

## Cytoarchitecture of the Hippocampal Formation in the African Giant Rat (*Cricetomys gambianus*, Waterhouse)

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**Summary:** The African Giant Rat, AGR is an indigenous nocturnal rodent noted for its unique olfactory and cognitive abilities. They have been deployed more recently in the detection of landmines and diagnosis of tuberculosis – two scourges that have had a tremendous negative impact on the African landscape. This olfactory-aided cognition has been linked to the hippocampus. While the anatomical infrastructure of the olfactory bulb of the AGR has been elucidated, little is known about the adaptive cytoarchitecture of the AGR hippocampal formation. This study describes the histological features, including subfields and stratifications of the AGR hippocampus using Nissl and Golgi stains. The basic cytoarchitecture of the AGR hippocampus observed in this study, with respect to stratification, subfields and cell types, is similar to those reported in the laboratory rats. Cell types identified in the AGR hippocampus include pyramidal cells, granule cells and mossy cells with mossy fibers and Schaffer collaterals also delineated. Hippocampal proper subfields CA1 to CA4 were identified. CA3 pyramidal neurons formed a well-defined cell layer starting in between the upper and lower ends of the dentate gyrus and had larger, more distinct pyramidal cells and higher cell layer thickness ( $240.0 \pm 6.0 \mu\text{m}$ ) relative to subfields CA1 ( $87.0 \pm 2.0 \mu\text{m}$ ) and CA2 ( $109.0 \pm 4.20 \mu\text{m}$ ) with significant statistical differences at  $p < 0.001$ . The detailed, delicate arrangement of various cell types and subfields, intricate wiring with synapses and laminar organization of the hippocampal formation noticed in the AGR strongly supports the canonical trisynaptic circuitry of the hippocampus. It will however be necessary to carry out densitometric studies and detailed neurochemical profiling of the AGR hippocampus to fully elucidate the functional leverage of this unique rodent. We, therefore, suggest the suitability of this rodent as a model for olfaction-linked memory studies.

**Keywords:** African giant rat, brain, cornu ammonis, dentate gyrus, hippocampus, histology.

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### INTRODUCTION

The African Giant Rat (AGR) also called Sniff rat, is a large nocturnal rodent with very poor eyesight and so it depends on its keen sense of smell (Kalan, 2014). This adaptive feature has further been buttressed by possessing relatively large olfactory bulbs (Nzalak *et al.*, 2005; Olude *et al.*, 2014a; 2014b). In addition, this unique African rodent is also noted for its high cognitive abilities (Olude *et al.*, 2014a). These two attributes (olfaction and cognition) have been positively explored by a registered Belgian non-governmental organization, APOPO, in the detection of landmines and diagnosis of tuberculosis – two scourges that have had a tremendous negative impact on the African landscape (Weetjens *et al.*, 2009; Mahoney *et al.*, 2012; Carrington, 2014; Poling *et al.*, 2015). This olfactory-aided cognition has been linked to the hippocampus (Vanderwolf, 1992; Herz and

Engen, 1996). The hippocampal formation, a prominent C-shaped structure on the floor of the temporal horn of the lateral ventricle, consists of the hippocampal proper, dentate gyrus, subiculum and entorhinal area (Kunzle and Radtke-Schuller, 2001; Schultz and Engelhardt, 2014). Histologically, it is partitioned into region and sub-fields according to the neuronal cell body location, cell body shape and size, proximal terminations, complex spines, distal branching characteristics as well as afferent and efferent projections (Turner *et al.*, 1998).

Vanderwolf (1992) demonstrated an increased hippocampal activity, specifically at the hilus of the dentate gyrus, following an olfactory stimulus in rats and suggested sniffing as an olfactory input to the dentate gyrus of the hippocampus. Interestingly, he observed that these heightened hippocampal activities were not elicited by visual, auditory, or somatosensory

inputs and is not related to motor activity. Thus, olfaction seems to be the sensory modality that is physically closest to the limbic system of which hippocampus and the amygdala are a part (Herz and Engen, 1996). The direct connection established by the olfactory bulb and piriform/olfactory cortex on these two structures sustains the unique ability of odours to activate emotions and memory (Mouly and Sullivan, 2010). While the anatomical infrastructure of the olfactory bulb of the AGR has been elucidated by Olude et al., (2014a), little is known about the adaptive cytoarchitecture of the AGR hippocampal formation as it relates to the transfer of procedural learning into declarative long term memory. This work thus reports preliminary findings in the cytoarchitecture of the AGR hippocampal formation, as part of an ongoing work aimed at providing probable evidence-based explanation for its cognitive capacities. This study describes the histological features, including subfields and stratifications of the AGR hippocampus using Nissl and Golgi stains.

