

Histological investigation of amygdala in horned and hornless ewes

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Abstract

The amygdala is the nucleus of the brain that is largely responsible for perceiving danger and plays a role in emotion, behavior, control and learning. A small amygdala has been associated to aggression. Horned ewes are expected to be more aggressive and have a smaller amygdala. Both horned and hornless ewes exhibit intraspecific head-butting behavior and both species are at risk for traumatic brain injury. The aim of this study was to investigate the neuronal density, glial cells and blood-brain barrier (BBB) of the amygdala in horned and hornless ewes. Four horned and six hornless ewe heads (age: 16.00 ± 4.00 months) were obtained from the abattoir. The brains were carefully removed and preserved in 10.00% formalin for 5 days. Bilateral amygdalae were sectioned. The samples were stained with Hematoxylin and Eosin, immunohistochemical (glial fibrillary acidic protein) and Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling methods, and the histological structures of the amygdala were examined by light microscopy. The Mann-Whitney U test was used to analyze the data. Neuronal density was estimated to be 143,230 ± 12,540 *per mm*³ in horned and 152,230 ± 18,430 *per mm*³ in hornless ewes. Horned subjects had reduced numbers of neurons, damaged BBB and localized inflammatory areas. More apoptotic neurons were observed in horned ewes. Further studies are needed to determine whether these differences in neuronal density, glial cells, and BBB are acquired (due to trauma) or congenital. The results of this study might need further similar studies to be conducted in the future.

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Introduction

The amygdala is a brain nucleus that forms a small but prominent protrusion at the rostral end of the parahippocampal gyrus or rhinencephalon and plays a role in emotion, behavior and learning.¹ It expresses fear and its small size is associated with aggression and causes some personality disorders in humans.² Furthermore, the amygdala plays a role in social recognition by processing olfactory information in ewes,³ and is involved in the development of olfactory memory in lambs.⁴

Horns are used by animals to fight or play with each other, and these animals are exposed to head trauma due to their natural structure. The size and shape of the horn is important for shock absorption, and removing half of the horn in bighorn sheep reduces shock absorption and increases brain acceleration by about 50.00%.⁵ The horn structure of ewes is much smaller than that of male bighorns. Therefore, they may not function adequately to absorb blows to the skull. In this case, the risk of traumatic brain injury (TBI) in these ewes may be higher than in

bighorn sheep. Although hornless females are thought to be less confrontational, these ewes also engage in head-butting behavior within the species. In these ewes, the lack of horn structure to absorb impact may result in a higher risk of brain damage in the event of trauma.

Research has shown that animals with a calm temperament are less anxious than animals with an irritable temperament.⁶ Horned ewes are more likely to initiate and win aggressive interactions.⁷ A small amygdala has been associated to aggression.² Therefore, it could be argued that horned ewes may have a smaller amygdala and are more likely to suffer TBI. Studies have shown the negative effects of trauma on neuronal density in brain tissue.^{8,9} Repeated head trauma can cause neuronal degeneration, damage to the blood-brain barrier (BBB) and astrogliosis leading to various neurobehavioral disorders.⁹ In a study conducted on rams, it was stated that measurements of brain damage could help assess the actual criteria for brain damage in human head impacts.¹⁰

Although horned ewes are more aggressive, both horned and hornless females display intraspecific head-

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butting behavior and both species are at risk of developing TBI. In the literature review, no study was found that examined the neuronal density and other morphologies of the amygdala between horned and hornless ewes. Therefore, the aim of this study was to investigate whether there was a difference between horned and hornless ewes in neuronal density, glial cells and BBB in the amygdala, which was associated with aggression, fear and many other behaviors.

Materials and Methods

Collection of specimens. The heads of six hornless and four horned purple ewes aged 16.00 ± 4.00 months living in rural Eastern Anatolia were obtained from the abattoir and the brain tissues were carefully removed. Local Animal Experiments Ethics Committee of our University decided that ethical approval was not required due to the use of dead animals in the study (decision date: 22.06.2023/2300192174). The study was performed in accordance with the National and Animal Research Reporting of *In Vivo* Experiments guidelines.

Histological procedure. The localization of the amygdala in the right and left temporal lobes was determined and sections were taken from both amygdalae (Fig. 1).

