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Structure of the Main Olfactory Bulb and Immunolocalisation of Brain-Derived Neurotrophic Factor in the Olfactory Layers of the African Grasscutter (*Thryonomys swinderianus* - Temminck, 1827)

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Key words:
African grasscutter, Brain derived neurotrophic factor, Main olfactory bulb, Mitral cells, olfactory glomerulus

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ABSTRACT

The present study has provided some novel findings on the structure of the olfactory bulb in the African grasscutter, by basic neuro-anatomical technique and immunhistochemical technique, using antibody specific to brain-derived neurotrophic factor. Brain samples extracted from neonates on postnatal day 3, juveniles on postnatal day 72 and adults on postnatal day 450 were utilised for the study. There was a consistent increase in the relative weight of the olfactory bulb with advancement in age, but a significant ($P < 0.05$) decrease in the relative length from 20.81 ± 0.37 on postnatal day 3 to 17.75 ± 0.45 on postnatal day 72. The concentric lamina organization of the olfactory bulb was evident on postnatal day 3, but the glomerular layer was completely absent. The layer was evident on postnatal days 72 and 450. The mean diameters of each glomerulus on postnatal days 72 and 450 were 86.82 ± 0.25 µm and 117.14 ± 0.32 µm, respectively. The difference was significant ($P < 0.05$). The zones of the external plexiform layer were undifferentiated on postnatal day 3. There was a decrease in the number of mitral cell layer, but increase in size of each mitral cell, with advancement in age. Brain-derived neurotrophic factor was moderately immunolocalised in the olfactory nerve layer on postnatal day 3, but negative on postnatal days 72 and 450. Cell bodies of the periglomerular short-axon neurones mildly expressed brain-derived neurotrophic factor on postnatal days 72 and 450. The granule cells did not express brain-derived neurotrophic factor on all postnatal periods. The functional significance of the results was discussed.

1. INTRODUCTION

The olfactory system has been subdivided into two components, the main olfactory bulb and the accessory olfactory bulb. The accessory olfactory bulb is a lentiform area on the dorsal surface of the main olfactory bulb. The accessory olfactory bulb is involved in the processing of non volatile chemical stimuli, while the main olfactory bulb processes volatile chemical stimuli (Kosaka and Kosaka 2009). The histological structure of the main olfactory bulb includes a multi-layered concentric cellular architecture consisting, in a dorso-ventral fashion, the olfactory nerve layer, olfactory glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer, granule cell layer and ependymal zone (Wei *et al*., 2008). All of these layers are distinct in a developed olfactory bulb, but the formation of the olfactory glumerular layer is age-dependent (Greer *et al*., 1982; Luskin, 1993; Wei *et al*., 2008). The olfactory glomerular layer is referred to, by some authors, as the functional unit of the mammalian main olfactory bulb. The layer receives input primarily from olfactory receptor neurones in the nasal epithelium; ends of the axons of the olfactory receptor neurones cluster to form the spherical glomeruli of the glomerular layer, where the axons make synaptic contacts with the glomerular arborisations of the dendrites of mitral, tufted, and periglomerular cells (Wei *at al*., 2008) Thus, the glomerular layer of the olfactory bulb is

the first level of synaptic processing and its organisation defines the acuity of olfaction (Hamilton *et al*., 2005).

The immunomarker, brain-derived neurotrophic factor is an active neurotrophin involved in the stimulation and regulation of postnatal neurogenesis (Benraiss *et al*., 2001). It is also very active in the reguation of synaptic plasticity (Cohen *et al*., 2011). Such synaptic plasticity is necessary to maintain impulse conduction between the olfactory receptor axons and mitral cell dendrites via the glomerular layer. Thus, to test the acuity of olfaction in the different postnatal periods in the African grasscutter, spatial distribution of brain-derived neurotrophic factor in the different layers was accessed by immunohistochemistry.

The functional significance of the adult olfactory system in reproduction (Polese *et al*., 2015), emotional response (Mouly and Sullivan, 2010), mate recognition (Keller *et al*., 2010), predator and prey detection (Johannesen *et al*., 2014), flavour perception (Trelea *et al*., 2008), cancer diagnosis (Amundsen *et al*., 2014) and salmonelosis diagnosis (Mahoney *et al*., 2014a) has been established. Conversely, the variability in the acuity of olfaction due to age in animal models is yet to be fully investigated. Basic to this investigation is an understanding of the neuroanatomical organisation of the olfactory system at different postnatal periods of an animal's development. The present study has filled some existing gap in the neuroanatomy of the olfactory system in the African grasscutter at different developmental periods.