## MATERIALS AND METHODS

### Experimental Animals, Perfusion and Brain Harvest

A total number of 5 adult male AGRs acquired from the wild by local hunters in Southwestern Nigeria were purchased from a local market for the purpose of this experiment. They were observed physically to exclude any physical deformities that may interfere with the study; stabilized for 48 hours and fed *ad libitum*. The body weights of the AGR were obtained using a dial spring scale (CAMRY® J1610494297). Animals were anaesthetized with Ketamine (50mg/kg) and Xylazine (5mg/kg), perfused with 4% paraformaldehyde transcardially and sacrificed. Their brains were carefully harvested from the cranium with the use of a bone nipper as described by Olude *et al.*, (2014a). Harvested brains were subsequently post-fixed in 4% paraformaldehyde for 48 hours. Coronal sections through the optic chiasma at the temporal lobes were made for all brain samples and were embedded in paraffin blocks for Nissl (Cresyl Violet) staining to illustrate the neuronal somata and cytoarchitecture of the hippocampus. Golgi Silver impregnation stain was used for highlighting individual neuronal morphology, as well as their axonal and dendritic arborizations.

### Histological Staining

#### Nissl (Cresyl Violet)

Brain sections were mounted on frosted glass slides and were baked in a pre-heated oven for 20-30 minutes prior to staining. The slides were initially deparaffinized for 5 minutes each in Xylene twice and then in equal parts of xylene and absolute alcohol. They were subsequently hydrated in descending grades of alcohol (100%, 90%, 70% and 50%) and immersed in Cresyl violet solution (2.50g Cresyl violet

in 500 mls distilled water) for 8 minutes, rinsed in distilled water for one minute. Sections were then dehydrated through ascending grades of alcohol (70% Ethanol; 95% Ethanol + glacial acetic acid until satisfactory differentiation was observed; 95% Ethanol; 100% Ethanol). Stained slides were then coverslipped with DPX mountant

#### Golgi Stain

The tissue blocks were placed in the fixation solution (60mls of 3% potassium bichromate in 20mls of 10% formalin) for 24 hours after which they were transferred into the 3% potassium bichromate for 7 days in the dark. The solution was replaced with a fresh one each day. After the 7 days, the tissue blocks were transferred into 2% silver nitrate solution for 3 days at room temperature in the dark (filter paper was used to absorb excess solution on the blocks before putting into silver nitrate solution). The sections were then cut at 60µm thick into distilled water, mounted on glass slides and air dried for 10 minutes. Sections were dehydrated through 95% and 100% alcohol then cleared in xylene and coverslipped with Entellan (Sigma Aldrich).

