



CYTOARCHITECTURE OF GREATER CANE RAT (*Thryonomys swinderianus*) HABENULAE AND MAMMILLARY BODY

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ABSTRACT

This study was conducted to investigate expression of neurons, glia and connective tissue markers in the habenulae and mammillary body of the Greater Cane Rat (GCR) using histological and immunohistochemical techniques. Four apparently healthy males GCR obtained from Lagos Nigeria were used for this experiment. The brains were harvested after perfusion, carefully dissected and post-fixed in 10% neutral-buffered-formalin (NBF) at 4°C for 48 hours. Subsequently, tissue sections were prepared and stained using Haematoxylin and Eosin (H&E), Cresyl-violet, ionized calcium-binding-adaptor molecule 1 (Iba1; microglia), glial fibrillary acidic protein (GFAP; astrocytes), neuronal nuclei (NeuN; neurons) and collagen type 1 (Collagen1) antibodies. H&E and Cresyl-violet staining showed the neuronal nuclei and their fibres in the habenulae, mammillary body, and pial capsule that surround the mammillary body ventrally. Astrocytes in the habenulae showed positive immunoreaction to anti-GFAP while the mammillary body revealed expression of perivascular astrocytes and capsular astrocytes. Also, in the mammillary body, the perivascular microglia and meningeal microglia in the pial capsule showed immunopositive reaction to anti-Iba1 while the fibres within blood vessels and capsule were immunopositive to collagen type 1 antibody in the same structure. This work has provided the first cytoarchitectural features and clear description of expression of these specific markers in habenulae and mammillary body in the GCR, which are similar to other rodents but with slight variations.

Key words: morphology, astrocytes, microglia, habenulae, mammillary body, greater cane rat.

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INTRODUCTION

Cane rat has two species these are: *Thryonomys swinderianus* and *Thryonomys gregorianus*. The *Thryonomys swinderianus* are also known as Greater Cane Rat (GCR) which are usually found in African south of Sahara while the *Thryonomys gregorianus* known as lesser cane rats are found in Cameroon, Southern Sudan and Zimbabwe (Adu et al., 2017). The animal commonly lives among dense grasses mostly along river banks and swamps and is rampant among non-woody vascular plants where there is a good covering (Aluko et al., 2015).

The habenulae and mammillary bodies are epithalamic and hypothalamic structures

respectively and are both derived from the diencephalon embryologically. The habenulae are found along with the pineal gland at the caudodorsal part of the thalamus. The name habenula is derived from Latin word habene, meaning: "little rein", because of its elongated shape. Originally its function was thought to be related to the regulation of the nearby pineal gland. More recent evidence, however, demonstrates that the habenula acts as critical neuroanatomical hub that connects and regulates brain regions important for divergent motivational states and cognitive function (Namboodiri et al., 2016). They are

surrounded by the thalamus and located very close to the midline, bordering on the third ventricle. They are divided into two main subregions in mammals; the medial and lateral habenulae. These two subregions display distinct gene expression profiles and anatomical connectivity and hence, are thought to subservise different functions. In lower, vertebrates, the habenulae are often split into dorsal and ventral habenulae (Hikosaka et al., 2008). Habenulae main input pathway is the stria medullaris, whereas their main output pathway is the fasciculus retroflexus, also known as habenulo-interpeduncular tract (Hikosaka et al., 2008). Originally the mammillary bodies were referred to as the "testicles of the brain", they have subsequently come to be known as the "breast of the brain" (Jones, 2011). The mammillary bodies have long been considered to play an important role in memory (Delay and Brion, 1969). They comprise just two major nuclei groups, the lateral and medial nuclei, with a narrow array of cell types in each (Vann and Aggleton, 2004). The mammillary bodies have major connections with a limited number of regions, and most of these pathways can be readily seen in a dissected brain (Vann et al., 2007). The mammillary bodies receive a major input from the hippocampus, via the

fornix, whereas their main output is to the anterior thalamic nuclei, via the mammillothalamic tract (Saunders et al., 2012).

