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NEUROGENESIS IN THE BRAINS OF SUBADULT AND ADULT SOUTH AFRICAN GROUND SQUIRRELS (*XERUS INAURIS*)

By:

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand in fulfilment of the requirements for the degree of Master of Science in Medicine

School of Anatomical Sciences

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Co-supervisor: Professor PR Manger

DECLARATION

I ……., hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise). It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. I declare that neither the whole work nor any part of it has been submitted for another degree or examination at this or any other university.

Signature: …….

Date: …….

DEDICATION

Here is to a long life and a merry one

A quick death and an easy one

A pretty wife and an honest one

A university degree and another one….

(Author unknown, adapted and modified)

ABSTRACT

Neurogenesis is the ability of the brain to generate new neurons. Neurogenic sites and rates of neuronal proliferation were investigated in the brains of subadult and adult South African ground squirrels. Seven female ground squirrels were trapped; euthanised and their ages determined using body masses and paired dry lens masses. Their brains were perfusion fixed with 4% paraformaldehyde before sectioning at 50 µm in a sagittal plane from which every fifth section was stained with cresyl violet to determine the architecture of the brain. Immunolocalisation of Ki-67 for neuronal cell proliferation and doublecortin (DCX) for immature neurons was also carried out on adjacent sections to those stained for cresyl violet. Ki-67 immunopositive neurons were counted in the dentate gyrus of the hippocampus to determine the changes in the rate of cell proliferation with age. Proliferating and immature neurons, DCX immunopositive, were observed in the subventricular zone (SVZ) of the lateral ventricles, the subgranular zone (SGZ) of the dentate gyrus, the rostral migratory stream (RMS), olfactory bulb, piriform cortex and neocortex of both age groups. Adult squirrel brains were heavier than the subadult brains, but showed significantly lower numbers of proliferating neurons in the dentate gyrus when compared to subadult brains.

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ABBREVIATIONS

3V – third ventricle

ac – anterior commissure

Amyg – amygdala

AOB – accessory olfactory bulb

C – caudate nucleus

CA – cornu ammonis

Cb – cerebellum

cc – corpus callosum

CNS – central nervous system

CT – cortex

D – diencephalon

DCN – deep cerebellar nuclei

DCX – doublecortin

DG – dentate gyrus

DT – dorsal thalamus

DV – dorsal vagal complex

EPL – external plexiform layer

f – fornix

fr – fasciculus retroflexus

GC – central grey matter of the midbrain

GCL – granule cell layer

GL – glomerular layer

GP – globus pallidus

Hb – habenular

Hyp – hypothalamus

IC – inferior colliculus

IPL – internal plexiform layer

LV – lateral ventricle

MCL – mitral cell layer

Med – medulla

Mid – midbrain

ML – molecular layer

N.Acc – nucleus accumbens

NEO – neocortex

OB – olfactory bulb

oc – optic chiasm

OE – occipital extension of the SVZ

ON – optic nerve

ONL – olfactory nerve layer

OT – olfactory tract

P – pons

PCL – Purkinje cell layer

PIR – piriform cortex

Pta – pretectal area

R – reticular nucleus of the dorsal thalamus

RMS – rostral migratory stream

S – septal area

SC – spinal cord

SC – superior colliculus

scp – superior cerebellar peduncle

SGZ – subgranular zone of the dentate gyrus

SN – substantia nigra

ST – striatum

SVZ – subventricular zone of the lateral ventricle

TOL – olfactory tubercle

VIIt – facial nerve tract

Vmot – motor nucleus of the trigeminal nerve

WM – white matter

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Adult neurogenesis refers to the ability of the adult central nervous system (CNS) to generate new neurons involving an interplay of endogenous and exogenous factors that affect the proliferation, migration, maturation, differentiation and integration of adult generated neuronal cells (Cameron and McKay, 2001, Kempermann *et al*, 2004 and Ihunwo, 2011). The concept of adult generated neuronal cells was rejected by most early scientists and only became widely accepted within the last 30 years.

1.2 Background

For a period exceeding 100 years, authorities in neuroscience maintained the view that no new neurons are added to the mammalian brain once development was complete (Gross, 2000). This was founded on the observation that "neurons with mitotic figures were absent in the adult CNS of most higher vertebrates" (Altman, 1962). However, Altman produced lesions in the brains of adult rats and combined this with autoradiography for radioactive ^{3}H thymidine administered intracranially and found ^{3}H thymidine labelled glial cells as well as neurons (Altman, 1962). Radioactive ^{3}H thymidine is taken up by dividing cells and incorporated into their DNA, thus it became possible to label any new cells that had just developed (Gross, 2000). This work was further advanced by Kaplan and Hinds, (1977) who carried out intraperitoneal injections on non-lesioned adult rats with ^{3}H thymidine and used autoradiography combined with electron microscopy to demonstrate that indeed some of the stained cells in the olfactory bulb and hippocampus had the ultrastructural characteristics of neurons. The use of non-lesioned brains showed that adult neurogenesis was a morphophysiological process. Further work in female canary brains and other avian species

showed that new neurons also possessed synapses (Goldman and Nottebohm, 1983). Gage and his colleagues in the 1990s were the first to utilise the more advanced techniques of bromodeoxyuridine (BrdU) labelling and immunocytochemistry to positively and conclusively identify the proliferating cells as neurons (Gage *et al*, 1995).

1.3 Active sites of adult neurogenesis

Adult neurogenesis is now a widely accepted phenomenon that has been observed consistently in two main zones of the brain in a number of mammalian species studied including rodents, namely the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone of the dentate gyrus of the hippocampus (SGZ) (Kuhn *et al*, 1996). The SVZ is considered the most active neurogenic site and contains neuronal cells that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB) where they become granule neurons and periglomerular interneurons (Taupin, 2006, Whitman and Greer, 2009). The SGZ generates neural progenitors that migrate into the adjacent granule cell layer (GCL), where they differentiate into mature granule cells that send axons to Ammon's horn (area CA3) and dendrites into the molecular layer of the dentate gyrus (Gould *et al*, 1999 and Kempermann *et al*, 2004). At this stage it is important to note that to date, adult hippocampal neurogenesis has been observed in over 70 mammalian species (Patzke *et al*, 2013b) including humans Eriksson (1998) and also in non-mammalian species such as birds (Goldman and Nottebohm, 1983) and reptiles (Kaslin *et al*, 2008) in which significantly more neurogenic sites were observed.

1.3.1 Stages of adult neurogenesis

Adult neural progenitor cells pass through the stages of proliferation, migration, maturation and integration before they become fully functional neurons (Ihunwo, 2011). Advances in

neuroscience have now allowed for the immunocytochemical identification of cell specific markers at different stages of neural progenitor development (von Bohlen und Halbach, 2007). Radial glia-like neural stem cells expressing glial fibrillary acidic protein (GFAP) and nestin are believed to be the source of neural progenitor cells (Kempermann *et al*, 2004). A neural stem cell is "a multipotent cell that retains the ability to proliferate and generate the main adult CNS phenotypes (neurons, oligodendrocytes and astrocytes)" (Ihunwo, 2011). Neural progenitors undergo proliferation and begin to express Ki-67, a marker of proliferating neurons. After proliferation, developing neurons tend to migrate (to the OB or GCL of the DG of the hippocampus depending on their site of origin) during which process they express doublecortin (DCX) and poly-sialylated neural cell adhesion molecule (PSA-NCAM) which are both markers of migrating and maturing neuroblasts (von Bohlen und Halbach, 2007). Developing neurons undergo morphological changes such as change in nuclear shape from irregular to circular and from possessing numerous stout dendritic processes to one strong apical dendrite and continue to express DCX (Seri *et al*, 2001). Maturing neurons begin to express NeuN, a marker of post-mitotic neurons and calretinin, a calcium binding protein, which is later exchanged for calbindin in mature granule neurons (Kempermann *et al*, 2004). Complete development takes 4 to 7 weeks in adult mice during which time the new neurons form functional connections with interneurons and with other pre-existing neurons, this process is called integration (Kempermann *et al*, 2004).

1.3.2 Potential sites of adult neurogenesis

Various reports have been made over the years concerning the identification of adult generated neurons in regions other than the SVZ and the SGZ (Bonfanti and Ponti, 2008). Reynolds and Weiss, (1992) isolated neural progenitor cells from the mouse striatum while Zhao *et al,* (2003) identified neurogenic cells in the substantia nigra in adult rodent brains.

Xu *et al*, (2005) demonstrated neural progenitors migrating from the subependymal layer of the third ventricle to the hypothalamus. Multipotent stem cells have also been reported in the spinal cord, amygdala, cerebral cortex and the dorsal vagal complex as summarised in the table below (Table 1.1).

Table 1.1: A summary of the reported neurogenic sites in adult mammalian brains

Animals	SVZ	DG	ST	SN	3V	SC	Amyg	CT	OB	DV	Cb
Rodents											
Rats											
Sprague-Dawley				*	*						
Wistar		*						*			
Fisher			*								
Transgenic Tg2576		*									
Mice											
CD1		*				*					
C57BL/6		*		*							
BALB/c		*									
C3H/H3J		*									
Albino									*		
DBA/2J		*									
129/SVJ		*									
Squirrels											
Red squirrel		*									
Fox squirrel		*									
Eastern grey squirrel	*	*							*		
Other species											
Megabats	*	*					*	*	*		*
Rabbits	*	*	*					*			*
Macaque monkeys	*	*					*	*			
Squirrel monkeys	*	*					*				
Humans	*	*						*	*		

Adapted and modified from Ihunwo and Pillay, (2007). 3V – third ventricle, Amyg – amygdala, Cb – cerebellum, CT – cortex, DG – dentate gyrus, DV – dorsal vagal complex, OB – olfactory bulb, SC – spinal cord, SN – substantia nigra, ST – striatum, SVZ – subventricular zone

1.4 Rodents that have been studied

Hippocampal neurogenesis has been studied extensively in wild eastern grey squirrels and chipmunks (Barker *et al*, 2005) and red squirrels (Johnson *et al*, 2010). This current study

focuses on the South African ground squirrel. Kempermann and Gage, (2002) reported variations in the rate of adult neurogenesis in the brains of different strains of the same species of mice implying a possible genetic role. This would suggest that the South African ground squirrel would most likely be unique, presenting differences from other studied closely related species of squirrels owing solely to a different genetic constitution. Furthermore Amrein *et al*, (2004) demonstrated significant differences in the rates of cell proliferation in phylogenetically closely related bat species, some even showing no neurogenesis at all (Amrein *et al*, 2007). Both hippocampal and parenchymal neurogenesis have been studied in other laboratory and wild rodents as well such as rats [(Altman, 1962, Kaplan and Hinds, (1977), Kuhn *et al*, (1996) and van Praag *et al*, (1999)] and mice (Reynolds and Weiss, 1992 and Zhao *et al*, 2003) and the results of those studies are as depicted in the table above (Table 1.1). The rabbit, despite being closely related to rodents, showed remarkable neurogenesis in the cortex, the cerebellum and the corpus striatum in contrast to most laboratory rodents (Luzzati *et al*, 2006 and Bonfanti and Ponti, 2008).

While much of the understanding of adult neurogenesis is derived from studies conducted on laboratory bred animals, the complex nature of the regulation of adult neurogenesis makes studies on animals in their natural habitats more important if its occurrence and significance in nature is to be understood (Boonstra *et al*, 2001, Amrein *et al*, 2004). The wild environment differs significantly from the laboratory environment and provides significant positive and negative pressures influencing the rate at which neurons proliferate and survive throughout adulthood (Ihunwo, 2011). Factors such as predator stress, search for food and adverse climatic conditions which may influence neurogenesis are eliminated in the laboratory setting. Owing to the aforementioned reasons, all the South African ground squirrels (*Xerus inauris*) used in this study were obtained from their natural habitats.

1.5 The effect of age on adult neurogenesis in rodents

Various researchers have observed that neurogenesis reduces significantly with age in captive bred and wild living rodents (Kuhn *et al*, 1996, Barker *et al*, 2005, Epp *et al*, 2009, Johnson *et al*, 2010). Adult red squirrels had 80 % fewer proliferating cells per unit volume than juveniles (Johnson *et al*, 2010), whereas adult eastern grey squirrels showed 50 % less proliferating neurons in the dentate gyrus when compared to the juveniles of the same species (Barker *et al*, 2005). Adult laboratory rats showed an 80 % reduction in proliferating neuron number after 12 hours and a 90 % decrease in immature neuron survival after 6 weeks, as compared to juvenile rats (Kuhn *et al*, 1996). Epp *et al*, (2009) also observed negative correlation between age and both the cell proliferation rate and immature neuron survival rates in wild and captive bred rats.

1.5.1 Other factors affecting adult neurogenesis

Various other factors influence the rate of adult neurogenesis and Ihunwo (2011) groups them into genetic, environmental and pathological factors. Kempermann and Gage, (2002) reported variations in the rate of adult neurogenesis in the brains of different strains of mice implying a possible genetic role. Environmental factors such as physical activity like running and swimming, (van Praag *et al*, 1999) increase the rate of neuronal proliferation. An enriched environment, such as that in which wild animals live and hippocampal dependent learning are thought to increase neurogenesis by promoting the survival of newly generated neurons (Kempermann *et al*, 1998; van Praag *et al*, 1999 and Boonstra *et al*, 2001). Kempermann et al, (1998) went on to show that a greater proportion of newly generated cells differentiated into neurons in mice living in an enriched environment when compared against laboratory controls. Environmental influences such as stress, social isolation and maternal separation decrease the rate of neurogenesis (Lajud *et al*, 2012). Diseases such as diabetes, stroke and

epilepsy are known to increase the rate of neurogenesis (Danzer, 2008) whereas Alzheimer's disease has negative effects in its early stages but shows increased levels of neurogenesis with disease progression, presumably due to the body's compensation for damaged tissue (Mu and Gage, 2011). Parkinson's disease (PD) results in the destruction of dopaminergic neurons and human PD patients have been observed to present with "reduced neurogenesis in the DG which is related to the duration of their illness" (Sullivan *et al*, 2011). Numerous chemical factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) steroid and peptide hormones have also been implicated as increasing the levels of neurogenesis in rabbits and various other mammals (Bonfanti and Ponti, 2008). Adrenal steroid hormones decrease the levels of neurogenesis (Gould *et al*, 1998).

1.6 The South African Ground Squirrel (*Xerus Inauris*)

The South African ground squirrel (*X. inauris*) (figure 1.1) is a relatively large rodent that is found in semi-arid and dry sandy pans in parts of Namibia, central South Africa and Botswana, where temperature ranges from 0 to over 30 ^{0}C and with mean annual rainfall of 100 to 500 mm (Skinner and Chimimba, 2005). This is in sharp contrast to the eastern grey and red squirrels found in North America that come from generally colder and wetter climatic conditions.