The organization of the olfactory system of some mammals has been documented. Anatomical studies by Nzalak *et al*. (2008), Ibe *et al*. (2014) and Olude *et al*. (2015) have confered a well developed olfactory system in the African giant pouched rat. This high acuity of odour perception has been attributed to the use of the rodent in the diagnosis of salmonelosis and detection of tobacco products (Mahoney *et al*., 2014). Similarly, Wei *et al*. (2008) has attributed the good olfactory sense in dogs to the histological organization of their main olfactory bulb. The glomerular layer of the olfactory bulb has been reported to be developed in rabbits (Smitka *et al*., 2009) and Wistar rat (Bukar, 2015). Unfortunately, anatomical description of the olfactory system of the African grasscutter is pauce in the available literature. Apart from the cerebellum that has been tested (Ibe *et al*., 2016), no part of the African grasscutter brain has been tested with antibody specific to brain-derived neurotrophic factor. Therefore, the present study will bridge some existing gap in the neuroanatomy of the olfactory system of the African grasscutter. The study will also serve as a lead for further behavioural studies of the African grasscutter with regards to olfaction.

The main aim of the present study was to expound the structural organization, and immunolocalise brain-derived neurotrophic factor in the main olfactory bulb of the African grasscutter at defined postnatal periods. Specific objectives were to detrermine the gross size of the African grasscutter olfactory bulb, describe the lamina organisation of the main olfactory bulb, describe the histomorphology of the neurons in the different layers and immunolocalise brain-derived neurotrophic factor in the olfactory bulb neurones on postnatal days 6, 72 and 450.

1. MATERIALS AND METHODS

2.1 Experimental Animals and Management: Twenty seven African grasscutters, comprising of 9 neonates of 3 days old, 9 juveniles of 72 days old and 9 adults of 450 days old were utilized for the study. They were purchased from a commercial grasscutter farm in Elele, Rivers State, Nigeria. Locally made wooden cages with adequate ventilation, and measuring 1.5 m x 1.5 m x 1.5 m were used to transport them, by road, to the Veterinary Histology Laboratory of the Michael Okpara University of Agriculture, Umudike. In the laboratory, there were physically examined under careful restraint and apparently healthy ones were utilized for the study. They were fed twice daily. Drinking water was provided *ad libitum*. The feeding troughs and drinkers were sterilized daily using Milton® (Laboratoire Rivadis, Louzy, France; active ingredient: sodium hypochlorite 2% w/w). The cages were also swept and disinfected daily using Milton®, and a broad spectrum bactericidal, fungicidal and virucidal agent.

The experimental protocol was approved by the Ethical Committee of Ahmadu Bello University, Zaria, Nigeria. Management of the experimental animals was as stipulated in the Guide for the Care and Use of Laboratory Animals, 8th Edition, National Research Council, USA (National Academic Press, Washington D.C.: www.nap.edu.).

2.2 Brain Extraction: The African grasscutters were weighed using a digital electronic balance (Citizen Scales (1) PVT Ltd., sensitivity: 0.01 g), and sedated by intraperitoneal injection of 20 mg/kg Thiopental Sodium (Rotexmedica, Trittau, Germany). Thereafter, each animal was placed on a dissection table on a dorsal recumbency, and perfused, via the left ventricle, with 4% paraformaldehyde fixative, using the method of Gage *et al*., 2012. Immediately after the perfusion fixation, the head was separated from the rest of the

body at the atlanto-axial joint, using a pair of scissors and knife. Thereafter, each skull containing the brain was obtained after skinning and stripping off all the facial muscles. Then, craniotomy preceded brain extraction. Specifically, brain extraction was performed in a caudo-rostral and dorso-ventral direction, using scalpel blades, thumb forceps, rongeur and a pair of scissors. The meninges and underlying blood vessels were gently removed to expose the intact brain.

2.3 Gross Structural Study of the Olfactory Bulb: The structural characteristics of all the olfactory bulbs were examined with the naked eyes and with the aid of a hand lens. The position, shape, size and surfaces of the bulb were observed and recorded. The absolute brain weight and weight of the olfactory bulb were obtained using the Mettler balance P 1261 (Mettler instrument AG. Greifensee, Switzerland; sensitivity: 0.01 g). For gross-morphometric study, the absolute brain length and length of the olfactory bulb was obtained using a vernier caliper MG6001DC (General Tools and Instruments Co., New York; sensitivity of 0.01 cm) and converted to millimeter. Landmark for the absolute brain length was the rostro-caudal extent of the intact brain, from the tip of the olfactory bulb to the caudal end of the medulla oblongata. The relative weight of the olfactory bulb was calculated by dividing the absolute weight of the olfactory bulb by the absolute brain weight, expressed in percentage. The relative length of the olfactory bulb was calculated by dividing the absolute length of the olfactory bulb by the absolute brain length, expressed in percentage.

