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Histomorphology and Histomorphometrics of the retina in Juvenile and Adult African Giant Rats (*Cricetomys gambianus*)

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ABSTRACT

Aging is accompanied with various forms of functional ultrastructural and morphological changes in the eye, including the retina, leading to vision deterioration. However, age related retinal degenerative changes requires further elucidation especially as all previous studies on age-related change of the retina were done in mutant mouse strain models of hereditary retinal degenerations. The potential of using African giant rats (AGRs) as models for ageing in the eye, especially retina, was explored in this study. A total of 14 AGRs divided into two age groups (juvenile and adult) were used to study the histomorphology and histomorphometrics of the retina as well as retinal astrocyte morphology and heterogeneity. Histological findings included retinal atrophy and hypoplasia, with cellular swellings of neuronal cell populations and astrocytes soma and ramifications in the retina of adult compared to juvenile AGR. We suggest that AGR be used as animal model for translational research into normal aging process of the retina, as well as to elucidate the process of age-related neuronal cells loss.

Keywords: African giant rats; Aging; Eye; Retina; Morphological changes

INTRODUCTION

Aging is accompanied with various forms of functional, ultrastructural and morphological changes in the eye (Weisse 1995), and Telegina *et al.* (2018) reported that age-related macular degeneration (AMD), a prime cause of irreversible loss of vision, is a multifaceted retinal neurodegenerative disease seen in 60 years and above persons. The major characteristic of eye aging process is the significant loss of neuronal cells of the retina (Weisse 1995).

Report have shown that visual functionality decrease is an integration of typically aging changes in populations of neuronal cells of the visual system, ocular media changes, and pupillary myosis (Salvi *et al.,* 2006). Furthermore, the retinal pigments epithelium (RPE) vital for rods and cones integrity, shows pleomorphism with increasing age (Salvi *et al.,* 2006). Other major findings related to aging in the eye are decreased cell populations in the retinal layers and cytoplasm volume, content of melanin and increased content of lipofuscin (Weisse 1995; Salvi *et al.,* 2006; Telegina *et al.,* 2018). Age alters, among other patho-physiological changes, the retinal layers thicknesses with loss of photoreceptor rod cells especially in the human subjects (Curcio *et al.,* 1993), and RPE inflammatory cells infiltrations (Damani *et al.,* 2008; Telegina *et al.,* 2018). However, age related retinal degenerative changes, requires further clarifications especially as all the observations made concerning age-related changes of the retina are in few models of mutant mouse strains of hereditary retinal degenerations (Sanyal *et al.,* 1980; Hawkins *et al.,* 1985; Smith 1992; 1995; Smith *et al.,* 1994; Shoji *et al.,* 1998) and laboratory rats (Salvi *et al.,* 2006; Nadal-Nicolás *et al.,* 2018). Specifically, age-related neuronal loss in retinal outer nuclear layer was demonstrated in conventional mice (Ferdous *et al.,* 2021) while photoreceptor cone populations were reduced with age in pigmented rats (Salvi *et al.,* 2006; Nadal-Nicolás *et al.,* 2018). Moreover, thicknesses of retinal plexiform and nuclear layers were reportedly notably reduced in studies using the rat model (Cano *et al.,* 1986; Nadal-Nicolás *et al.,* 2018).

African giant rat (AGR) (*Cricetomys gambianus*) is wide-ranging in sub-Saharan Africa (Usende *et al.,* 2017; 2018a, b; 2020; 2022a, b) and because of its high olfactory acuity, AGR is used in tuberculosis diagnosis in Europe and land mines detection in Mozambique (Ibe *et al.,* 2014). Additionally, in late nineties, the retina structure of the AGR was firstly demonstrated to possess no photoreceptor cones adapted to short wavelengths (Peichl and Moutairou, 1998) which had been suggested