Figure 1.1 A photograph of the South African ground squirrel. (Taken at the Lichtenburg breeding centre, Lichtenburg, South Africa)

1.6.1 Scientific classification

Scientific Classification (species authority Zimmerman 1780 cited by Skinner and Chimimba, 2005), Kingdom – Animalia, Phylum – Chordata, Class – Mammalia, Order – Rodentia, Family – Sciuridae, Genus – *Xerus*, Species – *inauris*

1.6.2 Physical description

The typical defining features of the South African ground squirrel are white stripes on the sides, which run from the shoulders to the thighs, a thin white ring around the squirrel's eyes and a long bushy tail, with long hairs (up to 60mm long) that have a black band close to the base and white tips (Herzig and Starchil, 1978). Adult male body mass ranges from 511 – 1022 g and the total body length is 412 – 508 mm, as compared to 511 – 795 g and 410 – 487 mm for the same parameters in the adult female (Skinner and Chimimba, 2005). The South African ground squirrel is a diurnal animal that occupies underground burrows, it leaves the burrow system about an hour after sunrise and spends 70% of the day foraging since it does not hoard food (Herzig and Starchil, 1978). Its reliance on memory will thus be different from the eastern grey squirrel which stores up food in multiple storage sites and the yellow

pine chipmunk which utilises a single food cache to last throughout the winter months (Barker et al, 2005). The home ranges of *X. inauris* were calculated to average 12.5 hectares for adult males and 4 hectares for females, mainly because males interact and breed with females from more than one colony. It may be predicted that males would have better spatial memory than the female squirrels and may display more hippocampal neurogenesis, but this is unknown. Male and female squirrels form distinct social groups that are independent of each other and have a strict dominance hierarchy in males that is age dependent. Individual social groups occupy a burrow system that is distinct from that of the next group. Little is known about their longevity in the wild but they are estimated to survive for several years. Adult size is reached 153 days after birth but sexual maturity will not be reached until the 8^{th} month for males and the 10^{th} month for females (Skinner and Chimimba, 2005). These squirrels communicate vocally, through various sounds such as chirps, squeaks, growls, alarm and high pitched shrill calls that are all correctly interpreted by members in the group. Squirrels have also been noted to live with yellow mongooses and to live a mutualistic life with meerkats (Skinner and Chimimba, 2005). They respond to alarm calls of the meerkats and will react by running quickly for shelter. Herzig and Starchil, (1978) observed a characteristic habit of urinating close to the entrance of the burrow system, which they believed to be some sort of marking behaviour. Mating is not seasonal and breeding occurs throughout the year. An adult female will have about 1 to 3 litters every year, with each litter consisting of 1 to 3 young (Skinner and Chimimba, 2005).

1.7 Justification for the study

No information exists on the active and potential sites of neurogenesis in the South African ground squirrel. No studies have investigated the changes in the rates of hippocampal neurogenesis in this species with increasing age. This study placed all ground squirrels into

specific age groups and investigated the pattern of transition of neurogenesis from the subadult to adult age groups. Habitat has now been demonstrated to influence adult neurogenesis in wild rodents of closely related species (Cavegn *et al*, 2013), therefore all ground squirrels in this study were obtained from a predominantly natural habitat with distinct climatic and geographical characteristics. The adult squirrel covers a lot of ground during feeding, but has been shown to flee back to its own burrow when attacked (Skinner and Chimimba, 2005), demonstrating good spatial memory, which has been linked to increased hippocampal neurogenesis (Kempermann *et al*, 1998, van Praag *et al*, 1999). South African ground squirrels may be good subjects for studying neurogenesis in a wild rodent species owing to their relatively long life, allowing for the study of a wider age range from a species that lives in a complex social interaction setting.

1.8 Aim

To establish the sites of occurrence of adult neurogenesis and rates of cell proliferation in the brains of wild-caught South African ground squirrels, *Xerus inauris*, from a range of ages.

1.8.1 Specific objectives

i) To determine the relationship between the eye lens mass, the brain mass and the total body mass

ii) Briefly describe the histological structure of the brain of the South African ground squirrel

iii) To identify the active neurogenic sites in the brains of subadult and adult South African ground squirrels using immunohistochemical staining for Ki-67 a marker for neuronal cell proliferation and DCX marker for immature and migrating neurons

iv) To identify the potential neurogenic sites in the brains of subadult and adult South African ground squirrels using immunohistochemical staining techniques for Ki-67 and DCX

v) To estimate the rate of cell proliferation in the dentate gyrus of the hippocampus of subadult and adult South African ground squirrels by the quantification of Ki-67 immunopositive neurons

CHAPTER 2: MATERIALS AND METHODS

2.1 Ethical clearance and study setting

Ethical approval was obtained from the University of the Witwatersrand Animal Ethics Screening Committee (AESC) (clearance number: 2012/30/01, Appendix A). Permission to trap and capture the squirrels was received from the North West Province and Lichtenburg Municipality (Appendix B). Export permits were obtained from the office of the North West provincial government Environmental Services Biodiversity Management and Conservation department and import permits from the Directorate of Nature Conservation in the office of the Premier of the Gauteng province (Appendix C).

2.2 Trapping and capture of the South African ground squirrels

Seven female free living ground squirrels (5 adults and 2 subadults) were trapped and captured as a sample of convenience from the Lichtenburg Breeding Centre in the North West Province through the use of animal handling humane live cage traps (60 x 19 x 21 cm). The squirrels were divided into subadult and adult groups based on their body masses, where 500 - 510 g was the critical mass range (Skinner and Chimimba, 2005). Each captured squirrel was weighed on a hand held scale in the field and the lowest body mass for admission into the study was 200 g. The mass of each cage was determined prior to trapping, after trapping the mass of each squirrel was obtained by subtracting the mass of the cage from the combined mass of the cage and the captured squirrel. All captured squirrels between 200 and 500 g were considered subadults and those from 510 g and upwards were adults. The captured squirrels were transported to the Central Animal Services of the University of the Witwatersrand Faculty of Health Sciences for euthanasia and perfusion fixation before the removal of the brains.

2.3 Perfusion and fixation

The selected squirrels were euthanized by intraperitoneal injection of euthanase (1 ml/kg). Thereafter, each squirrel was weighed to obtain an accurate body mass before dissection to expose the heart. A midline incision was made down the skin of the anterior chest wall of each squirrel to expose the ribcage. The sternum and adjoining parts of the ribcage were removed to expose the heart. After removal of the fibrous pericardium around the heart, the right atrium was cut and 0.9% cold normal saline was perfused through the left ventricle of the heart. Thereafter, 4% paraformaldehyde solution in 0.1M PB (PFA), was passed through the circulatory system before the eyes and brains were dissected out and post fixed in PFA.

2.4 Processing of the eye lenses

Eye lens masses were used along with body masses, as methods for determining the ages of the ground squirrels. All the eyes were treated after the manner described by Fischer and Perry, (1970). Following removal, the eyes were immediately placed in 4% PFA for 3 weeks for the lens to harden. The lenses were then dissected from the eyes, cleaned and air dried overnight. All lenses were then dried in an oven at 80 ^{0}C for 48 hours. Beale (1962) and Fischer and Perry (1970) reported that after 48 hours no significant amount of lens weight loss occurs with increased time spent in the drying oven. Thereafter the paired lenses were immediately weighed on a scale and recorded to the nearest 0.1 mg.

2.5 Cryoprotection and sectioning of brain tissue

Whole brains were transferred from 4 % PFA to a 30 % sucrose solution and kept at 4 ^{0}C until equilibrium was reached. The brains were then transferred to antifreeze (1:1 mixture of ethylene glycol and glycerol in 0.244M PB) where they were kept until equilibrium was reached once again before storage at -20 ^{0}C until the time for sectioning. Each whole brain

was split in half along the midline and each left half brain was then transferred into a solution containing 30 % sucrose in 0.1M PB for cryoprotection. The right half of each brain was stored in antifreeze for use in later studies. When ready for sectioning, brains were frozen in crushed dry ice, mounted on a stage with 30% sucrose and sagittal sections were cut at a 50 µm thickness.

2.6 Nissl staining for cytoarchitecture of the brain

Every fifth brain section was mounted onto 0.5 % gelatine coated slides and stained using the cresyl violet method to reveal Nissl bodies. This series was used for anatomical orientation and cytoarchitecture of the brain. The mounted sections were left to air dry, thereafter they were placed in defatting solution (1:1 of 100 % chloroform and 100 % ethanol) overnight then partially rehydrated by placing the slides in decreasing concentrations of alcohol down to 50 % alcohol; (2 changes in 100 % alcohol for 5 minutes each and 2 minutes each in 95 %, 70 % and 50 % alcohol). Slides were dipped in 1 % cresyl violet stain for one minute before transfer to distilled water. Cresyl violet stains ribonucleic acid (RNA) molecules (Nissl bodies) blue. Dehydration was then carried out in alcohol concentrations from 50 % to 100 %, with 70 % alcohol being the differentiation step. Dehydrated sections were cleared in xylene for ten minutes, mounted in DPX mountant (Merck chemicals (Pty) Ltd, Wadeville South Africa) and cover slipped.

2.7 Immunohistochemistry

One in every five sections was chosen for immunohistochemical staining against each of the newly generated neuronal markers, Ki-67 and Doublecortin (DCX). Ki-67 is a mitosis related protein, which is expressed in proliferating cells (G_1, S, G_2 and mitosis), but not in the G_0 resting phase (Lu et al., 2005). Immunohistochemistry for Ki-67 was done to identify

proliferating neuronal cells. DCX is a microtubule associated protein that promotes microtubule polymerisation and is expressed by immature and young neurons (von Bohlen und Halbach, 2008). Immunohistochemistry for DCX was carried out to determine the presence of young and immature neurons.

2.7.1 Immunohistochemistry for Ki-67

Free floating brain sections were placed in appropriately labelled trays with wells containing 0.1M phosphate buffer (PB). The sections were transferred to endogenous peroxidase inhibitor (EPI) a solution containing 49.2 % methanol, 49.2 % PB and 1.6 % of 30% hydrogen peroxide (H_2O_2) by volume. The sections were rinsed 3 times for 10 minutes each in PB before transfer to blocking buffer solution (2 % bovine serum albumin (BSA), 3 % normal goat serum (NGS) and 0.25 % Triton X in PB) for 2 hours at room temperature. Immediately afterwards, sections were transferred to the primary antibody solution [polyclonal rabbit NCL- Ki-67 (Novocastra laboratory (NCL), Wetzlar, Germany; 1:1000) supplemented with 2 % BSA, 3 % NGS and 0.25 % Triton X in PB] for 48 hours at 4 ^{0}C under gentle agitation.

Thereafter, the brain sections were rinsed 3 times for 10 minutes each in PB at room temperature then transferred to secondary antibody solution [biotinylated goat anti-rabbit (Vector lab, CA, USA; 1:250) supplemented with 2% BSA, 3% NGS in PB] for 2 hours at room temperature. Sections of brain tissue were then washed in 0.1M PB and then transferred to avidin-biotin complex (ABC) (Vector lab, CA, USA; 1:125, A and B) for 1 hour at room temperature. Afterwards the sections were rinsed then immersed in a 0.05 % solution of diaminobenzene (DAB) to which H_2O_2 solution was added after 5 minutes, and observed under a stereomicroscope until a strong immunohistochemical staining was obtained. The

reaction was stopped by rinsing sections in PB 3 times for 10 minutes under gentle shaking. Sections were then mounted on 0.5 % gelatin coated slides and left to dry. After drying, brain sections were dehydrated in increasing concentrations of alcohol from 70 % (2 hours) 95 % (2 minutes) then twice in 100 % (5 minutes each) after which the sections were cleared in xylene for ten minutes then mounted in DPX mountant (Merck chemicals (Pty) Ltd, Wadeville, South Africa) and cover slipped.

2.7.2 Immunohistochemistry for DCX

Brain sections were transferred to endogenous peroxidase inhibitor (EPI) to quench endogenous peroxidase activity. The brain sections were rinsed 3 times for 10 minutes each in PB before transfer to blocking buffer solution [2 % BSA, 3 % normal rabbit serum (NRbS) and 0.25 % Triton X in PB] for 2 hours at room temperature. Immediately afterwards, brain sections were transferred to the primary antibody wells containing a solution of goat anti-DCX [(C-18), (Santa Cruz biotechnology, Inc. CA, USA; 1:250)] supplemented with 2 % BSA, 3 % NRbS and 0.25 % Triton X in PB] for 48 hours at 4 ^{0}C under gentle agitation.

Thereafter, the brain tissue was rinsed 3 times for 10 minutes each in PB at room temperature then transferred to secondary antibody solution [biotinylated rabbit anti-goat (Vector lab, CA, USA; 1:250) supplemented with 2% BSA, 3% NRbS in PB] for 2 hours at room temperature. Sections of tissue were then washed then transferred to ABC (Vector lab, CA, USA; 1:125, A and B) for 1 hour at room temperature. The sections were rinsed PB and immersed in 0.05% solution of diaminobenzene (DAB) to which H_2O_2 solution was added after 5 minutes, and observed under a stereomicroscope until a strong immunohistochemical staining was obtained. The reaction was stopped by rinsing sections in PB 3 times for 10 minutes under gentle shaking. Sections were then mounted on 0.5 % gelatin coated slides and left to air dry. Once dried, the brain sections were dehydrated in increasing concentrations of alcohol, from

70 % to 100 % then cleared in xylene for ten minutes, mounted in DPX mountant (Merck chemicals (Pty) Ltd, Wadeville South Africa) and cover slipped.

2.8 Analysis under the microscope

The Nissl stained sections were viewed under the microscope to determine the histology and cytoarchitecture of the squirrel brain. Ki-67 stained sections allowed for the determination of the distribution of proliferating neurons in the squirrel brains and for the proliferating cell numbers in the dentate gyrus of the hippocampus. DCX stained sections showed the distribution of immature and migrating neurons in the squirrel brain.

2.8.1 Morphology of DCX positive cells

DCX immunopositive cells were reviewed for shape of the cell body and polarity depending on the number of processes from the cell body. DCX immunopositive cells in the dentate gyrus were classified according to presence or absence of cell processes, cell process length and branching pattern from cell types A to F (Table 2.1) (Plumpe *et al*, 2006).

Table 2.1 Classification of DCX immunopositive cells in the dentate gyrus

Cell type	Description
A	No processes
B	Short plump processes, less than a nucleus width in length
C	Medium length processes extending into the granule cell layer
D	Cell processes extending as far as the start of the molecular layer
E	One strong dendrite branching in the molecular layer
F	Delicate dendritic tree branching in the granule cell layer

Adapted from Plumpe *et al*, (2006)

2.8.2 Photomicrographs of stained tissue sections

Representative photomicrographs of selected brain regions were captured using the Zeiss Axioshop (Carl Zeiss Microscopy GmbH, Jena, Germany) with Axiovision software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.8.3 Proliferating cell count in the dentate gyrus

The proliferating cell count was conducted to obtain an estimate of the total number of proliferating neuronal cells in the whole dentate gyrus. Proliferating cells were counted on a Zeiss Axioshop (Carl Zeiss Microscopy GmbH, Jena, Germany) with Axiovision software (Carl Zeiss Microscopy GmbH, Jena, Germany) and a 63X objective lens. Counting was done on a one in five series of brain sections involving the hippocampus and the result was multiplied by the inverse of the sampling fraction to obtain the total estimated number of proliferating cells in the hippocampus (Ajao *et al*, 2010).

