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ADULT NEUROGENESIS IN THE FOUR-STRIPED MOUSE *(RHABDOMYS PUMILIO)* AND COMMON MOLE RAT *(CRYPTOMYS HOTTENTOTUS)*

By:

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A dissertation submitted to Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science.

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Johannesburg, 2010

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DECLARATION

I declared that this dissertation is my own unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Signature of candidate:

Date:

ABSTRACT

Adult neurogenesis was investigated in captive bred four-striped mouse *(Rhabdomys pumilio)* and wild caught common mole-rat *(Cryptomys hottentotus).*

Eight individuals per species were used in the study. The animals were anaesthetized and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed and post fixed in the same fixative overnight. Following equilibration in 30% sucrose in PB, 50 μm frozen sections were cut in the saggital planes followed by BrdU immunohistochemistry. Ki-67 and doublecortin (DCX) staining was undertaken as an additional confirmatory staining for cell proliferation.

BrdU, Ki-67 and DCX immunostainings confirmed adult neurogenesis in the subventricular zone (SVZ) of the lateral ventricle and dentate gyrus (DG) of the hippocampus of the four-striped mouse and common mole rat. The existence of adult neurogenesis was observed in potential sites namely, the striatum in the four-striped mouse and substantia nigra in both species. Immature neurons were observed in the cerebral cortex only in the common mole rat.

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DEDICATION

TO THE GENTLE VOICE THAT ASSURES ME.

THE THICK CLOUD THAT SHIELDS ME

MY DAYSPRING AND COMPANION IN LONLELY NIGHTS

MY WISDOM, STRENGTH AND STAFF

BLESSED JESUS,

UNTO YOU I SUBMIT THIS CROWN

AND IN LOVING MEMORY OF MY LATE FATHER, SAMUEL ABIODUN OLALEYE

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2.9. Abbreviations

ABC- avidin biotin complex

AMG- amygdala

BrdU- bromodeoxyuridine

BSA- bovine serum albumin

DAB- 3, 3'-diaminobenzidine tetrahyrochloride

DCX- doublecortin

DG- dentate gyrus of hippocampus

H_2O_2- hydrogen peroxide

NGS- normal goat serum

NRbS- normal rabbit serum

OB- olfactory bulb

PB- phosphate buffer

PBS- phosphate buffer saline

RMS- rostral migratory stream

SC- spinal cord

SN- substantia nigra

STR- striatum

SVZ- subventricular zone

TB- tris buffer

TBS- tris buffer saline

TBST- tris buffer saline triton-X

CHAPTER 1: INTRODUCTION

1.1 Introduction

One of the great achievements of the past three decades is the discovery that the mammalian brain has the capacity to generate new neurons throughout its lifespan (See review in Ihunwo and Pillay, 2007). Neurogenesis has been defined as the ability of brain cells to regenerate themselves (Gage, 2000). Brain cells that possess regenerative abilities are called neural stem cells (Okano, *et al.*, 2002). The concept of adult neurogenesis began as early as 1912 when scientists discovered that mitotically active cells reside in the mammalian central nervous system throughout life (Watts *et al*., 2005; Rakic, 2002). In the 1930s and 1940s, cytological investigations revealed the presence of these cells in the postnatal and adult rodent brain (Alvarez-Buylla and Garcia-Verdugo, 2002; Lennington, *et al.*, 2003). In 1965, Joseph Altman, in a similar study reported that adult neurogenesis occurs in discrete areas like the wall of the subventricular zone and dentate gyrus of the hippocampus of the adult brain in rodents (Watts *et al*., 2005). With the advent of new methods for labelling and identifying dividing cells such as Bromodeoxyuridine (BrdU) labelling, retroviral labelling and confocal microscopy, studies have confirmed the findings of Joseph Altman (1965). Adult neural stem cells are self renewing, multipotent cells that normally generate the main phenotypic cells of the nervous system namely, neurons, astrocytes and oligodendrocytes (Taupin, 2006a). However, neurons behave as non renewable epithelia unlike most somatic cells that are continuously renewed or can be regenerated (Peterson & Leblond, 1964). Adult neurogenesis has been investigated in several mammalian brains from rodents to human. Details of these