Tissue processing procedure Brains preserved in 10.00% formalin for 5 days were washed in running water for 4 hr. Tissues were passed through increasing alcohol series for dehydration, passed through a xylene series to ensure transparency, infiltration was carried out by passing through a paraffin series, and paraffin and tissue blocks were prepared. Tissue blocks were cooled at 4.00°C and five $5.00\text{-}\mu\text{m}$ sections were cut from each block using a Leica RM 2145 microtome (Leica Microsystems, Wetzlar, Germany).

Hematoxylin and Eosin (H&E) staining. The samples were placed on polylysine slides and kept in an oven at 65.00°C for 15 min. The samples were passed through a xylene series to remove paraffin. Tissue samples washed with distilled water were stained with H&E. Tissue sections passed through alcohol and xylene series were cover-slipped with Entellan (Merck KGaA, Darmstadt, Germany) for microscopic examination.

Immunohistochemical staining for glial fibrillary acidic protein (GFAP). This method was carried out according to the study of Karadeniz *et al.*¹¹ Under a positively charged microscope slide, five microsections were taken from the tissue. Sections were placed in the Leica Bon-Max immunohistochemistry instrument (Leica). Paraffin was melted at 60.00°C for 30 min and removed by immersion in immunohistochemistry solution for 15 min. Samples were then rehydrated by immersion in 99.00% alcohol for 15 min. Samples were washed with buffer solution for 3 min, antigens were collected in Epitope 2

solution and washed again with buffer solution for 3 min. Samples placed in 3.00% hydrogen peroxidase were washed with buffer solution for 3 min. Anti-GFAP was then added and waited for 60 min, then washed with buffer solution for 3 min. The samples dripped with post-primer solution were allowed to stand for 10 min and then washed with buffer solution for 3 min. The samples to which a polymer solution was added were left for 10 min and then washed with buffer solution for 3 min. Then, diaminobenzidine + Chromogen was then added to the samples washed in deionized water for 3 min and waited for 3 min. The samples washed with distilled water were stained with an immunohistochemistry staining device for 5 min and washed with distilled water, alcohol and xylene. The samples removed from the device were covered with a thin coverslip for microscopic examination.

Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) method. This method was carried out according to the study of Sun *et al.*¹² Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling staining for brain tissue was performed using an *in situ* Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. After brain sections were deparaffinized and rehydrated, sections were permeabilized with proteinase K ($20.00\ \mu\text{g mL}^{-1}$) at 37.00°C for 10 min and then incubated with TUNEL reaction mixture at 37.00°C for 2 hr in the dark. Then, sections were incubated converter-peroxidase at 37.00°C for 30 min and washed with phosphate-buffered saline three times for 5 min. Finally, the slides were developed with diaminobenzidine tetrahydrochloride and counter-stained with Mayer's Hematoxylin. In each amygdala section, TUNEL-positive and intact cells nuclei were analyzed.

Examination of the preparations. The preparations were evaluated and photographed by computerized light microscopy (Eclipse E600; Nikon, Tokyo, Japan equipped with DP72 camera (Olympus Tokyo, Japan)).

Examination of H&E preparations. In these preparations, the localization of the amygdala in histological sections was shown (Figs. 1 and 2) and neuronal density was estimated (Fig. 3).

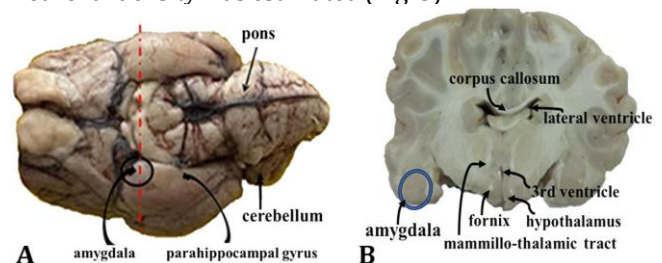


Fig. 1. Localization of the amygdala (AG) in ewe brain. **A)** Inferior view of the brain tissue of the ewe, the section passing through the temporal pole and the AG is shown with a red dashed line; **B)** Localization of the AG within the temporal pole in the coronal section of the brain tissue passing through the blue circle.