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to be linked to the nocturnal behavior of certain mammals (Jacobs and Deegan, 1992; Szél *et al*., 1996; von Schantz *et al*., 1997). Since this first report, little has been done to further elucidate on the AGR visual structure. Moreover, previous studies by Olude *et al.* (2014) showed that the periventricular and granule cell layers of the AGR olfactory bulb had numerous astrocytes and are involved with prominent migratory activities of newly born cells from the subventricular zone to glomerular layer. Interestingly, there is scarce literature report on sex related differences in astrocytes morphology in the retina, and especially in the AGR. Moreover, sex have been implicated in difference in astrocytes morphology in specific brain regions. In the preoptic area of newborn rats, males exhibit more complex astrocytes morphology compared to the arcuate area where astrocytes are seen to have increased dendritic spines (Amateau and McCarthy, 2001). Also, in the ventromedial nucleus of the brain, there is no sex related differences in astrocyte complexity and dendritic spine density between male and female rats (Amateau and McCarthy, 2001). Thus, the mechanisms regulating astrocytic morphology appear to be unique for some brain regions and needs further investigation (McCarthy *et al.,* 2002.) Furthermore, aging has been shown to influence astrocytes population in distinct brain regions (Olude *et al.,* 2015). We thus sought to explore the histomorphology and histomorphometrics of the retina and retinal astrocytes in juvenile and adult African giant rats (*Cricetomys gambianus*).

MATERIALS AND METHOD

Study Area

This research was conducted in Neuroscience Unit of the Veterinary Anatomy Department, University of Abuja, Nigeria. Abuja is located at latitude 8° and 25° of the equator and Longitude 6°45° East of Greenwich meridian covering 1,043kmin terms of territory and falls within the semi-seasonal equatorial climatic zone and contrasting the periods of wet and dry seasons (Ujofe *et al*., 2010; Usende *et al.,* 2017).

Sample collection and preparation

Fourteen male and female African giant rats of two age groups were used for this study. The aging of the AGR was as described by Olude *et al*. (2015). According to Olude *et al.* (2015), AGR are aged based on body weight and body weight of 70 to 500 were considered juvenile while body weight of 500 and above were considered Adult. All AGR (adult and juvenile) used for this study were obtained from local hunters in Gwagwalada Area Council of Abuja, FCT, Nigeria using a food trap that does not cause injury to them. And they were transported by road in a well-ventilated cage to Department of Veterinary Anatomy, University of Abuja. The AGR were acclimatized for at least 48 hours in laboratory condition before the study commenced. During the period of acclimatization, the AGR were housed individually in metal cages in ventilated animal core facility of Neuroscience Unit of the Department and fed *ad-libitum* with normal rodent chow, yam, watermelon and groundnuts (Usende *et al.,* 2022 a, b). Clean waters were also given *ad-libitum*.

All experimental protocol used was approved by University of Abuja Ethics Committee on Animal use (UAECAU/2017/0007) and in conformity to ethical standards of the Declaration of Helsinki National Institute of Health guide for care and use of laboratory Animals (NIH Publication N080-23) and European Committees Council Directive of November 24, 1986(86/609/EEC).

All animals used were given a lethal injection using Ketamine (100mg/kg) and Xylazine (10mg/kg) combination and immediately perfused transcardic using 0.1M PBS4% paraformaldehyde (Usende *et al.,* 2016, 2022a). The eyes were enucleated based on techniques described by Olopade *et al*. (2005). Subsequently, the eyes were harvested, and immersion fixed in same solution for 48hrs (Usende *et al.,* 2013). Well-fixed eyes were prepared to paraffin blocks and serial sections were obtained for routine histochemistry (Cresyl violet) and immunohistochemistry for astrocytes.

Cresyl violet/Nissl Staining

The serial sections of the eyes destined for cresyl violet staining were transferred into a chloroform/ethanol 4:1 solution for one hour before placing in solution of cresyl violet acetate (Sigma-Aldrich, Germany) for 10 minutes. Tissues were checked at intervals so it does not get too dark. Tissues were dehydrated in ascending grades of ethanol, and clearance was done in 2 changes of xylene, coverslipped with dibutylphthalate xylene (DPX), viewed and micrographs taken.