After a thorough search of literature, there is however a scarceness of data on histology and immunohistochemistry of habenulae (an epithalamic structure) and mammillary body (a hypothalamic structure) of this cane rat. Due to the increasing use of GCR as an animal research model (Mustapha et al., 2020), there is the need to establish more baseline research data on the cellular architecture and neuroanatomical profile. Previous histoarchitectural studies reporting on the nervous system of the GCR include: Dwarika et al., 2008 (on putative catecholaminergic and serotonergic neurons); Spruston, 2008 (on pyramidal neurons); Obadiah and Obadiah, 2014 (on cerebellum); Elson and Manger, 2014 (on pyramidal cells); Mustapha et al., 2017 (on spinal cord) and Gilbert et al., 2020 (on pineal and pituitary glands). Therefore, this study aims to describe briefly the gross morphology, basic histological structures, and finally identify some immunohistochemical features of the habenulae and mammillary body of the young adult male GCR.

MATERIALS AND METHODS

Cadaveric Experimental Animal, Perfusion, Brain Extraction and Fixation

A total of four apparently healthy young adult male GCR, 7-8 months old with average body weight of 2.13 ± 0.09 kg were obtained from Panvemgo Grasscutter Farm in Lagos, Nigeria, a farm reputable for keeping birth records. They were transported in a well-ventilated metallic cage and were acclimatized for 48 hours in Animal House of the Neuroscience Unit, Department of

Veterinary Anatomy, University of Ibadan, Nigeria. They were provided with free access to water and food. At the time of sacrifice, the body weight of each rodent was obtained with a digital electronic balance (Zhongshan Camry Electronic Co. Ltd, China) for the purpose of anaesthesia. They were anaesthetized by intraperitoneal injection of ketamine and xylazine combination (100/10mg/kg), and perfused intracardially with normal saline followed by 10% neutral buffered formalin (10% NBF) as described by Olopade et al., 2011. The brains were

harvested from the skull and both the habenulae and mammillary body were carefully dissected. This was followed by post-fixation of these tissues in 10% NBF for 48 hours at 4°C.

Histological Staining

Paraffinized brain tissues were sectioned at a thickness of 5µm, and Haematoxylin and Eosin (H&E) and Cresyl violet (Nissl) staining of the habenulae and mammillary body was carried out following the methods described by Olopade et al., 2011 and Mustapha et al., 2019. Prepared sides were viewed and photomicrographs taken with a Leica DM500 HD digital microscope (New York, USA) with both low and high-powered objectives (i.e. x4, x10 and x40 magnification).

Immunohistochemistry

The habenulae and mammillary body tissues were prepared for immunohistochemistry following the protocol described by Azeez et al., (2016). Briefly, 5µm thick paraffinized brain sections were mounted on adhesive glass slides, well labeled with pencil and baked in an oven set at 60°C for 1 hour 30 minutes to melt the wax. Deparaffinization of the sections was done in 2 changes of xylene, after which the sections were hydrated in decreasing concentration of ethanol to water. Sections were then rinsed in double distilled water before retrieval of antigen was achieved by incubating the

