In the current study, we aimed to examine neurogenesis (cell proliferation) by histological and stereological methods in rat brains, as well as the possible effects of age and sex. In our histological assessment, cortical layers were clearly shown and cortex-medulla boundaries were distinguished easily from each other in all groups. The findings obtained from the current study showed that neurogenesis of neurons in rat brains begins to take shape in the early stages of pregnancy and in the organized structure of the neocortex after birth. The primary and secondary thickenings of the cerebral cortex were determined between 1- and 5-week periods, especially in the intrauterine 20th-daygroup and the postnatal 1-week-old groups. These results suggest that adult neurogenesis may also occur in regions within the brain and that the postnatal neurogenesis capacity of neural precursors in the neocortex continues to increase under suitable conditions depending on age.

Key Words: brain, embryology, neurogenesis, cerebral cortex, stereology

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1. Introduction

The brain begins developing with a rapid multiplication of cells within the front end of the neural tube. With the closing of the neural tube, some changes occur within its walls. The neural tube walls consist of a single layer at first, and then the walls of the cells grow and become multilayered as the cells expand in number. Cell division occurs in the zone of proliferation, toward the innermost region of the neural tube. Dividing cells migrate outward, far and near, grabbing onto

something and then pulling the rest of the cell body along. Migrating neurons are led to their final destinations by a complex array of molecular signals and mechanisms. When arriving at their destinations, neurons adjust themselves within specific tissue locations. They undergo distinct changes in structure and molecular composition as they prepare to form synaptic connections within the rapidly growing nervous system (Schatz, 1992; Gross, 2000). Embryonic development of the brain has a critical stage of neurogenesis; neuronal precursors positioned in the primitive ectoderm of the neural tube and the neural crest, able to differentiate in response to neural-inducing signals, migrate and form proscribed functional circuits, which creates the central and peripheral nervous systems, respectively. In the adult brain, on the other hand, the capacity for neurogenesis is dramatically limited (Altman, 1969; Kaplan and Hinds, 1977; van Praag *et al.*, 2002).

Corresponding author: Atakan Yücel, MD., Departments of Psychiatry, Medical Faculty of Ataturk University, Erzurum, Turkey.

Address: *Departments of Neurology, Medical Faculty of Gaziantep University, Gaziantep; Turkey. †Departments of Histology, Medical Faculty of Ataturk University, Erzurum; Turkey. ‡Departments of Psychiatry, Medical Faculty of Ataturk University, Erzurum; Turkey. §Departments of Histology, Veterinary Faculty of Ataturk University, Erzurum; Turkey. ||Departments of Child Psychiatry, Medical Faculty of Ataturk University, Erzurum; Turkey.

Phone: +90 442 231 74 10

Fax: +90 536 624 51 52

✉ dr_atakanyucel@hotmail.com

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The investigation of nervous system mechanisms in prenatal and postnatal development helps us understand the basic principles of developmental abnormalities. For decades, the belief was that adult brain cells were not able to generate new nerve cells because they simply lacked the capacity. Nowadays, with the increasing number of neuroscience studies, this neurogenesis is better understood in the adult brain and particularly for newly generated cells in the neurogenic zones (Morshead and van der Kooy, 1992; Cameron and McKay, 2001).

Since the presentation of the dissector principle (Gundersen, 1986), stereological techniques have been used for scientific studies. These techniques include simple and efficient instruction in estimating quantities of three-dimensional (3D) unbiased objects from two-dimensional (2D) images (Serarslan *et al.*, 2009). A number of methods can be used to estimate numerical density, volume, and total number of neurons in a considered area. In this study, we used stereological methods, which comprise a serial tool for quantifying the features of 3D objects from their 2D sections through an object (such as microscopic slides of a solid specimen or consecutive *magnetic resonance imaging* sections) (Unal *et al.*, 2010). Stereological methods ensure that these estimates are unbiased, objective, reliable, and easy.