2.4 Histological Study of the Main Olfactory Bulb Layers: The fixed samples of the olfactory bulb were trimmed and placed in labeled tissue cassettes. The cassettes were transferred into a semi-enclosed bench-top tissue processor TP 1020 (Leica Biosystems, Nußloch, Germany) which dehydrated the tissues in graded alcohol (70 %, 80 %, 90 %, 100 %, 100 % and 100 %) and cleared in toluene. The tissues were then infiltrated with molten paraffin wax (BDH Chemicals Ltd. Poole, England) at 50ºC, blocked in paraffin and labeled. Sagittal sections of 5 µm thick of the olfactory bulb were cut from the embedded tissues using Jung rotary microtome 42339 (Berlin, Germany) and floated unto adhesive charged slides. The slides were heated in an oven at 60 ^{0}C for 2 hours, deparaffinised in 3 changes of xylene and alcohol, each, and taken to water for rehydration. Every 4th, 5th and 6th sagittal sections of each sample were floated unto adhesive charged slides. Every 6th section on the slide was reserved for immunohistochemistry, while every 4th and 5th sections were stained thus: every 4th section was stained with haematoxylin and eosin stain for general histological study and every 5th section was stained with cresyl fast violet stain for the Nissl substance. Slides were oven-dried for 15 minutes and cover-slipped with DPX as the mountant. The cytoarchitecture of the olfactory bulb was studied microscopically, and photographed with a digital eyepiece (Scopetek® DCM500, Resolution: 5M pixels) attached to a light microscope (OLYMPUS® EUROPA GmbH, XSZ107BN, Hamburg, Germany). Photomicrographs were taken at x 40, x 100, x 250 and x 400 magnifications. For histo-morphometric study, the diameter of the glomeruli and thickness of the plexiform layers were obtained using a calibrated ocular micrometer (Leitz Wetzlar, Germany) following appropriate calibration of the light microscope with a stage micrometer (Graticules Ltd., London, U.K.). *Nomina Anatomica Veterinaria* (2005) was used for nomenclature.

2.5 Immunolocalisation of Brain-derived Neurotrophic Factor: To immunolocalise brain-derived neurotrophic factor in the olfactory bulb layers, the slides of every 6th serial sagittal section of the olfactory bulb were incubated in 100X Citrate Buffer (Abcam PLC., Cambridge UK; Product number: ab93678; pH: 6.0) at 65 ^{0}C, heated to 95 ^{0}C and maintained for 20 minutes, for antigen retrieval. Thereafter, the antigen retrieval solution containing the slides was placed on the bench for 20 minutes, to attain room temperature. The slides were placed in the immunohistochemistry staining chamber, washed with Tris buffered-saline and 0.05 % Tween 20 (TBST 10-0028; Genemed Biotechnologies Inc., California USA; pH: 7.4) for 2 minutes and hydrogen peroxide block from a Rabbit Specific ABC/DAB detection IHC kit (Abcam PLC., Cambridge, UK; Product number: ab64261) was applied and incubated for 10 minutes at room temperature. After washing, protein block from the Rabbit Specific ABC/DAB detection IHC kit was applied and incubated for 10 minutes at room temperature. The slides were washed again and the Anti-brain-derived neurotrophic factor primary antibody (Abcam PLC., Cambridge, UK; Product number: ab101748; Dilution: 1:1000) was applied to the slides and incubated for 60 minutes at room temperature.

After another round of washing, biotinylated goat anti-rabbit secondary antibody from the Rabbit Specific ABC/DAB detection IHC kit was applied to the slides and incubated for 10 minutes at room temperature. Slides were rinsed in Tris buffered-saline and 0.05 % Tween 20 for 2 minutes. Thereafter, streptavidin peroxidase from the Rabbit

Specific ABC/DAB detection IHC kit was applied and incubated for 10 minutes at room temperature. The slides were rinsed again, and 3, 3-diaminobenzidine chromogen plus diaminobenzidine substrate (1drop:1.5ml) from the Rabbit Specific ABC/DAB detection IHC kit was applied to the slides and incubated for 3 minutes. The slides were washed and counterstained with haematoxylin for 30 seconds. Slides were dehydrated, oven-dried for 15 minutes and cover-slipped with DPX.

The slides were examined under the light microscope at final magnification of x 400. Positive immunostaining of brain-derived neurotrophic factor was observed as a brown cytoplasmic colour. For assessment of the immunostaining, a semi-quantitative scoring was performed by three independent observers. The number of immunoreactive cells was classified as: - = none, + = mild (positive cells constituted less than 10%); ++ = moderate (10–50% of cells were positive); and +++ = strong (more than 50% of cells were positive).

The manufacturer of the anti-brain-derived neurotrophic factor primary antibody (Abcam PLC., Cambridge, UK), stated in the product datasheet, that mouse, rats and humans are immunoreactive to the primary antibody, and the immunomarker is highly expressed in the cerebral cortex, among other tissues. Thus, the cerebral cortex of Wistar rat was harvested and treated with the same primary antibody, at the same time with the test slides, and used as the positive control. A negative control was developed to validate the positive results; some control slides of the Wistar rat cerebral cortex were not treated with the primary antibody, and served as the negative control.

2.6 Statistical Analysis of the Data: Data obtained from the gross- and histo-morphometrics was expressed as mean ± SEM (Standard Error of the Mean) and presented on a graph. Values were subjected to one-way analysis of variance (ANOVA), followed by Turkey's post-hoc test to determine significance of the mean. Values of $P < 0.05$ were considered significant. GraphPad Prisms, version 4 for Windows 8 was used for the statistical analysis.