sections in 10 mM citrate buffer at pH of 6.0 for 25 minutes to unmask the hidden antigenic site. In order to reduce non-specific antibody binding and to hinder endogenous peroxidase activities, sections were treated with 30% hydrogen-peroxide/methanol. Sections were subsequently blocked by incubating in 2% phosphate buffered saline (PBS) milk while on a rocker for 60 minutes. Consecutive sections were immune-labeled with the following antibodies: rabbit anti-Iba1 (dilution 1:1000, Wako Pure Chemical Industries Ltd., Japan) for microglial cells, rabbit anti-GFAP (1: 1000; Dako, Denmark), to visualize astrocytes; rabbit anti-NeuN (dilution 1:500, EMD Millipore, USA) to visualize neurons and rabbit anti-collagen type 1 (1:200, Abcam Inc, USA), for collagen connective tissue. The antibodies were diluted in 1% PBS milk and 0.1% Triton X detergent (to facilitate quick penetration of antibody) and the samples incubated overnight at 4°C. The end product of the entire reaction was visualized with 3, 3'-diaminobenzidine a chromogen (1:25 dilution, Victor Laboratories, USA) for 5 minutes. Subsequently, sections were dehydrated in solutions of graded ethanol concentrations, and the ethanol was removed by passing through two changes of xylene (5 minutes each), then mounted wet in permount (toluene based mountant, Atom Scientific, Manchester), cover slipped and allowed to dry. Microscopic examination was carried out using Leica DM500 HD digital microscope (New York, USA).

RESULTS

Gross Examination

The habenulae of the GCR are paired bilaterally and elongated. They are located rostral to the pineal gland in the GCR. They are also seen lying at the most caudal and dorsal part of the thalamus and lateral to the third ventricle in the GCR (Figure 1). The mammillary body of the GCR is a rudimentary structure located at the base of the caudal

margin of the hypothalamus at the ventral part of the brain, with the median eminence being rostral to it (Figure 2).

Histological and Immunohistochemical Examination

Examination of the habenular nuclei in GCR habenulae revealed no distinct demarcations between the medial habenular and lateral

habenular nuclei. Furthermore, H&E and Cresyl violet stains revealed the entire habenular nuclei and their fibres in the two habenulae (figure 3). The ependymal cells are also seen lining the third ventricle which separates the two habenulae. Both H&E and Cresyl violet showed neurons and their fibers within the parenchyma of the mammillary body in the GCR. The blood vessels within the parenchyma and capsule around the mammillary body were also seen (Figure 4). GFAP immunostaining of the GCR's habenulae revealed the expression of astrocytes within the parenchyma of both habenulae (Figure 5). The habenular nuclei were immunoreactive to NeuN antibody (Figure 6). The mammillary body showed the expression of astrocytic-like cells within the parenchyma, around the blood vessel and within capsule of the GCR (Figure 7). The mammillary body also had the expression of perivascular microglia round the blood vessels and microglia in the capsule (Figure 8). The GCR mammillary body showed the expression of collagen type 1 connective tissue in the layers of blood vessels and capsule (Figure 9).

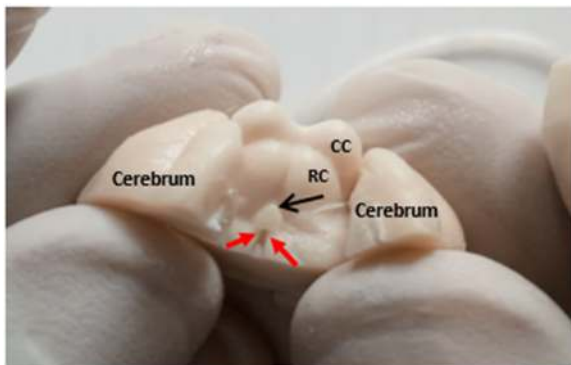


Figure 1: Photograph of the brain of GCR showing the bilaterally paired habenulae (red arrows) and the pineal gland (black arrows). RC, rostral colliculus; CC, caudal colliculus

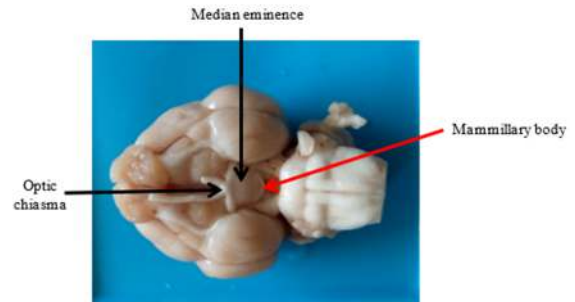


Figure 2: Ventral view of the GCR brain showing the mammillary body, median eminence and optic chiasma.