The aim of the present study was to evaluate possible changes in brain volumes among the intrauterine 16th-day group, intrauterine 20th-day group, newborn group, postnatal 1-week-old male group, 1-week-old female group, 5-week-old male group, and 5-week-old female group via histological methods and the Cavalier principle, which is a stereological estimating method.

2. Material and Methods

Thirty-five Sprague Dawley rats were used in the present study. They all were born and raised in the Ataturk University Experimental Research and Application Center, Erzurum, Turkey. The animals were divided into seven groups equal in number. Although our samples were classified, intrauterine and newborn samples were not divided by sex; only the 1- and 5-week-old postnatal groups were separated according to both age and sex. All animals were provided with a standard pelleted rat diet (Bayramoglu, Erzurum,

Turkey) and water ad libitum, and room temperature and humidity were preserved at 22–24°C and 60–65%. Lighting was provided with a 12-hour light and 12-hour dark automatically controlled photoperiod. Experiments for animal studies were realized according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. The protocol of this study was reviewed and approved by and conducted in accordance with the Institutional Animal Care and Use Committee of Ataturk University.

All animals were anesthetized with Sevorane® (Abbot; Istanbul, Turkey). After the anesthesia, the animals were intracardially perfused initially with 4% formaldehyde at room temperature. The brains were removed and stored in the same fixative overnight at 4°C. On the following day, the samples were put through a graded alcohol series and then embedded in paraffin. Suitable sections were cut at a thickness of 5 µm. From each block, sections were collected on slides for histological examination and stereological analysis. All sections were obtained from each block with sampling procedures for use in stereological analyses. Volumes and the number of neurons were estimated by the Cavalier principle using Stereo Investigator software (version 6.0, MicroBrightField; Colchester, VT). Stained sections were ready for review preparations and were examined under a light microscope, which was an Olympus BH-40 brand with manual camera attachment, and the necessary photographs were taken of the different growth.

A statistical program for statistical analysis estimated using a one-way ANOVA test was used in this study, followed by an LSD test using SPSS® 17.0 for Windows (SPSS, Inc., Chicago, IL, USA).

3. Results

Histological Findings

Results of the intrauterine 16th-day group

The cortex-medulla border was clearly identified in 2X magnification. So, aforementioned magnification was used to assess the section profiles. In the same cross-section images, brain membranes (meninges) were distinguished. However, it was observed as the normal subdural range under both the diameter and subarachnoid space above the piamater. Six layers of cortex were the first to



be distinguished from the others by layers in the cross-section of the brain (Fig. 1). In large magnifications, an array of many small and intense neurons could be seen in the external granular layer. There were no significant differences ($p < 0.05$) between the intrauterine 16th- and 20th-day groups in granular layer thickness. However, there were highly significant differences between the intrauterine groups and the newborn group (Table 1). According to the stereological and histological results of this study, non-neuronal structures were seen in the brain of the rat fetuses during the last pregnancy.