### Photomicrography, Image Analysis, Quantification and Data Analysis

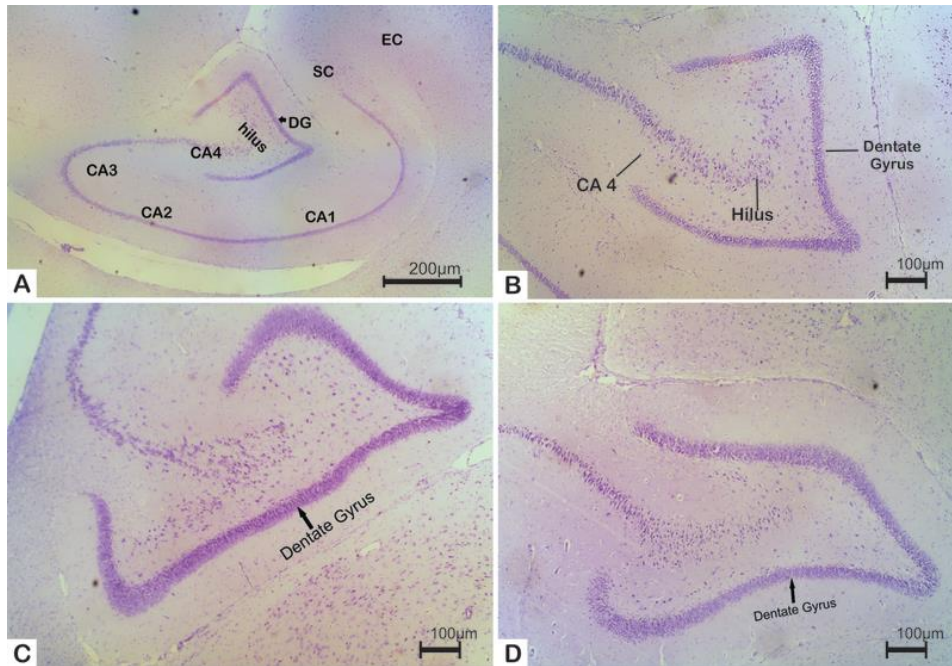
All stained sections were viewed under the light microscope (Olympus® CX21FS1). Images of several fields of each stained tissue sections and the observed histological features of the hippocampal formation including subfields, stratification and cytoarchitecture were captured in a clock-wise manner and then, stitched together to produce a single photomicrograph of each hippocampus using AmScope® Digital Camera Software. All images were processed and labelled with Coreldraw version X7. Heat map of the dentate gyrus was generated using an image processing application (Picassa Version 3.9.0) to further highlight dendritic arborizations of the dentate granule neurons in the molecular layer of the dentate gyrus and outline the dentate somata in the granule cell layer. Average pyramidal cell layer heights of the hippocampal subfields (CA1, CA2 and CA3) were quantified by randomly measuring seven different points along each subfields using Image J 1.46r application package (1.60\_20, NIH, USA).

### Statistical Analyses

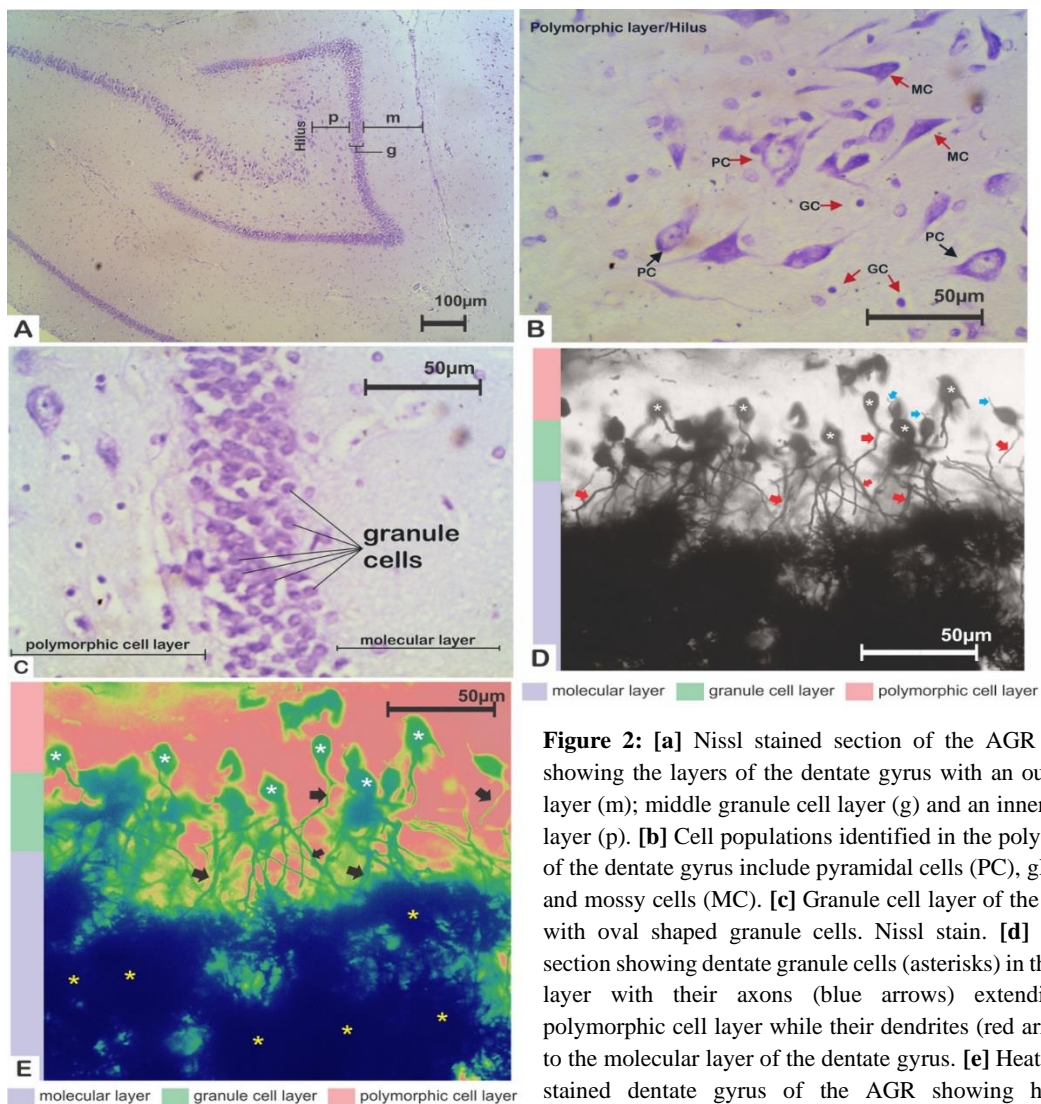
Data generated were expressed as Mean±SEM, and analyzed for significant difference ( $p < 0.05$ ) with one-way ANOVA using Graphpad Prism® version 4.0.