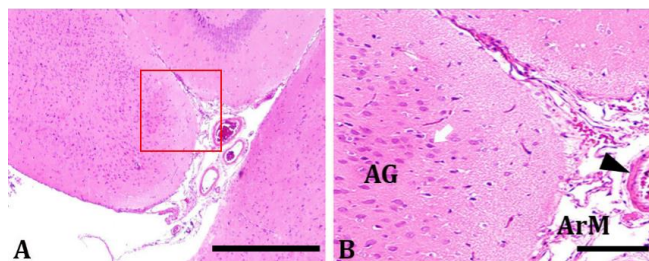


Fig. 2. A) Histological section of the amygdala (AG) in the temporal pole (H&E staining; bar = 200 μm); **B)** Macro magnification of red square in the section A illustrating the AG neurons (white arrow) and the arachnoid membrane (ArM) and the subarachnoid space underneath, and the artery feeding the AG (black arrowhead), (H&E staining; bar = 100 μm).

Stereological cell counting method. This method was carried out according to the study of Kanat *et al.*¹³ The number of neurons in the amygdala was assessed using the physical disector method. Two consecutive sections (separated by 5.00 μm) of tissue were placed on each slide (a pair of sections consisting of two consecutive sections used for cell counting in stereology is called a pair of dissectors). The first of the paired sections was used as the reference section and the other as the look-up section. The nuclei of neurons present in the reference section and absent in the look-up section were counted (if the nucleus of a neuron whose nucleus was visible in one section was also visible in the other section, the nucleus in the other section was excluded from the count to avoid double counting). The procedure was then repeated in reverse to increase the sample, so that the first section was considered the look-up section and the second the reference section. Counting frame areas were determined as shown in Figure 3. In the counting frame, the top and right lines indicated inclusion lines, and the bottom and left lines (with their extensions) indicated exclusion lines. Nucleoli in contact with the inclusion line were not counted. Nucleoli within the frame despite touching the inclusion line were counted as disector particles unless visible in the reference section. The number of neurons obtained from two sections was calculated in a volume formed by the product of the frame area and the segment thickness. The mean numerical density of amygdala neurons *per* mm^3 was estimated using the below formula:

$$NvAGN = \Sigma QN/t \times A$$

where, $NvAGN$ = mean numerical density of amygdala neurons *per* mm^3 ; ΣQN = total number of neurons counted that appeared only in the reference sections; t = section thickness; and A = area of the counting frame).¹³

Examination of immunohistochemical staining for GFAP preparations. In these preparations, degenerated neurons in the amygdala and BBB were evaluated by the light microscopy; (Fig. 4). The number of degenerated neurons was calculated using the stereological method. The BBB structures were assessed directly by observation.

