An examination of blind mole-rat *(Nannospalax xanthodon)* brain, cerebellum, and spinal cord tissues: A histological and stereological study

Research Article

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ABSTRACT

The purpose of this study was to perform a histological examination of blind mole-rat (*Nannospalax xanthodon*) brain, cerebellum, and spinal cord tissues. Six blind mole-rats were caught in a natural environment, anesthetized with ether, and sacrificed. Brain, cerebellum, and spinal cord tissues were then removed. All tissues were kept in 10% formaldehyde for one week, at the end of which they were subjected to routine histological procedures and embedded in blocks. Five micron-thick sections were taken from the blocks (5 and 15 micron thick from spinal cord tissues). All sections were then stained with hematoxylin-eosin, Cresyl Violet, and DAPI. These sections were then evaluated under light and fluorescent microscopes. The blind mole-rats weighed 201.3±61 g, the brains and cerebella weighed 1.8±0.3 mg and 0.32±0.05 mg, respectively, and the brain, cerebellum, and spinal cord volumes were 1.49±0.46 ml, 0.33±0.08 ml, and 2.53±0.19 µm^3, respectively. No histological variation was observed in the brain or cerebellum tissues. However, examination of the spinal cord tissue revealed differences compared to other rodents. The spinal cord exhibited a segmented, lobulated appearance, each lobe itself exhibiting the characteristics of a small spinal cord. No butterfly appearance was observed, and white and gray matter transitions were irregular, with less white and more gray matter. The location of the anterior and posterior horns was unclear. The motor neuron cells were also small in size. No significant variations were observed at nuclear organization (DAPI signals) between any tissues. In conclusion, the blind mole-rats compared to rats were normal in weight, increased brain and cerebellum tissue weight and volumes were observed, while a decrease was determined in spinal cord tissue volumes. The brain and cerebellum were normal at histological examination, while structural differences were detected in the spinal cord.

Keywords: Brain, Cerebellum, Spinal Cord, Nanosplax xanthodon, Stereology, Blind mole-rat

INTRODUCTION

Blind mole-rats are organisms adapted to a subterranean life. They spend their entire lives in tunnels they build underground and rarely emerge above the surface. They feed on the roots, rhizomes, and bulbs of root plants they encounter when digging their tunnels (Sözen, 2005). Blind mole-rats are long-lived (>20 years) rodents with tolerance to hypoxia (O_2 as low as 3%) (Figure 1). They have therefore recently been used as a model organism in medical studies. These animals exhibit no aging or age-related disorders or symptoms. Studies of blind mole-rats over an extended period (50 years) have reported no spontaneous tumors among thousands of individuals (Hadid et al., 2012; Kardong, 1995; Keleş et al., 2020; Manov et al., 2013; Nevo et al., 1995; Tian et al., 2013).

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Such studies have involved various branches of science, such as histology, anatomy, embryology, morphology, ecology, geography, behavior, biochemistry, cytogenetics, molecular biology, and geology. Although numerous studies have been carried out, research into these organisms is continuing due to the fact that they exhibit chromosomal changes, the difficulty of maintaining their habitats in the clinical environment, and that the reasons for their resistance to diseases have not been fully resolved. Some studies of *Nannospalax* species examining the central nervous system have described enlargement of the brain, cerebellum, and motor structures (Frahm et al., 1997). Another study reported that the spinal nerves forming the brachial plexus in the spinal cord and the interconnection of these spinal nerves differ from those of other rodents and mammals (Aydın and Karan, 2012). Although several studies involving the optic nerve have been performed, adequate information has not been obtained. Studies on this subject have reported that although the animal is blind, a structure has developed instead of the eye, and that this is connected to the brain through optic nerves and even a retina (Cernuda-Cernuda at al., 2002; Keleş at al., 2020). It is thought that the absence of visual organs may have led to greater development of the nervous system.

The above factors have all led to blind mole-rats being employed in scientific research. The starting point for the present study was to determine whether any difference exists in the central nervous system of these unique organisms. Our scan of the literature revealed some studies on the subject, although no clarity has been achieved. The purpose of this study was therefore to perform a histological examination of the brain, cerebellum, and spinal cord of the blind mole-rat *(Nannospalax xanthodon)*.