2. RESULTS

3.1 Gross Structure of the Olfactory Bulb at Different Postnatal Periods: The gross structural characteristics of the olfactory bulb were the same throughout the postnatal periods. The olfactory bulbs were evident as ventral protrusions of the olfactory cortex. Each olfactory bulb was conical in shape, with the base continuing as the olfactory tracts on the ventral surface of the brain, and the apex protruding into the frontal bones.

The absolute weights of the brain and olfactory bulbs, relative weight of the olfactory bulbs, absolute lengths of the brain and the olfactory bulbs and relative length of the olfactory bulbs, for each postnatal period, was represented in Table 1.

Table 1: Mean Values (± SEM) of the Brain and Olfactory Bulb Gross-morphometric Parameters in the African Grasscutter at Different Postnatal Periods

Parameter	Day 3 (n = 9)	Day 72 (n = 9)	Day 450 (n = 9)
Absolute brain weight (g)	4.19 ± 0.08	6.77 ± 0.04	12.22 ± 0.23
Absolute olfactory bulb weight (g)	0.03 ± 0.01	0.06 ± 0.02	0.13 ± 0.01
Relative olfactory bulb weight (%)	0.61 ± 0.04^{a}	0.90 ± 0.04^{a}	1.03 ± 0.06^{c}
Absolute brain length (mm)	39.12 ± 0.46	53.18 ± 0.52	63.74 ± 1.47
Absolute olfactory bulb length (mm)	7.30 ± 0.09	9.30 ± 0.06	11.20 ± 0.09
Relative of olfactory bulb length (%)	20.81 ± 0.37^{a}	17.75 ± 0.45^{b}	17.51 ± 0.24^{b}

Values on the same row with different superscripts are significantly different ($P < 0.05$)

There was a consistent increase in the relative weight of the olfactory bulb as the animal advanced in age, but the difference in the value between day 3 and day 72 was not significant. However, the relative weights of the olfactory bulbs on postnatal days 72 and 450 were significantly ($P < 0.05$) different. There was a significant ($P < 0.05$) decrease in the relative length of the olfactory bulb from 20.81 ± 0.37 on postnatal day 3 to 17.75 ± 0.45 on postnatal day 72. The further decrease in the relative length of the olfactory bulb between postnatal days 72 and 450 was insignificant.

3.2 Histological Structure of the Main Olfactory Bulb on postnatal day 3 (Neonatal Period): The concentric lamina organization of the olfactory bulb was evident on postnatal day 3. The most superficial layer, the olfactory nerve layer (Fig. 1: a) was made of few cell bodies and axons. The glomerular layer was completely absent. Deep to the olfactory nerve layer was the external plexiform layer (Fig. 1: c), measuring 95.34 ± 0.26 µm in thickness. This layer comprised of scanty cell bodies, including some mitral cells, but numerous axons. Deep to the external plexiform layer was the mitral cell layer (Fig. 1: d), well delineated into a distinct thick layer of several mitral cell bodies. Inner to this layer was the internal plexiform layer (Fig. 1: e), a layer of low cell density, measuring an average of 91.99 ± 0.22

µm in thickness. The granule cell layer (Fig. 1: f) was next to the internal plexiform layer. The layer was distinctly divided into an external zone, composed of granule-containing-cell bodies arranged in 3-4 distinct layers; and an internal zone made up of scattered granule cells.

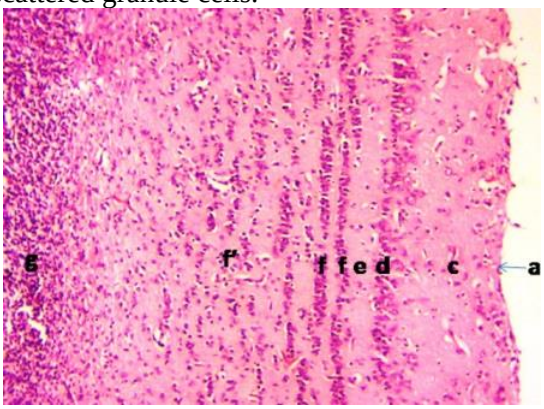

Fig. 1: Sagittal section of the main olfactory bulb of the African grasscutter on postnatal day 3
a: Olfactory nerve layer; Note the absence of b (Glumerular layer); c: External plexiform layer; d: Mitral cell layer; e: Internal plexiform layer; f: Granule cell layer (external zone); f': Granule cell layer (internal zone); Haematoxylin and Eosin stain, x 100.