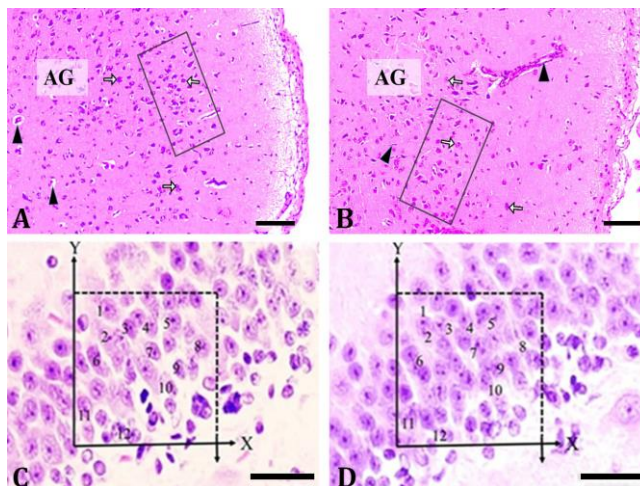


Fig. 3. Histological structure and stereological neuron counting of the amygdala (AG) in hornless and horned ewes. **A)** Macro magnification of the AG neurons of a hornless ewe, white arrows indicate higher neuronal density, and black arrowheads indicate capillaries supplying the AG, filled with cerebrospinal fluid (Virchow-Robin distance); **B)** Macro magnification of the AG neurons of horned ewe, white arrows indicate lower neuronal density and black arrowheads indicate vascular structures supplying the AG, surrounded by dense cerebrospinal fluid (Virchow-Robin distance), some of which have occluded capillaries; **C and D)** Stereological cell count of ewe AG. The number of neurons was estimated using the physical disector method described in the Materials and Methods section. The top and right lines represent inclusion lines, and the bottom and left lines represent exclusion lines. The nucleoli labeled 1, 2, 3, 4, 6, 8, and 10 in section C are disector particles because they are not visible in section D. The nucleoli labeled 5, 7, 9, 11, and 12 in section I are not disector particles because they are also visible in section D, (H&E staining; bar = 100 μm in A and B, 20.00 μm in C and D).

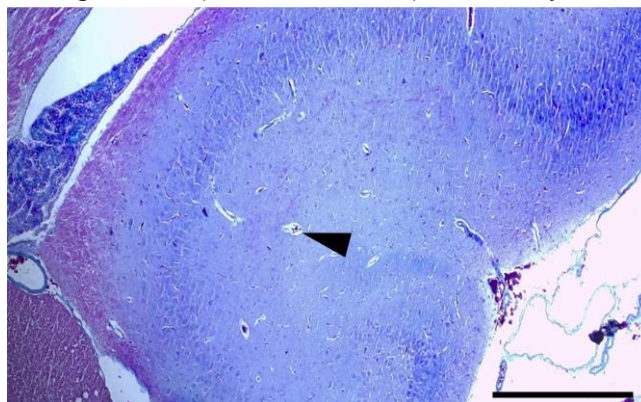


Fig. 4. Histological section of the amygdala in the temporal lobe is observed in a hornless ewe. The feeding capillaries are indicated by the black arrowhead (H&E staining; bar = 100 μm).

Examination of TUNEL method preparations. Neuronal apoptosis was evaluated in these preparations. Neuronal shrinkage, cytoplasmic and nuclear darkening were accepted as criteria for apoptosis, and darker and brown cells were considered apoptotic cells.

Statistical analysis. SPSS Software (version 20.0; IBM Corp., Armonk, USA) was used to analyze the data. The study groups were not normally distributed according to the Kolmogorov-Smirnov and Shapiro-Wilk tests ($p < 0.05$). The Mann-Whitney U test was used to analyze the results. The p value < 0.05 was accepted as statistically significant.

Results

Stereology. Stereological analyses of neuronal density using the physical dissector method were performed on preparations obtained with H&E stain. A higher neuronal density was observed in hornless ewes (Fig. 3A) compared to horned ewes (Fig. 3B). The difference in neuronal density between the right and left side in both horned and hornless ewes was not statistically significant ($p > 0.05$; Table 1). Neuron density was estimated to be $143,230 \pm 12,540$ per mm^3 in horned ewes and $152,230 \pm 18,430$ per mm^3 in hornless ewes, and the difference between hornless and horned ewes in these values was statistically significant ($p < 0.005$; Table 1; Figs. 3C and 3D).

Immunohistochemistry. Degenerating neurons were more abundant in horned than hornless ewes. The difference in neuronal degeneration between the right and left side in both horned and hornless ewes was not statistically significant ($p > 0.05$; Table 1). The number of degenerating neurons was $19,840 \pm 2,180$ in horned and in $6,370 \pm 850$ hornless ewes, and the difference between hornless and horned ewes in these values was statistically significant ($p < 0.001$; Table 1; Fig. 5). The sample vessel of the amygdala is indicated by the black arrowhead in Figure 5 in H&E preparations. The BBB was examined by the GFAP method in all groups.

The damaged BBB features accepted as reduced size of the vessel lumen, atrophied endothelium and smooth muscle, clogged lumen with blood elements, fragmented and reduced number of glial cells, and ischemic damage and inflammatory pathology were observed around the disrupted BBB. Normal BBB was more prominent in hornless ewes than in horned ewes, whereas, disrupted BBB was more prominent in horned ewes.