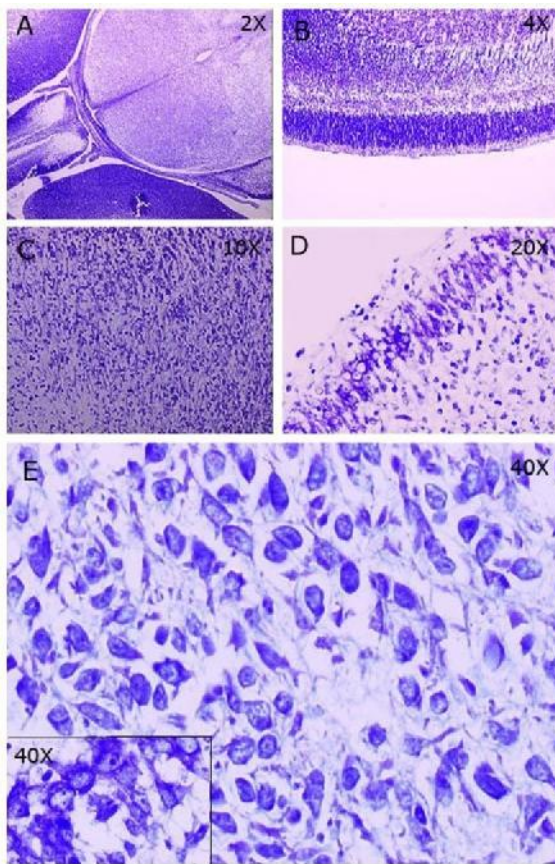


Figure 1. Light microscopic sections obtained from rats in the intrauterine 16th-day group. Staining: Cresyl Fast Violet.

Results of the intrauterine 20th-day group

The mature choroid plexus was observed in the intrauterine 20th-day group, and it was different from other groups in cross-sectional images. Higher magnifications revealed the number of glia cells as well as the number of neurons, which were big nucleus with euchromatic stained and central replacement (Fig. 2).

Table 1. Statistical comparison of the average volume values in rat brains from all groups

Paired Samples Statistics		
Comparisons-1	Significance (2-tailed)	P
IU 16 th day vs. IU 20 th day	0,080	P>0,05
IU 16 th day vs. NB	0,000	P<0,05
IU 16 th day vs. PN 1-week	0,000	P<0,05
IU 16 th day vs. PN 5-week	0,000	P<0,05
Comparisons-2		
IU 20 th day vs. NB	0,001	P<0,05
IU 20 th day vs. PN 1-week	0,001	P<0,05
IU 20 th day vs. PN 5-week	0,001	P<0,05
Comparisons-3		
NB vs. PN 1-week	0,202	P>0,05
NB vs. PN 5-week	0,001	P<0,05
Comparisons-4		
PN 1-week vs. PN 5-week	0,000	P<0,05

IU: Intrauterine, NB: Newborn, PN: Postnatal, SEM: Standard Error Mean, SD: Standard Deviation

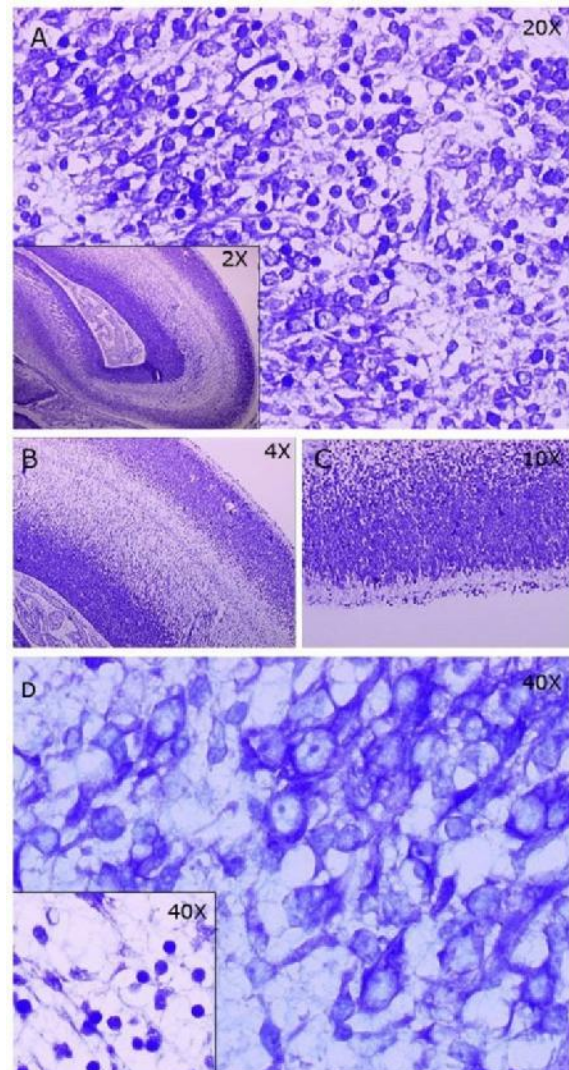


Figure 2. Light microscopic sections obtained from rats in the intrauterine 20th-day group. Staining: Cresyl Fast Violet.