2.9 Drawing of stained tissue sections

A series of representative brain sections were selected for drawing using a stereo microscope (Leica MZ75, Leica Microsystems Ltd, CH – 9435 Heerbrugg, Switzerland). The drawings were traced using canvas software version 7 (Deneba Software, Miami, Florida, USA) and used to represent on a larger scale, the cytoarchitecture of the brain of the South African ground squirrel and the distribution of Ki-67 and DCX stained areas of the brain.

2.10 Statistical Analysis

Statistical analysis was conducted using Stata (version 11.1) and the Pearson coefficient of determination (R^2) calculated to determine if there was correlation between the eye lens mass,

total body mass, brain mass and the Ki-67 cell count. Calculations to determine if there was a statistically significant correlation between the estimated numbers of Ki-67 positive cells against brain mass and body mass in the subadult and adult age groups were also conducted.

CHAPTER 3: RESULTS

3.1 Body mass, Eye lens mass and Brain mass

Seven female South African ground squirrels were captured for this study. As per age classification by body mass, 5 were adults with body masses ranging from 520 g to 600 g, and 2 were subadults weighing 230 g and 250 g (overall mean 471 and sd 160.772) (Table 3.1). The brain masses ranged from 5.5 g to 5.7 g in the subadult squirrels and 6.4 g to 7.5 g in adults and were directly proportional to the body masses showing a corresponding increase with an increase in body mass (Table 3.1). Figure 3.1 below shows a typical adult brain from the South African ground squirrel. With increase in age in the South African ground squirrel, the percentage of brain mass to total body mass showed a decrease averaging 2.3 % in the subadult animals and 1.2 % in the adult animals. The paired dry lens mass increased with an increase in body mass and the rate of increase slowed down as the body mass of the squirrels increased to 600 g (Figure 3.2).

Table 3.1 A summary of the body mass, brain mass and paired lens mass of the South African ground squirrels

Animal	Body mass (g)	Brain mass (g)	Paired lens mass (mg)
Subadult			
Xa3	230	5.5	32
Xa4	250	5.7	31
mean	**240**	**5.6**	**31.5**
Adult			
Xa1	600	7.5	56
Xa2	600	7.5	53
Xa5	550	6.5	58
Xa6	550	6.5	56
Xa7	520	6.4	46
mean	**564**	**6.88**	**53.8**

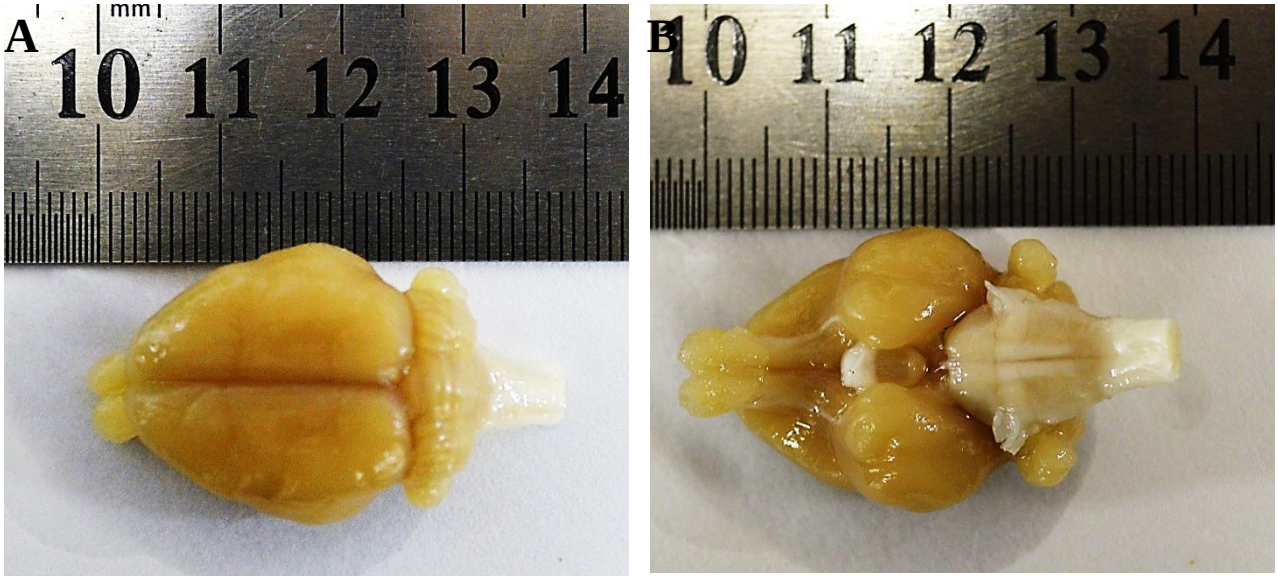


Figure 3.1: Photograph showing the brain of an adult South African ground squirrel. A – dorsal view, B – ventral view

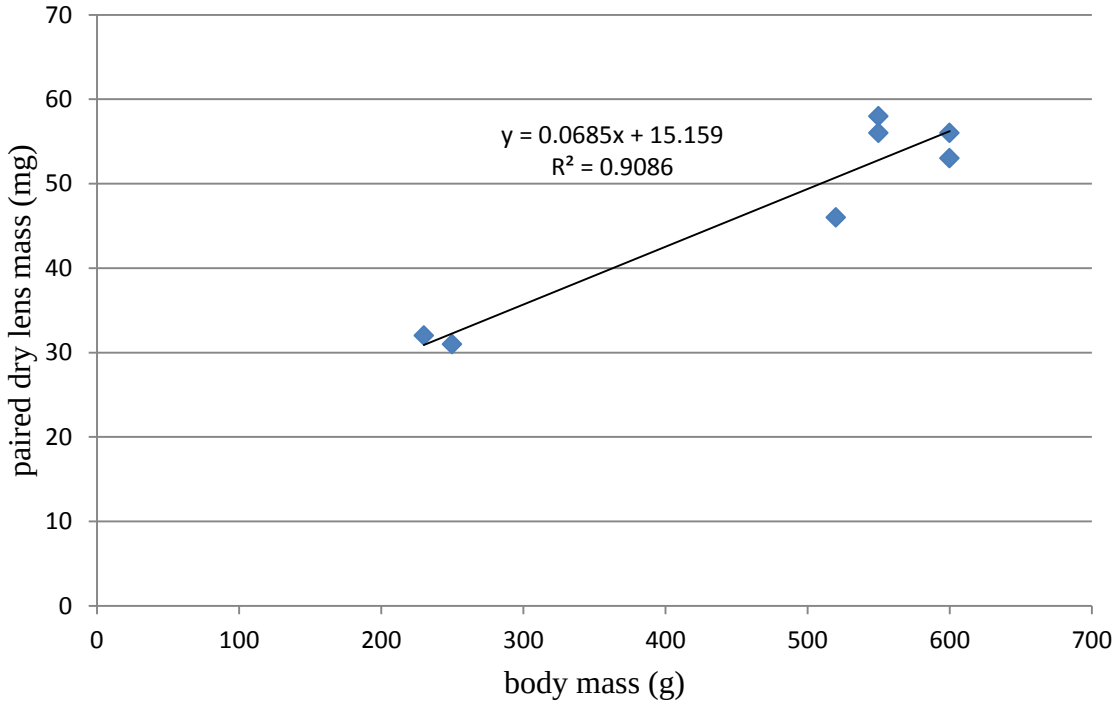


Figure 3.2: The relationship between body mass and paired dry lens mass. (p=0.0416).

3.2 Nissl staining

Nissl stained sections were used to show the architecture of the squirrel brain (figure 3.3). The adult South African ground squirrel has a typical rodent brain showing all major brain regions such as the cerebrum with the cerebral hemispheres and diencephalon, the brainstem, clearly showing the midbrain, pons and medulla oblongata and a relatively large cerebellum (figure 3.3). It also presented with a prominent olfactory bulb, in common with most other rodent brains. The squirrel olfactory bulb showed six layers that could be differentiated microscopically (figure 3.4 A). The outermost layer consisted of fibres from the olfactory epithelium, the olfactory nerve layer (ONL) entering the olfactory bulb. Deep to it was the glomerular layer (GL) showing basket-like aggregations of cells with periglomerular cells. The GL was separated from the mitral cell layer (MCL) by the (external plexiform layer (EPL), which contained few nuclei of granule cells. In between the MCL and granule cell layers (GCL) was the internal plexiform layer (IPL). The MCL consisted of relatively large cells whereas the GCL had numerous cells with medium sized to small nuclei.

The neocortex showed the typical mammalian six-layered structure and most easily distinguishable were layers 1 and 2, the molecular and external granular layer (figure 3.4 B). Layers 3 to 6, the external pyramidal, the internal granular, internal pyramidal and multiform layers had partly overlapping components. The hippocampus and dentate gyrus were intensely stained and readily visible on Nissl stained sections (figure 3.4 C) as were the layers of the cerebellum, the molecular, Purkinje cell and granule cell layers (figure 3.4 D).

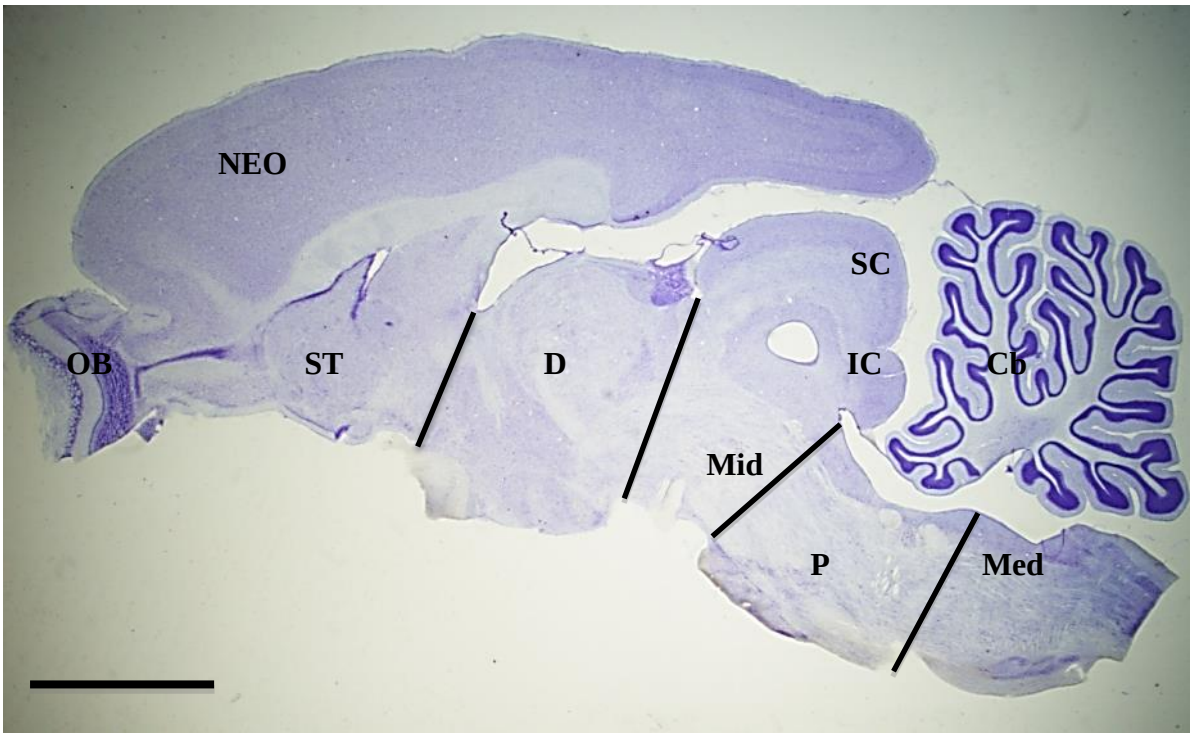


Figure 3.3: Representative photomicrograph of a sagittal section of the squirrel brain. Cb – cerebellum, D – diencephalon, IC – inferior colliculus, Mid – midbrain, Med – medulla, NEO – neocortex, OB – olfactory bulb, P – pons, SC – superior colliculus, ST – striatum. Nissl stain, scale bar 5 mm.

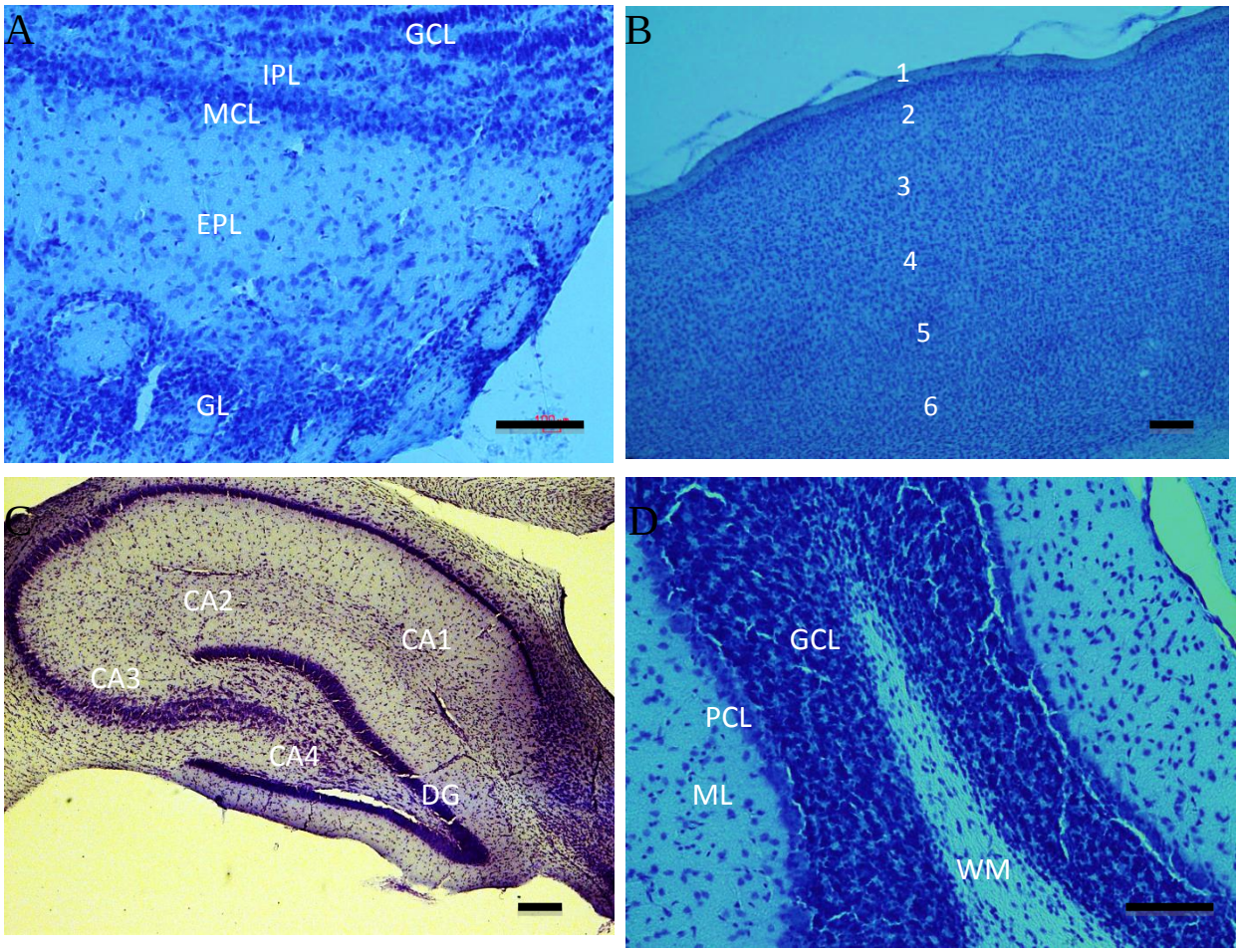


Figure 3.4 Representative photomicrographs of the adult squirrel brain showing various brain areas. A – olfactory bulb, B – neocortex, C – dentate gyrus and the hippocampus and D – cerebellum, CA1-4 – cornu ammonis 1 – 4, DG – dentate gyrus, EPL – external plexiform layer, GCL – granule cell layer, GL – glomerular layer, IPL – internal plexiform layer, MCL – mitral cell layer, ML – molecular layer, PCL – Purkinje cell layer, WM – white matter, 1 – molecular layer, 2 – external pyramidal layer, 3 – external granular layer, 4 – internal pyramidal layer, 5 – internal granular layer, 6 – multiform layer. Cresyl violet staining for Nissl substance of the adult squirrel brain Scale bar is 200 µm for all diagrams.