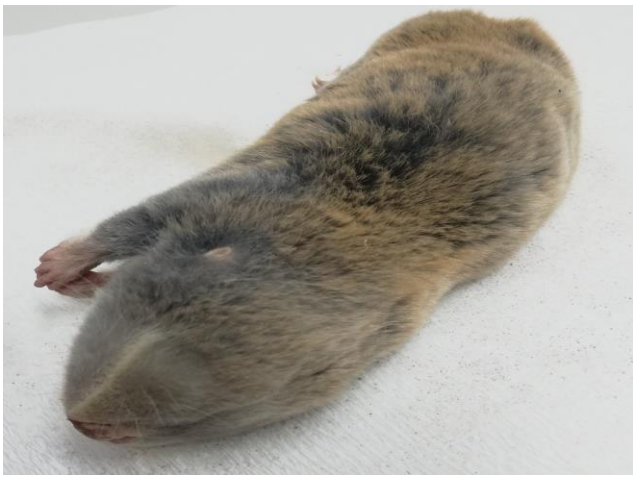

Figure 1: A photo of a blind mole-rat, Nannospalax xanthodon

MATERIAL and METHOD

Location, animals and feeds

Ethics statements and animal care

All animal procedures were approved by the institutional ethics committee at Niğde Ömer Halisdemir University (protocol number 2019/24 dated 10.09.2019) and were carried out in accordance with the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Wild-type *Nannospalax xanthodon* blind mole-rats were obtained from Niğde/Turkey. Six animals were weighed and sacrificed via ether inhalation without being exposed to any treatment. The spinal cord in the upper thoracic region, the brain, and the cerebellum were removed and also weighed.

Histological procedures

The harvested brains and cerebella were fixed in 10% formaldehyde for a week. Paraffin-embedded blocks were prepared through routine procedures. The spinal cord was kept in 10% formaldehyde overnight and subjected to decalcification [formic acid (5 ml), 10% formalin (5 ml), and distilled water (90 ml)] (Boncroft, 1996). At the end of the decalcification process, the tissues were kept in running water overnight. The spinal cord was

then subjected to routine histological procedures.

Five micron-thick transverse sections were cut from the brain, cerebellum, and spinal cord tissues using a Leica Biosystems RM 2245 Microtome (Germany). All sections were stained with Eosin Y (Product Code: HST-EOQ-0500, Histoplus) and hematoxylin Harris (Product Code: HEMH-OT-100, Biognost) for histological evaluation and with cresyl violet (Product Code: C5042). All sections were evaluated under a light microscope (Olympus, BX53, digital camera: DP 80, Olympus and cellSens standard software version 1.17, Japan).

DAPI procedures

Five micron-thick transverse sections were cut from the brain, cerebellum, and spinal cord tissues using a Leica Bio systems RM 2245 Microtome (Germany). Tissue sections were then deparaffinized, washed in phosphate buffered saline washed and kept in 0.4% Triton-X-100 (1x10 minutes). Sections were processed consecutıvely in Twin 20 (3x1 minutes), 2% Paraformaldehyde (1x40minutes), and Twin 20 (3x1 minutes). Next, the sections were blocked with 4% bovine serum albumin (1 hour) and Twin 20 (3x1 minutes). 4′,6-diamidino-2-phenylindole (DAPI) was kept in the dark for 15-20 minutes and then sealed and stored at 4°C in the dark. The resulting sections were evaluated histologically with the aid of a fluorescent microscope attached to a light microscope (Olympus, BX53, Japan) equipped with a digital camera (DP 80, Olympus, Japan) and cellSens standard software (version 1.17).

Brain and cerebellum volume measurement

Archimedes' principle (Keleş et al., 2020) was used to measure the volume of the brains and cerebella. Both organs were submerged separately into a graduated cylinder filled with water. The volume of each tissue was equal to that of the displaced water.

Spinal cord volume measurement

Five tissue sections per animal each 15 microns in thickness, were taken and stained with hematoxylin-eosin (H&E for stereological analyses. The section sampling fraction was 1/10. Volumetric evaluation of the spinal cord tissues was performed using the Cavalieri principle (Keleş and Biterge Süt, 2021) (Table 1) (Figure 2)

Table 1: Parameters used in stereological analysis

Stereological analysis parameters	Values
Section sampling fraction	1/10
Sampled sections from each spinal cord	10
Paraffin section thickness (µm)	15
Objective lens for thickness	x20

Spinal cord volume was calculated manually using a point grid. The following formula was employed when calculating the results -

Ai=Σ Pi x a / p

where **Ai** represents the total section area, **Σ Pi** the total number of points on the section image of interest, and the center of each “plus” (+) sign on the grid represents a specific unit area **(a/p)** (Keleş, 2019).