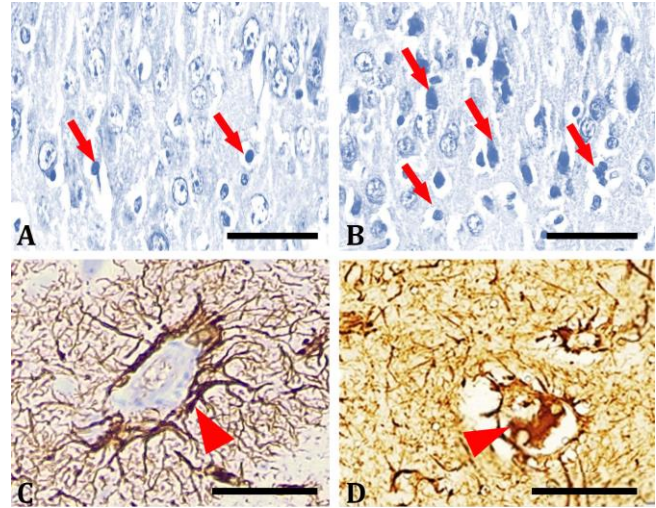


Fig. 5. Histopathological sections reveal that **A)** hornless ewes have fewer degenerated neurons and **B)** horned ewes have more degenerated neurons, red arrows indicate degenerated neurons. In horned subjects, there is a decrease in the number of neurons, shrinkage of nuclear structures, neuronal angularity and localized inflammatory areas; **C)** normal blood-brain barrier (BBB) is observed (red arrowhead) in hornless ewes, and **D)** damaged BBB is observed (red arrowhead) in horned ewes. Ischemic damage and inflammatory pathology are observed around the disrupted BBB (GFAP staining; bars = 20.00 μm).

Table 1. Neuronal density and degeneration of amygdala in hornless and horned ewes (n: case number)

Variables		Neuronal density per mm^3	Neuronal degeneration per mm^3
Amygdala of hornless ewes	Right (n:6)	151,140 \pm 18,300	6,210 \pm 830
	Left (n:6)	153,320 \pm 18,560	6,530 \pm 870
	Total (n:12)	152,230 \pm 18,430 *	6,370 \pm 850 **
Amygdala of horned ewes	Right (n:4)	141,350 \pm 12,375	19,160 \pm 2,110
	Left (n:4)	145,110 \pm 12,705	20,520 \pm 2,250
	Total (n:8)	143,230 \pm 12,540 *	19,840 \pm 2,180 **

* $p < 0.005$ and ** $p < 0.001$: The difference between hornless and horned ewes, respectively.

$p > 0.05$: There is no difference between the right and left side of both ewes.

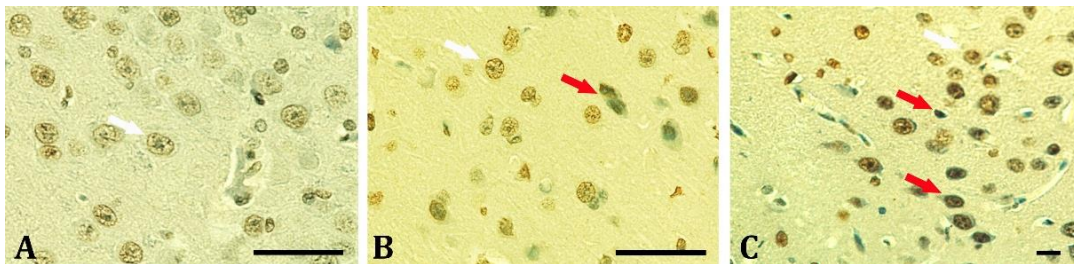


Fig. 6. Detection of apoptotic neurons (AN; red arrows) using the Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) method. **A)** Normal amygdala neurons (NN; white arrows); **B)** The AN with slightly darker nuclei and reduced size in hornless ewes; and **C)** Darker, smaller and more AN in horned ewes (TUNEL staining; bars = 20.00 μm in A and B; 5.00 μm in C).

TUNEL. Normal neurons in the preparations obtained by the TUNEL method are shown in Figure 6. More apoptotic neurons were observed in horned than in hornless ewes.