3.3 Active and potential neurogenic sites in the South African ground squirrel

Immunolocalisation for Ki-67 and DCX showed that the subventricular zone of the lateral ventricles had the largest number of new neurons as compared to any other region of the squirrel brain. The DG of the hippocampus was identified as the other active neurogenic site and these were observed in both subadult and adult brains. Potential neurogenic sites in the South African ground squirrel were the neocortex and the piriform cortex across all age groups. The occipital extension of the subventricular was identified as a potential neurogenic site only in the brains of subadult squirrels.

3.3.1 Subventricular zone (SVZ) of the lateral ventricle and the rostral migratory stream (RMS)

The subventricular zone consistently showed the presence of Ki-67 immunopositive neurons in all the brains studied (figure 3.5). Ki-67 immunopositive neurons were, however, less numerous in the adult brains as compared to those of the subadults (Figure 3.5A and C). Both proliferating and immature neurons were distributed in the walls of the lateral ventricles, including the area immediately dorsal to the corpus striatum. The subventricular zone of the lateral ventricles also consistently showed the presence of DCX immunopositive immature neurons (figure 3.5 B). Once again, subadult brains showed a denser distribution of the immunostained cells as compared to the adult brains (figure 3.5 B and D).

Cells from the SVZ migrated along the rostral migratory stream (RMS) that coursed rostral to the corpus striatum and slightly ventral to enter the olfactory tract and terminate in the OB (figure 3.6). Numerous Ki-67 immunopositive neurons were observed migrating along the RMS as chains of neuroblasts (figure 3.6 A). Proliferating neurons were significantly more in

the subadult squirrel brains as compared to the adult brains (figure 3.6 A and C). The DCX immunopositive cells showed a very dense distribution of fibres in this region with cell processes oriented parallel to the RMS (figure 3.6 B). The majority of DCX immunopositive cells had small to medium sized cell bodies surrounded by dense aggregation of cell processes, especially in the subadult brains. Cell process density appeared greater in subadult brains as compared to adult brains.

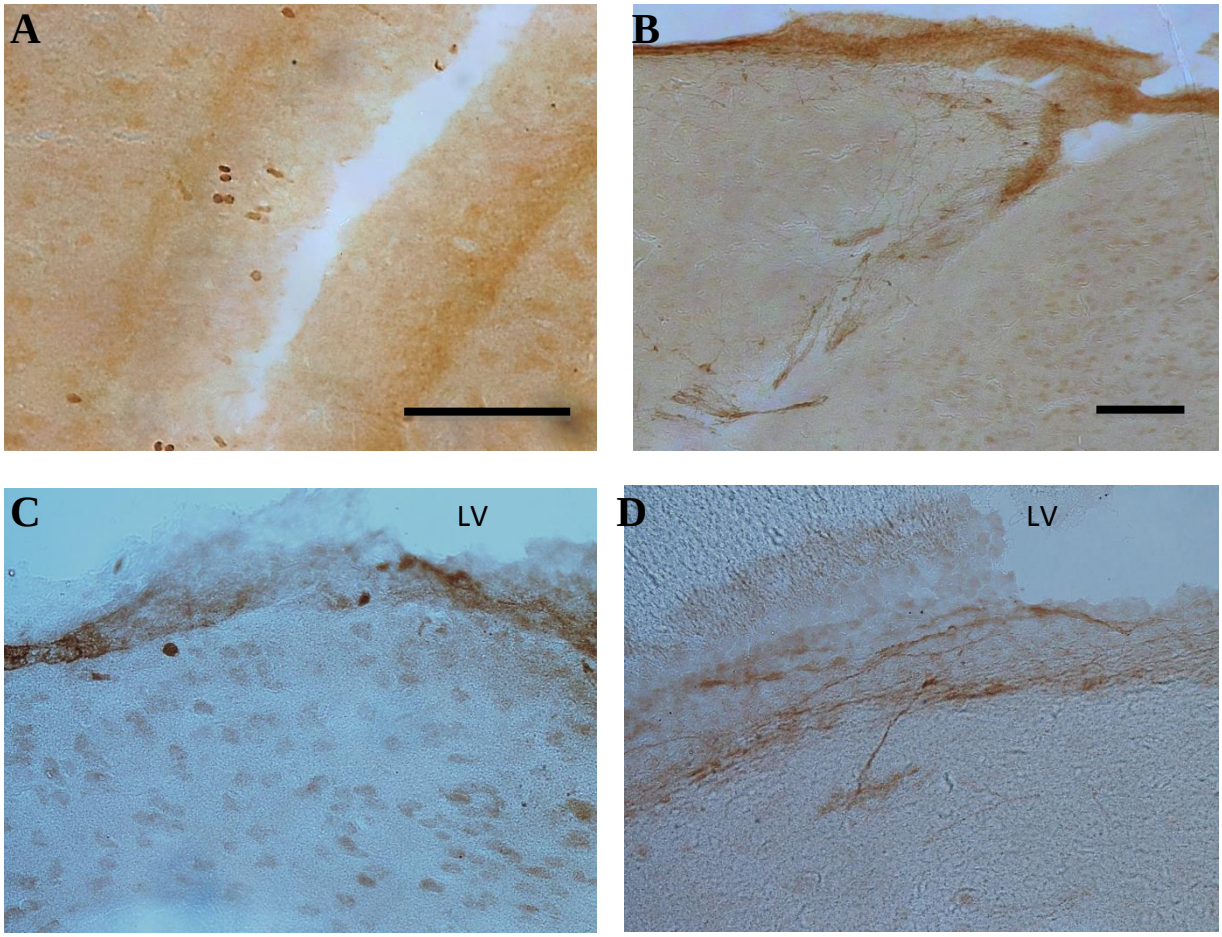


Figure 3.5: Representative photomicrographs showing the SVZ of the lateral ventricles. A – subadult brain stained for Ki-67, B – subadult brain stained for Ki-67, C – Adult brain stained for Ki-67and D – adult brain stained for DCX. LV – lateral ventricle, Scale bar is the same for A, C and D = 100 µm, scale bar B = 200 µm.

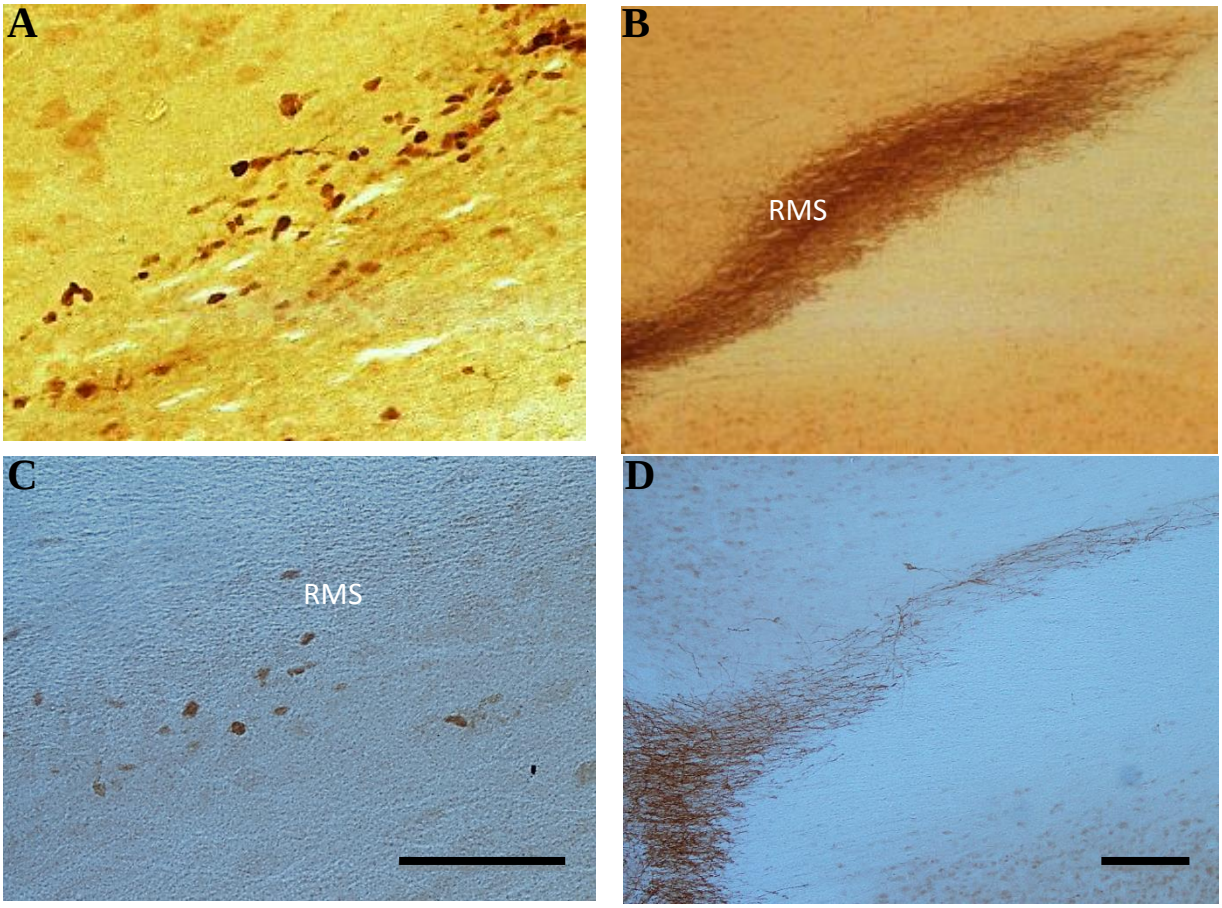


Figure 3.6 Representative photomicrographs showing the RMS of the ground squirrel. A – Subadult brain stained for Ki-67; B – Subadult brain stained for DCX; C and D – Adult brains stained for Ki-67 and DCX respectively. RMS – rostral migratory stream, Scale bar for A and C is 100 µm, scale bar for B and D is 200 µm.

3.3.2 Olfactory bulb

Ki-67 immunopositive proliferating cells as well as DCX immunopositive immature neurons were observed in the olfactory bulbs of all brains studied (figure 3.7 B to D). The subadult squirrel brains presented more proliferating neurons than the adult brains. The majority of Ki-67 immunopositive cells were found in the granule cell layer; however there was a more generalised but less dense distribution in almost all the other layers. The olfactory bulb showed a dense distribution of ovoid DCX immunopositive cells with short processes (figure 3.7 D), abundant in the granule cell layer. Fewer cells with longer processes were observed in the internal plexiform layer with even less dense processes observed in the glomerular layer. The majority of cell processes were oriented towards the olfactory nerve layer.