Immunohistochemistry

The sections destined for immunohistochemistry were processed following the modified protocol of Usende*et al.,* (2022 a, b). In brief, prepared slides were pencil labeled and oven baked at 60^0C for 15mins to dewax, deparaffinized in xylene (2 changes) and hydrated in graded ethanol (decreasing grades). Retrieval of antigen was done for 25mins in 10mM citrate buffer (pH = 6.0). Peroxidase quenching was done for 10mins using hydrogen peroxide before protein blocked in 3% PBS milk for 1hourat room temperature in humidity chamber. Every section was immuno-labeled with the anti-GFAP (Dako) diluted in 1.5% PBS milk in 0.10% Triton X detergent and incubated over night at 4^0C. Bound antibody was detected using HRP-conjugated secondary antibody and according to manufacturer's protocol. 3, 3'-diaminobenzidine (DAB) was used to enhanced reaction product for 5minutes. Thereafter, dehydration was done with graded ethanol, cleared in two changes of xylene, and tissues were mounted using dibutylphthalate xylene (DPX), cover slipped carefully and left to air dry before examination with bright field microscope (Leica DM 300) connected to Excelis HDS (1080P) Camera and Monitor.

Retinal histomorphometry

The retinal histomorphometry were performed on the Cresyl and GFAP immunostained slides using software Image J (NIH, Bethesda, MD, USA) software at X100 magnification. The following parameters were measured:

Thickness of full Retinal layer: The entire retinal layers thickness was quantified by drawing a line from the height of the base of the retinal pigmented epithelium

through all the retinal layers to retinal nerve fibrous layer at x100 and 10 fields from each sample from each group was examined.

***Thickness of Photoreceptor layer**:* This was obtained by drawing a line across the maximum height of the photoreceptor layer at x100 and 10 fields from each sample from each group was examined.

Thickness of Outer nuclear layer: This was obtained by drawing a line across the maximum height of the outer nuclear layer at x100 and 10 fields from each sample from each group was examined.

***Thickness of Outer plexiform layer*:** This was obtained by drawing a line across the maximum height of the outer plexiform layer at x100 and 10 fields from each sample from each group was examined.

Thickness of Inner nuclear layer: This was obtained by drawing a line across the maximum height of the inner nuclear layer at x100 and 10 fields from each sample from each group was examined.

Thickness of Inner plexiform layer: This was obtained by drawing a line across the maximum height of the inner plexiform layer at x100 and 10 fields from each sample from each group was examined.

***Thickness of Ganglionic cell layer*:** This was obtained by drawing line across the maximum height of ganglionic cell layer at x100 and 10 fields from each sample from each group was examined.

Circumference of the Retinal ganglionic cells: This was obtained by drawing a circle using computer software that covers the complete circumference of the ganglionic cells and figures automatically generated were recorded. At least 6 retinal ganglionic cells from each field at x100 were examined and four fields from each sample were evaluated from all samples in each group.

Total retinal GFAP positive Astrocytes counts: A quantification of the GFAP+ immunostain slides for the inner plexiform and ganglionic cell layers astrocytes in the age groups studied was as described by Usende *et al.* (2022b) with minor modifications (Usende *et al.,* 2024). For quantification of cellular population, we performed quantitative analyses done by investigator blinded to experimental conditions using bright field microscope (Leica DM 300) connected with Excelis HDS (1080P) Camera and Monitor and equipped with Image J software (NIH, USA) at X10 magnification, using all eyes from the juvenile and adult groups of AGR. Astrocytes (GFAP immunoreactive cells) count was done unbiased in layers of the retina (inner plexiform and ganglionic cell layers) in all sections. Cell counting was performed according to the method described by Gaykema and Goehler (2009) and Gerashchenko *et al.* (2001) using gridlines with some modifications according to the region of interest (ROI). The optical fractionator method described by West (1993) was followed. Each region selected for astrocytes cell count was divided into 100 counting frames (100µm by 100µm counting frame size). A define dissector option was used for the counting. The counting unit was a GFAP immunopositive cell profiles counted only when a cell was entirely contained within the frame (Palomba *et al.,* 2015).

