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Changes in MAP-2 and GFAP immunoreactivity in pup hippocampus during prepubertal and pubertal periods caused by maternal subclinical hypothyroidism

Aysegul B. Yildirim[1], Saim Ozdamar[2], Betul Yalcin[3], Derya Karabulut[3]

[1]Department of Histology and Embryology, Kahramanmaras Sutcu Imam University, Kahramanmaras Turkey ,[2]Department of Histology and Embryology, Pamukkale University, Denizli, Turkey, [3]Department of Histology and Embryology, Erciyes University, Kayseri, Turkey

SUMMARY

An absence of the thyroid hormone during the critical period of brain development causes delayed maturation of glial cells and neurons and decreases the number of hippocampal cells. This study aims to examine the impact of maternal subclinical hypothyroidism (SCH) on pup hippocampus during the prepubertal and pubertal periods by means of assessing the histopathological changes in astrocytes and neurons.

Twelve Wistar albino pregnant rats were divided into two groups, Group H and Group C. Group C was designated as the control group and nothing was added to their drinking water. SCH was induced in Group H by administering 6-propyl-2-thiouracil (PTU) at a final concentration of 0.01% in the drinking water of pregnant rats for 21 days. Male pups for each group were divided evenly and evaluated on either day 15 (prepubertal) or 60 (pubertal) (7 pups in each group). At the end of the experimental period, the rats were sacrificed for analysis of brain tissue.

Immunoreactivity intensities of MAP-2 and GFAP were evaluated in hippocampus tissue. Thyroid function was determined using ELISA. The structure of hippocampus was evaluated by hematoxylin-eosin staining. Finally, the TUNEL method was utilized to show apoptosis of hippocampus tissue. The results were analyzed statistically.

The findings show that maternal SCH causes disruption in hippocampal cytoskeleton and dendritic organization, especially during the pubertal period, as well as a decrease in MAP-2 expression. We observed structural deformation in astrocytes, reduced astrocyte survival and GFAP expression. Finally, we found that the number of neuronal apoptotic cells tended to increase in the pubertal period.

Key words: GFAP – Hippocampus – Subclinical hypothyroidism – IHC – MAP-2 – Propylthiouracil

INTRODUCTION

Thyroid hormones (THs) play an important role in embryogenesis and fetal maturation (Anselmo et al., 2004). For fetal brain development and functioning, thyroid hormone (TH) levels must be sufficiently high during pregnancy (Casey and Leveno, 2006). Insufficient production of TH is termed hypothyroidism, and occurs in 2.5% of pregnant women (Andersen et al., 2003; Pop et al., 1999). There are some types of hypothyroidism. One of these, subclinical hyperthyroidism (SCH), T3 and T4 levels of patients are normal and TSH levels are higher than normal (Cooper, 2001). The prevalence of SCH in the general population is 4-10%, and can reach 5% in pregnant women (Zhang et.al., 2018).

Corresponding author: Dr Aysegul Burcin Yildirim. Department of Histology and Embryology, Kahramanmaras Sutcu Imam University, Kahramanmaras Turkey

E-mail: aysegulburcin@gmail.com

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In the critical phase of brain development, a lack of TH leads to delayed maturation of glial cells and neurons, abnormal distribution of dendritic spines, decreased synaptic density, decreased hippocampal cell proliferation and abnormalities such as myelination defects (Alvarez-Dolado et al., 2000).

The hippocampus has an important role in mental functions, especially learning and memory, and displays high levels of functional and structural plasticity (Gould et al., 1991). Proper organization of the hippocampus during its development is of critical importance for these functions. Furthermore, the hippocampus is an important site for adult neurogenesis (Barry et al., 2008), and the production of newborn neurons is important to adult hippocampal function (Schinder and Gage, 2004). Studies have reported a decreased number of adult hippocampal neurons due to hypothyroidism during the prepubertal and pubertal periods (Alvarez-Dolado et al., 2000; Alva-Sanchez et al., 2009; Gong et al., 2010a). Hypothyroidism has also been reported to cause changes in axonal transport of microtubules and neurofilaments (Rahaman et al., 2001), and may change neuronal cytoskeleton and neuronal growth by means of cytoskeletal proteins and various mechanisms (Zamoner et al., 2008).

MAP-2 proteins are important to the cytoskeleton, as they connect microtubules to one another; they are particularly located in perikarya and dendrites (Kaufmann et al., 2000; Sanchez and Diaz-Nido, 2000). MAP-2 also has an important place in the dendritic growth, development, branching, and synaptogenesis of hippocampal neurons (Mundy et al., 2008; Tucker, 1990).

During the development phase, astrocytes play a key role in synaptic transmission, neuronal differentiation, neuronal migration, and axon growth (Trentin, 2006). Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed in mature astrocytes. It is important for adaptation to neural activities and changes during brain development. GFAP is also known to have a role in neuronal-glial interaction. Changes in GFAP levels may result in disruptions in neuron-neuron and neuron-glia links (Kim et al., 2011; Gomes et al., 1999; Dönder et al., 2010; Bezzi et al., 2001). Studies also show that congenital hypothyroidism leads to down-regulation of GFAP in astrocytes of the cerebral cortex (Zamoner et al., 2008), as well as reduced concentration of GFAP in the hippocampus (Cattani et al., 2013).

Given the high prevalence of hypothyroidism in the human population, it is important to understand the cellular and molecular mechanisms that contribute to the neural changes caused by hypothyroidism. This study aims to examine the impact of maternal SCH on pup hippocampus during the prepubertal and pubertal periods by means of assessing the histopathological and immunohistochemical changes in astrocytes and neurons using the glial cell marker GFAP and the neuronal cell marker MAP-2.

MATERIALS AND METHODS

Animals

The study was conducted in the Erciyes University Hakan Çetinsaya Experimental and Clinic Research Center. Twenty-four adult, eight to ten weeks old Wistar albino rats (12 female and 12 male) were used for this study. They were housed in plastic cages in a well-ventilated rat house, allowed *ad libitum* access to food and water. They were housed in a quiet and temperature- and humidity controlled room (21 ± 3°C and 60 ± 5%, respectively) in which a 12 h light:12 h dark cycle was maintained (07:00–19:00 h light). All animals received humane care according to standard guidelines. Ethical approval for the study was obtained from the Erciyes University Animal Research Ethics Committee. Ethical regulations were followed in accordance with national and institutional guidelines.

Experimental protocol

The animals were mated prior to the beginning of the experiment. Vaginal smears of female rats were examined daily for a period of two weeks. Female rats were housed separately in cages during proestrus. Then, during estrus, females were housed in the same cage with male rats overnight and separated in the morning. Pregnancy was confirmed by the presence of sperm in subsequent vaginal smears, and pregnant rats were considered to be at day 0.5 of gestation, at which point the experiment was initiated.

Twelve pregnant, 10-week-old Wistar albino rats were used in the study. The pregnant rats were divided into two groups, Group C and Group H. Group C was designated as the control and nothing was added to their drinking water. Group H was designated as the treated group, and had SCH induced by the administration of 6-propyl-2-thiouracil (PTU, Sigma, P3755-25G) at a final concentration of 0.01% in the drinking water of mothers for 21 days from the first day of pregnancy.