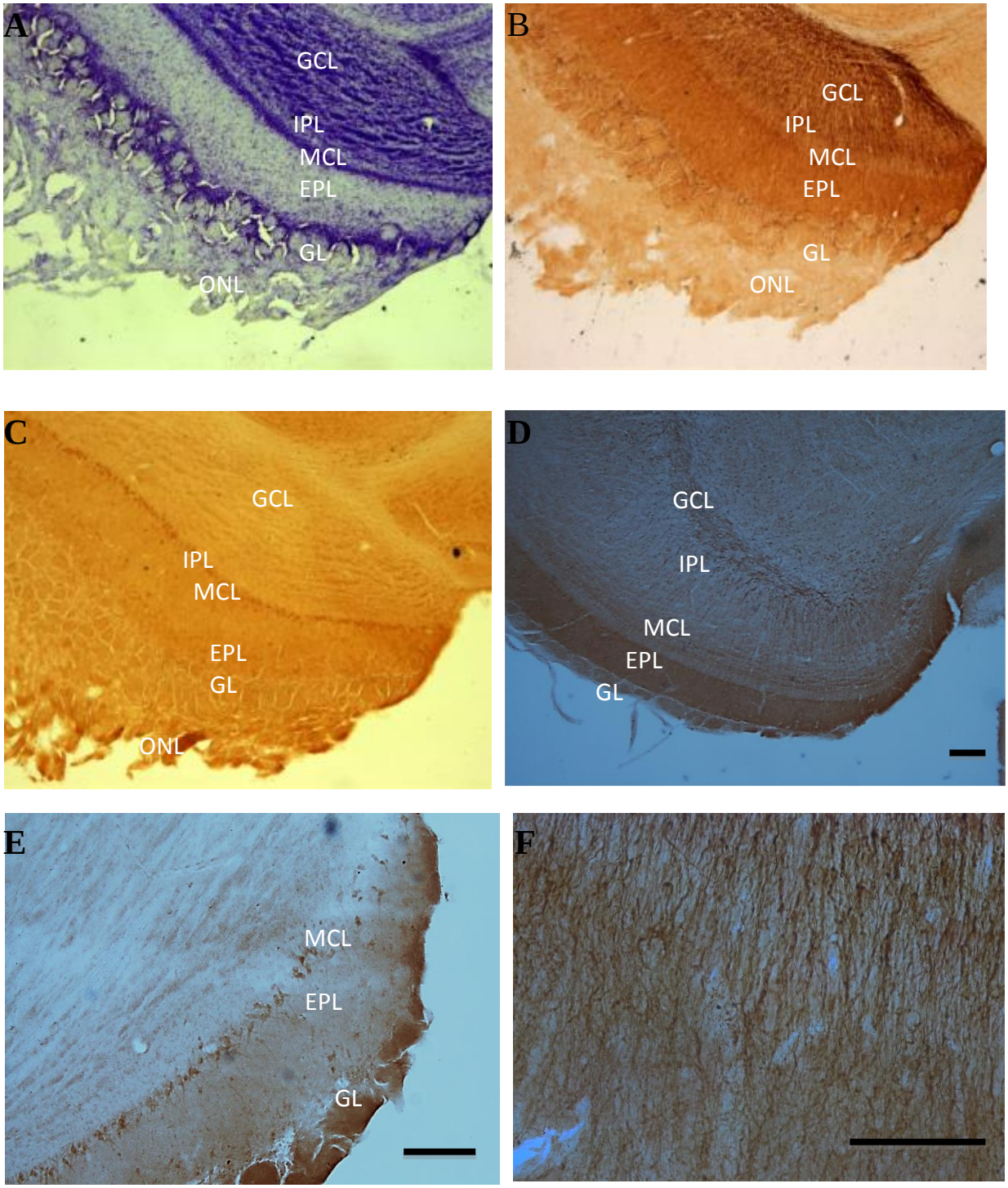


Figure 3.7 Representative photomicrographs of the olfactory bulbs of the South African ground squirrels. A – subadult squirrel, Nissl; B – subadult DCX; C – subadult Ki-67; D – adult DCX and E – Adult Ki-67, F – subadult DCX, with GCL showing small ovoid cell bodies and dendritic processes. EPL - external plexiform layer; GCL-granule cell layer; GL - glomerular layer; IPL- internal plexiform layer; MCL- mitral cell layer; ONL - olfactory nerve layer. Scale for A to E = 200 µm and F = 100 µm

3.3.3 Dentate gyrus of the hippocampus

Proliferating neurons were mainly identified in the subgranular zone (SGZ) and to a lesser extent, the granule cell layers of the dentate gyrus (figure 3.8 A, C). Very few Ki-67 immunopositive neurons were observed in the hilar region and the molecular layer of the dentate gyrus of subadult squirrels, whereas none were observed in the adult brains. More Ki-67 immunopositive cells were evident in the subadult ground squirrel when compared to the adult brains. DCX immunopositive cells were localised only to the SGZ and the GCL (figure 3.8 B, D). The majority of immature neurons in the dentate gyrus were bipolar displaying ovoid cell bodies each with a prominent apical dendritic process (Figure 3.8 D). DCX immunopositive cells were classified according to the presence or absence of the apical dendrite, its length and branching pattern (Table 2.1). Type A had no cell processes whereas type F was the most complex showing extensive branching within the granule cell layer and extending to the molecular layer. Regardless of the age of the animal, all cell types (types A to F) were observed in the DG of all the brains studied. It was also observed that the numbers of more mature cell types D, E and F increased as brain sections were analysed in a medial to lateral direction. Subadult brains evidently had greater numbers of DCX immunopositive cells and denser distribution of DCX immunopositive cell processes than adult brains (figure 3.8 B and D); however this did not seem to affect the relative proportions of cell types (types A to F) within the DG.

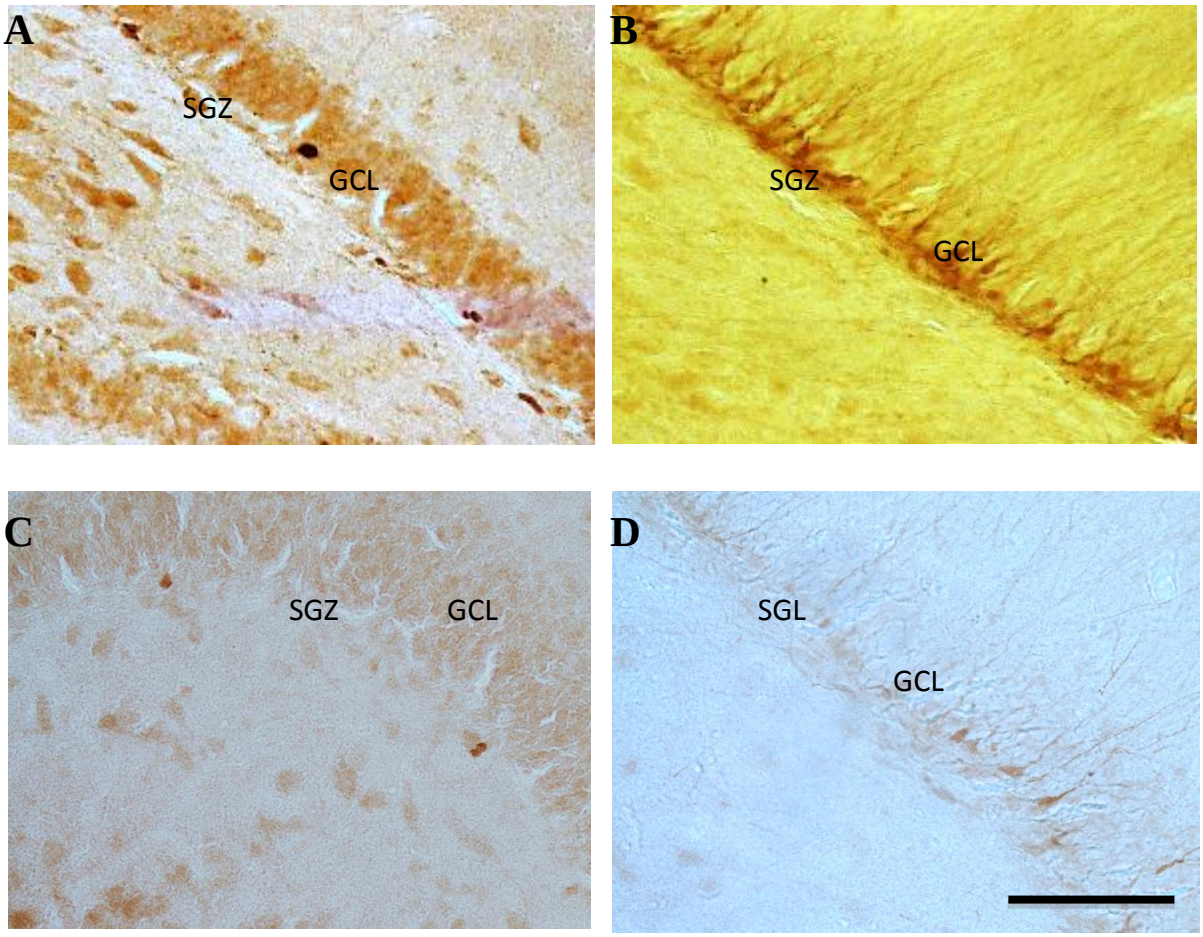


Figure 3.8 Representative photomicrographs of the DG of the hippocampus. A – Subadult brain stained for Ki-67; B – subadult brain stained for DCX; C – adult brain stained for Ki-67 and D - Adult brain stained for DCX. GCL – granule cell layer, SGZ – subgranular zone, Scale bar is 100 µm and applies to all pictures

3.3.4 Neocortex

No Ki-67 immunopositive neurons were observed in the neocortex of both subadult and adult squirrels; however, immature neurons immunostained for the DCX marker were identified and were almost exclusively limited to layer 2 of the neocortex (figure 3.9). Cells appeared to migrate from the RMS into the layers of the neocortex. The observed DCX immunopositive cells were bipolar and multipolar neurons, some of whose processes ran parallel to the cortical surface. The rest pursued perpendicular courses towards the cortical surface into layer 1 of the cerebral cortex or deeper into layer 2. Once again it was apparent that the intensity of staining was greater in subadult squirrels and cell numbers more numerous, than they were in the adult animals (figure 3.9 B and D).

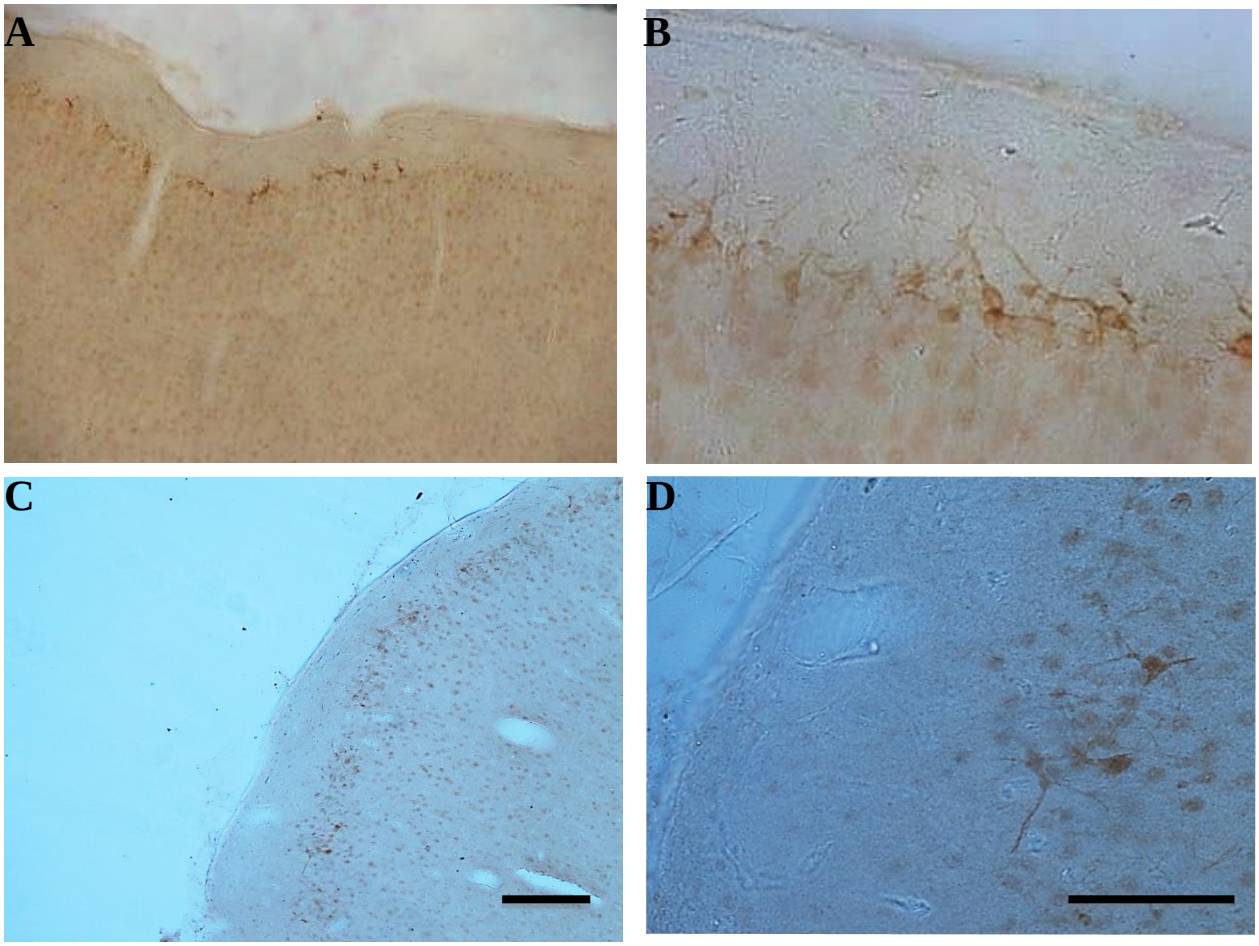


Figure 3.9: Representative photomicrographs of the frontal neocortex of the South African ground squirrel. A and B show subadult squirrel brain stained for DCX and C and D show and adult brain stained for DCX. Scale bar for A and C is 200 µm and C and D = 200 µm

3.3.5 The Occipital extension of the SVZ of the lateral ventricle

A line of proliferating cells, immunopositive for Ki-67 was observed deep in the white matter of the occipital lobe in the subadult brains only (figure 3.10). A similar stream was present in the adult brains as well but was devoid of neurogenic cells (figure 3.10 D). No DCX immunopositive cells were observed in this area in both subadult and adult squirrels.

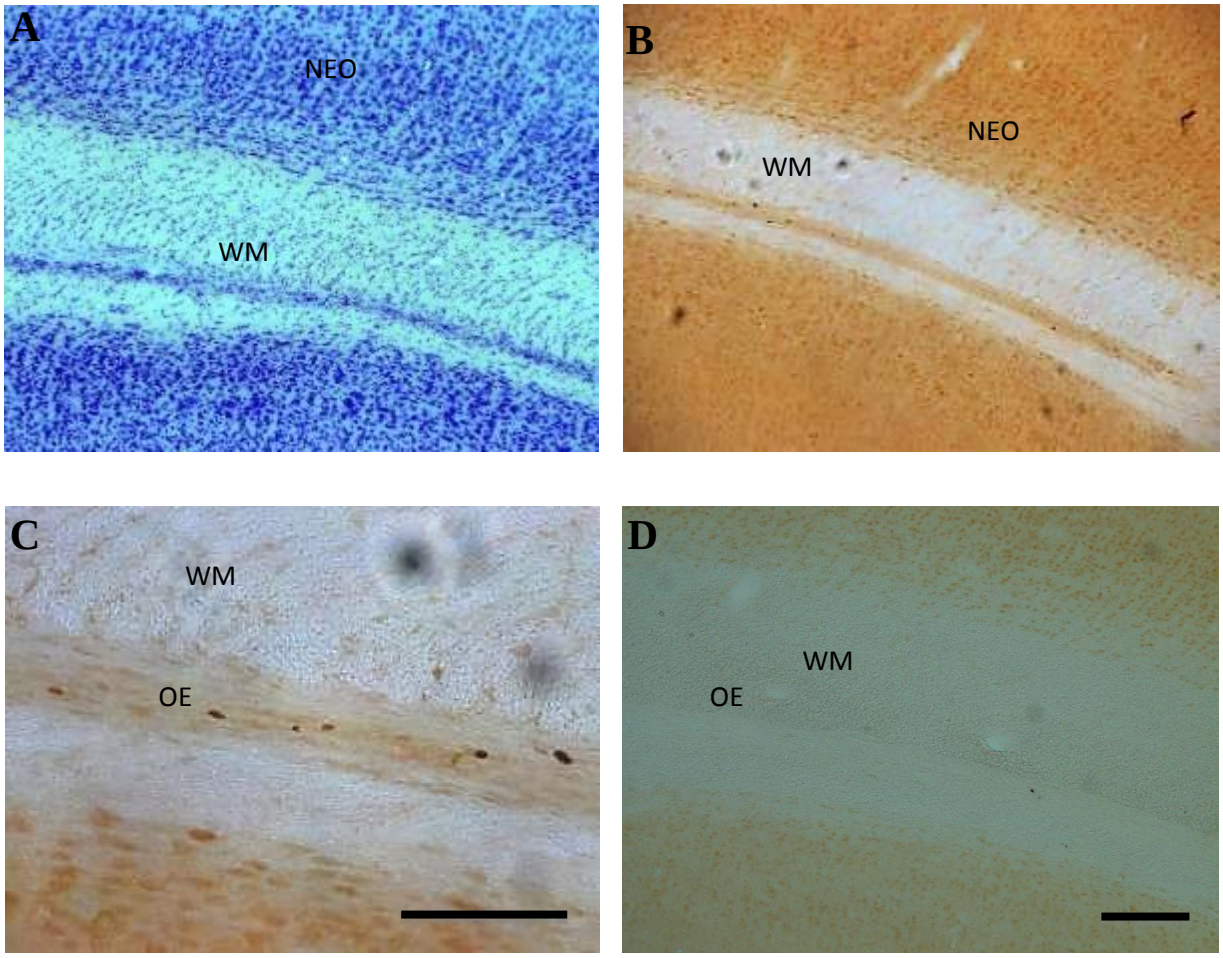


Figure 3.10: Representative micrographs showing the occipital extension of the SVZ into the white matter of the occipital cortex. A – Subadult brain, Nissl stain, B – subadult brain stained for Ki-67 C – subadult brain stained for Ki-67 and D – adult brain stained for Ki-67, LV – lateral ventricle, NEO – neocortex, OE – occipital extension of the SVZ, WM – white matter. Scale bar for A, B and D is 200 µm and For C scale bar = 100 µm