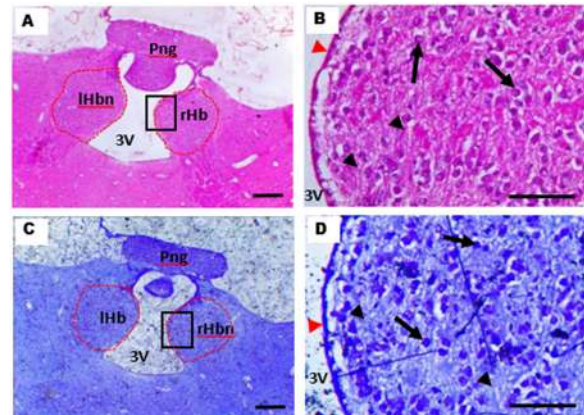


Figure 3: Photomicrograph of the GCR habenulae. A&C. The habenular nuclei (Hbn) is divided into left (IHbn) and right (rHbn) habenulae separated by third ventricle (3V). Png = pineal gland (H&E and Cresyl staining; x4 Magnification; A&C: Scale bar = 200 μ m); B&D. Higher magnification showing habenular neuronal nuclei (black arrows) and neuronal fibres (arrow heads). The ependymal cells (red arrow) are also seen lining the third ventricle. l, left; r, right. (H&E and Cresyl staining; x40 Magnification; B&D: Scale Bar = 50 μ m).

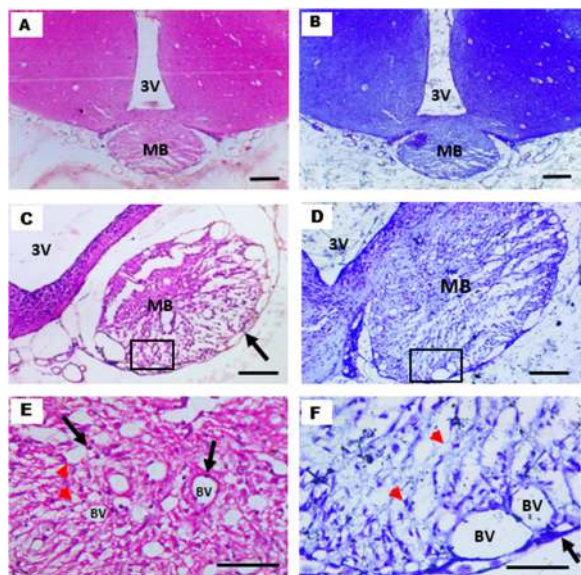


Figure 4: Photomicrograph of mammillary body of the GCR. A-D. The mammillary body (MB) is located below the third ventricle (3V) and covered with pial capsule (black arrow) (H&E and Cresyl staining; x4 and x10 Magnification; A: Scale bar = 200 μ m, B: Scale bar = 200 μ m); E. The parenchyma of the mammillary body showing the neuronal nuclei (long black arrow), nerve cell fibers (red arrow), and blood vessel (BV). (H&E and Cresyl staining; x40 Magnification; C: Scale Bar = 50 μ m). F. The parenchyma of the mammillary body showing the neuronal nuclei (red arrow), blood vessel (BV) and capsule (black arrow). (Cresyl staining; x40 Magnification; C: Scale Bar = 50 μ m).

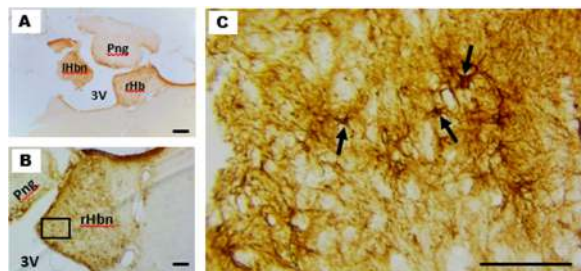


Figure 5: Photomicrograph of the habenulae of the GCR. A. The two habenular nuclei (Hbn) is divided into left (IHbn) and right (rHbn) habenulae separated by third ventricle (3V) (Anti-GFAP x4 Magnification; A Scale bar = 200 μ m); B&C. The habenula showing astrocytes (black arrows). l, left; r, right. (Anti-GFAP immunostaining; x10 and x40 Magnification; B: Scale bar = 100 μ m, C: Scale bar = 50 μ m).