Infant rats in each group were sex-assigned at birth. Only male pups were used in the experiment. Pups in the control and treated groups were divided as follows:

Group C-15: 15-day-old male pups of Group C (n=7)

Group C-60: 60-day-old male pups of Group C (n=7)

Group H-15: 15-day-old male pups of Group H (n=7)

Group H-60: 60-day-old male pups of Group H (n=7)

At the end of the 15th or 60th postnatal days, the rats were anesthetized by intraperitoneal injection of the combination of xylazine (1 mg/100 g; Rompun, Bayer, Leverkusen, Germany) and ketamine (8 mg/100 g; Ketalar, Parke-Davis, Detroit, MI, USA). Brains of the anesthetized rats were quickly removed from the skull and prepared for analysis.

Histological analysis

For microscopic analysis, brain tissue samples taken from the rats were fixed for 48 hours in a 10% formaldehyde solution. After fixation, tissue samples were dehydrated in a series of increasing grades of alcohol. They were made transparent with xylene and stored overnight at 60°C in liquid paraffin. Paraffin-embedded tissues were cut into serial coronal sections (5 µm) using a microtome. Sections were stained with hematoxylin-eosin (H&E) to provide a morphological overview of the tissue.

Immunohistochemistry

The Avidin-Biotin-Peroxidase Complex (ABC) method and a couple of immunohistochemical methods were used to determine MAP-2 and GFAP immunoreactivities in the hippocampus. Paraffin sections (5 µm thick) were placed on poly-(L-lysine)-coated slides and stored overnight in an oven at 60°C. Then they were deparaffinized with xylene and rehydrated with a series of decreasing grades of alcohol (100%, 96%, 80%, 70%). After rehydration, the sections were washed in distilled water 3 times for 5 minutes. For antigen retrieval, tissues were heated for 5 minutes in 5% citrate buffer in a microwave oven at 600 W. The sections were washed with phosphate buffered saline (PBS) and treated with 3% hydrogen peroxide (H_2O_2) for 12 minutes in order to block endogenous peroxidase. For the next stages, the Large Volume Detection System (Thermo Fisher Scientific, Waltham, MA, USA, Catalog no: TP-125-HL) immunochemistry staining kit was used. To prevent background staining, the sections were treated with Ultra V block for 5 minutes. Then, the sections were incubated overnight at 4°C with MAP-2 and GFAP antibodies (5 µg/mL diluted in PBS). (MAP-2, GFAP, Santa Cruz Biotechnology, Dallas, TX, USA. Negative controls were treated with PBS in place of the primary antibodies. Processes in the other stages mentioned above were carried out in the same way. After primary antibody incubation, the sections were rinsed. Reverse staining was performed using appropriate biotinylated secondary antibodies (biotinylated goat anti-polyvalent, TP-060-BN, Thermo Scientific), Streptavidin-HRP (Horse Radish Peroxidase), DAB (3,3'-Diaminobenzidine) chromogen, and Gill's Hematoxylin. Finally, the sections were cleared in xylene before being coverslipped with Entellan® (Merck, Kenilworth, NJ, USA). The stained sections were examined for MAP-2 and GFAP immunoreactivity under an Olympus BX-51 light microscope.

Quantitative immunohistochemistry

To determine the immunoreactivity intensities of MAP-2 and GFAP, images of the hippocampus were captured for local regions and as a whole using a light microscope. Local examination was performed on two regions: the cornu ammonis (CA1) and the dentate gyrus (DG). General examination was performed on the whole hippocampus. Images of the CA1, the DG, and other regions were taken through a light microscope equipped with a digital camera (DP71, Olympus, Center Valley, PA, USA). The mean of immunoreactivity intensities and the number of immunoreactive cells were separately determined from totally 25 microscopic fields in the five hippocampus slides for each animal. For determining MAP-2 immunoreactivity intensity, five images were taken for each region. For determining GFAP immunoreactivity intensity and the number of GFAP immunoreactive cells, seven different cells were measured in each image. (CA1 region: 175; DG region: 175; other regions: 175; total of 525 microscopic fields or cells were analyzed). Measurements were performed at the same section level for each group at an original magnification of X400. The mean immunoreactivity intensities and numbers of immunoreactive cells were calculated using Image J software (Yang et al., 2012). Measurements were statistically analyzed (see below).

Apoptosis (TUNEL)

The TUNEL assay was used to determine the presence of cells undergoing apoptosis in the hippocampus, using an *in situ* Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland). First, 5-6 µm thick brain tissue sections were deparaffinized, rehydrated, and washed with PBS. Tissues were heated in 0.01 M sodium citrate buffer for 5 minutes in a microwave oven at 350 W, and then cooled at room temperature for 10 minutes. The TUNEL kit reaction mixture was prepared and dropped on the slides. The slides were then incubated in a humid and dark environment for 1 hour at 37°C. At the end of the incubation period, they were washed three times in PBS and stained with 4',6-diamidino-2-phenylindole (DAPI). The slides were then coated with glycerol and examined using an Olympus BX-51 fluorescence microscope (450-500 nm wavelength). The process was carefully carried out in a dark environment. For quantification of TUNEL-positive cells, 10 fields per section were examined and counted at 400-fold magnification.

Elisa

Blood samples were taken from the mother rats immediately after giving birth to measure their T3, T4, and TSH levels. Samples (1 ml) were collected into vacuum gel tubes by intracardiac puncture under anesthesia. For anesthesia, a combination of xylazine and ketamine was administered intraperitoneally. The mothers survived after the blood collection process. The blood samples were kept for half an hour, then centrifuged at 5,000 rpm for 10 minutes to collect serum. Serum samples were stored in Eppendorf tubes at -80°C until analysis. Measurements of T3 (Sunred Biological Technology, Shanghai, China, Catalog no: 201-11-0256), T4 (Sunred Biological Technology, Shanghai, China, Catalog no: 201-11-0257) and TSH (YH Biosearch, Shanghai, China, Catalog no: YHB1050Ra) in the serum samples were per-

Table 1. TSH and thyroid hormone levels in mothers after pregnancy.

Groups	**TSH** (mIU /	**T4** (ng/ml)	**T3** (ng /L)
C-Mother	3,10±0,53	59,84±4,23	602,34±173,80
H-Mother	4,08±0,58*	49,07±10,24	531,34±180,53

n =6 for all groups. Values are expressed as mean±SD.
* p< .05 indicates significantly different from control.

formed spectrophotometrically using commercial ELISA (Enzyme-Linked Immuno Sorbent Assay) kits in accordance with manufacturer protocols.

Statistical analysis

The obtained data were analyzed with SPSS for Windows, version 20.0. Before, skewness and kurtosis values of the data were examined to determine whether they showed normal distributions. T-test and ANOVA were used to determine statistical significance.

RESULTS

Induction of SCH

Statistically non- significant changes in maternal T3 and T4 levels and statistically significant increases in TSH levels reveal the presence of PTU-induced maternal SCH (Table 1).

Changes in Weight

Tables 2 and 3 show changes in the body weight of mothers by week of pregnancy and changes in pup body weights between postnatal days 1, 15, and 60, respectively. Mothers in Group H were found to gain less weight in the early weeks of pregnancy compared to mothers in the control group. Newborns in the treated group were also observed to be under-weighted respective to controls. Weight of newborns in the treated group was significantly decreased when compared with the control group. On the 15th day, the treated group had a statistically significant increase in pup weight when compared to the control group. But, on the 60th day; there was no statistically significant difference in the weight change of the treatment group compared to the control group (Table 3). Approximately 15% of pups from hypothyroid mothers died, either stillborn or dying one or two days after birth.