3.3.6 Piriform cortex

Very few Ki-67 immunopositive neurons were observed in this region and all of them were found in the subadult brains. No Ki-67 immunopositive neurons were observed in the piriform cortex of adult squirrels. DCX immunopositive cells were also observed predominantly in the area immediately ventral to the amygdala and extending in the cortex rostral and ventral to this area (figure 3.11). The distribution of DCX cells thinned out away from this area so that immature neurons were once again restricted to layer 2 just as in the rest of the cortex where DCX immunopositive cells were found. DCX staining of the piriform cortex was denser in the subadult brains as compared to the adult brains (figure 3.11 B and D)

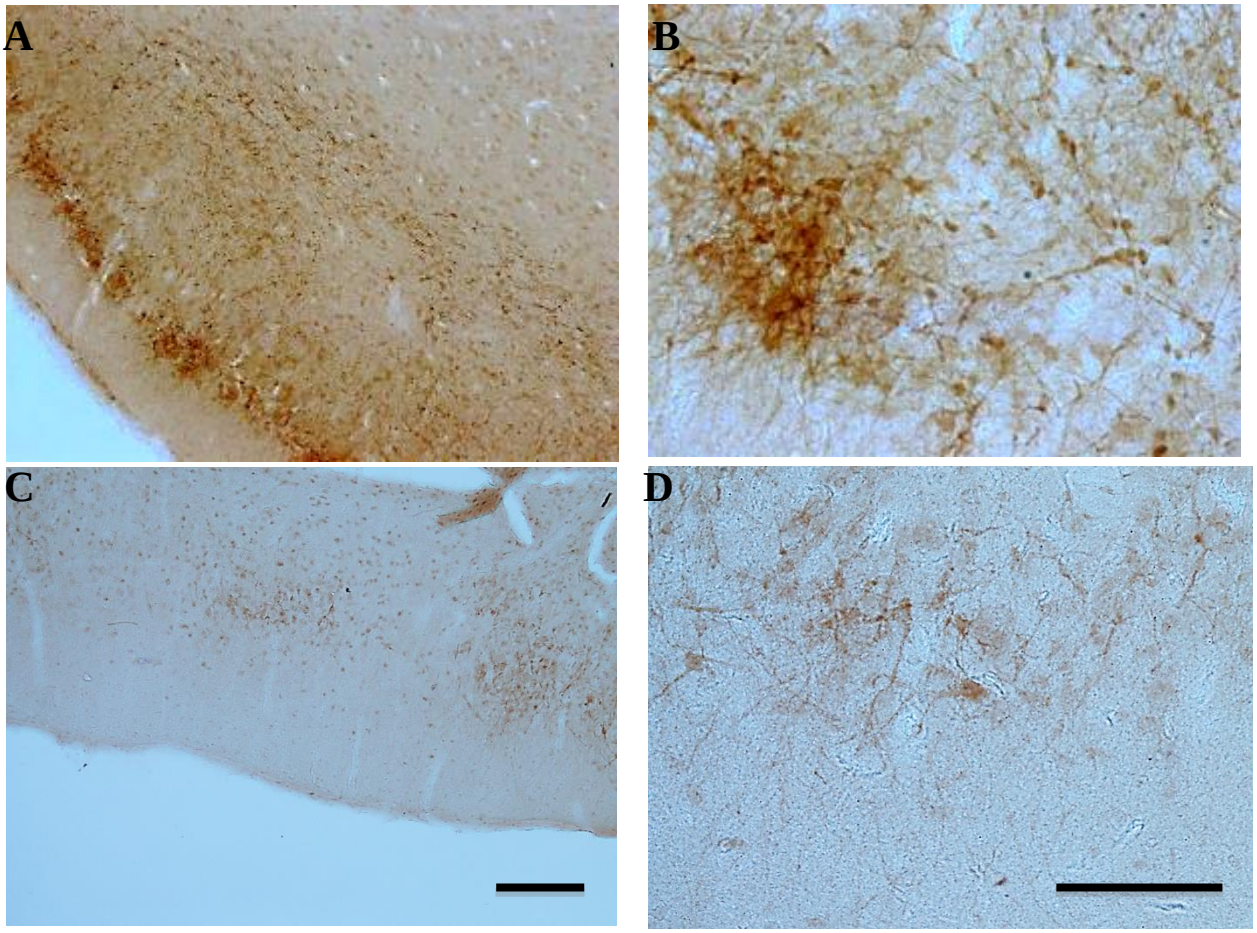


Figure 3.11 Representative micrographs showing the piriform cortex of the South African ground squirrel. A and B – subadult brain stained for DCX and C and D – adult brain immunostained for DCX showing the cell bodies and dendritic processes. Scale bar for A and C = 200 µm and for B and D = 200 µm

3.4 Summary of the identified active and potential neurogenic sites

Table 3.2 and figure 3.12 summarise the active and potential neurogenic sites that were identified in the brains of subadult and adult South African ground squirrels.

Table 3.2: Areas in which Ki-67 and DCX positive cells were identified

				Ki-67			
Region	SVZ	RMS	OB	DG	NEO	PIR	OE
Subadult	+	+	+	++*	+	+	+
Adult	+	+	+	+	+	-	-
				DCX			
Subadult	+	++*	++*	++*	+	+	-
Adult	+	+	+	+	+	+	-

++* shows a higher number of cells than +. DG – dentate gyrus, NEO – neocortex, OB – olfactory bulb, OE – occipital extension of the SVZ, PIR – piriform cortex, RMS – rostral migratory stream, SVZ – subventricular zone

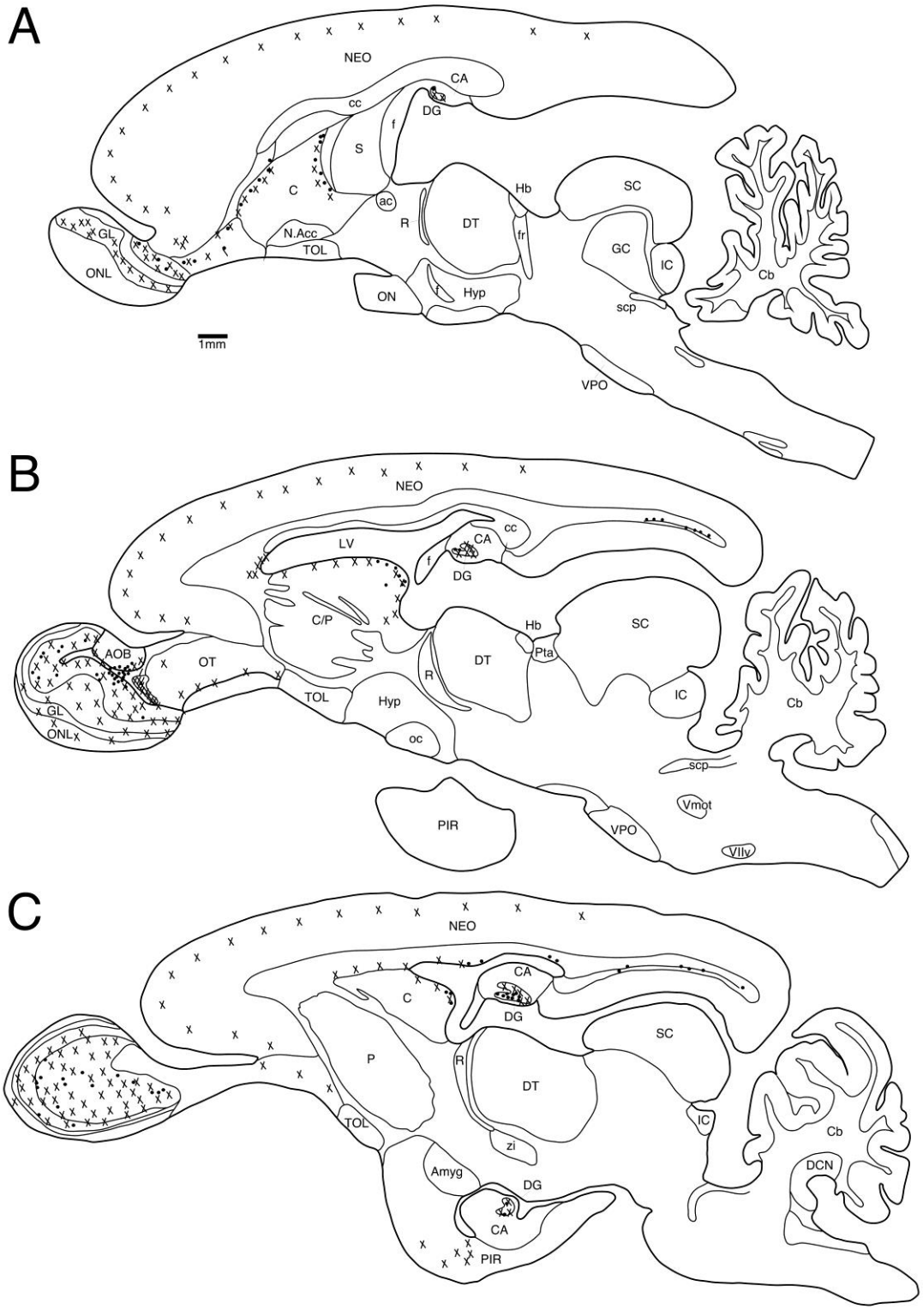


Figure 3.12: Representative stereomicrographs showing all the potential and neurogenic sites in the brain of a subadult South African ground squirrel. The sections are 1 mm apart with A being the most medial and H the most lateral. 'X'- DCX staining and '•'- Ki67

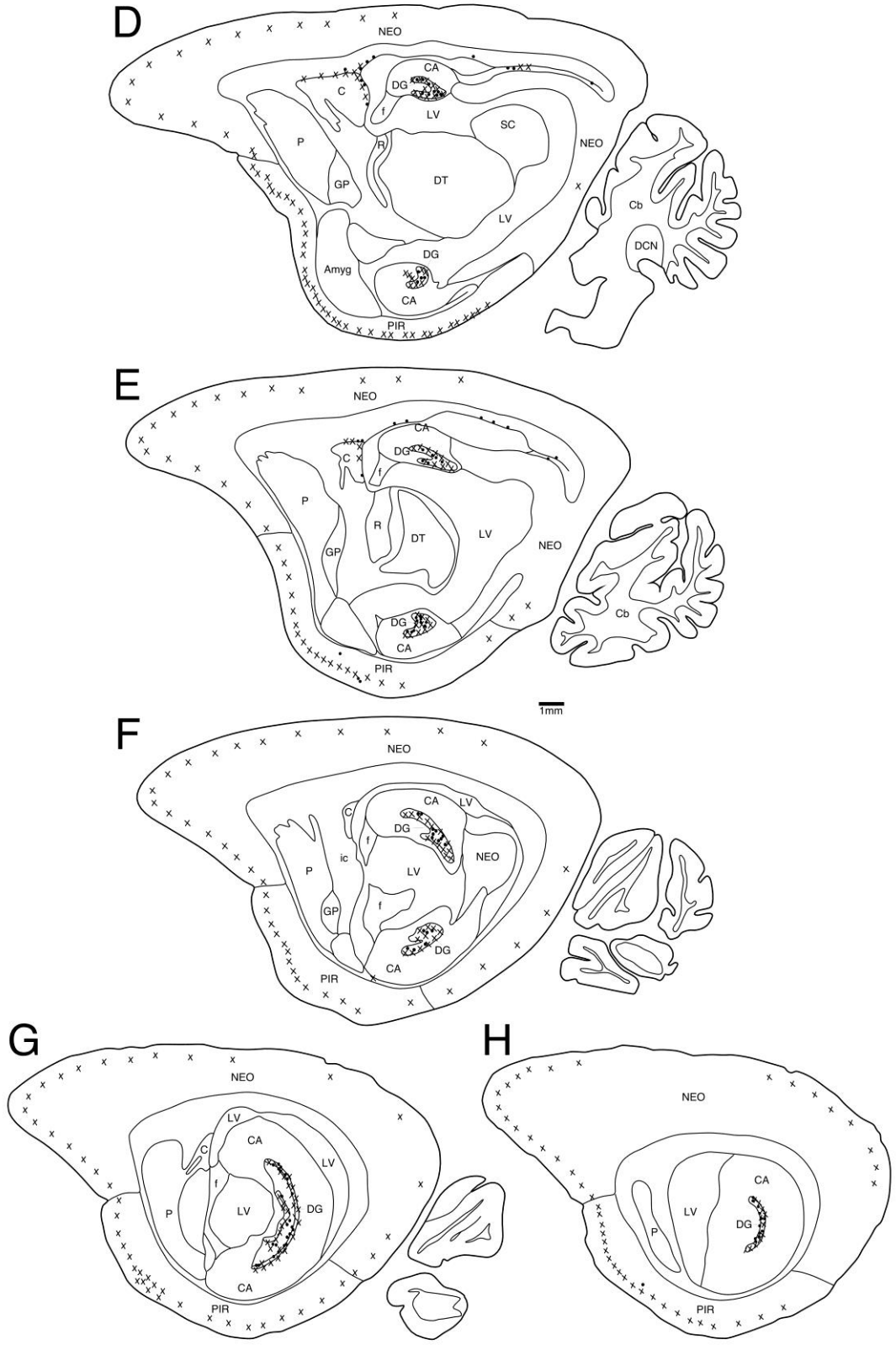


Figure 3.12 contd: Representative stereomicrographs showing all the potential and neurogenic sites in the brain of a subadult South African ground squirrel. The sections are 1 mm apart with A being the most medial and H the most lateral. 'X'- DCX staining and '•'- Ki67

3.4 Estimates of the numbers of Ki-67 positive neurons in the dentate gyrus

Proliferating cell counts in the dentate gyrus were conducted in all the brains used in this study. The numbers of proliferating cells also decreased significantly as the body masses of the squirrels increased (figure 3.13). As the paired dry lens mass of the animals increased, there was an initial sharp decline in the total estimated number of proliferating cell in the dentate gyrus (figure 3.14). As the paired lens mass continued to increase, this decline became less marked. A similar graph was obtained when the estimated proliferating cell count for the brain was plotted against brain mass (figure 3.15). As the brain masses and consequently, the sizes of the brains increased, the number of proliferating cells in the hemisphere of the respective brain decreased. Adult squirrels showed a decrease of 87 % in number of proliferating cells as compared to the subadult squirrels (figure 3.16).