Integrated density of soma of GFAP positive retinal Astrocytes in the inner plexiform & ganglionic cell layers: Viewing of GFAP positive astrocytes soma from each eye section was achieved with the aid of bright field microscope (Leica DM 300) connected to Excelis HDS (1080P) Camera and Monitor(X40; zoom factor 1.9) and images were captured. To evaluate GFAP+ cells soma mean integrated density, we quantified the intensity of stain usingImage J (NIH, USA) software. In short, we averaged mean staining intensities around the GFAP+ soma. For every of the 4 adjacent eye sections for each animal of each group, 4 different astrocytes were evaluated by two investigators blinded to the experimental grouping. Thereafter, the mean intensities of all the 4 astrocytes from 4 eye slices from five (5) AGRs of each group were averaged to get the mean ± SEM for statistical analysis.

Diameter of Retinal GFAP Astrocytes soma in the ganglionic cell layer: To measure the diameter of GFAP positive astrocytes in the ganglionic cell layer of the retina, a straight line was drawn vertically to cover the full length of the cell bodies running from one edge through the midpoint to the other edge (Igwenagu*et al.,* 2016). Four (4) astrocytes cell bodies from 4 eye slices from five (5) AGRs of each group were averaged to get the mean ± SEM used for statistical analysis.

Circumference of Retinal GFAP Astrocytes cell bodies in ganglionic cell layer: This was obtained by drawing a circle round the astrocytes cell body using the Image J computer software. The circle covered the entire cell body and figures were generated automatically and recorded. Four (4) astrocytes cell bodies from 4 eye slices of five(5) AGRs of each group were averaged to obtain mean ± SEM for statistical analysis.

Statistical Analysis: Numerical data generated are presented as Mean ± SEM and subjected to student *t-test*using the GraphPad Prism for windows version 9.3.1. A p-value of $p \leq 0.05$ was set as statistically significant.

RESULTS

Histological findings

On histological examination, the juvenile retina appeared normal (Fig. 1A). Adult AGR retina had marked reduced cell number in the INL and ONL (hypoplasia). There was evidence of retinal degeneration characterized by cellular swellings in the ganglionic cell layer of all adult AGR (Fig. 1B). The choroid layer of the adult AGR also showed a marked thickening (Fig. 1C). Comparative histomorphometrics revealed a significant reduction in all layers of the adult AGR compared to the juvenile group. Taken together, the retinal layers of the adult AGR had a significant reduced thickness (-47.27%; *** $P<0.001$) compared to juvenile group (Fig. 1D). Comparing the various layers, the photoreceptor layer (PL) data revealed a significant reduced thickness (-20.71%; ***$P<0.001$) in adult AGR in comparison to juvenile group (Fig.1E). Similar patterns were seen in the outer nuclear layer (ONL) (-9.07%; *** $P<0.001$; Fig. 1F), outer plexiform layer (OPL) (-19.36%; *** $P<0.005$; Fig 1G), inner nuclear layer (INL) (-28.55%; *** $P<0.001$; Fig. 1H) and in the inner plexiform layer (-16.36%; ** $P<0.011$; Fig. 1I).

Stereological cell count of ganglionic cells of the retinal ganglionic cell layer revealed significant reduced ganglionic cell populations in the adult AGR retina compared to juvenile (Fig. 2A). Interestingly, significant increased ganglionic cell diameter and circumference were seen in the adult AGR retina compared to the juvenile (Fig. 2B and C).

Upon immunohistochemical examination of the anti-GFAP stained slides, GFAP immuno-positive retinal astrocytes were observed to be confined to two layers of the retina; the inner plexiform and the ganglionic cell layers in both ages studied. Specifically, about 75% were in the ganglionic cell layer while the remaining 25% were seen in the inner plexiform layer. Moreover, the astrocytes soma and ramifications in both the inner plexiform and the ganglionic cell layers of the adult AGR appeared hypertrophied, with a likely bushy appearance indicating their activation compared to the juvenile (Fig. 3A-C). Morphologically, the AGR retina astrocytes presented heterogeneity in the two (ganglionic and inner plexiform) layers. Whereas, in the juvenile, the ganglionic layer had protoplasmic types of astrocytes characterized by highly branched bushy processes (Fig. 3b) and are widely distributed,and in the inner plexiform layer presented fibrous form of astrocytes characterized by straight and long processes (Fig. 3a); in the adult AGR, this type of heterogeneity is not defined as astrocytes from both layers presented the protoplasmic type with highly branched bushy processes (Fig. 3A-C).