3.3 Histological Structure of the Main Olfactory Bulb on Postnatal day 72 (Juvenile Period): The concentric lamina organisation of the olfactory bulb, observed in the neonatal period was maintained in the juvenile, with key differentiations. The glomerular layer (Fig. 2: b), characterized by the periglomerular short axon neurones (Fig. 2: line arrows), surrounding a meshwork of neurites, was evident at this period. The glomerular layer was located deep to the olfactory nerve layer. The mean diameter of each glomerulus was 86.82 ± 0.25 µm. The external plexiform layer (Fig. 2: c and c′) was thicker than that of the neonates, measuring an average of 98.58 ± 0.34 µm in thickness. The external and internal zones of the external plexiform layer have differentiated at this period, unlike in the neonates. The external zone was characterized by scanty round to oval shaped neurones, just deep to the glomeruli. The internal zone, just superficial to the mitral cell, was of lower cell density but more numerous neurites. There were numerous blood vessels in the external plexiform layer. The mitral cell layer (Fig. 2: d) was made of distinct cell bodies. The thickness of the mitral cells has reduced to about 2-3 cell lines, unlike the several cell lines observed in the neonates. However, at higher magnification, the size of the mitral cells was more than that observed in the neonates. The internal plexiform layer (Fig. 2: e), interposed between the mitral body layer and the granule cell layer, was thinner at this period than in the neonates, with an average thickness of 84.10 ± 0.12 µm. The external and internal zones of the granule cell layer (Fig. 2: f) was maintained. The external zone was composed of granule containing cell bodies arranged in 3-4 layers, as observed in the neonatal samples.

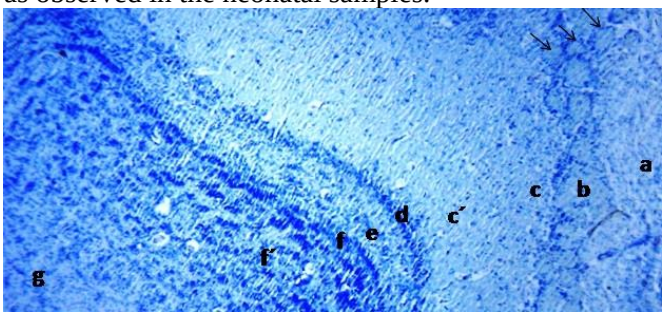

Fig. 2: Sagittal section of the main olfactory bulb of the African grasscutter on postnatal day 72
a: Olfactory nerve layer; b: Olfactory glomerular layer; c: External plexiform layer (external zone); c′: External plexiform layer (internal zone); d: Mitral cell layer; e: internal plexiform layer; f: Granule cell layer (external zone); f′: Granule cell layer (internal zone); g: Ependymal zone. Arrows indicate periglomerular cells of the glomerular layer. Cresyl fast violet stain x 100.

3.4 Histological Structure of the Main Olfactory Bulb on Postnatal day 450 (Adult Period): On a sagittal section, the main olfactory bulb presented a 6-layered concentric cellular architecture. From the outermost to the innermost layer, they consist of the olfactory nerve layer (Fig. 3: ONL), olfactory glomerular layer (Fig. 3: GL), external plexiform layer (Fig. 3: EPL), mitral cell layer, internal plexiform layer and the granule cell layer. The olfactory nerve layer contained mostly of axons and very few neuronal cell bodies. The olfactory glomerular layer was a 2-3 layered glomeruli (Fig. 3: line arrows), which were made up of clusters of axons surrounded by periglomerular cells. The glomeruli were spherical to oval in shape, each measuring 117.14 ± 0.32 µm in diameter. The diameter of the glomeruli in the adult was significantly ($P < 0.05$) higher than the value for the juveniles (Fig. 4). The periglomerular cells contained astrocytes and juxtaglomerular neurones. The external plexiform layer was a very thick layer, measuring 246 ± 0.58 µm in thickness, and containing mostly axons and a few cell bodies. The mean thickness of the external plexiform layer in the adult was significantly ($P < 0.05$) higher than the value for the juveniles or neonates (Fig. 4). The external and internal zones of the external plexiform layer were more distinct in the adult than in the juveniles. The external zone was characterized by

round to oval shaped neurones, while the internal zone, just superficial to the mitral cell, was of lower cell density, but containing more neurites. The mitral cell layer was deep to the external plexiform layer, and contained mitral cell bodies whose thickness was less than that observed in the juveniles and neonates, but with increased neuronal size. Inner to the mitral cell layer was the internal plexiform layer; which was more distinct in the adult as a layer of very low cell density, separating the mitral body layer and the granule cell layer. The mean thickness of the adult internal plexiform layer was 99.50 ± 0.17 µm. The granule cell layer was also a thick layer. The two sub-layers of the granule cell layer, which were first observed in the neonates, were maintained in the adult. The innermost layer was the ependymal zone, which was made of very few cells in the adult.

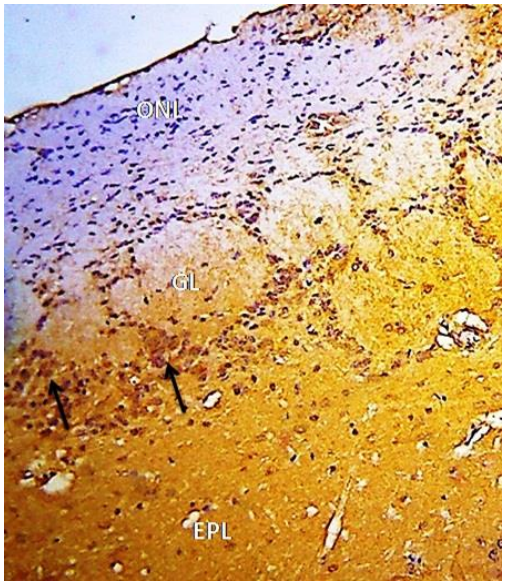

Fig. 3: Immunoreactivity of brain-derived neurotrophic factor on a sagittal section of the olfactory layers in the African grasscutter on postnatal day 450. ONL: Negative immunoreactivity of olfactory nerve layer cells; GL: Mild immunoreactivity of periglomerular short axon cells (black arrows) in the glomerular layer; EPL: Negative immunoreactivity in the external plexiform layer cells; magnification: x 100.