Histopathological findings

In the sections stained with H&E, neurons and glia cells with large, intensively dispersed, round nuclei were observed in the hippocampi of control groups (Figs. 1A, 1B, 1C, 1G, 1H and 1I). Group H -15 showed no histopathological changes related to structural damage (Fig. 1D) and similar DG (Fig. 1F) compared to the control group. In Group H-60, decreased neuronal density and flattened neuronal nuclei were observed in the CA1 pyramidal layer, along with capillary expansion combined with vacuolization in some regions (Fig. 1K, 1L). Moreover, degeneration and vacuolization was observed in the morphological structure of neurons (Fig. 1L).

Immunohistochemical findings

The Avidin-Biotin-Peroxidase Complex (ABC) method was performed on the coronal sections of brain tissues to determine MAP-2 and GFAP immunoreactivities in the hippocampus. MAP-2 was observed to be expressed in the neuron cytoplasm and dendrites. The control groups showed notably strong expression of MAP-2 in hippocampal neurons (Figs. 2A, 2B, 2C, 2G, 2H and 2I). Conversely, MAP-2 expression was observed to be extremely weak in hippocampal neurons for both groups H-15 and H-60 (Figs. 2D, 2E, 2F, 2J, 2K and 2L). This was thought to be the result of a serious cytoskeletal defect. Dendritic spines were found to be uninterrupted in the control group, but both interrupted and less in number in the pups from hypothyroid mothers, indicating a dendritic organization disorder. Moreover, MAP-2 expression in the neural perikarya was found to be weak (Figs. 2F, 2L). Overall, across the whole hippocampus and the CA1 and DG regions, groups H-

Table 2. Changes in the body weight of mothers based on the weeks of pregnancy (average) ± standard deviation.

Mother Groups	First day of pregnancy	First week	Second week	Third week
C-Mother(gr)	167,33±28,12	175,10±30,17	188,33±32,24	213,66±31,34
H-Mother (gr)	158,50±10,50	170,02±7,94	179,83±8,81	192,67±11,96

n =6 for all groups

Table 3. Changes in pup body weight (average) ± standard deviation

Pup Groups	1th day	15th day	60th day
C(gr)	6,35±0,19	14,25±0,64	193,61±9,93
H(gr)	3,36±0,30*	16,52±0,89*	194,51±21,69

n =7 for all groups. * p< .05 indicates significantly different from control.

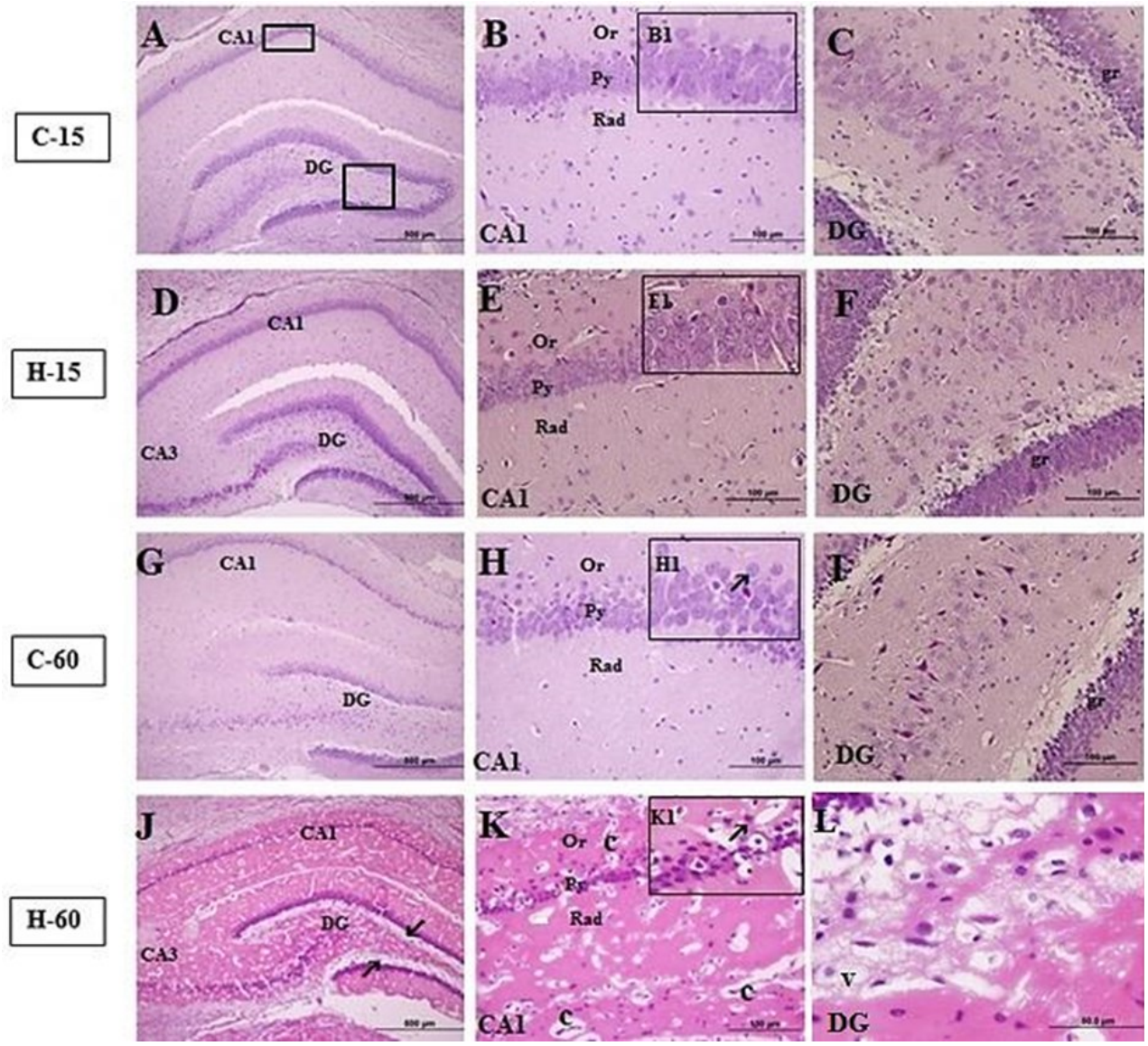

Fig 1. Light microscopy of hippocampus tissue in experimental groups. H&E stained sections are shown. Group C-15: the hippocampus (A), the CA1 region (B, B1), the DG region (C). Group H-15: the hippocampus (D), the CA1 region (E, E1), DG region (F). Group C-60: the hippocampus (G), the CA1 region (H), round neuronal nuclei in Py (arrow) (H1), the DG region (I). Group H-60: the hippocampus, separation between granular(gr) and polimorfica layers (po), (arrows) (J), enlarged capillaries (c) (K), flattened Py neuronal nuclei (K1), vacuolization (v) (L). *Stratum or ens (Or), St. pyramidalis (Py) and St. radiatum (Rad). A,D,G,J x10, Bar 500µm; B,C,E,F,H,I,K x40, Bar 100 µm; L x100, Bar 50 µm .*

15 and H-60 were found to have significantly decreased immunoreactivity of MAP-2 compared to control groups (Figs. 3-4).