Comparing the staining intensity between groups, the signaling intensity of astrocytes appeared to be significantly increased (+22.34%; ***$P<0.001$) in adult compared to juvenile (Fig. 3D). The circumference of the cell bodies of the retinal GFAP positive astrocytes in both ganglionic and inner plexiform layers of adult AGR appeared increased (+71.31%; ** $P < 0.01$) and this was significant statistically in comparison to juvenile group (Fig. 3E). Interestingly, diameter of the retinal GFAP positive astrocyte cell bodies in adult AGR was significantly increased (+62.02%; ** $P < 0.01$) compared to juvenile AGR (Fig. 3F). Concerning stereological GFAP immunopositive astrocytes cells count of both the ganglionic cell and inner plexiform layers, there was a significant increased cell count (+100.01%; *** $P < 0.001$) in adult AGR group compared to juvenile (Fig. 3G). This same pattern was noticed when these layers were taken individually.

DISCUSSION

This present study is the first detailed exploration of both the histomorphology and morphometrics of the retina as well as astrocytes heterogeneity of two age groups of African giant rats captured from their natural environment. This study showed a marked increase in integrated density, intensity of staining and in diameter of astrocytes positive cells in the adult AGR compared to the juvenile, corroborating findings in humans (Ramirez *et al.* 2001) and in Wister rat (Mansour *et al.,* 2008) retinas.

Histomorphology and Morphometrics of Retinal layer of Juvenile and Adult African Giant Rats

The retina is a multi-layered neuro-epithelial tissue, and retinal morpho-functional changes in aging have been said to be related to changes seen in early stages of age-related macular degeneration (AMD) described by Telegina *et al.* (2018). Also, apart from these morphological changes, several reports implicated neuronal cell loss in aged retina in human model (Feeney-Burns*et al.,* 1990; Gao and Hollyfield, 1992; Curcio and Drucker 1993; Curcio *et al.,* 1993; Panda-Jonas *et al.,* 1995) and in laboratory rats (Weisse and Stoetzer 1974; Lai *et al.*, 1978; Katz and Robinson 1986; O'steen *et al.,* 1987, 1995; Weisse *et al.,* 1990; O'steen and Landfield 1991; Imai and Tanakamaru, 1993; Spencer *et al.,* 1995; Weisse 1995; Shoji *et al.,* 1998).

We report herein for the first time such retinal age-related morphological changes (marked atrophy) in adult African giant rat when compared to the juvenile. Specifically, we have shown that the adult (aged) AGR captured from their natural environment had marked reduced thickness of retinal layers. Report have described that with age, the retinal layers thickness reduced (Cano *et al.,* 1986) but may not be associated with reduction of retinal volume *per se* (Feng *et al.*, 2007). The present study indeed showed that retinal layers was altered with age like earlier reports (Zeng and Yang, 2015; Geng *et al.* 2011). We also showed that this reduced retinal layer thickness is associated with marked reduced number of cells in the INL, ONL and ganglionic cells layer of the retina. Interestingly, we showed that the reduced ganglionic cells numbers are associated with swelling and increase in diameter and circumference of these cells, a compensatory mechanism. These changes seen in the adult AGR herein are considered normal physiologic retinal age-related changes (Shoji *et al.* 1998). In laboratory mouse for example, neuronal cell loss is an age-related phenomenon (Shoji *et al.,* 1998) similar to this present report. In the present study, we have demonstrated a striking retinal atrophy in the adult AGR relative to the juvenile, especially in photoreceptor cells loss (in INL and ONL) and ganglion cells layer, and we hypothesize that this rodent is a well-suited model to study aging process of retina, as well as to elucidate the mechanistic pathways of age-related neuronal cells loss. Furthermore, this study used both female and male AGRs to avoid bias, since gender is known to affect aging process because estrogens (more in females) are known antioxidant, neuroprotectant (Zhang *et al.*, 2009), and influences functionality of the retina (Kobayashi *et al.*, 1998; Chaychi *et al.*, 2015).