are presented under the literature review section. Adult neurogenesis is regarded as an exciting area in the field of neuroscience despite the controversies in the evidences in adult neurogenesis occurring in human and experimental animals. This research area however, is believed to provide clues to the treatment of various neurological disorders (Rackic, 2002; Kempermann & Kronenberg, 2003; Duman, 2004; Feldmann *et al.*, 2007; Vollmayr *et al.*, 2007). It is a dynamic process that can be modulated by various exogenous stimuli (Siwak- Tapp *et al.*, 2007). So the need for the study of adult neurogenesis in the captive bred four-striped mouse and wild caught common mole-rat is important to have a broad understanding of adult cell proliferation not only in laboratory rodent but in wild rodents as well which are readily available. And also, exposure to more stimuli results in different/ more pronounced neurogenesis in captive-bred and wild caught rodents compared to laboratory rodents.

1.2. Objective of the study

The main objective of the study is to provide and describe qualitative evidence of adult neurogenesis in the four-striped mouse (*Rhabdomys pumilio*) and common mole-rat (*Cryptomys hottentotus*). The specific objectives of this study are:

- To establish whether adult neurogenesis occurs in the active neurogenic sites; subventricular zone and dentate gyrus in the four-striped mouse and common mole-rat.
- To establish whether adult neurogenesis occurs in other reported potential sites such as the striatum, substantial nigra, ependymal wall of the third

ventricle, amygdala, cerebral cortex and olfactory bulb as this is not a common occurrence in all species.

- Describe the morphology of the proliferating cells as revealed by bromodeoxyuridine (BrdU), Ki-67 and doublecortin (DCX) immunohistochemistry at the different locations.

1.3. Literature review

Adult neurogenesis is a complex process involving the proliferation, survival, differentiation and functional integration of new cells into the hippocampus (Warne-Schmidt and Duman, 2006). Neurogenesis has been reported in the mammalian brain of rats, mice, tree shrews, guinea pigs, rabbits, cats, monkeys and humans (Khun *et al.,* 1996; Kempermann *et al.,* 1997; Gould *et al.,* 1997; Altman & Das, 1967; Gueneau *et al.,* 1982; Wyss & Sripanidkulchai, 1985; Gould *et al.,* 1998; Gould *et al.,* 1999; Rakic & Nowakowski, 1981; Eriksson *et al.,* 1998). It is low or absent in bats due to spatial behaviour (Amrein *et al.,* 2004). Though, cellular and molecular mechanisms that regulate adult neurogenesis remain unclear (Schauwecker, 2006), the process can be modulated by a variety of factors including glutamate receptor activation (Cameron *et al.,* 1995, 1998; Gould *et al.,* 1997; Bernabeu and Sharp, 2000), dietary restriction (Lee *et al.,* 2002a,b), growth factors (Scharfman *et al.,* 2005; Palmer *et al.,* 1995), stress (Brunson *et al.,* 2005; Nichols *et al.,* 2005) and neuronal injury (Parent, 2003; Cooper-Kuhn *et al.,* 2004). Enriched environments (Kempermann *et al.,* 1997), running wheel exercise (van Praag *et al.,* 1999), hippocampal-dependent learning (Gould *et al.,* 1999), and dietary restriction (Lee, Seroogy, & Mattson,

2002), all increase neurogenesis in the adult hippocampus, while stress (Gould *et al.,* 1997, 1998) and social isolation (Lu *et al.,* 2003, Lievajova *et al.,* 2010) reduces neurogenesis. Even though estradiol alters; and neurogenesis does not. A decrease in hippocampal neurogenesis does not always correlate with the development of learned helplessness in male rats (Pawluski & Galea, 2006; Vollmayr *et al.,* 2003).

Likewise adult neurogenesis also occurs in all non rodents mammals studied to date (Taupin, 2006a). The concept of adult neurogenesis is now widely accepted in the scientific community (e.g. Kaplan and Bell, 1984; Kaplan and Hinds, 1997; Alvarez-Buylla, *et al*, 2002; Zhao *et al.,* 2003; Lennington *et al*, 2003; Watts *et al.,* 2005; Takemura, 2005; Luzzati *et al.,* 2006) and Table 1 below shows a list of some strains of rats and mice investigated to date.

TABLE 2: List of some strains of rats and mice investigated to date.