Discussion

Traumatic brain injury is a major health problem that causes death and disability in thousands of people each year, and mild TBI accounts for the majority of these cases.¹⁴ Studies have shown that repetitive head trauma results in decreased neuronal density, glial cell degeneration and disruption of the BBB.^{8,9} The TBI can cause damage to the amygdala as well as other brain structures. One study in concussed rugby players showed smaller hippocampus and left amygdala volumes compared to healthy individuals.¹⁵ Another study reported that the volume of the amygdala in a soldier with at least one mild TBI was decreased in direct proportion to the time since the injury.¹⁶ In professional fighters, each increase in impact exposure score resulted in a 0.90% decrease in amygdala volume,¹⁷ but there was no significant difference in amygdala volume between amateur boxers and healthy subjects.¹⁸ Structural changes in the amygdala have been linked to many diseases, including depression, post-traumatic stress disorder, schizophrenia, autism, epilepsy, Alzheimer's and Parkinson's disease.¹⁹ The emotional behavioral disorders mentioned are disorders closely related to the amygdala, and this is an indication that the amygdala is affected in mild head trauma. In previous studies, bilaterally smaller amygdala volume was found in individuals with post-traumatic stress disorder compared to healthy individuals,²⁰ and reduced amygdala volume was reported to cause some personality disorders in humans.²

Horns are important structures for absorbing head impacts, but in small horned animals, the horn structure is not sufficient to absorb impacts⁵, and the risk of brain injury during head trauma is high in these animals. Although horns appear to be a structure unique to rams, some ewes are known to have small horns. Male bighorn sheep (rams) are thought to be more aggressive, however, horned females are also known to be more aggressive than hornless females.^{2,7} Although this suggests that horned females are more confrontational and suffer more head trauma, hornless females also exhibit intraspecific head banging behavior and are not completely free of trauma. This means that these sheep are not completely trauma free and have a higher risk of developing brain damage if exposed to head trauma, especially since they are hornless. This raises the question of whether there is a structural difference in the amygdala, which is associated with aggression, depending on whether ewes have horns or not. Therefore, this study investigated whether there was a

difference between horned and hornless sheep amygdala of the same species (ewes).

In the present study, no statistical difference was found between the right and left sides of amygdala in terms of neuronal density and neuronal degeneration of the amygdala in both horned and hornless ewes. It has been reported in the literature that the left amygdala grows volumetrically earlier, however, the right amygdala reaches more volume over a longer period of time.²¹ One study reported that the amygdala was smaller on the left side in people with mild cognitive impairment.²² Another study reported that there was no significant difference between the sides of the amygdala in Alzheimer's patients.²³ Similarly, a laterality study in rats found no significant difference between the right and left sides of the amygdala.²⁴ In the literature search, no study was found examining the side differences in terms of neuronal degeneration and neuron density in the right and left amygdala in ewes. In this study, neuronal degeneration was found to be higher in horned ewes, while neuronal density was higher in hornless ewes. In horned ewes, degenerated neurons were calculated as $19,840 \pm 2,180$ and neuronal density as $143,230 \pm 12,540$ per mm^3 . In hornless ewes, degenerated neurons were calculated as $6,370 \pm 850$ and neuronal density as $152,230 \pm 18,430$ per mm^3 . The fact that horned ewes are more aggressive suggests that the amygdala of these ewes is smaller than that of hornless ewes.⁷ The lower neuronal density in horned ewes in our study may be related to this. However, the presence of more degenerating neurons in horned ewes also supports the idea that these sheep are exposed to more head trauma and develop TBI due to the inability of the small horns to absorb the impact sufficiently. In the literature, there are studies examining amygdala structure among different species and according to gender.^{7,25-27} However, no study was compared the structure of the amygdala in horned and hornless ewes. A previous study found that there were differences in the sexual behavior of domestic rams, with rams with poor sexual performance gravitating towards males rather than females, and the number of estrogen receptors in the amygdala of these rams being lower than in other rams.²⁸ The same study found no difference in the number of estrogen receptors in the amygdala between low sexually active rams and ewes.²⁸ In a study conducted in the Nigerian population, the neuron density of the basolateral amygdala of goats was found to be higher than sheep.²⁷ According to their study, goats recognized danger earlier than sheep and had fewer accidents than sheep.²⁷ One study reported that the superficial and center-medial nuclei of the amygdala were larger in men and that there is also a significant shrinkage in the amygdala with age.²⁶ However, in a study performed on monkeys, no difference was found between genders in amygdala sizes.²⁵