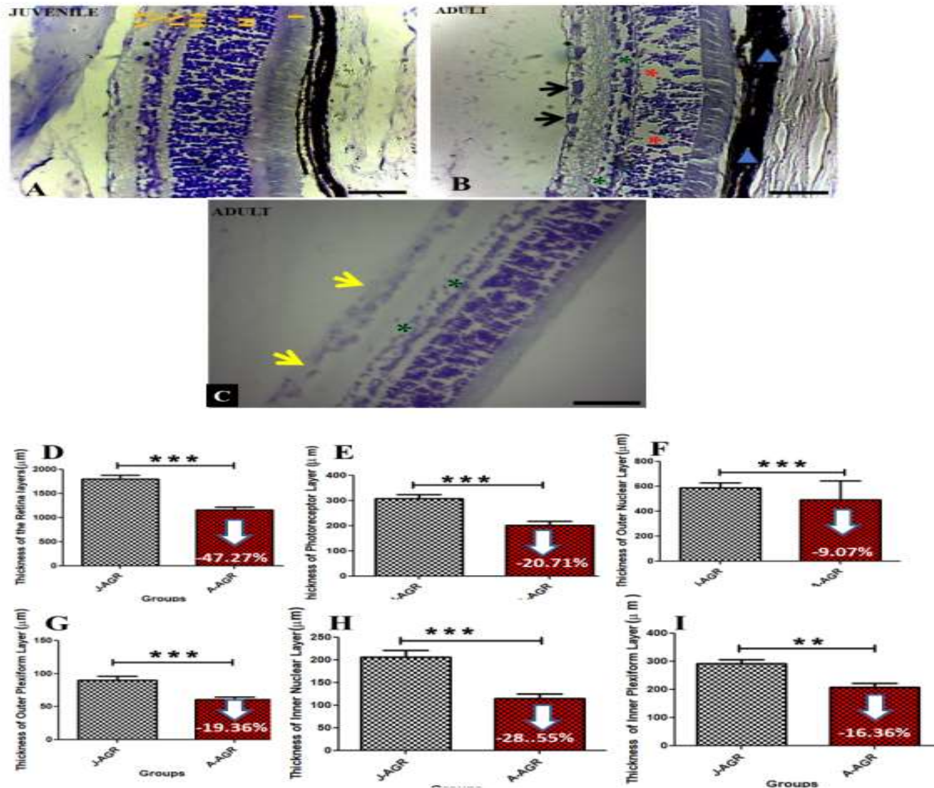

Figure 1: Micrographs showing the retinal layers of juvenile (A) and adult (B and C) AGR. I, Receptor outer and inner segments; II, Outer nuclear layer; III, Outer plexiform layer; IV, Inner nuclear layer; V, Outer plexiform layer; VI, Ganglionic cell layer. Note the severe loss of cells in the outer (red asterisks) and inner (green asterisks) nuclear layers and the swollen (B, black arrows) and lost (C, yellow arrows) ganglion cells with thickened choroid layer (B, blue arrow head) in the adult AGR. Scale bar: 20μm.Bar charts showing statistically significant reduction in the overall thickness of the retinal (D), photoreceptor (E), outer nuclear (F), outer plexiform (G), inner nuclear (H) and inner plexiform (I) layers of adult AGR compared to juvenile. ***P< 0.001; **P< 0.01