Results of the newborn group

The newborn group was different from the intrauterine groups. We observed larger neurons and more thickness in the layers of the brain cortex (especially the ganglionic and external granular layers). In addition to these findings, the hippocampus was distinguished in this group from the first image (Fig. 3).

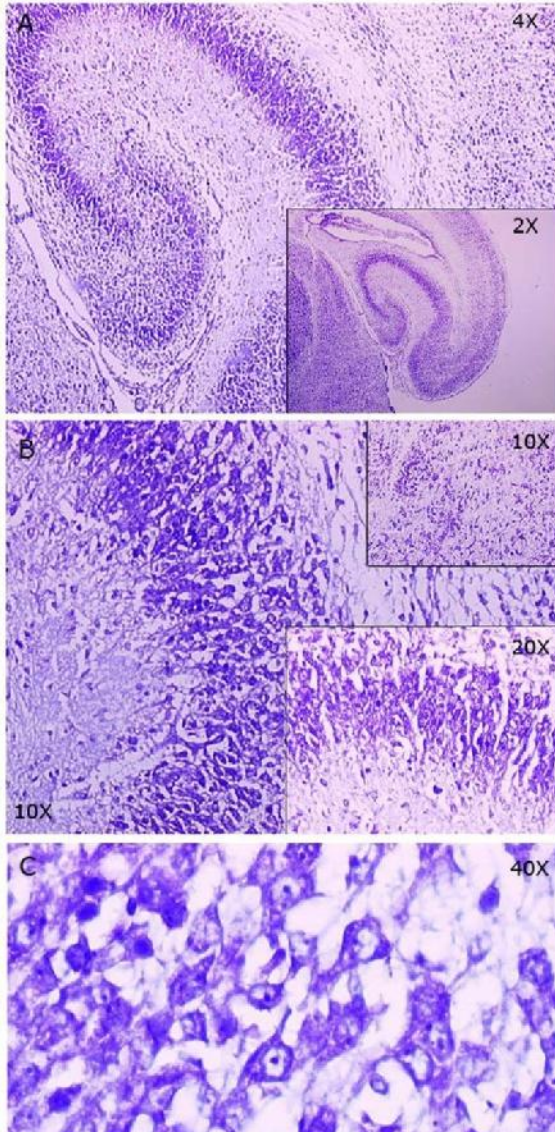


Figure 3. Light microscopic sections obtained from rats in the newborn group. Staining: Cresyl Fast Violet.

Results of the 1-week-old postnatal groups

The images of these groups and the newborn group were more similar to each other. Moreover, there were no significant differences determined between the female and male groups (Figs. 3, 4A and 4B).

Results of the 5-week-old postnatal groups

In these groups, the increased size of neurons and layer thickness were determined and showed differences from other groups. Furthermore, no significant differences were determined between the female and male groups (Figs. 5A, 5B).

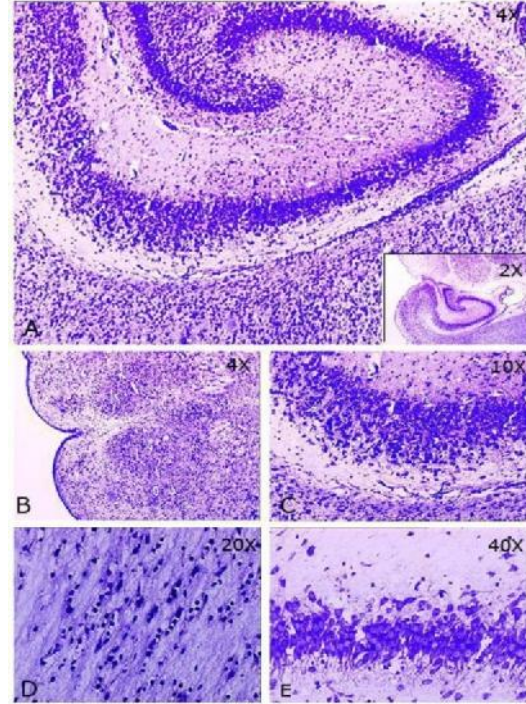


Figure 4A. Light microscopic sections obtained from male rats in the 1-week-old group. Staining: Cresyl Fast Violet.