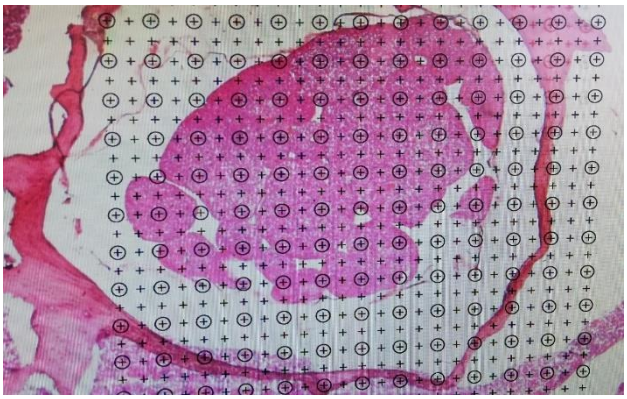

Figure 2: Placing the point-counting probe on the spinal cord tissue (x4, Hematoxylin & Eosin)

Statistical analysis

Statistical analysis was performed on Statistical Package for the Social Sciences (SPSS) version 22.0 software (BM Corporation, Armonk, NY, USA). Values were calculated as mean± standard deviation.

RESULTS

Brain, cerebellum, and spinal cord volume analysis results

The body, brain, and cerebellum volumes of the six blind mole-rats in the study are shown in Table 2**.**

Body, brain, and cerebellum weight

The brain, cerebellum, and spinal cord weights of the six blind mole-rats are shown in Table 2.

Table 2: Weight and volume measurements (n=6)

Measured parameters	
Blind mole-rats (n=6)	**Mean±standard deviation**
Body weight (g)	201.3 ± 61
Brain weight (mg)	1.8 ±0.30
Cerebellum weight (mg)	0.32 ±0.05
Cerebellum volume (ml)	0.33 ±0.08
Brain volume (ml)	1.49±0.46
Spinal cord volume (µm3)	2.53± 0.19

Histopathological evaluation of brain, cerebellum, and the spinal cord tissues

Analysis following H&E and cresyl violet staining of the brain, and cerebellum tissues of the blind mole-rat revealed no histological difference in the brain and cerebellum tissues of the other rodent (Figure 3-4).

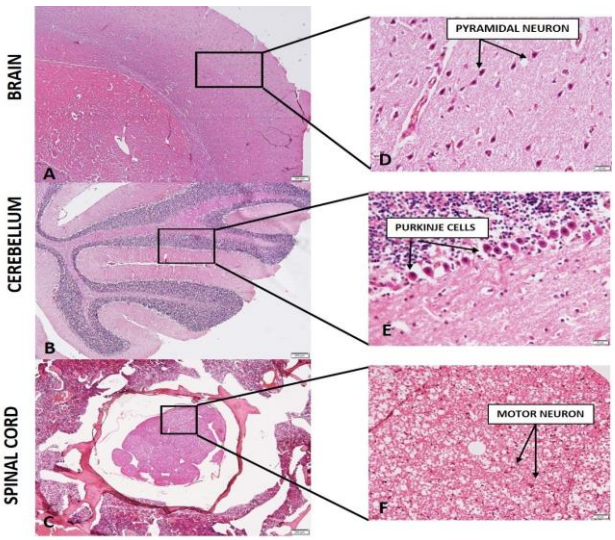

Figure 3: Representative micrographs of the brain (prefrontal cortex), cerebellum, and spinal cord (A, B, C x4; D, E, F x40, Hematoxylin & Eosin)

However, examination of spinal cord tissues revealed differences compared to both humans and other rodents. The pia mater surrounding the spinal cord was normal and easily visible. In general terms, it exhibited a segmented and lobulated appearance. However, the butterfly appearance that would normally be expected in the spinal cord was not observed. White and gray matter transitions were irregular, with less white and more gray matter. The site of the anterior and posterior horn was unclear. Examination of the cellular structures of the spinal cord revealed neuroglial cells and motor and sensory neurons, although their location (in the anterior and posterior horn) was unclear, and the motor neurons were smaller in size. Each lobe contained a cord resembling a small spinal cord, white matter, gray matter, neurons, and glial cells. Ependymal cells were observed around the medulla spinalis. The epithelium of the ependymal cells was single-layered and cuboidal (Figures 3-4).