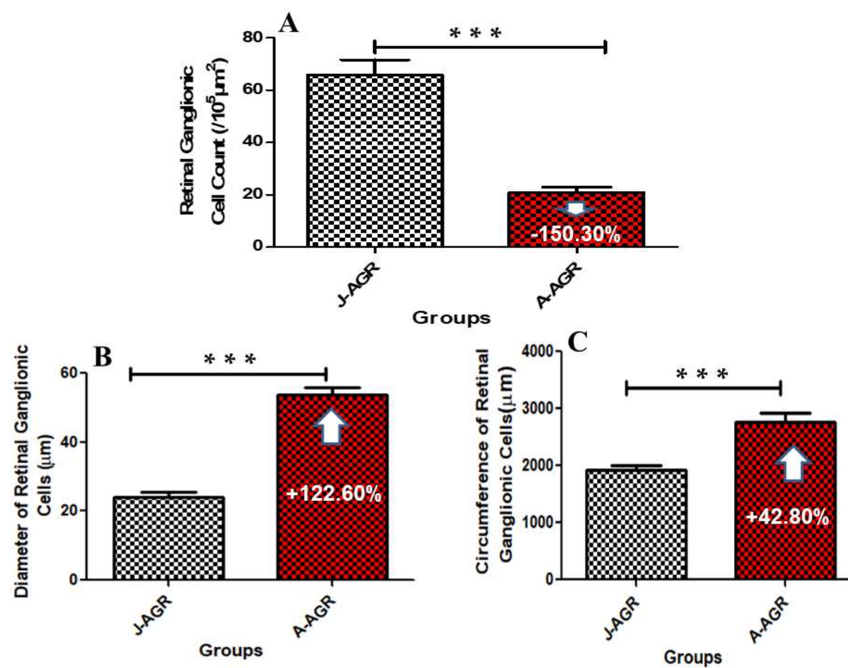

Figure 2: Bar chart showing statistically significant decrease cell count (A) but increase diameter (B) and circumference (C) of retinal ganglionic cells of the adult GR compared to juvenile. ***P< 0.001

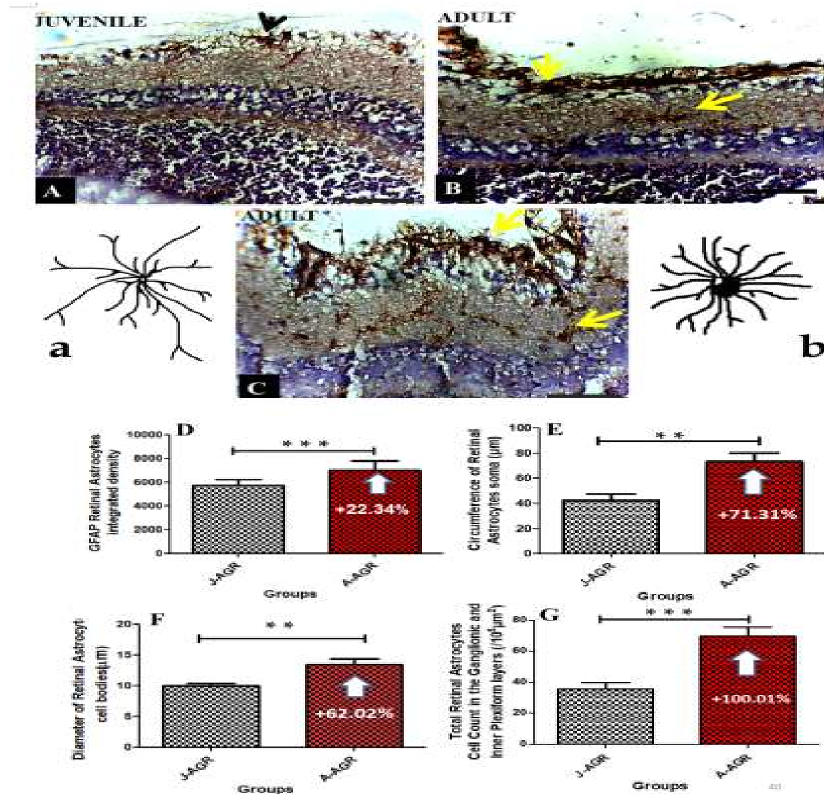

Fig. 3: GFAP-immunostained retina of juvenile (A) and adult AGR (B and C). Adult AGR had astrocytic activation identified by highly branched bushy and extended processes, increased number of cells and thickened cell body (yellow arrow). Scale bar: 20µm. Schematic representation of fibrous (a) and Protoplasmic (b) types of astrocytes.Bar chart showing statistically significant increase GFAP retinal astrocytes staining integrated density (D), circumference of retinal astrocytes soma (E), diameter of retinal astrocytes soma (F) and GFAP positive astrocytes cell count (G) of retina of the adult GR compared to juvenile. **P< 0.01; ***P< 0.001