GFAP was observed to be expressed in astrocytes (Fig. 5). No histopathological changes were observed in astrocytes between Group H-15 and Group C-15 (Figs. 5A, 5B, 5C, 5D, 5E and 5F). The morphological structure of a single astrocyte of each group has also been determined (Figs. 5C1, 5F1, 5I1, 5L1). Astrocyte spines were found to be fewer and reduced in number in Group H-60 compared to Group C-60 (Figs. 5K, 5K1, 5L1). Notably, groups H-15 and H-60 were both found to have significantly decreased immunoreactivity of GFAP compared to control groups (Figs. 6-7).

Number of GFAP+ Cells

GFAP+ cells were counted and statistically analyzed. No significant difference was found in the general number of GFAP+ astrocytes between Group H-15 and Group C-15 (Fig. 8A). However, the general number of GFAP+ astrocytes was significantly reduced in Group H-60 compared to Group C-60 (Fig. 8B). Similarly, no significant difference was observed between Group H-15 and Group C-15 in the number of GFAP+ cells in the CA1 and DG regions (Fig. 9A), but Group H-60 showed a statistically significant decrease in both regions compared to Group C-60 (Fig. 9B).

Apoptotic Findings

TUNEL staining was performed to determine

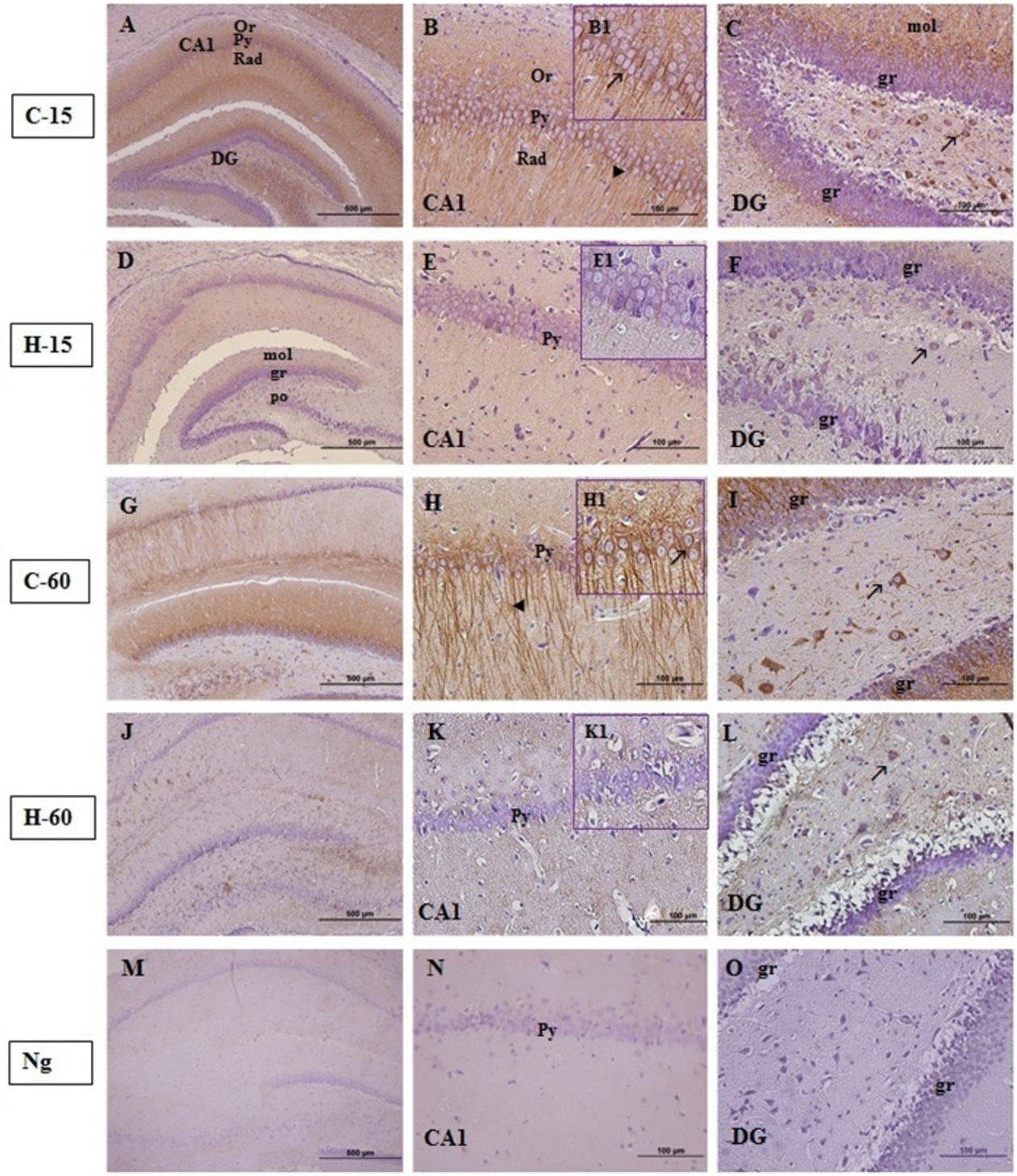

Fig 2. MAP-2 immunostaining in the experimental groups. Group C-15: the hippocampus (A), the CA1 region (B), MAP -2 expression (arrow) in the dendrites (head of the arrow); (B1), in the neuronal cytoplasm (arrow) and in the neuronal cytoplasm of DG (C). Group H-15: the hippocampus (D), the CA1 region (E, E1), weak MAP-2 expression (arrow) in the neuronal cytoplasm of DG (F). Group C-60: the hippocampus (G), the CA1 region, strong MAP-2 expression in the dendrites (head of the arrow) (H), in the neuronal cytoplasm (arrow) (H1) and in the neuronal cytoplasm of DG (arrow) (I). Group H-60: the hippocampus (J), the CA1 region (K, K1), weak MAP-2 expression (arrow) in the neuronal cytoplasm of DG (L). Negative control: the hippocampus (M), CA1 the CA1 region (N), negative MAP-2 expression in the DG region (O). *Stratum moleculare (mol), St. granulosum (gr), St. pol morf ca (po). A,D,G,J,M x10, Bar 500µm; B,C,E,F,H,I,K,L,N,O x40, Bar 100 µm.*

apoptotic cells in hippocampus tissue (Fig. 10). The numbers of apoptotic cells in group C-15 were similar to those of group H-15, but increased in group H-60 in comparison to group C-60 (Table 4).