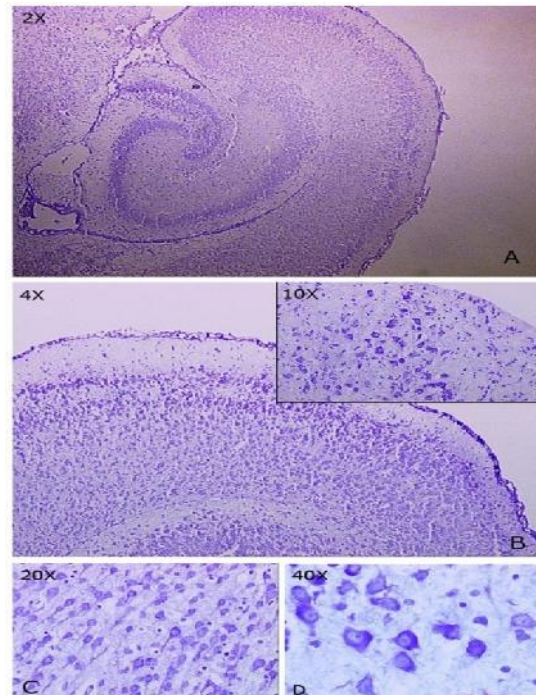


Figure 4B. Light microscopic sections obtained from female rats in the 1-week-old group. Staining: Cresyl Fast Violet.



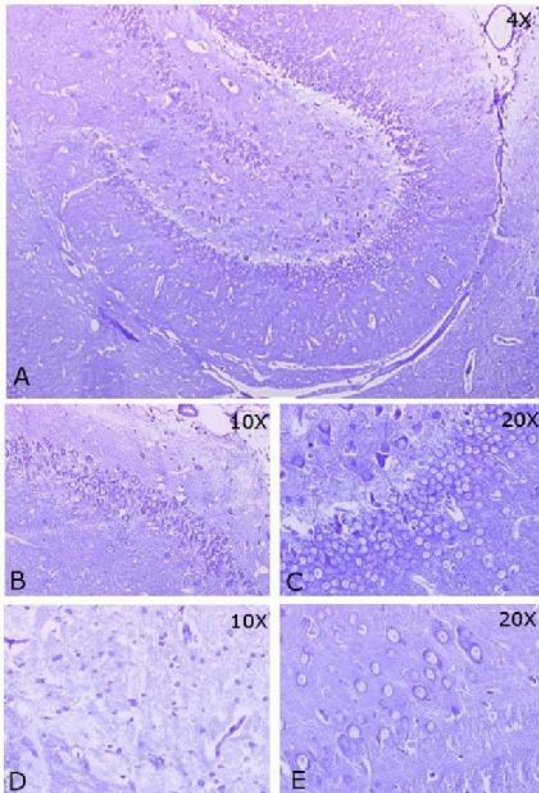


Figure 5A. Light microscopic sections obtained male rats in the 5-week-old group. Staining: Cresyl Fast Violet.