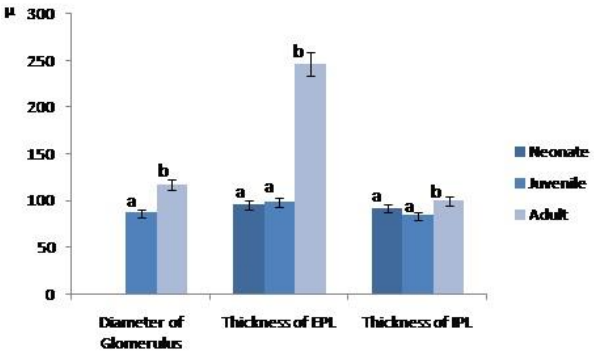

Fig. 4: Morphometric result of the glomerular and plexiform layers in the African grasscutter olfactory bulb ar different postnatal periods

Bars of the same index with different superscript are significantly ($P < 0.05$) different

3.5 Immunolocalisation of Brain-Derived Neurotrophic Factor in the Main Olfactory Bulb Layers at Different Postnatal Periods: The scoring of brain-derived neurotrophic factor immunoreactive neurones in the different layers of the olfactory bulb is represented in Table 2. In the olfactory nerve layer, brain-derived neurotrophic factor immunoreactive cells were observed only on postnatal day 3. Brain-derived neurotrophic factor was observed in the cell bodies of the positive neurons. No brain-derived neurotrophic factor immunoreactive cell was observed in the olfactory nerve layer on postnatal days 72 and 450 (for slide of postnatal day 450, see Fig. 3: ONL). Olfactory glomerular layer was absent on postnatal day 3; thus, the immunostaining of brain-derived neurotrophic factor was not accessed at the period. The cell bodies of the periglomerular short-axon neurones mildly expressed brain-derived neurotrophic factor on postnatal days 72 and 450 (for slide of postnatal day 450, see Fig. 3: black arrows). No brain-derived neurotrophic factor was immunolocalised in the external and internal plexiform layers of all the postnatal periods (see Fig. 5 for postnatal day 3). About 50% of mitral cells were brain-derived neurotrophic factor immunoreactive on postnatal days 3 and 450, while no observable mitral cell expressed the neurotrophin on postnatal day 72. The granule cells did not express brain-derived neurotrophic factor on postnatal day 3 (Fig. 5). The same result was observed in the granule cell layer of all other postnatal periods.

Table 2: Quantitative Analysis of Brain-Derived Neurotrophic Factor Immunoreactive Neurones in the Olfactory Bulb Layers of the African Grasscutter at Different Postnatal Periods

	Olfactory Bulb Layers

	Day 3	Day 72	Day 450
Olfactory nerve layer	++	-	-
Olfactory glomerular layer	Absent	+	+
External plexiform layer	-	-	-
Mitral cell layer	++	-	++
Internal plexiform layer	-	-	-
Granule cell layer	-	-	-

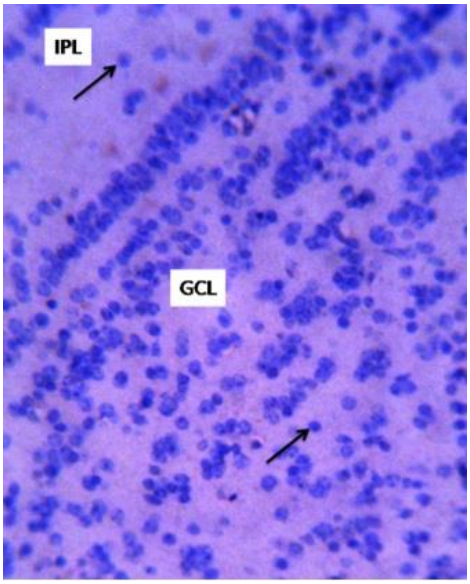

Fig. 5: Negative immunoreactivity of brain-derived neurotrophic factor in the internal plexiform layer (IPL) and granule cell layer (GCL) of the African grasscutter on postnatal day 3. The same result was observed on postnatal days 72 and 450; magnification: x 400.