## RESULTS

The term “hippocampal formation” which is a complex of the dentate gyrus, the hippocampus proper (CA1 – CA4), the subiculum and the entorhinal cortex were observed in all coronal sections of the AGR brains stained by the Nissl technique (Figure 1a).



**Figure 1:** Nissl stained sections of the AGR hippocampus [a] showing various subfields (CA1-CA4) of the hippocampus proper, the dentate gyrus (DG), subiculum (SC) and entorhinal cortex (EC). Morphological plasticity was noticed in dentate gyrus presenting with three different shapes: [b] angled C-shaped dentate gyrus [c] wedge-shaped dentate gyrus and [d] V-shaped dentate gyrus.



**Figure 2:** [a] Nissl stained section of the AGR hippocampus showing the layers of the dentate gyrus with an outer molecular layer (m); middle granule cell layer (g) and an inner polymorphic layer (p). [b] Cell populations identified in the polymorphic layer of the dentate gyrus include pyramidal cells (PC), glial cells (GC) and mossy cells (MC). [c] Granule cell layer of the dentate gyrus with oval shaped granule cells. Nissl stain. [d] Golgi stained section showing dentate granule cells (asterisks) in the granule cell layer with their axons (blue arrows) extending into the polymorphic cell layer while their dendrites (red arrows) extends to the molecular layer of the dentate gyrus. [e] Heat map of Golgi stained dentate gyrus of the AGR showing high dendritic arborizations of dentate neurons in the molecular layer of the dentate gyrus and outline of dentate somata in the granule cell layer.