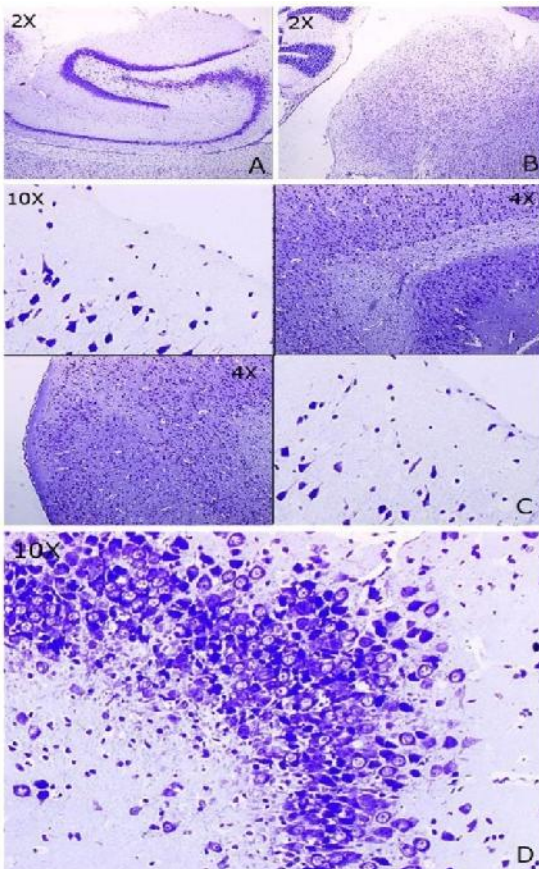


Figure 5B. Light microscopic sections obtained from female rats in the 5-week-old group. Staining: Cresyl Fast Violet.

Stereological Results

The average rat brain volumes in the intrauterine 16th- and 20th-day groups were measured as 32.4 and 38.6 mm³, respectively, by using the stereological estimating method. When compared statistically with the Independent Samples T-Test (two tailed, $\alpha=0.05$), there was no significant difference found between the two groups (Table 2, Fig. 6).

Table 2. Brain volume values

	IU	IU	NB	PN	PN	PN	PN
	16th day	20th day	0th day	1-w F	1-w M	5-w F	5-w M
Brain Volume (mm ³)	32,40	38,60	83,50	86,80	92,00	358,00	422,00
SEM	1,98	2,55	3,42	3,42	9,63	24,14	37,23
SD	4,40	5,70	7,70	7,60	21,50	53,90	83,30

IU: Intrauterine, NB: Newborn, PN: Postnatal, SEM: Standard Error Mean, SD: Standard Deviation, M: Male, F: Female, w: week.

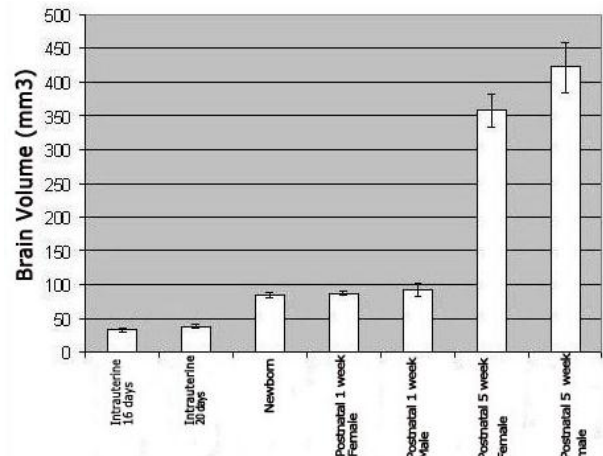


Figure 6. Brain volume graphic.

The average rat brain volumes of the newborn, 1-week-old post natal, and 5-week-old postnatal groups were calculated as 83.5, 90.4, and 390 mm³, respectively, by using the stereological estimating method. When compared statistically with the Independent Samples T-Test (two tailed, $\alpha=0.05$), there was no significant difference found between the newborn group and the 1-week-old postnatal groups (Table 1).

Conversely, there was a significant increase in rat brain volumes between the newborn group and the 5-week-old postnatal



groups when compared statistically with the Independent Samples T-Test (two tailed, $\alpha=0.05$). Moreover, when compared statistically with the Independent Samples T-Test (two tailed, $\alpha=0.05$), there was a significant increase in rat brain volumes between the 1-week-old postnatal and 5-week-old postnatal groups (Table 1).