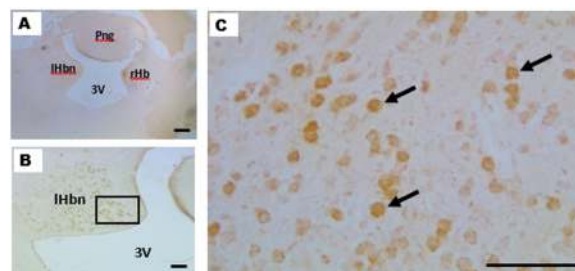


Figure 6: Photomicrograph of the habenula of the GCR: A&B. The habenulae showing the two habenular nuclei (Hbn) that is divided into left (IHbn) and right (rHbn) habenulae separated by third ventricle (3V). Png = pineal gland (Anti-NeuN immunostaining; x4 and x10 Magnification; A: Scale bar = 200 μ m, B: Scale bar = 100 μ m) C. The habenula is showing the expression of neuronal nuclei (black arrows). l, left; r, right. (Anti-NeuN; x40 Magnification; C: Scale bar = 50 μ m).

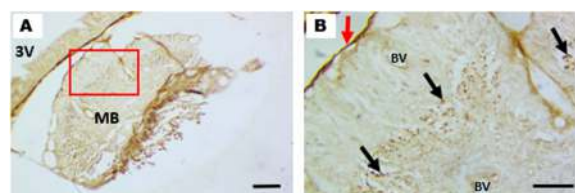


Figure 7: Photomicrograph of mammillary body of the GCR. A. The mammillary body found below the third ventricle (3V) (Anti-GFAP; x10 Magnification; A: Scale bar = 200 μ m). B. The mammillary body showing the expression of astrocytes found around the blood vessel (BV) and astrocytes in the capsule (red arrow). The astrocytic-like cells (black arrow) are also seen in the parenchyma of the mammillary body (Anti-GFAP; x40 Magnification; B: Scale bar = 100 μ m).

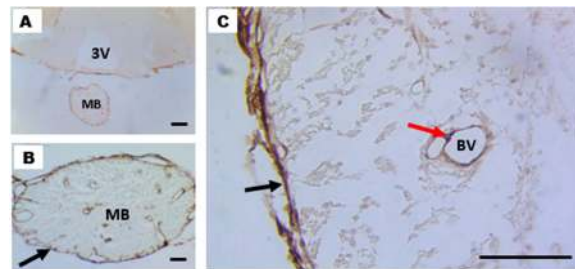


Figure 8: Photomicrograph showing the GCR mammillary body: A&B. The mammillary body (MB) is seen below the third ventricle (3V) (Anti-Iba1 immunostaining; x4 and x10. Magnification; A Scale bar = 200 μ m; B Scale bar = 100 μ m); C. The parenchyma of the mammillary body showing the expression of perivascular microglia (red arrow) round the blood vessels (BV) and capsular microglia (red arrow) (Anti-Iba1 immunostaining; x40 Magnification; C: Scale bar = 50 μ m).