DISCUSSION

This study revealed that maternal SCH causes slight histopathological changes in the prepubertal pup hippocampus, but serious changes during the

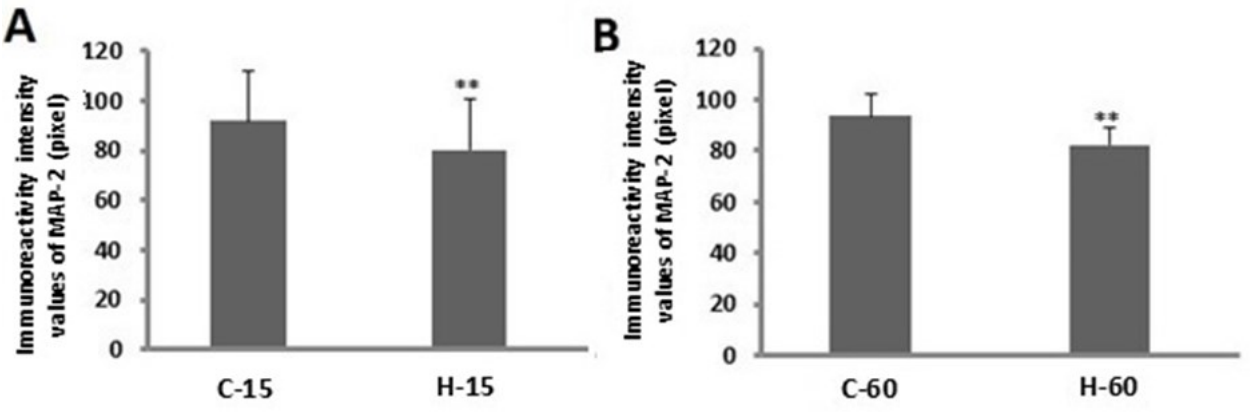

Fig 3. A: 15-day-old groups, B: 60-day-old groups, immunoreactivity intensity values of MAP-2 in the whole hippocampus: ** p< 0.01 indicates significantly different from control.

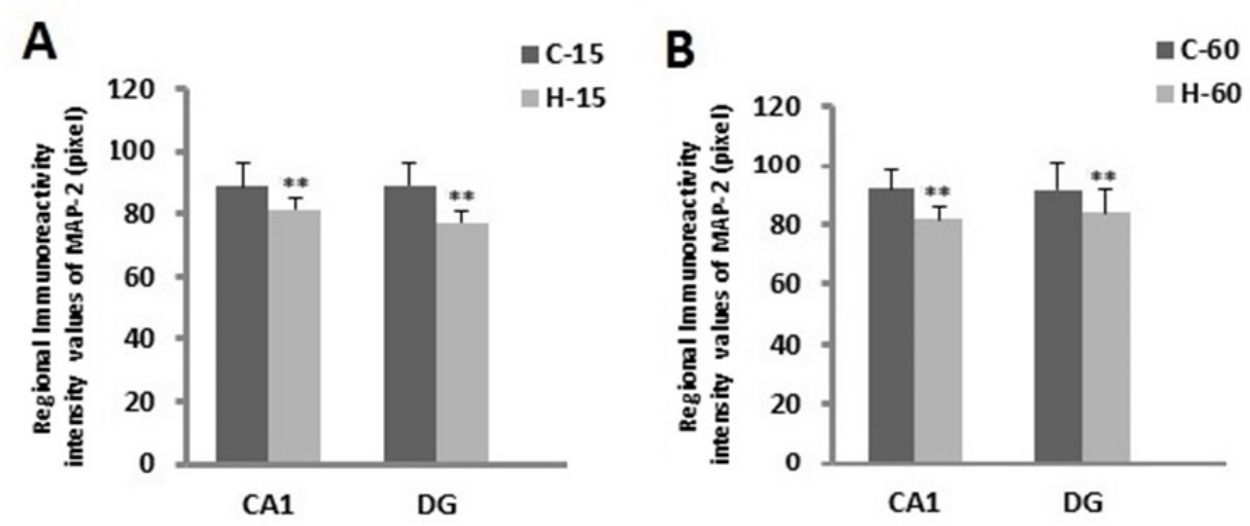

Fig 4. A: 15-day-old groups, **B**: 60-day-old groups, immunoreactivity intensity values of MAP-2 in the CA1 and DG regions. ** p< 0.01 indicates significantly different from control.

Table 4. Apoptotic cells number of groups

	Group C-15	Group H-15	Group C-60	Group H-60
Apoptotic cells number	0,29±0,493	0,42±0,991	0,00±0,000	0,23±0,422*

Values are expressed as mean±SD. *p <. 05 compared to group C-60. (H-15 group was compared to C-15 group, H-60 group was compared to C-60 group).

pubertal period. In addition, a significant decline in MAP-2 and GFAP immunoreactivity and a decreased number of GFAP+ cells were observed for the pubertal period. In contrast, the number of apoptotic cells in hippocampus tissues tended to increase in the pubertal period for pups of maternal subclinical hypothyroid mothers.

The hippocampus is one of the brain regions sensitive to disruption of TH function during development (Rami et al., 1986). Decrease in or lack of TH during brain maturation causes molecular, morphological, and functional changes in the hippocampus (Gong et al., 2010a; Lee et al., 2003; Koromilas et al., 2010). Studies have reported reduction of hippocampal and brain volume in hypothyroid rats (Hasegawa et al., 2010). Rodents with gestational TH deficiencies and children with congenital hypothyroidism also show abnormal hippocampal development (Willoughby et al., 2014).

In addition to global effects, hypothyroidism has been shown to impact specific brain regions. Neonatal hypothyroidism is reported to decrease the number of dendritic branch points of pyramidal cells in the CA region of the hippocampus (Rami et al., 1986) and to reduce the area of and number of cells in the granular layer of the hippocampal dentate gyrus (DG) (Rami et al., 1986). THs in adults are also reported to play a role in hippocampal neurogenesis and hypothyroidism cause damage in nerve fibers in the later stages of brain development as well as decreasing the number of healthy nerve fibers (Desouza et al., 2005; Kobayashi et