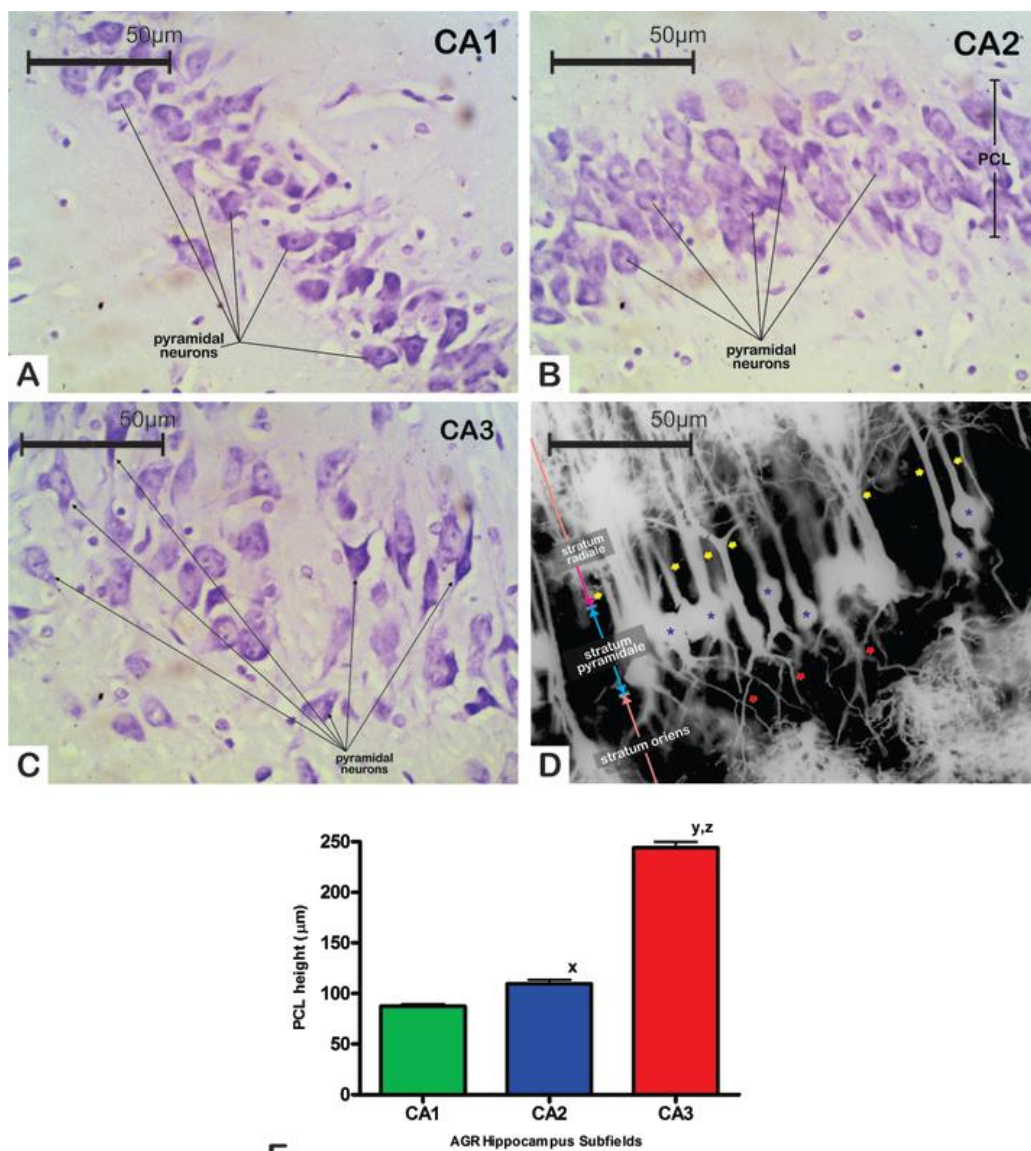
### Dentate Gyrus

The dentate gyrus is seen as a separate structure, it appeared as V-shaped in some sections, wedge-shaped and 'angled' C-shape in others with its concave part opened towards the hippocampus proper (Figures 1b, c and d). It is made up of the fascia dentate and the hilus. The dentate gyrus of the AGR was noted to be three-layered namely: the inner multiform/polymorphic layer, the middle granule cell layer and the outer molecular layer (Figure 2a).