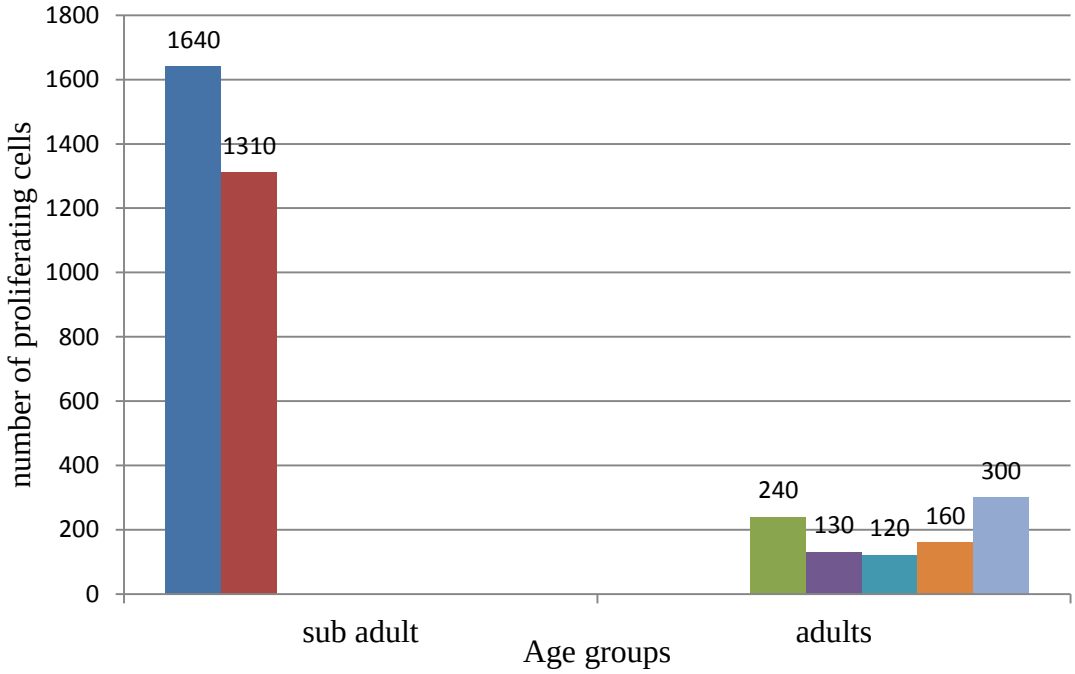


Figure 3.13: Graph showing the proliferating cell numbers in each of the subadult and adult brains studied.

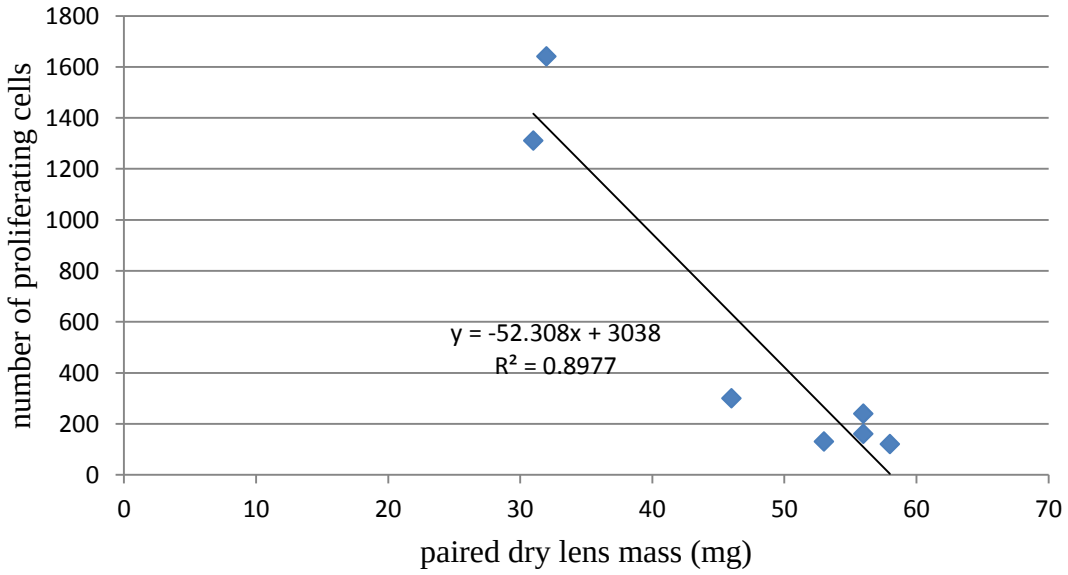


Figure 3.14 The relationship between the paired dry lens mass and the proliferating cell number. (p=0.0162)

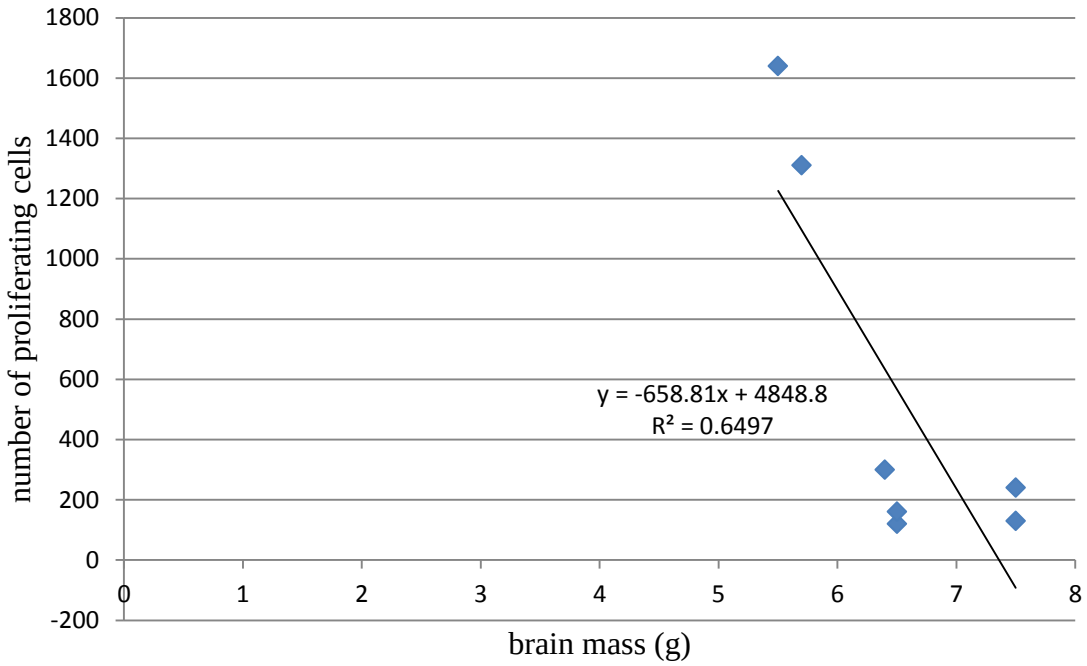


Figure 3.15 The relationship between the brain mass and the proliferating cell number. (p=0.0457)

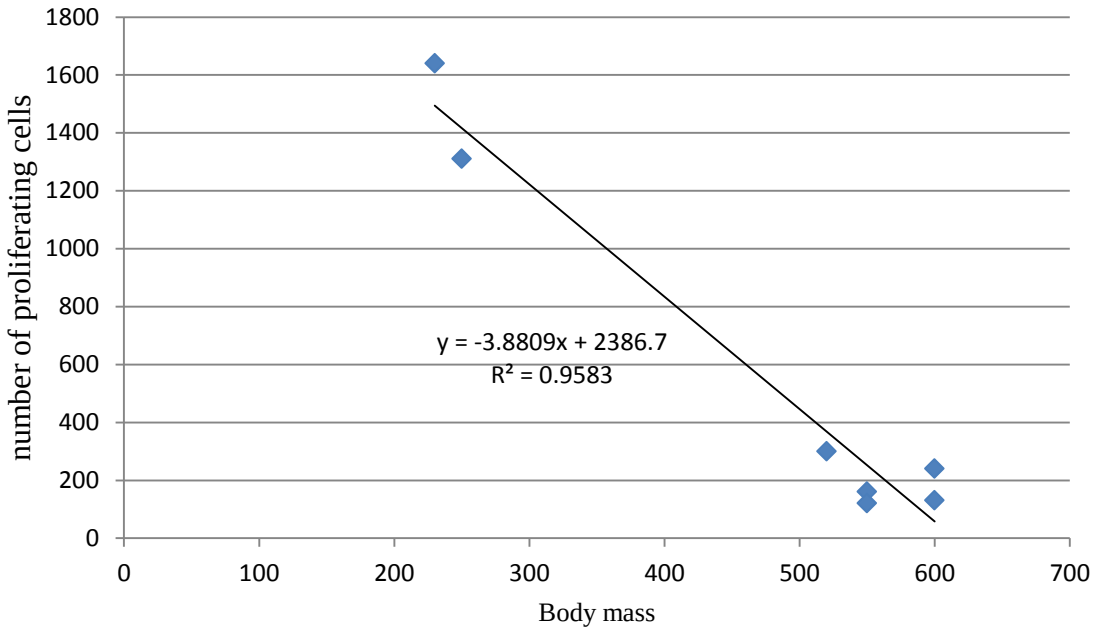


Figure 3.16: Graph showing the body mass plotted against the number of proliferating cells. (p=0.3851).

CHAPTER 4: DISCUSSION

4.1 General considerations

All the seven ground squirrels were female so the influence that sexual differences in circulating sex hormone levels have on the rate of neurogenesis were mitigated. Some species of animals have shown seasonal variations in neurogenic rates; however, trapping all animals within four days ensured that seasonal variation could be ruled out as a factor for this sample. It may, however, be important to note that no significant seasonal variations in the rate of new neuron generation, survival rates or total granule cell numbers were observed in the eastern grey squirrel (Lavenex *et al*, 2000). All South African ground squirrels were trapped from two colonies within a two kilometre radius of each other, ensuring they existed under the same general environmental conditions with presumably the same levels of competition for food and predator stress. The matching of as many variables as possible for animals in this sample ensured that age was most probably the single most important factor accounting for the differences in the levels of neurogenesis observed.

4.2 Relationship between eye lens mass, brain mass and body mass

The proportion of the brain mass to the total body mass decreased from an average of 2.3 % in the subadults to about 1.2 % in the adults; however, the absolute mass of the brain of each squirrel increased with an increase in body mass. The paired lens mass showed continued increase with increased in body mass in the South African ground squirrel, as is reported in the literature for other rodent species such as the eastern grey squirrel (Fisher and Perry, 1970), fox squirrel (Beale, 1962), Norway rats (Donaldson and King, 1937 and Epp *et al*, 2009). According to Lord (1959), "New lens fibres are continuously being proliferated by the growth and elongation of the epithelial cells at the lens equator and the most central ones will

undergo cell death and lose their water content." This therefore means that the dry lens mass of the South African ground squirrel continued to increase as the animals got older, thus making paired dry lens mass a reliable indicator for age in this species.

4.3 Active neurogenic sites in subadult and adult South African ground squirrels

The SVZ of the lateral ventricle and the SGZ of the dentate gyrus of the hippocampus were identified as the active sites of neurogenesis in both the subadult and adult ground squirrels.

4.3.1 The subventricular zone, rostral migratory stream and olfactory bulb

The SVZ of the lateral ventricle formed a stream of proliferating and immature neurons that entered the RMS rostral to the corpus striatum. This concurred with previous reports based on studies in laboratory rodents (Kaplan and Hinds, 1977, Doetsch *et al*, 1997, Peretto *et al*, 1999, Mobley *et al*, 2013). Cells migrating along the RMS formed chains of neuroblasts, in rodents, which chains disintegrate upon entry into the OB. Ki-67 immunopositive neurons were observed in the SVZ and RMS of both subadult and adult squirrels, however, they were more numerous in the subadult squirrels. DCX immunopositive neurons were also present in both age groups but showed a qualitative decrease in density, by means of a decrease in the intensity of the staining of neural processes, with a transition from the subadult to the adult age group. Chains of neuroblasts in the RMS migrate tangentially along the RMS, but within the OB individual neuroblasts migrated radially through the layers of the OB and densely packed DCX immunopositive cells were observed in the granule cell layer and to a lesser extent, the glomerular cell layer. Other than laboratory rodents, similar observations have also been reported in the OB of the African elephant (Ngwenya *et al*, 2011), megabats (Chawana *et al*, 2013), and giant otter shrew (Patzke *et al*, 2013a). Migrating neuroblasts formed granular interneurons that mainly lodged in the deeper layers of the granule cell layer.

Relatively fewer adult generated neuroblasts migrated to the deep regions of the glomerular layer where they mature into periglomerular interneurons which synapse mainly with the secondary processes of the tufted cells (Mobley *et al*, 2013). Ki-67 immunopositive cells were easily identifiable in the layers of the OB of animals in both age groups. It was not immediately clear as to whether they were more numerous in one age group relative to the other; however, DCX immunostaining was evidently more intense in the OB of subadult animals, demonstrating either larger numbers of immature neurons or larger numbers of developing neural processes.