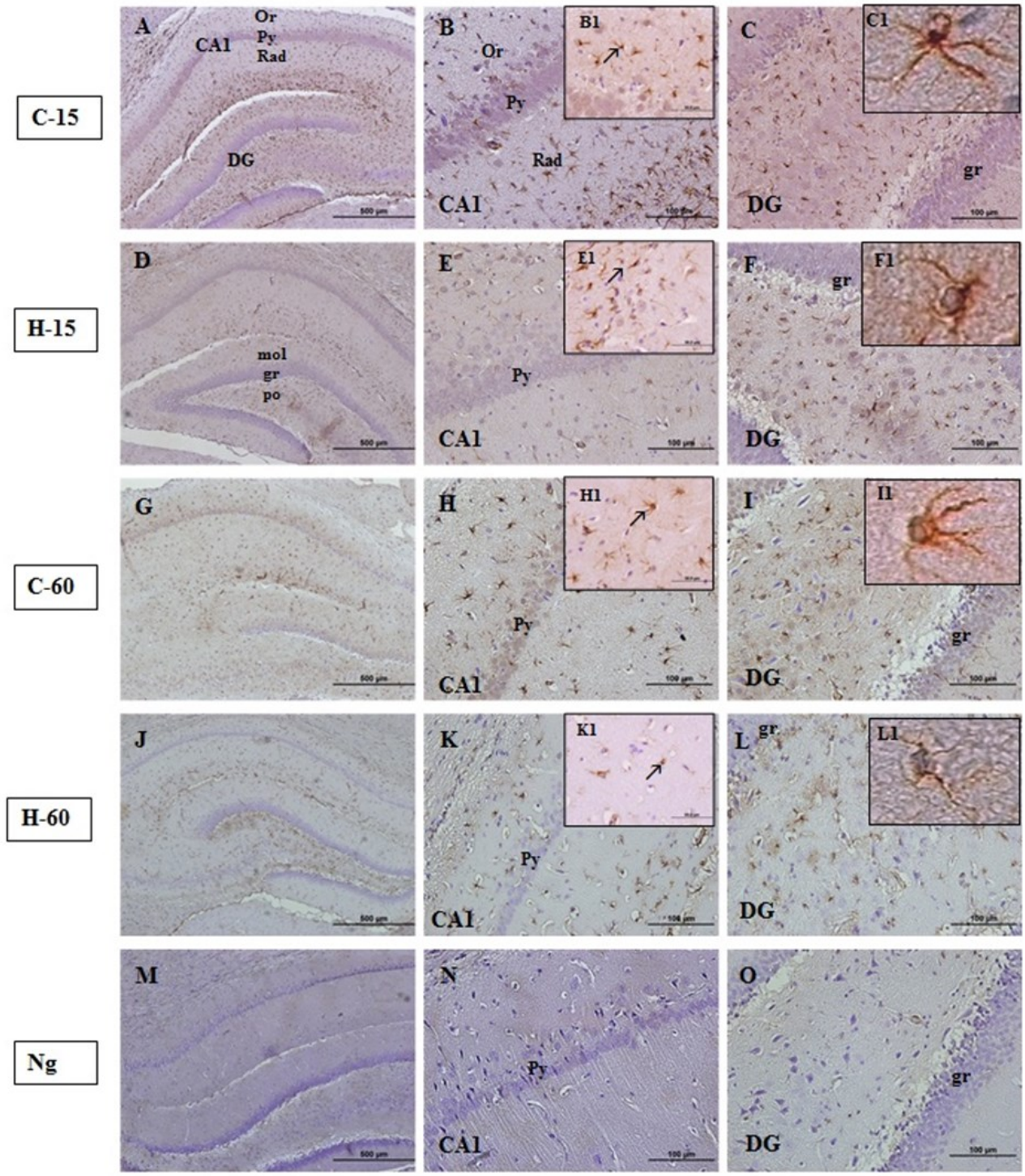

Fig 5. GFAP immunostaining in the experimental groups. Group C-15: the hippocampus (A), the CA1 region, GFAP expression in astrocytes (arrow) (B, B1), in DG (C). Group H-15: the hippocampus (D), the CA1 region, GFAP expression in astrocytes (arrow) (E, E1), in DG (F). Group C-60: the hippocampus (G), CA1 region, strong GFAP expression (arrow) in astrocytes (arrow) (H, H1), in the neuronal cytoplasm of DG (I). Group H-60: the hippocampus (J), the CA1 region, GFAP expression in astrocytes (arrow) (K, K1), in DG (L). The morphological structure of a single astrocyte of each group (C1,F1,I1,L1). Negative control: the hippocampus (M), the CA1 region (N), negative GFAP expression in DG (O). *Stratum or ens (Or), St. pyram dal s (Py) and St. rad atum (Rad). A,D,G,J,M x10, Bar 500μm; B,C,E,F,H,I,K,L,N,O x40, Bar 100 μm; B1,E1,H1,K1 x100, Bar 50 μm.*

al., 2005). Additionally, adult-onset hypothyroidism was found to decrease survival and neuronal differentiation of dentate granule cell progenitors (Desouza et al., 2005). Hypothyroidism also reduces newborn hippocampal granule cells and innervation of nerve fibers, which is thought to disrupt neuronal connection, excitability of pyramidal cells and memory formation (Gong et al., 2010b; Kempermann et al., 2004). In addition to replicating the findings of previous studies, this study revealed a

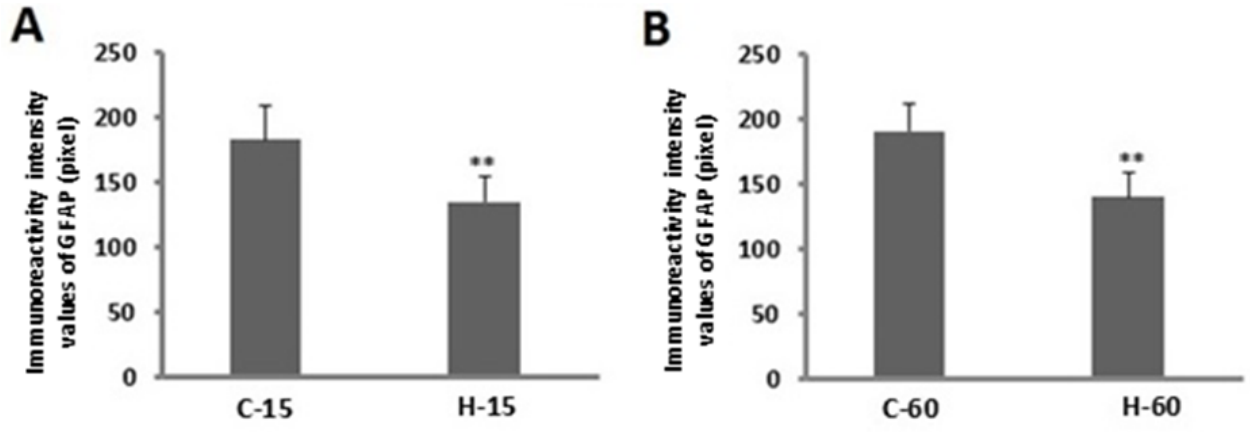

Fig 6. A: 15-day-old groups, B: 60-day-old groups, immunoreactivity intensity values of GFAP in the whole hippocampus. ** p< 0.01 indicates significantly different from control.

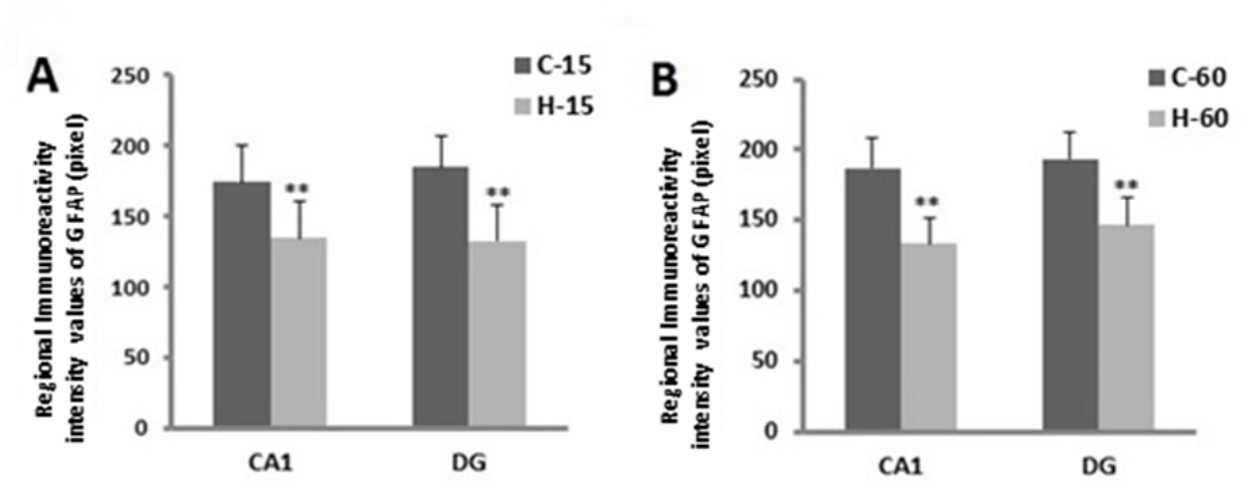

Fig 7. A: 15-day-old groups, B: 60-day-old groups, immunoreactivity intensity values of GFAP in the CA1 and DG regions. ** p< 0.01 indicates significantly different from control. n=7 for all groups.