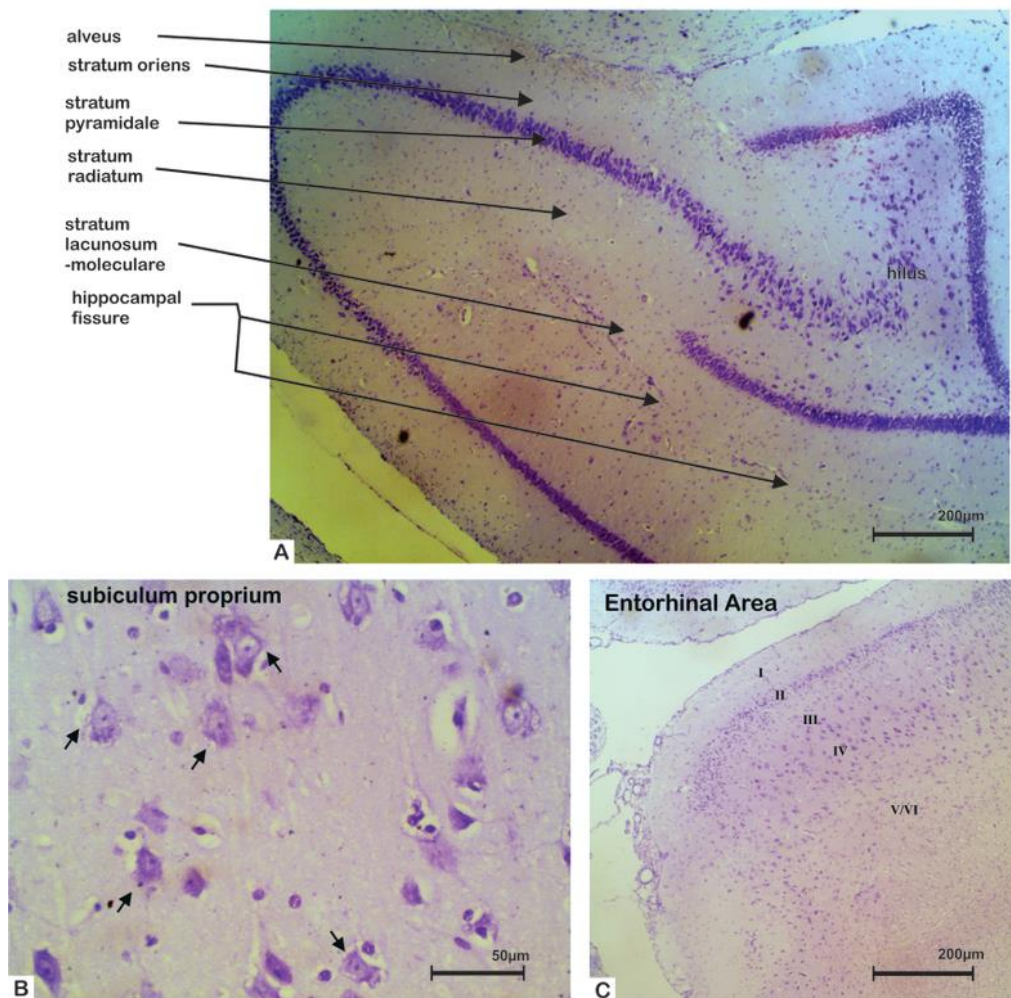
**The multiform or polymorphic layer** is the innermost layer containing polymorphic nervous cells. It is continued into the hilus, after the granule cell layer of the dentate gyrus. Cell populations identified include the hilar mossy cells, glial cells and pyramidal cells (Figure 2b).

**The granule cell layer** of the dentate gyrus is the middle layer that contains oval shaped dentate granule cells with Nissl stain (Figure 2c). Mossy fibers (axonal projections of the granule cells) were projected through the polymorphic cell layer and hilus of the dentate gyrus to the hippocampus proper. The dendrites of these dentate granule cells were observed to be directed towards the molecular layer of the dentate gyrus (Figure 2d and 2e).

**The molecular layer** of the dentate gyrus of the AGR with Nissl stains revealed scanty cellular populations relative to its neuropil (Figure 2c) and also contains a synapse of dendrites. This was further corroborated by the Golgi stains showing majorly spiny dendrites of granule cells of the dentate gyrus (Figure 2d and 2e).



**Figure 3:** Nissl stained sections of the AGR hippocampal proper subfields showing [a] the pyramidal cell layer of CA1 subfield with less densely packed pyramidal neurons of approximately 3-4 cell layer thick; [b] the pyramidal cell layer (PCL) containing tightly stacked pyramidal neurons of the CA2 subfield and [c] abundant and relatively larger CA3 pyramidal neurons with intense cytoplasmic stain compared to CA1 and CA2 subfields. [d] Golgi stained section of CA3 subfield showing somata pyramidal cells (blue asterisks) with their axons (Schaffer collaterals; yellow arrows) and dendrites (red arrows) extending into the stratum radiale and stratum oriens respectively (image inverted). [e] Pyramidal cell layer (PCL) height of the different AGR hippocampal proper subfields. x = statistically significant difference between CA1 and CA2; y & z = statistically significant differences between CA1 & CA3, and CA2 & CA3 respectively at p value < 0.01.