In this study, remarkable damaged BBB structures (atrophic capillary endothelium and smooth muscle, lumen occluded with blood elements, ischemic damage and inflammatory pathologies in glial cells around the BBB) were observed in horned ewes. The horn protects against brain damage by absorbing blows to the skull. However, the small horn structure of horned ewes may be insufficient to absorb impact. Therefore, the risk of TBI and BBB disruption increases in these sheep as a result of repeated head trauma. The results of this study also supported this hypothesis by showing BBB damage and neuronal degeneration in horned ewes. In cases where TBI develops, damage to the BBB is followed by perivascular inflammation, hemorrhage, coagulation, thrombotic necrosis, immune cell infiltration and neurodegeneration.²⁹ One study emphasized that BBB damage was common after head trauma and that this condition might occur several days, weeks and even years after the acute event.³⁰ Disruption of tight junctions in the BBB may cause neuroinflammatory disorders.³¹ Proinflammatory cytokines such as tumor necrosis factor- α and interleukin-1 β released in TBI increase endothelial permeability in the BBB,³² and secondary brain damage occurs as a result of components in the bloodstream coming into contact with the brain parenchyma.³³ This literature information supports that the neuronal degeneration, decrease in inter-neuronal glial cells, and local inflammatory areas in the horned ewes found in our study are associated with the damaged BBB found in the same samples. However, in our study, although there were noticeable damaged BBB structures in the horned ewes, the damaged BBB count and analysis was not performed in all areas in the horned and hornless ewes. Therefore, further study is required to determine if there is a statistical difference. No study was found comparing the BBB and other characteristics of the amygdala in horned and hornless ewes.

Although mechanical damage occurs first in TBI, secondary brain damage also occurs as a result of delayed neurochemical processes. The apoptotic mechanism has been implicated in the pathology of TBI in animal and human studies.^{34,35} Neuronal apoptosis to eliminate unnecessary neurons after traumatic injury may be a physiological and protective response to damage,³⁶ however, premature or impaired apoptosis is observed in the pathological process of many neurodegenerative diseases.³⁷ Apoptosis, also known as programmed cell death, can occur both physiologically and pathologically, and cell shrinkage, condensation and fragmentation of chromatin and apoptotic bodies containing organelle contents are features of apoptosis.³⁸ In the present study, it was observed that the number of apoptotic neurons in the amygdala was higher in horned ewes than in hornless ewes. In a study on rats, more apoptotic cells were detected in the amygdala of subjects exposed to a single episode of prolonged stress than in the normal group.³⁹

Another study in mice showed that mild to moderate TBI had significant effects on amygdala function and that amygdala excitability was decreased after TBI.⁴⁰ Apoptotic processes in brain tissue after TBI have been demonstrated in many animal models in the literature,⁴¹ however, there is no comparative study of apoptotic processes in the amygdala of horned and hornless ewes, which limits the discussion.

The differences observed in the amygdala of horned and hornless ewes can be explained as follows: Horned ewes are more confrontational; however, their small horn structures are insufficient to absorb blows to the head, increasing the risk of TBI. Because hornless ewes are calmer and less combative, they are not exposed to as much trauma and are at lower risk for TBI. On the other hand, hornless ewes are known to engage in head banging for play or fighting purposes. The lack of horn structure to absorb impact in these ewes would be expected to increase the risk of TBI resulting from head trauma. However, it was observed that amygdala neuron density, astrocyte number and branching were higher and the BBB was more intact in these ewes. This can be interpreted as the absence of horns to absorb impact in hornless ewes, which means that astrocyte structures are better organized against concussion during development. Nevertheless, the more intact amygdala of hornless ewes may be due to their less aggressive behavior. Another question is whether horned ewes become aggressive because their amygdala is structurally small or because the amygdala shrinks over time as a result of trauma. On the other hand, many conditions other than trauma, such as genetic or acquired causes, socio-emotional competencies, psychiatric diseases, age and gender can affect amygdala neuron density.⁴²⁻⁴⁴ Therefore, comparative studies of embryonic, neonatal and developmental stages are needed to better understand this situation. The results of this study may shed light on similar studies to be carried out in the future.

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Conflict of interest

The authors declared no conflicts of interest.

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