Astrocyte's morphological changes in the retina of of Juvenile and Adult African Giant Rats

Retinal neuronal integrity and homeostasis (herein reported to be compromised in adult AGR captured from their natural environment) is sustained partly by glia cells including astrocytes, although these glia cells exclusively constitute a smaller fragment of the retina (Goldman, 2014). Specifically, eye diseases experimental models have revealed neuronal damage due to experimentally induced pathology of glia dysfunctions (Coorey *et al.,* 2012). Reports on the effects of age on astrocytes population in the retina is scarcely investigated (Mansour *et al.,* 2008) and is still controversial. Indeed, some findings have reported that aging is linked with decrease astrocytes glia cell population (Peters *et al.,* 1991; Berciano *et al.,* 1995;Nishimura *et al.,* 1995;Desjardins *et al.,* 1997; Sabbatini *et al.,* 1999; Shetty *et al.,* 2005; Lasn *et al.,* 2006) in the CNS generally, and specifically, in the retinas from age humans (60 years and above) compared with those of younger age (40 years and below) (Ramirez *et al.* 2001). Others showed that aging is linked with increasing density and intensity of staining of astrocytes in different brain regions and retina in human models and other species of mammals (Lolova, 1991; Jalenques *et al.,* 1995; Sheng *et al.,* 1996; Amenta *et al.,* 1998; Peinado *et al.,* 1998; Cotrina and Nedergaard, 2002; Wu *et al.,* 2005). These reports on increase are like our present report of increased number of GFAP positive astrocytes and intensity of staining (statistically significant) in the adult AGRs in comparison to juvenile group. Astrocyte density is known to be critically regulated by astrocytic proliferation and their death; both processes occurring during development and in some aging related pathological states (Krueger *et al.,* 1995; Li *et al.,* 1997; Sandercoe *et al.,* 1999; Su *et al.,* 2000; Chu *et al.,* 2001; Takuma *et al.,* 2004). We also reported morphological changes such as astrocytes soma and ramifications hypertrophy, with a likely bushy appearance indicating their activation in the adult AGR compared to juvenile. Earlier Olude *et al,* (2015) demonstrated that the morphology of astrocyteand their density and intensity of staining are dependent of age in different regions of the brain of AGR. They (Olude *et al.,* 2015) also showed that astrocytic numbers increase with age and hypothesized that (a) the increase seen could be as a response to injury or damage to neurons as a result of aging process and (b) the increase is vital in providing some neuroprotection present in brains of younger animals compared to aged animal. The age-related structural changes in astrocytic populations described herein are consistent with earlier studies in Wistar rat retina and indicates that astrocytes undergo severe hypertrophy with other reactive gliosis-like

morpho-functional changes during aging (Mansour *et al.* 2008; Olude *et al.,* 2015).

Conclusion

We reported herein, significant histomorphological and histomorphometrical differences including retinal atrophy and hypoplasia, with cellular swellings of neuronal cell populations and astrocytes soma and ramifications in the retina of adult compared to juvenile African Giant Rats (*Cricetomys gambianus*). The AGR could be a suitable model to study aging process of the retina

Conflict of Interest

The authors declare no conflict of interest

Author's Contribution

UIL, RAA, OJO, OFO were involved in conceptualization. UIL, RAA, FIO, SI, AOR, TSZ, OFO were involved in carrying out the investigation, methodology and formal data analysis. RAA and UIL did the original draft preparation of manuscript while UIL, RAA, FIO, OFO and OJO did the reviewing and editing of manuscript.

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