**Figure 4:** Nissl stained section of the AGR hippocampus showing [a]: the layers of the hippocampus proper, [b]: loosely packed deep layer pyramidal cells in the region of the subiculum proprium and, [c]: the entorhinal area of the hippocampal formation with the six layers (I-VI) of the entorhinal cortex

### Hippocampus Proper

The hippocampus proper of the AGR had four subfields namely: Cornu Ammonis 1 (CA1); Cornu Ammonis 2 (CA2); Cornu Ammonis 3 (CA3) and Cornu Ammonis 4 (CA4) (Figures 1a and 3).

**Subfield CA1:** It is the first region of the hippocampal circuit that yields significant output pathway. Cells of the pyramidal cell layer of this subfield were observed to be less densely packed. Higher magnification of the pyramidal cell layer reveals pyramidal neurons of approximately 3-4 cell layer thick with an average thickness of  $87.0 \pm 2.0 \mu\text{m}$  (Figure 3a). This subfield continues into the CA2 of the hippocampal proper (Figure 1a).

**Subfield CA2:** This is a narrow subfield appearing transitional between subfields CA1 and CA3 with an average pyramidal cell layer thickness of  $109.0 \pm 4.20 \mu\text{m}$  (Figures 1a and 3b).

**Subfield CA3:** CA3 extends from the CA4 and continues with CA2. CA3 pyramidal neurons form a well-defined cell layer starting in between the upper and lower ends of the dentate gyrus (Figures 1a). This subfield revealed larger, more distinct pyramidal cells and higher cell layer thickness ( $240.0 \pm 6.0 \mu\text{m}$ ) relative

to the remaining subfields with significant statistical differences at  $p < 0.001$  (Figures 3c - e). Schaffer collaterals were delineated as axonal extensions of the CA3 pyramidal cells extending into the stratum radiatum (Figure 3d).

**Subfield CA4:** CA4 subfield is a direct continuation of the hilus of the dentate gyrus and thus presents similar histological features.

### Stratification of the Hippocampus proper

Five layers of the hippocampus proper were outlined with the Nissl and Golgi stained sections. They include from without inwards: alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum-moleculare (Figures 3d and 4a).

### Subiculum and Entorhinal Area

The subiculum, a component of the hippocampal formation, was observed as a continuation of the CA1 subfield and contained loosely packed deep layer of pyramidal cells (Figures 1a and 4b). The subiculum was noted to connect the hippocampal proper to the entorhinal cortex. The entorhinal cortex was

delineated as a six-layered cortical area based on the varying size of the pyramidal cells (Figure 4c).

## DISCUSSION

The hippocampus is an organized laminar structure which receives sensory inputs mainly from the entorhinal cortex (Karger and Basel, 2014). The hippocampal formation is thought to play a role in memory, spatial navigation and control of attention (Andersen *et al.*, 2007). The hippocampal formation is generally accepted to consist of the dentate gyrus, hippocampus proper, subiculum and entorhinal cortex (Amaral and Lavenex, 2006). All these structures were identified in the AGR hippocampal formation from this study.

The basic cytoarchitecture of the AGR hippocampus observed in this study, with respect to stratification, subfields and cell types, is similar to those reported in the laboratory rats (Westrum and Blackstad, 1962; Seress and Ribak, 1990; Caeser and Aersten, 1991; Falougy *et al.*, 2008; Hussein and George, 2009). Cell types identified in the AGR hippocampus include pyramidal cells, granule cells and mossy cells. Pyramidal cells which are the principal cells of the hippocampus proper were found in the pyramidal cell layer extending their dendrites to the stratum oriens and axons (Schaffer collaterals) into the stratum radiatum. This is consistent with observations in the rat hippocampus (Hussein and George, 2009). The oval-shaped granule dentate cells of the AGR hippocampus were found in the distinct granule cell layer of the dentate gyrus where they send their dendrites to the molecular layer of the dentate gyrus and synapse with afferent inputs from the entorhinal cortex (Hargreaves, 2007). Mossy cells were found at the hilus/polymorphic layer of the dentate gyrus, where they have been recognized as the second principal glutamatergic (excitatory) cells of the dentate gyrus after the granule dentate cells. They are thought to have intrinsic and circuitry properties that make them suitable to activate granule dentate cells (Scharfman and Myers, 2013).