3. DISCUSSION

The olfactory bulb of the African grasscutter was observed as a rostral outgrowth of the cerebral cortex into the floor of the frontal bone, similar to that observed in other rodents like the African giant pouched rat (Olude *et al*., 2014) and the Wistar rat (Bukar, 2015). Similar to the result of the present study, Byanet *et al*. (2009) reported that the olfactory bulbs of the adult African grasscutter were visible on the dorsal brain view, and not hidden under the frontal lobes. In higher mammals, such as the elephant, the olfactory bulb is not visible on the dorsal brain view, but hidden under the frontal lobes (Shoshani *et al*., 2006). The acuity of olfaction has been reported to be reduced in such higher animals, unlike in rodents. However, the acuity of olfaction differs among rodents, partly due to the varied sizes of the olfactory bulbs. Nzalak *et al*. (2008) reported a higher relative length of the olfactory bulb of the African giant pouched rat, than the value reported in the present study for the adult African grasscutter. This may point to a better olfactory sense in the African giant pouched rat than in the African grasscutter.

Results of the morphometric features indicated continuous increase in the relative weight and absolute length of the olfactory bulbs as the animal advances in age. This was indicative of increased neuronal and neuroglial cell size. However, result of relative length of the bulb indicated a significant decrease between the neonates on day 3 and the juveniles on day 72. The value further decreased between the juvenile and the adult, though not significantly. The decrease in the relative length of the olfactory bulbs is evidence that the bulbs did not grow simultaneously, but at a slower rate than the increasing frontal bone. Thus, the length of the bulb, relative to the nose-rump length decreased with increase in postnatal age.

The lamina organisation of the olfactory bulb differed during development in the African grasscutter. Results of the present study indicated that the olfactory glomerular layer was not organised in the 3-day-old neonates. There were observed in the juveniles of 72 days' old. Similarly, Greer *et al*. (1982) reported that the glomerular layer was poorly differentiated in newborn Sprague-Dawley rat pups, and that the demarcation of individual glomeruli by their ring of periglomerular cells started on postnatal day 3. The results of the work done by Luskin (1993) showed that the periglomerular cells are incompletely differentiated and yet to migrate to the glomerular layer at birth. According to Wei *et al*. (2008), the structure of the glomerular layer of the olfactory bulb was developed in juvenile dogs. The olfactory glomerular layer is the most important histological layer, referred to, by some authors, as the functional unit of the mammalian main olfactory bulb. This layer receives input primarily from olfactory receptor neurones in the nasal epithelium. The ends of the axons of the olfactory receptor neurones cluster to form the spherical glomeruli of the glomerular layer, where the axons make synaptic contacts with the glomerular arborisations of the dendrites of mitral, tufted, and periglomerular cells. Thus, the glomerular layer of the olfactory bulb is the first level of synaptic processing and its organisation defines the acuity of olfaction (Hamilton *et al*., 2005). The absence of well organised glomeruli in the olfactory bulbs of

neonatal African grasscutter as reported in the present study may result to low olfaction acuity in the age group.

The number of layers of olfactory glomeruli has been extrapolated to be inversely proportional to the acuity of olfaction in adult mammals (Bukar, 2015). Although a maximum of three layers were observed in the present study, Bukar (2015) observed a maximum of two layers in the adult African giant pouched rat and three layers in the Wistar rat. Ngwenya *et al*. (2011) observed a maximum of four layers in the African elephant. Furthermore, the increase in diameter of the olfactory glomeruli from juveniles to adult African grasscutter as observed in the present study is similar to the observations of Greer *et al*. (1982) and Wei *et al*. (2008) in the Sprague-Dawley rat and dog, respectively.

Further laminar changes observed in the olfactory bulb of the African grasscutter was the differentiation of the external and internal zones of the external plexiform layer, the varied cell density of the mitral cell layer with age, and the organisation of the granule cell bodies during development. The external and internal zones of the external plexiform layer were not observed in the neonatal period, but were observed in juveniles. Greer *et al*. (1982) reported that they were not organised on postnatal day 3, but delineated on postnatal day 9 in the Sprague-Dawley rats.

The mitral cells are the principal output cells of the olfactory bulb. They make synapses with the periglomerular cells and relay their axons to the anterior olfactory nucleus and higher olfaction centre, the piriform cortex via the olfactory tracts. The reduced thickness of the mitral cell layer during development of the African grasscutter, from the present study, was reported in the Sprague-Dawley rat by Greer *et al*. (1982) and developing dogs by Wei *et al*. (2008). The reduced thickness, resulting from the decline in the number of cell lines may be indicative of apoptosis, necessary for increased neuronal size of existing mitral cells in the adult, relative to the cell sizes in the neonates and juveniles. This finding again, may be one of the reasons acuity of olfactory sense in adults is better than neonates.

The internal plexiform layer was observed in the African grasscutter from foetal day 90 up to adulthood, as a layer of low neuronal density, between the mitral and the granule cells. Although this layer was not reported in the adult African giant pouched rat (Olude *et al*., 2014), it has been reported to be present in other mammals, such as the Sprague-Dawley rat (Greer *et al*., 1982), dog (Wei *et al*., 2008), rabbit (Smitka *et al*., 2009) and Wistar rat (Bukar, 2015). The functional significance of this layer is yet to be elucidated.