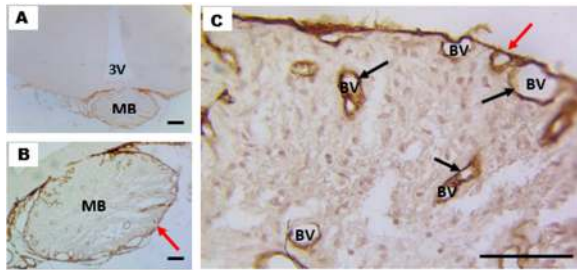


Figure 9: Photomicrograph showing the GCR mammillary body: A-B. The mammillary body (MB) is visible below the third ventricle (3V) (Anti-Collagen1 immunostaining; x4 and x10 Magnification; A: Scale bar = 200 μ m. B: Scale bar = 100 μ m); C. The parenchyma of the mammillary body showing the expression of collagen type 1 in the layers (black arrow) of blood vessels (BV) and mammillary capsule (red arrow) (Anti-Collagen1 immunostaining; x40 Magnification; C: Scale bar = 50 μ m).

DISCUSSION

The habenulae of the GCR are located rostromedial to the pineal gland as epithalamic structures just as reported by Hikosaka et al., 2008 in rhesus monkey. The habenular commissure runs between the two bilateral paired habenulae of the GCR as reported by Namboodiri et al., 2016 in monkey, mice and zebrafish. In the GCR, bilaterally paired habenular nuclei are positioned adjacent to third ventricle as described by Bianco and Wilson in 2009. The demarcation between medial and lateral habenular nuclei is not distinct as seen in many mammals such as monkeys, mice and zebrafish (Bianco et al. 2008; Namboodiri et al., 2016). However, Company et al., 2021, showed immunoreactivity of both medial and lateral habenulae using contactin 2 (CNTN2) antibody as a marker for medial habenular axonal projections and fem protein (NFEM) antibody for lateral habenular axonal projections in the mice embryos. Therefore, the medial and lateral habenular projections in GCR may be expressed with contactin 2 and fem antibodies as markers for medial and lateral projections respectively. There was also no obvious difference in size between the left and right habenulae, unlike what is observed in reptiles and fishes, as size asymmetry is said to be more subtle in mammals (Bianco and Wilson, 2009). The astrocytes expression to GFAP antibody seen in the GCR habenulae parenchyma is in agreement with what was reported by Cui et al., 2014, where they demonstrated the expression of astrocytes using GFAP antibody in the habenulae of mice. Furthermore, the habenular nuclei of the

GCR showed the expression of neuronal nuclei to NeuN antibody in the two habenulae as reported by Cui et al., 2014 and Cui et al., 2018.

The mammillary body of the GCR was located at the base of the brain caudal to the median eminence as reported by Allen and Hopkins (1988) in rats and Vann and Aggleton (2003) in man. Cresyl violet stain showed neuronal nuclei and their fibers in the parenchyma of the mammillary body as revealed by Allen and Hopkins 1988 using the rats. However, we did not see any observable demarcations between the lateral and medial mammillary nuclei as reported by Vann and Aggleton (2003) in man. The immunohistochemical results in the GCR mammillary body, showed the expression of GFAP of perivascular astrocytes around the blood vessels and astrocytes expression was also seen in the capsule of mammillary body. Furthermore, astrocytic-like cells expression to GFAP was seen in the parenchyma of the mammillary body, which is similar to the findings of Kalman and Hajos (1989), where they showed that mammillary nuclei do not stain effectively with GFAP, however, a weakly labeled bundle of astrocytes was seen separating the medial and lateral mammillary nuclei.

CONCLUSION

This to the best of our knowledge, is the first report on cytoarchitecture of the habenulae and mammillary body of the GCR that showed unique histomorphology. It was

observed, that the general microanatomy is similar to other rodents. Furthermore, we were able to show the following in the GCR: astrocytes/astrocytic-like cells in the habenulae and mammillary body, perivascular and capsular microglia in the mammillary body, neuronal nuclei in the parenchyma of habenulae using NeuN antibody, collagen type 1 connective tissue fibers in the mammillary body which are found around the perivascular and capsular

regions. As the GCR continues to attract attention as a spontaneous model of laboratory research in Africa, baseline data on habenulae and mammillary body morphophysiology will increasingly become important.

CONFLICTS OF INTEREST

The authors affirmed that there are not any conflicts of interest.

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