The hippocampus proper of rats as documented by Falougy *et al.* (2008) is subdivided into four regions (CA1-CA4) according to density, size and branching of the axons and dendrites of the pyramidal cells. Similarly, these four regions of the hippocampus proper were distinguished in the Nissl stained sections of the AGR brains.

The features of the hippocampal stratification of the AGR observed in this work using the Nissl and Golgi stains based on cell body location, cell body shape and size, proximal terminations, distal branching characteristics were consistent with reports for the laboratory rats (Turner *et al.*, 1998). The stratum pyramidale, a layer of the principal excitatory neurons of the hippocampus proper, was delineated across all subfields. Here, the pyramidal cells extend their axons

to the stratum radiatum – the Schaffer collaterals. The pyramidal layer expands significantly along its course from CA1 – CA3 and become sparse as it enters the CA4 and hilus area of the dentate gyrus. The observed significant difference in the average heights of the CA subfields with maximum values favouring CA3 is deemed necessary as CA3 has been noted to be a major recipient of sensory inputs from the entorhinal cortex via perforant pathway through the dentate gyrus (Hargreaves, 2007; Knierim and Neunuebel, 2016).

The granule cell layer of the dentate gyrus has been well established as one of the sites for adult neurogenesis in the AGR (Olude *et al.*, 2014b). This noted neural plasticity may account for the different shapes of the dentate gyrus seen in this study. Veena *et al.*, (2011) posited that several factors such as stress, acetylcholine and dopamine levels play significant roles in the regulation of adult neurogenesis. For example, stress was stated to decrease neurogenesis while enriched environment and exercise increases neurogenesis with associated improvement in memory functioning and enhanced synaptic plasticity.

The detailed, delicate arrangement of cell types and subfields, intricate wiring with synapses and laminar organization of the hippocampal formation noticed in the AGR strongly supports the canonical trisynaptic circuitry of the hippocampus as described by Witter (1989) and Knierim and Neunuebel, (2016). Firstly, afferent inputs from the entorhinal cortex projects via axons of the perforant path into granule cells of the dentate gyrus. Secondly, the granule dentate cells project via mossy fibers into the pyramidal CA3 cells. Lastly, the latter project into the pyramidal cells of CA1 by means of the Schaffer collateral system. While the subiculum serves as the main hippocampal output portal, the entorhinal cortex forms the major link between the hippocampal formation and the neocortex (Ding, 2013). It has been well established that olfactory information is transmitted from the primary olfactory cortex to several other parts of the forebrain, including the hippocampus (Biella and de Curtis, 2000). Interestingly, Price (2009) remarked there is an especially strong olfactory input to the hippocampus of rats because more than half of their entorhinal cortex receives olfactory inputs. These olfactory inputs on getting to the entorhinal cortex project into the hippocampus proper via the trisynaptic circuit for memory consolidation. This pathway probably alludes to the connexion in the unique neurobehavioural attributes of this rodent: olfaction and memory.

For this study, two staining techniques (Nissl stain and Golgi impregnation) were deployed to elucidate the histology of the AGR hippocampus. Although traditional stain such as Nissl stain (Cresyl violet) gives an indication of the size and packing density of the cells and cell layers, it cannot distinguish between the various "protoplasmic processes", or trace their full extent. However, Golgi impregnation offers the

advantage of identifying the shape of the cell, and therefore its orientation, and of course the full extent of the dendritic arborization (Cimino, 2000). It will however be necessary to carry out densitometric studies and detailed neurochemical profiling of the AGR hippocampus to fully elucidate the functional leverage of this unique rodent.

The previous and widely accepted hypothesis that hippocampus is widely involved in olfaction was negated by a series of studies that could not find direct projections from the hippocampus to the olfactory bulb (Finger, 2001). However, much work done later confirmed the projections of the olfactory bulb to the ventral part of the entorhinal cortex, the CA1 field, to the main olfactory bulb (Van Groen and Wyss, 1990, Biella and de Curtis, 2000). It has now been noted that the hippocampus belongs to a larger learning system in the brain (Horel, 1994). Therefore, ideas of the hippocampus have developed into ideas of the hippocampal system (Petri and Mishkin, 1994) which is believed to play some roles in learning and memory process. Thus, AGR may serve as a suitable indigenous model for researches in olfaction-linked memory pathologies.

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