Adult generated neurons may play an important role in olfactory learning since mice required to complete an olfactory learning task showed higher rates of granule cell survival (Whitman and Greer, 2009). They may also function in odour discrimination, as adult mice that lived in an odour enriched environment showed increased rates of survival of new granule interneurons (Rochefort *et al*, 2002). Olfactory cues are obviously important for the survival of the South African ground squirrel, enabling it to navigate its surroundings in search of food and to communicate with and identify other members of the colony. A considerable amount of olfactory learning would be expected from the different scents produced by the various predators and the seasonal change of odour in mates, and this would probably account for the presence of OB neurogenesis in both the subadult and adult South African ground squirrels. The same would explain the presence of adult neurogenesis in the piriform cortex, which is also a part of the olfactory system. The origin of piriform cortical neurons in the South African ground squirrel is discussed below (section 4.4.1).

4.3.2 Subgranular zone of the dentate gyrus

The distribution of the cell bodies of both Ki-67 and DCX immunopositive neurons in this region was similar, with cells appearing on the outer edge of the SGZ or inner edge of the GCL. The discovery of immature neurons in the South African ground squirrel confirms the findings from numerous studies in rodents, especially the study by Rao and Shetty (2004) in which they provided evidence that DCX immunopositive cells in the DG of adult rats were indeed newly generated neurons. DCX immunopositive cells were denser in all areas of the subadult DG as compared to corresponding areas in the DG of adult squirrel brains. Previous studies in red squirrels (Johnson *et al*, 2010), eastern grey squirrels and chipmunks (Barker *et al*, 2005) and wild and captive bred rats (Epp *et al*, 2009) also provide similar results. Amrein *et al*, (2004) state that "neurogenesis increases with novelty rather than continued complexity" meaning environmental enrichment increased survival of new neurons albeit for a limited time period. It is assumed that as older individuals get more accustomed to their environment, the influence of environmental enrichment decreases. This would account for the lower levels of neurogenesis observed in the adult squirrels DG by DCX immunostaining. Encinas *et al*, (2011) propose that the population of quiescent neural progenitors undergoes asymmetric division throughout life, which leads eventually to a depletion of the stem cell population. This would invariably lead to a decrease in the numbers of newly generated cells as the South African ground squirrel gets older.

New neurons from the subgranular zone are believed to migrate a short distance into the neighbouring granule cell layer of the dentate gyrus in which they mature and develop functional connections with other areas of the hippocampus. Cells in the granule cell layer send dendrites that synapse in the molecular layer and their axons project into Ammorn's horn area CA3 (Kempermann *et al*, 2004). The function of newly generated neurons in the

dentate gyrus is not fully understood but is believed to be associated with spatial learning and memory. Increased survival of dentate gyrus neurons has been shown to improve performance in tasks related to spatial learning and memory (Kempermann *et al*, 1998). Reduced neurogenesis also impaired long-term retention of spatial memory as well as object recognition tasks (Jessberger *et al*, 2009). Environmental enrichment increases the survival of DG neurons while physical exercise, such as running, increases their rate of proliferation (Kempermann *et al*, 1998, van Praag *et al*, 1999). Learning is a stimulus for neurogenesis; however it has to be the learning of hippocampal dependent tasks and should occur within a certain 'critical period' to increase new neuron survival (Epp *et al*, 2007). The South African ground squirrel covers a relatively large home area but when under attack, will flee directly to its own burrow even if it has to run past burrows belonging to other colonies (Skinner and Chimimba, 2005). This demonstrates good spatial orientation and spatial memory and this would possibly account for the very high neurogenesis levels observed in the subadults of this species.

4.4 Potential neurogenic sites in subadult and adult South African ground squirrels

The potential neurogenic sites observed in this species were the neocortex, piriform cortex and the white matter in the occipital cortex.

4.4.1 Migration of cells from the SVZ to the neocortex, piriform cortex and occipital extension of the SVZ

Developing neurons migrated from the rostral end of the RMS into the layers of the frontal neocortex. No Ki-67 immunopositive neurons could be observed migrating along this pathway in subadult or adult squirrels. DCX immunopositive neurons were mainly observed in the superficial part of cortical layer 2 where they appeared as aggregates. There were more

DCX immunopositive cells in the neocortex of subadult squirrels than there were in adult squirrels. In the subadults, aggregations of DCX cells tended to extend as far back as the occipital cortex whereas they only went as far as the frontal cortex in adult squirrels. Such observations were also made in the cortex of rats (Altman and Das, 1966), but no comparisons were made between subadult and adult species. Neocortical neurogenesis has also been reported in rabbits (Bonfanti and Ponti, 2006), megabats (Chawana *et al*, 2013) as well as primates (Gould *et al*, 1999). Neurogenesis was apparent in the layers of the piriform cortex, with very few Ki-67 immunopositive cells observed only in the subadult squirrels. No Ki-67 immunopositive cells could be identified in the piriform cortex of adult brains; however, DCX immunopositive cells were observed in both subadult and adult squirrels, with the subadult squirrels showing a denser distribution of DCX immunostaining. The majority of DCX immunopositive neuroblasts formed aggregates in layer 2 of the cortex in both subadult and adult squirrels. Shapiro *et al*, (2007) conducted studies using both rats and mice to determine the origin, route of migration and fate of adult generated neurons in the piriform cortex. Piriform cortical neurons originate from the SVZ of the lateral ventricles and migrate by two streams, a ventrocaudal stream, prominent during development and mainly populating the rostral piriform cortex and a caudoventral stream, prominent in adulthood and populating the caudal piriform cortex (Shapiro *et al*, 2007). Both subadult and adult ground squirrels had greater distributions of DCX immunopositive cells in the caudal piriform cortex suggesting a more prominent caudoventral stream. The caudoventral stream has been termed the temporal stream (TS) and has previously been documented in primates where it is believed to contribute neuroblasts that help ensure the plasticity of the olfactory system (Bernier *et al*, 2002 and Gould *et al*, 1999).

The SVZ of the lateral ventricles in this species also had a prolonged occipital extension in which Ki-67 immunopositive neurons were found only in subadult squirrels (figure 3.9).

There was no DCX immunostaining of this region even in the subadult brains. No other reports of such findings have been reported to date and it remains to be determined what the fate of these neurons might be. The absence of both Ki-67 and DCX immunostaining in this region in all the adult brains studied may suggest that progenitor cells in this region eventually give rise to glial cells, die off or alternatively become dormant in adulthood.

Although neurogenesis has been reported in the substantia nigra of adult mice (Zhao *et al*, 2003) and other brainstem regions such as the dorsal vagal complex (Moyse *et al*, 2006) in rodents, none of these and other potential sites were identified in the South African ground squirrel. In any case some of these potential sites are still under debate and the role that new neurons could play in these regions remains speculative.

4.5 Proliferating cell numbers and body mass

The numbers of Ki-67 immunopositive neurons showed a definite decrease as the body masses of the squirrels increased. An increase in body mass was taken to mean an increase in the age of the squirrel under study. The subadult squirrels had significantly higher numbers as compared to all the adult squirrels. There was a negative correlation between Ki-67 immunopositive cell numbers and both brain mass and eye lens mass. This was in agreement with what has been reported in the literature by various researchers where the numbers of proliferating neurons decreased with age (Amrein *et al*, 2004; Barker *et al*, 2005 and Epp *et al*, 2009). There was a calculated 87 % decrease in the numbers of proliferating cells between the subadult and adult South African ground squirrels. This was comparable to the 80 % reduction obtained in red squirrels (Johnson *et al*, 2010) and the 80 % obtained in laboratory rats (Kuhn *et al*, 1996) but significantly higher than the 50 % reduction obtained in the eastern grey squirrel. Amrein *et al*, (2011) concluded that the age of the animal could be the single most important factor regulating proliferation of neurons in the hippocampus and that

all other factors may primarily be involved in influencing differentiation and survival of the generated cells.

CHAPTER 5: CONCLUSIONS AND FURTHER STUDIES

5.1 Conclusions

There exists a statistically significant correlation between body mass and eye lens mass and both methods can be reliably used to determine age in the South African ground squirrel. The brains of both subadult and adult ground squirrels showed two active neurogenic sites, the SVZ of the lateral ventricles and the DG of the hippocampus, shown using Ki-67 and DCX immunolocalisation. The neocortex and piriform cortex were identified as potential neurogenic sites but showed decreasing levels of neurogenesis with the transition from the subadult to the adult age groups. The white matter of the occipital cortex exhibited Ki-67 immunopositive cells, but can only be considered a potential neurogenic site in subadult squirrels. The rate of cell proliferation in the DG of the hippocampus decreased significantly from the subadult to the adult age groups.

5.2 Research limitations

- Limitations in terms of time and financial resources to obtain a more evenly spread age distribution across the lifespan of the ground squirrel
- Inability to rule out some factors affecting neurogenesis such as capture stress since it was not possible to euthanise the animals in the field

5.3 Recommendations for further studies

Further studies that could be considered may focus on:

1) Determination of hippocampal volume and its changes with age
2) Determination of pyknotic cell numbers in the dentate gyrus and changes with age

3) Correlation between hippocampal volume and the numbers of proliferating and pyknotic cells
4) Estimation of the total granule cell number in each of the brains and to determine numbers of proliferating and pyknotic cells as fractions of the total
5) The use of squirrels from distinct populations to counteract the localised environmental effects and to allow for generalizability of findings to the entire species

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APPENDIX A: ETHICS CLEARANCE LETTER

AESC3

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/30/01

APPLICANT: **Mr S Chengetanai**

DEPARTMENT: **Anatomical Sciences**

PROJECT TITLE: **Neurogenesis in the brains of sub adult and adult South African ground Squirrels (Xerus inauris)**

Number and Species

Approved: 12 Ground Squirrels (*Xerus inauris*)

Approval was given for the use of animals for the project described above at an AESC meeting held on **31 July 2012**. This approval remains valid until **31 July 2014**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Conditions:

- Sample size is limited to 6 animals per group. If it transpires that this sample size is too small, further animals can be requested with an M&E.
- Details of traps and transport cages are provided (make, dimensions and mechanism).

Signed: _____ (Chairperson, AESC) Date: 13/8/12

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 13/8/12

cc: Supervisor:
Director: CAS

APPENDIX B: NORTHWEST PROVINCE AUTHORITY

The DEDECT

Department:
Economic Development, Environment, Conservation and Tourism
North West Provincial Government
Republic of South Africa

AgriCentre Building
Cnr. Dr. James Moroka and Stadium Rd
Private Bag X2039,
Mmabatho. 2735

Tel: (018) 389 5093
Fax: (018) 389 5640

ENVIRONMENTAL SERVICES
BIODIVERSITY MANAGEMENT AND CONSERVATION

PERMIT 058 NW- 12

ISSUED IN TERMS OF THE PROVISIONS OF THE TRANSVAAL NATURE CONSERVATION ORDINANCE 12/83, CAPE NATURE CONSERVATION ORDINANCE 19/74 AND THE BOPHUTHATSWANA NATURE CONSERVATION ACT 3 OF 1973.
In terms of and subject to the provisions of the Ordinances and Law and the regulations framed there under, the permit holder is hereby authorized subject to the conditions and requirements appearing on this permit to carry out the activity with the wild animals or plants as mentioned in the permit during the period of validity of this permit mentioned in the permit during the period of validity of this permit

VALIDITY: FROM 10.10.2012 TO 09.01.2013

1	Description of permitted activity	**CAPTURE AND EXPORT OF GROUND SQUIRRELS IN NORTH WEST PROVINCE FOR RESEARCH PURPOSE**
	Particulars of person; ID, Home address & telephone	Prof. Amadi O. Ihuhwo ID: 631128 5235 185 Lichtenburg Vakansie Oord (Lichtenburg Holiday Resort) PO Box 2929, Lichtenburg, 2740 Tel/Fax: 018 632 5826
2	Species	12x Ground squirrels (*Xerus inauris*)
3	Additional information	• A written permission must be obtained from the land owner/s. • This permit is subject to a valid import permit from Gauteng Province. • This permit authorises capture and export of species mentioned above to University of Witswatersrand Medical School, Parktown, Gauteng Province. • A report must be submitted to NW DACERD BSS Unit, D Buijs (dbuijs@nwpg.gov.za) after completion of the study (Mr Buijs must be informed about sampling trips (083 320 2727)).

CONDITIONS AND REQUIREMENTS.
THE PERMIT IS NOT TRANSFERABLE. ONLY A PERSON AUTHORIZED THERETO MAY MAKE AN ALTERATION ON THIS PERMIT. THIS PERMIT SHALL BE SUBJECT TO THE PROVISIONS OF ANY LAW IN FORCE DURING THE PERIOD OF VALIDITY OF THE PERMIT. THE HOLDER OF THE PERMIT WHO CONTRAVENES OR FAILS TO COMPLY WITH ANY ONE OF THE CONDITIONS OR REQUIREMENTS, TO WHICH THIS PERMIT IS SUBJECT, SHALL BE GUILTY OF AN OFFENCE. THE HOLDER OF THE PERMIT SHALL HAVE THE PERMIT WITH HIM/HER WHEN THE ACTIVITY IS CARRIED OUT

Drafted by
Permit Officer
DEDECT: North West Province

Nature Conservation Permit Office
NORTH WEST PROVINCE
DATE
2012 -10- 10
Private Bag X126, Mmabatho 2735
Tel. +27(0)18 3895040 Fax: +27(0)18 3895640

Authorized by
Senior Manager / Manager Permitting
DEDECT: North West Province

Nature Conservation Permit Office
NORTH WEST PROVINCE
2012 -10- 10
Private Bag X126, Mmabatho 2735
Tel. +27(0)18 3895040 Fax: +27(0)18 3895640

APPENDIX C: GAUTENG PROVINCE AUTHORITY

CPB6 003894

PREMIER OF THE PROVINCE OF GAUTENG

DIRECTORATE: NATURE CONSERVATION

PERMIT TO IMPORT INTO THE PROVINCE A LIVE WILD ANIMAL

(Issued in terms of the provisions of the Nature Conservation Ordinance 12 of 1983)

PARTICULARS OF THE PERMIT

Name and residential or business address of holder of permit: DATE STAMP

AMAKA OSONDA IUNNO
SCHOOL OF ANATOMICAL SCIENCES-WITS MEDICAL SCHOOL-7 YORK STREET PARK TOWN JNB

DETAILS OF PERMIT

FROM	DETAILS OF IMPORT	DESTINATION	ACTUALLY RECEIVED
LICHTENBURG. NORTH WEST PROVINCE	20 (TWENTY) GROUND SQUIRRELS (XERUS INAURIS) CONDITIONS. SUBJECT TO A VALID EX PORT PERMIT FROM NORTH WEST SEE ATTACHED CONDITIONS.		SOUTH AFRICAN

Date and signature of permit holder to be appended here .....

In terms of and subject to the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983) and the regulations framed thereunder, the above-mentioned person is hereby authorized, subject to the conditions appearing on this permit, to import into the Province the live wild animal/s, referred to above from the place referred to above during the period of validity of this permit and to convey it by the shortest route to the destination referred to above.

Period of validity of permit: From the date of issue to 04 / 12 / 2012.

FOR PREMIER

Signature of holder of permit

(See conditions on reverse side)

V&R Printing Works (012) 328 7180 (11930)