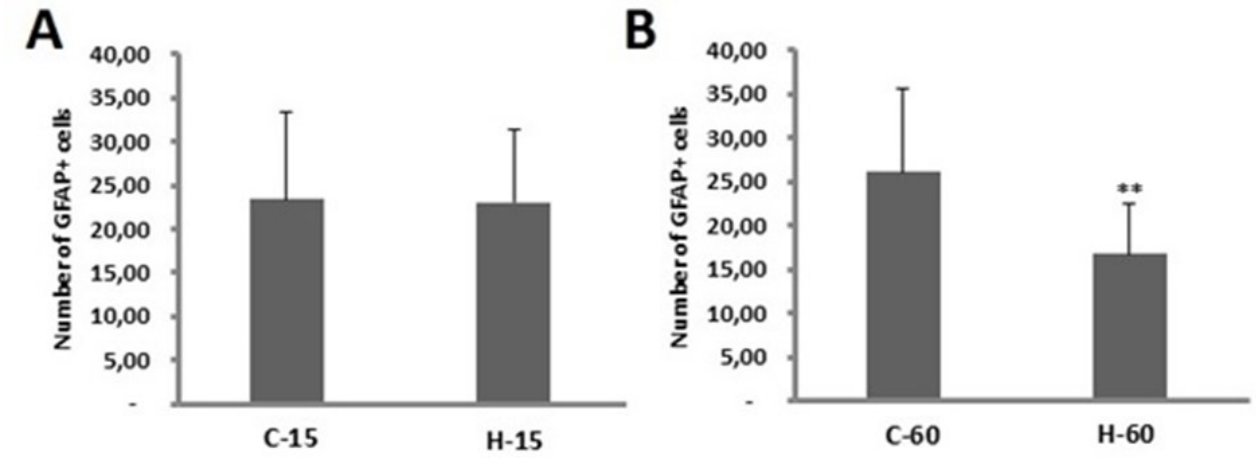

Fig 8. A: 15-day-old groups, B: 60-day-old groups, the number of GFAP+ cells across the whole hippocampus. ** p< 0.01 indicates significantly different from control.

reduction in the number of astrocytes during the pubertal period in rats with intrauterine TH deficiency.

TH deficiency causes deficiencies in cell acquisition, increases in neuronal death, and impaired dendritic arborization, which result in irreversible reductions in total granule cell number, volume of the granule cell layer, cell density, and synapse number (Ahmed et al., 2008). It has been shown that neurological abnormalities of hippocampal structures were irreversible, even if TH levels returned to normal after development of the central nervous system (Madeira et al., 1991). In this study, remarkable histopathological changes in

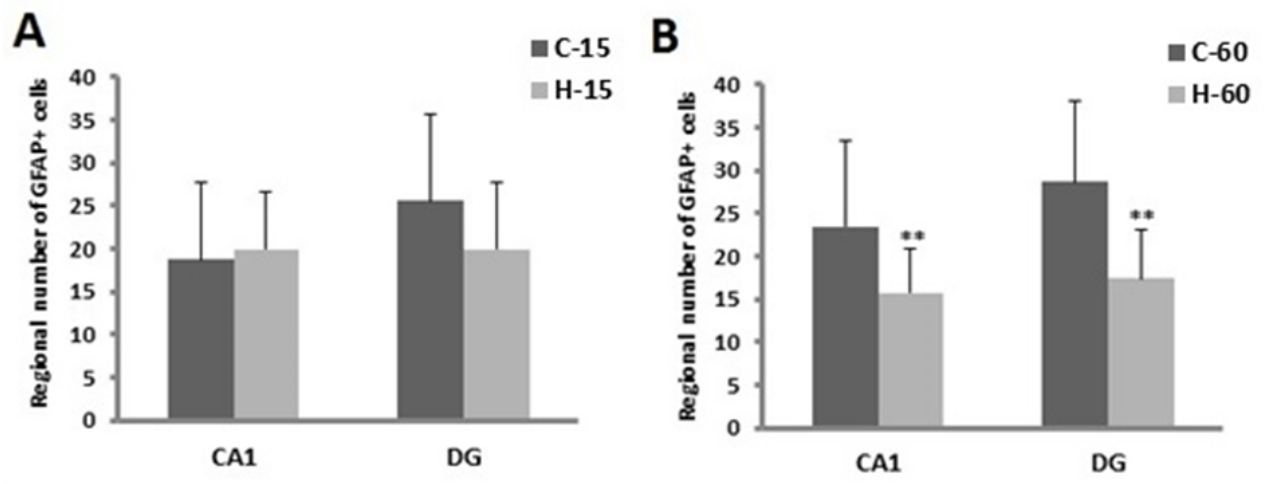

Fig 9. A: 15-day-old groups, B: 60-day-old groups, the number of GFAP+ cells in the CA1 and DG regions. ** p< 0.01 indicates significantly different from control.