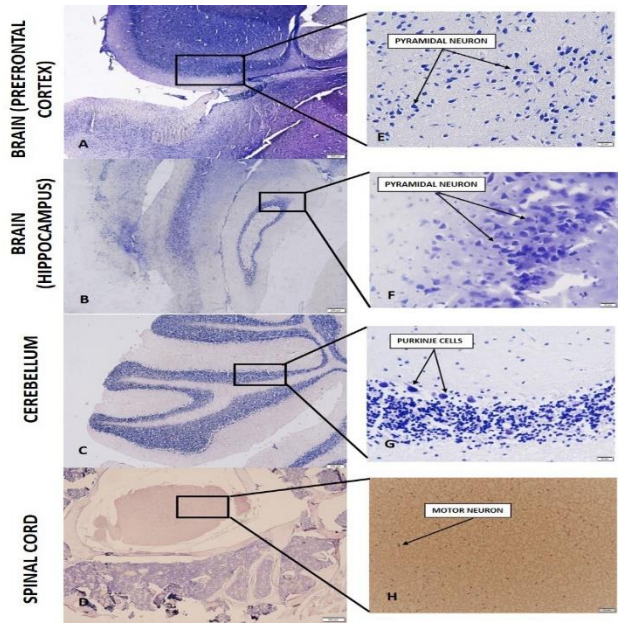

Figure 4: Representative micrographs of the brain (prefrontal cortex and hippocampus), cerebellum, and spinal cord (A, B, C, D x4; E, F, G, H x40, Cresyl viole

Immunofluorescence evaluation of the brain, cerebellum, and spinal cord

Cell signals were evaluated with DAPI staining in areas with dense cells in the brain (hippocampus and prefrontal region), cerebellum, and spinal cord tissues on immunofluorescence images. Cell signals were

localized in the nuclei of the cells in the prefrontal cortex, hippocampus [cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 3 (CA3), dentate gyrus and hilus], cerebellum, and spinal cord regions in the brain (Figures 5-6).

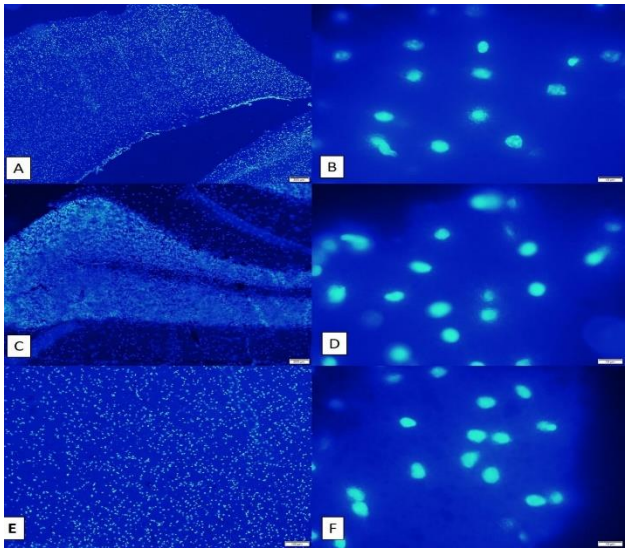

Figure 5: Representative micrographs of the brain (prefrontal cortex) (A, B), cerebellum (D, C), spinal cord (E, F). DNA stained with DAPI (blue) marks the nucleus) DAPI: 4′,6-diamidino-2-phenylindole. (A, C x4, E x10, B, D, F x100)

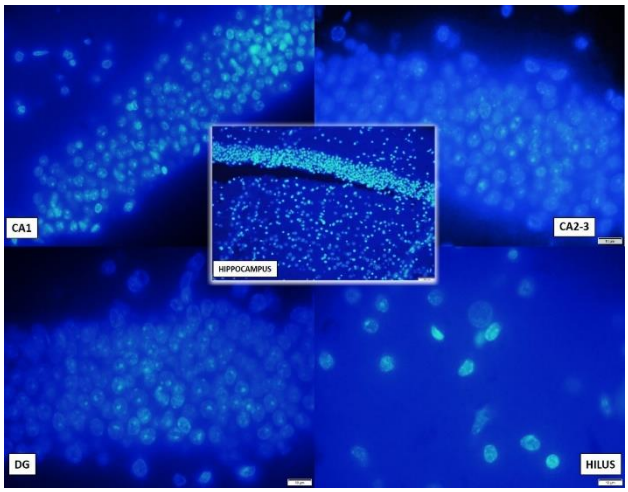

Figure 6: Representative micrographs of the brain. Hippocampus x20 and CA1, CA2-3, DG, Hilus x100. DNA stained with DAPI (blue) marks the nucleus. (C1, cornu ammonis 1; C2-3, cornu ammonis 2-3; DG, dentate gyrus) DAPI: 4′,6-diamidino-2-phenylindole

DISCUSSION

The histological structures of the brain, cerebellum, and spinal cord of the blind mole-rat were examined due to these animals' high tolerance to hypoxia (Nevo et al., 1994), behavior, emotion-perception, and sensitivity to different stresses (Avivi et al., 2005; Avivi et al., 1999), the fact they do not experience cancer (Altwasser et al., 2019), and are even resistant to induced carcinogenesis (Manov et al., 2013).