Average rat brain volumes of the postnatal 1-week-old females and males were measured as 86.8 and 92 mm³, respectively. When compared statistically with the Independent Samples T-Test (two tailed, $\alpha=0.05$), there was no significant difference between the sexes (Table 1).

Average rat brain volumes of the postnatal 5-week-old females and males were measured as 358 and 428 mm³, respectively, by using the stereological estimating method. When compared statistically with the Independent Samples T-Test (two tailed, $\alpha=0.05$), there was no significant difference between the sexes (Table 1, Figure 6).

4. Discussion

One part of the brain is called the archicortex and includes the large area of the hippocampus (CA1, CA2, CA3), which is derived from the ventricular zone (VZ). Also, the neocortex is another part of the brain derived from the subventricular zone (SVZ). It has been claimed that there are differences between the VZ and SVZ proliferation times; in other words, the cerebral cortex layers first appear in the VZ zone and then the related layers appear with SVZ activity (Nowakowski and Rakic, 1981; Takahashi *et al.*, 1995a). Our histological results parallel these findings.

In the literature, VZ and SVZ proliferation times are similar to each other, but our findings show that the hippocampal region was not organized in the embryos from the intrauterine 16th-day group, slightly organized in those from the intrauterine 20th-day group, and well organized in those from the newborn group.

In the SVZ, young neurons immigrate from the striatum to the neocortex. The SVZ produces both neurons and glia cells, but these differ both regionally and temporally (Garcia-Verdugo *et al.*, 1998; Anderson *et al.*, 2002). In our histological observation, we saw increased glia cell populations and changed glia cell sizes in the intrauterine 20th-day

group and the newborn group, particularly during consecutive periods (Figures 1 and 2).

In the VZ, the cell cycle dividing process consists of three different migration stages that are very important for neurogenesis. In previous studies, the cells migrated from the VZ to the neocortex on the 6th day. For instance, the rat embryo cell dividing time started on the 11th intrauterine day, and the cell cycles were completed in approximately 8 hours. In cell cycles, the G₁ phase took 3 hours, the S phase took 3 hours, and the G₂+mitosis phase took 2 hours. The cells needed 10 hours to recover. That is why the first neurons were observed in the neocortex layer of brain on the 16th intrauterine day (Takahashi *et al.*, 1995b). With respect to the results from our study, we also observed the first neurons in neocortex on the 16th intrauterine day similar to the above literature. Some researchers suggested that neocortex development may continue into the postnatal stage (Nowakowski and Hayes, 2005). But, regarding the results of our study, we can conclude that the neocortex development process is completed in 19 intrauterine days. The neocortex is a part of the brain or the cerebral cortex of mammals. It is the outer layer of the cerebral hemispheres and consists of six layers: (a) Layer I (molecular), (b) Layer II (external granular), (c) Layer III (external pyramidal), (d) Layer IV (internal granular), (e) Layer V (internal pyramidal), and (f) Layer VI (multiform) (Stevens and Lowe, 2005). Layer I (molecular) is closest to the pial surface; the cell density is relatively low, and the cells are called fusiform cells, which are located horizontally (Kierszenbaum, 2006). We determined in our study that in the intrauterine 16th-day group, the thickness of Layer I changed by a small amount during the progressive stages of development (intrauterine 20th day, newborn, postnatal 1week, postnatal 5weeks). In previous studies, Layer II (external granular) contained small cells or pyramidal cells as well as granular (stellate cell) and basket cells (Stevens and Lowe, 2005). Also, in our study these cells were located close to each other, and in progressive stages the external granular layer thickness of the rat brain in the intrauterine 16th-day group was increased; in relation to this finding, external granular layer neuron density was decreased. Although this increased layer thickness was not determined well in the intrauterine 16th- and 20th-day groups, it was



easily determined in the newborn group as well as the postnatal 1-week-old and 5-week-old groups. This thickness was associated with producing new neurons and increased axon and dendrite density, which probably came from neighboring layers. In the progressive stage of postnatal life (5 weeks), new neuron production was not possible; therefore, this layer thickness may be related to increasing axon and dendrite density or learning associated with synaptic formations. Furthermore, there were a number of synapses between Layer V pyramidal neurons' apical dendrites and ascending fibrils, and then these formations were increased (Gartner and Hiatt, 2001).