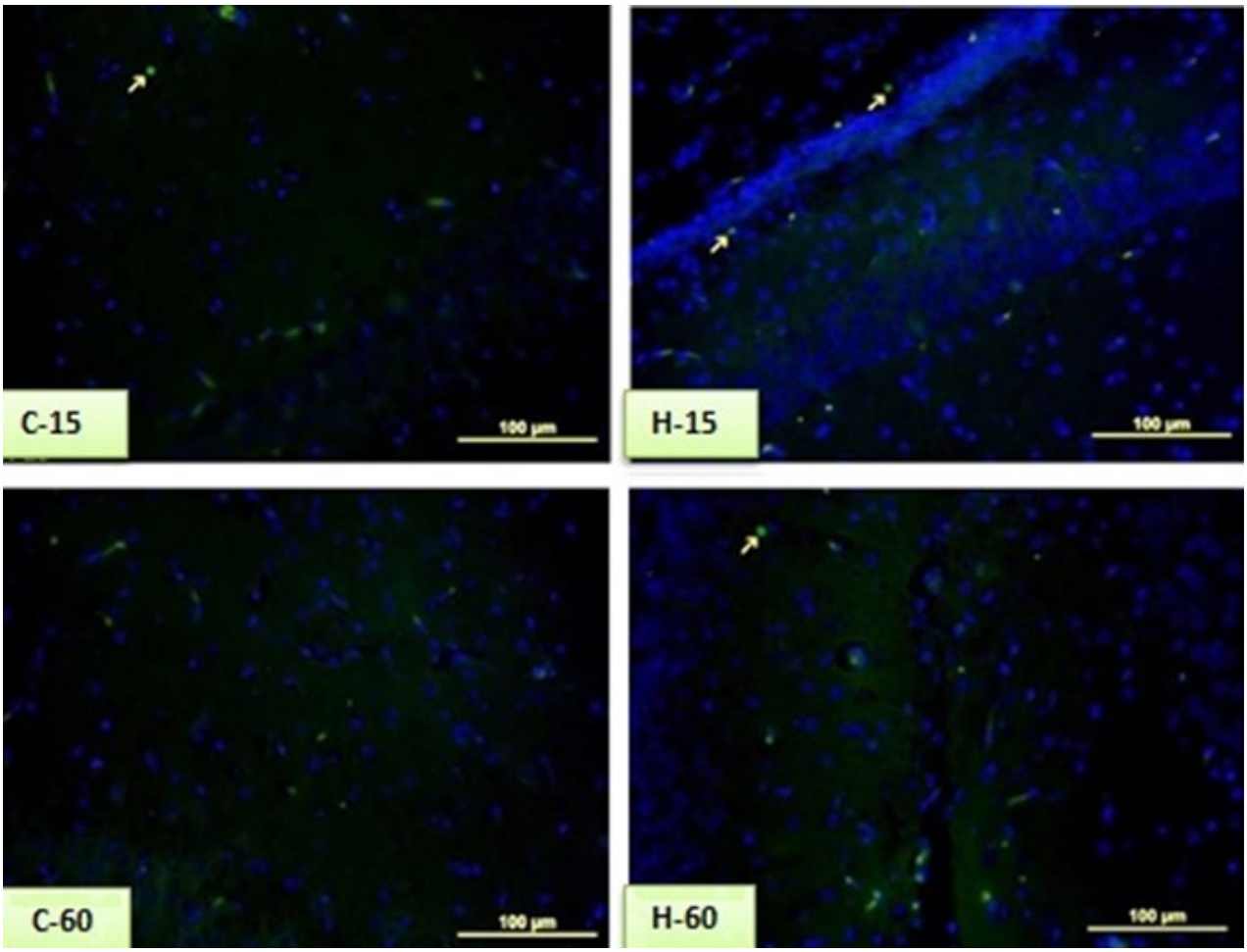

Fig 10. TUNEL staining of hippocampus tissue were observed. TUNEL-positive cells (arrows). Bars: 100 µm.

pups from subclinical hypothyroid mothers were observed for the neuronal cytoskeleton of the hippocampus, especially in 60-day-old pups. However, no remarkable changes in the hippocampus were recorded for 15-day-old pups. These findings indicate permanent damage to the developing hippocampus from maternal hypothyroidism during pregnancy. A previous study reported no nerve fiber damage during the prepubertal period, even though such damage was likely. The reason for delayed appearance of nerve damage remains unexplained, but it may have been because most brain structures of these rats are formed in the third postnatal week (Gong et al., 2010b; Koba-

yashi et al., 2005).

Microtubules are the main structural component that lie behind the morphology of dendrites. MAP-2 is a protein that interacts with microtubules and stabilizes them (Fujimoto et al., 2004). It is an important brain protein that plays key roles in the restructuring of dendrites during neurogenesis, synapse formation, learning, and memory processes (Quinian and Halpain, 1996; Woolf et al., 1999). As an important component of the cytoskeleton, MAP-2 has a defining role in the structure of the dendritic tree and its three-dimensional stability (Hurtado et al., 2015). Therefore, abnormal dendritic morphology has been associated with changes in MAP-2 expression for some neurological diseases (Johnson and Jope, 1992). There are a limited number of studies on changes in MAP-2 expression in the hippocampus caused by hypothyroidism. Studies show that MAP-2 deficiency causes decreased MT density in dendrites and reduced dendritic length, suggesting that MAP-2 has a role in dendrite elongation (Dehmelt and Halpain, 2005). It has also been suggested that a decrease in T4 induced by marginal ID might reduce MAP-2 activation and limited dendritic branching (Min et al., 2016).

In this study, maternal SCH was found to significantly and remarkably decrease MAP-2 immunoreactivity intensity in the pup hippocampus, especially in the pubertal period. Examination of the dendritic structure by means of MAP-2 immunostaining revealed some neuropathological conditions such as hippocampal cytoskeletal disruption and impaired dendritic organization.

GFAP is an intermediate filament protein primarily expressed in astrocytes, which are important cells that modulate neuronal differentiation and guide neuronal migration and axon growth during development (Trentin, 2006; Fields and Stevens-Graham, 2002). The effects of TH on neuronal proliferation and differentiation are primarily seen in astrocytes. TH has an effect on the synthesis and secretion of growth factors, as well as on regulating astrocyte proliferation, maturation and differentiation by means of its effect on cytoskeleton (Dönder et al., 2010; Gavaret et al., 1991). T3 has been shown to stimulate astrocyte proliferation in culture (Trentin et al., 1995). Moreover, it stimulates GFAP expression, which is the first step in astrocyte differentiation (Lima et al., 1998).

A study using rat model of maternal hypothyroidism examines the hypothesis that the observed reduction of GFAP under maternal hypothyroidism is due to alterations in the epigenetic marks on GFAP promoter. This study provides evidence for an epigenetic silencing of GFAP under TH insufficiency (Kumar et al., 2018).

In a study where maternal hypothyroidism was induced by PTU in the first, second, and third trimesters of pregnancy, a significant decrease was observed in GFAP levels for the hypothyroid groups in the second and third trimesters, compared to the control and first trimester hypothyroid groups (Dönder et al., 2010). The same study also revealed that maternal hypothyroidism prevented astrocyte proliferation and maturation. Maternal hypothyroidism has also been reported to reduce fetal brain levels of GFAP at 21 days of gestation, suggesting delayed astrocytic differentiation (Sampson et al., 2000). Studies have further shown that congenital hypothyroidism leads to down-regulation of GFAP in astrocytes of the cerebral cortex (Zamoner et al., 2008), as well as reduced concentration of GFAP in the hippocampus (Cattani et al., 2013). Neonatal TH deficiency has also been revealed to reduce GFAP concentration (Faivre-Sarrailh et al., 1991). Finally, radial glia in the fetal hypothyroid hippocampus have been shown to contain reduced amounts of GFAP (Martinez-Galan et al., 1997).

In this study, maternal SCH was found to reduce GFAP immunoreactivity in the pup hippocampus, both across the whole hippocampus and in the CA1 and DG regions specifically. No change was observed in the number of GFAP+ cells in the 15-day-old group, though the immunoreactivity intensity of GFAP was reduced. In the 60-day-old group, decreases were observed both in the number of GFAP+ cells and in immunoreactivity intensity.

Hypothyroidism is known to both directly and indirectly affect the proliferation, migration, differentiation, and apoptosis of various glial and neuronal cell types during postnatal brain development (Ahmed et al., 2008). Apoptotic mechanisms play a key role during postnatal and adult neurogenesis (Biebl et al., 2000; Zhang et al., 2009). In some studies, reduced TH levels were found to result in upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes during the prepubertal period (Rami et al., 1986). However, their expression returned to normal during the pubertal period. In addition, TH levels also returned to normal, suggesting the presence of intracellular conditions in favor of cell survival. Similarly, some other studies have reported that hypothyroidism increased expression of apoptotic signaling molecules in the hippocampus (Alva-Sanchez et al., 2009; Cortés et al., 2012). In our study, apoptotic cell counts were not changed in prepubertal pups of subclinical hypothyroid mothers, whereas counts were increased in pubertal pups of subclinical hypothyroid mothers.

In conclusion, the findings of this study are consistent with those of other studies, indicating that PTU-induced maternal SCH during brain development causes permanent damage to glial cells and neurons in the pup hippocampus, while at the same time reducing GFAP and MAP-2 immunoreactivity intensities. Maternal SCH negatively affects the neuronal cytoskeleton and dendritic organization in pups. It also causes reduction in the number of astrocytes and astrocytic deterioration. Such deterioration in astrocyte and neuron function might contribute to the physiopathology of hippocampal neurological disorders that affected pups might encounter in their future life. Further studies might provide more insight into the molecu-

lar mechanisms underlying such deformations.

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