The morphogenesis of the granule cell layer in the African grasscutter from the present study differed from that of the Sprague-Dawley rats (Greer *et al*., 1982). It was already organised into a definite granule cell lines by postnatal day 3 in the African grasscutter, but, in the Sprague-Dawley rats, it appeared as a field of scattered perikarya between 0 to 3 days postpartum, and as islets typical of adult granule layer by postnatal day 15 (Greer *et al*., 1982). The difference implies that granule cells differentiate earlier in the African grasscutter, thus, the dendro-dendritic synapse with mitral cell, which produces the lateral inhibition on mitral cells necessary for processing and perception of distinct odours (Scott *et al*., 1993), may be initiated earlier in the African grasscutter than in the Sprague-Dawley rat.

The differential distribution of a neurologically active protein, brain-derived neurotrophic factor, in the studied brain structures at the different postnatal periods was accessed by a sensitive immunohistochemical technique. The immunomarker, brain-derived neurotrophic factor was adopted for the present study due to its functional significance in the control of neurogenesis, dendritic growth and synaptic plasticity (Carter *et al*., 2002; Carim-Todd *et al*., 2009). The result of the present study revealed a wide-spread localisation of brain-derived neurotrophic factor protein in the olfactory bulb of the African grasscutter at different postnatal periods, and the protein was confined to some specific neurones. The implication of the positive cells is that brain-derived neurotrophic factor protein is synthesised by the cell in a significant quantity to bind with the primary antibody. Previous studies (Ohta *et al*., 2001; Willis *et al*., 2007) have indicated that some cells may not naturally produce significant quantity of neurotrophins to allow for a positive immuno-histochemical result, except when pre-treated with colchicine to force the accumulation of normally-exported proteins within the cell body.

The immunoreactivity of brain-derived neurotrophic factor in the olfactory nerve layer of the neonates, unlike juvenile and adult periods is indicative of the active synaptogenesis, expressed by the axons of the olfactory nerve receptors in neonates. These axons, which reside in the olfactory nerve layer, synapse with the mitral cells. Although the timing of synapse proliferation is region-dependent, Semple *et al*. (2013) emphasized that the critical period of synaptogenesis in rodents occurs

during the first three postnatal weeks of life, peaking during week 2. The negative reactivity of brain-derived neurotrophic factor in the juvenile and adult olfactory nerve layer may imply that synapse density and morphology have been achieved, thus the cessation of synaptogenesis. The lack of brain-derived neurotrophic factor protein in the plexiform neurones and the mild immunoreactivity in the periglomerular cells of the adult were consistent with the observations that Hofer *et al.* (1990) and Conner *et al*. (1997) made in the adult mouse and Sprague Dawley rat, respectively. Similarly, Conner *et al*. (1997) observed a few brain-derived neurotrophic factor immunoreactive mitral cells in the adult Sprague Dawley rat, similar to the adult African grasscutter. However, Hofer *et al*. (1990) reported mild expression of brain-derived neurotrophic factor in the granule cells of adult mice, unlike in the adult African grasscutter, from the present.

The absence of brain-derived neurotrophic factor immunoreactive granule cells of the olfactory bulb in the present study implies that either the granule cells does not synthesize the protein, or the protein was immediately transported from the granule cells to other adjacent cells, by anterograde axonal transport. This is possible as brain-derived neurotrophic factor has been implicated in anterograde axonal transport from the site of synthesis to their target cells (Conner *et al*., 1997). This paracrine action of brain-derived neurotrophic factor is employed in the regulation of synaptic plasticity. Further analysis of brain-derived neurotrophic factor mRNA in the olfactory granule cells of the African grasscutter is necessary as this will confirm or disprove the possibility of brain-derived neurotrophic factor synthesis by the cerebellar granule cells. This is because cells that were positive for brain-derived neurotrophic factor and its mRNA synthesize brain-derived neurotrophic factor; cells, negative for brain-derived neurotrophic factor but positive for brain-derived neurotrophic factor mRNA also synthesize brain-derived neurotrophic factor, but the protein has been lost by the cell to paracrine function. Cells positive to brain-derived neurotrophic factor and negative to brain-derived neurotrophic factor mRNA do not synthesize brain-derived neurotrophic factor, but receive the pool of brain-derived neurotrophic factor by axonal transport from adjacent cells that actually synthesize the protein (Conner *et al*., 1997).

4. CONCLUSION

The present research has, for the first time, provided information on some structural changes that occur in the brain of the African grasscutter during postnatal development. The lack of olfactory glomerular layer and reduced thickness of the mitral cell layer points to a lower acuity of olfaction in the neonatal African grasscutter. The positive immunoreactivity of brain-derived neurotrophic factor in the olfactory nerve layer of the neonates, unlike juveniles and adults, is suggestive of active synaptogenesis expressed by the axons of the olfactory nerve receptors in neonates. Further analysis of brain-derived neurotrophic factor mRNA in the olfactory granule cells is necessary as this will confirm or disprove the possibility of brain-derived neurotrophic factor synthesis by the granule cells.

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