Layer III (pyramidal) middle pyramidal cells and small pyramidal neurons were located in approximately two layers as well as horizontal and vertical fusiform neurons (Stevens and Lowe, 2005). In the assessment of our study, Layer III neurons began growing particularly during the newborn and 1-week stages. Also, in the 5-week-old groups Layer III had typical vesicular cell bodies with nuclei.

After Layer III, the high-density nuclei could be located in the intrauterine 16th-day group's internal granular layers, and the internal granular layers were distinguished more easily than other layers in the intrauterine 16th-day group and older groups. In relation to this layer, in the progressive stages a development line could be determined as an external granular layer.

In the internal granular (Layer IV), especially in the 5-week-old groups, intensive pyramidal neurons were detected with vesicular nucleus. Although larger neurons were seen in the developmental stage of the 1-week-old and 5-week-old groups, the increased numbers of neurons per unit area were determined in the developmental stage of the intrauterine 16th- and 20th-day groups and the newborn group.

In our experiment, postnatal rats were separated by sex because we hypothesized a difference between the sexes. However, there were no significant differences by the sexes in the newborn and older groups in our assessment (Table 2).

The stereological methods have been used in the morphometric evaluation of biological structures as new approaches for approximately 10-15 years (Gundersen, 1977;

Howard and Reed, 1998). The Cavalier principle of stereological methods is the most commonly used way to calculate the volume of unbiased structures (Gundersen and Jensen, 1987). In the present study, we used the Cavalier principle for the volumetric assessment of brain development. Measurements of the volume, with the help of the above-mentioned method, are summarized as follows: intrauterine 16th-day group = 32.4 mm³, Standard Error Mean (SEM) 1.98; intrauterine 20th-day group = 38.6 mm³, SEM 2.55; newborn group = 83.5 mm³, SEM 3.42; 1-week-old male and female groups = 90.4 mm³, SEM 3.41; and 5-week-old male and female groups = 390 mm³, SEM 37.23 (Table 2). There were no statistical differences between the intrauterine groups (16th day vs. 20th day) or between the newborn group and postnatal 1-week-old groups ($p > 0.05$) (Table 1). In addition, there were no significant differences between the 1-week-old and 5-week-old groups for both sexes ($p > 0.05$). In all other comparisons, we determined a statistically significant correlation between groups (Table 1). Looking at the results of the volume, we determined the first jump of the brain volume values in the newborn group (approximately 116% increase between the intrauterine 20th-day group and the newborn group). We observed the second significant jump in the transition period from 1 week to 5 weeks (approximately 433%). Furthermore, it has been observed that brain development increased at the end of the 3rd postnatal week (Dobbing, 1970; Dobbing and Sands, 1974). When we looked at the statistical results, we saw that the largest volume increase (~433%) was determined between the postnatal 1-week-old and 5-week-old groups (Table 1, Figure 6), and the second largest volume increase (~116%) was determined between the intrauterine 20th-day group and the newborn group (Figure 6). According to the literature, newborn rats' brains do not develop adequately; therefore, postnatal brain development processes increase rapidly at the end of the 3rd week (Dobbing, 1970; Dobbing and Sands, 1974). In our study, with these results, we have proved both increased cell size and numbers via histological and stereological methods. These results suggest that the prenatal and postnatal neurogenesis capacity of neural precursors in the brain continues under suitable conditions.



5. Conclusion

Neurogenesis of the adult brain is one of the most important issues in neuroscience studies for understanding not only prenatal and postnatal development processes but also the function of the central nervous system.

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