The bodies, brains, and cerebella of six randomly caught blind mole-rats were weighed. The mean body weight of these six blind mole-rats was 201.3 ± 61g. A previous study reported a mean body weight of 191.7 ± 26.5 g in 25-day-old Sprague Dawley rats (Keleş et al., 2019). Blind mole rats appear have a higher body weight. However, considering the biological, genetic, and environmental factors, we do not think it would be appropriate to directly compare the animals caught in this study with normal rats.

The mean weight of the brains of the blind mole-rats in this study was 1.8 ± 0.3 mg, and the mean weight of the cerebella was 0.32 ± 0.05 mg. A previous study of male Sprague Dawley rats weighing 191.7 ± 26.5 g determined a mean brain weight of 1.2 ± 0.05 mg (Keleş et al., 2019). Another study reported a mean brain weight of 1.43 ± 0.07 mg in female Sprague Dawley rats (mean weight 241.83 ± 20.10 g) (Aktürk et al., 2014). Another study reported a cerebellar weight of 0.19 g in 21-day-old female Wistar rats (Fang et al., 2020). Our findings indicate greater brain and cerebellum weights compared to those of normal rats.

The mean brain and cerebellum volumes of the blind mole-rats in this study were 1.49±0.46 ml and 0.33± 0.08 ml, respectively. Aktürk et al. (2014) determined rat weights of 241.83±20.10 g, with a mean brain volume of 4.61±0.16 mm^3 (0.004 ml). Another study reported a cerebellar volume of 1.4 mm^3 (0.001 ml) in 21-day-old female Wistar rats (Fang et al., 2020).

Frahm et al. (1997) compared the brain and cerebellum volumes of rats and blind mole-rats

and concluded that these were greater in the latter. The present study also suggests a volume increase. The findings show that the increases in the weights and volumes of the brain and cerebellum are directly proportional. Spinal cord volume was also evaluated in this study, and was calculated at 2.53± 0.19 μm^3. We encountered no previous studies investigating spinal cord volume in blind mole-rats. A rat study reported a volume of 2.88±0.26 μm^3 (Keleş and Biterge Süt, 2021). This was lower than the spinal cord volume of blind mole-rats. The volumetric decrease in spinal cord tissue appears not to be normal in the light of the rats' body weights. The volume would normally be expected to be greater than the value determined. In addition, the reasons for the decrease in spinal cord volume, despite greater brain and cerebellum weights and volumes, is unclear.

Histological examination of spinal cord tissue revealed differences compared to humans and other rodents. The pia mater surrounding the spinal cord was normal and clearly visible. It exhibited a segmented, lobulated appearance. However, the butterfly appearance that would normally be expected in the spinal cord was not present. The white and gray matter transitions were irregular, with less white matter being seen and more gray matter. The location of the anterior and posterior horn was unclear. Examination of the cellular structures of the spinal cords revealed neuroglial cells and motor and sensory neurons, although their locations (in the anterior and posterior horns) were unclear. Cells were also smaller than normal compared to other rodents. Each lobe contained a medulla spinalis, white matter, gray matter, neurons, and glial cells, resembling its own small spinal cord. Ependymal cells were observed around the medulla spinalis. The epithelium of the ependymal cells was single-layer and cubic in appearance. These findings suggest the presence of significant differences in the spinal cord structures. One previous study reported that the spinal nerves forming the brachial plexus and the interconnection of these spinal nerves in the spinal cord of blind mole-rats differ from those of other rodents and mammals (Aydın and Karan, 2012). Although this does not fully support the present study, we nevertheless think that it shows the presence of differences in spinal cord structures. Our examination of the literature in terms of the structures of the blind mole-rat spinal cord structures revealed no previous studies on the subject.

No difference was detected in the analysis of cell signals in brain, cerebellum or spinal cord tissues under fluorescent microscopy. No difference was found in terms of structural or genetic (DAPI signal) evaluation when nucleus organization was examined in these tissues.

CONCLUSION

In conclusion, based on a comparison with rata data in the literature, the weight of the blind mole-rats in this study was normal, an increase was observed in volume and weight in brain and cerebellum tissues, and a volumetric decrease in spinal cord tissue. Structural and volumetric differences were also observed in spinal cord tissue, and cells were smaller than other rodents.

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Ethical approval:

All animal procedures were approved by the institutional ethics committee at Niğde Ömer Halisdemir University (protocol number 2019/24 dated 10.09.2019) and were carried out in accordance with the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Conflict of interest: The authors declare that they have no conflicts of interest.

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