Rodent/ Strains	**Site**	**Reference**
Rats		
Sprague- Dawley	III VEN, SN	Xu *et al.*, 2005; Frielingdorf *et al.*, 2004.
Wistar	DG, CTX	Seaberg and Kooy, 2002; Takemura, 2005
Fisher 344	SEP/STR	Palmer *et al.*, 1995.
Pine vole	SGZ, DG	Amrein *et al.*, 2004
Transgenic Tg2576	SGZ, DG	Ihunwo & Schliebs, 2010
Mice		
CD1	DG, SC	Seaberg & Kooy, 2002; Weiss *et al*, 1996; Kemperman *et al.*, 1997.
C57BL/ 6	SN, DG, SGZ	Zhao *et al*, 2003; Schauwecker, 2006; Kemperman *et al.*, 1997.
A/J	SGZ	Kempermann and Gage, 2002
BALB/ c	SGZ	Kemperman *et al.*, 1997
C3H/ H3J	SGZ	Kemperman *et al.*, 1997
DBA/ 2J	SGZ	Kemperman *et al.*, 1997
129/ SVJ	DG	Kemperman *et al.*, 1997
Albino	OB	Gritti *et al*, 2002.
FVB/NJ	DG	Schauwecker, 2006
Wood mouse	DG	Hauser *et al.*, 2009

Key: OB, olfactory bulb; DG, Dentate gyrus; SN, Substantia nigra; SC, Spinal cord; SEP\STR, Septum and Striatum; CTX, Cerebral Cortex; III VEN, Third ventricle; SGZ, Subgranular zone.

1.3.1. Active neurogenic sites in the brain

Adult neurogenesis occurs predominantly in two active sites of the brain, the rostral subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus of the hippocampus (Kaplan and Bell, 1984; Kaplan and Hinds, 1997; Ihunwo and Pillay, 2007; Figure 1).

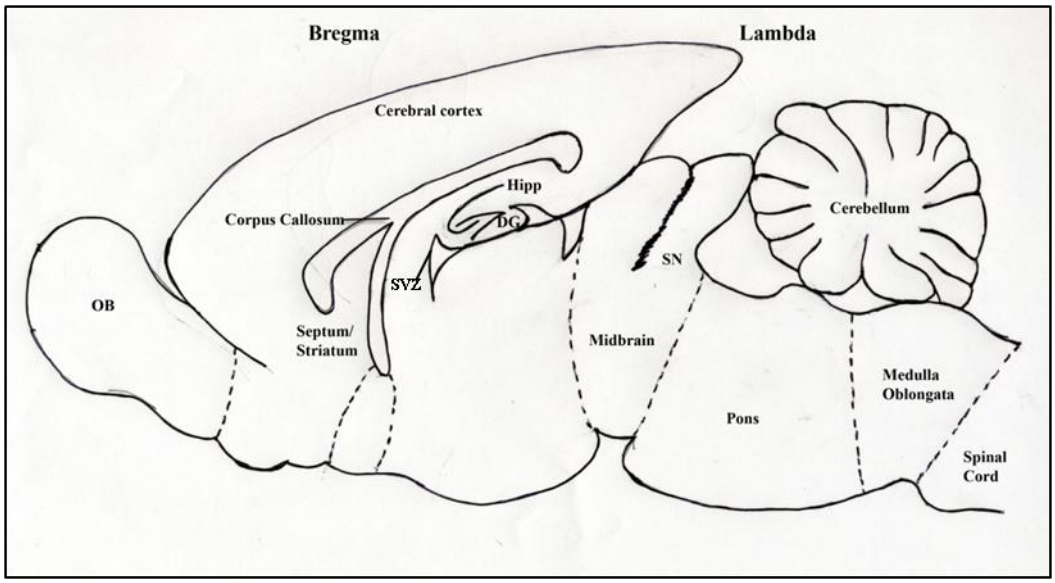


Figure 1: A schematic diagram of the saggital section of the rat brain showing the different regions of the brain. OB-olfactory bulb, DG- dentate gyrus, Hipp-hippocampus, SVZ-subventricular zone, SN-substantia nigra. Drawing modified from Paxinos and Watsons, (2006) 5th ed.

The subventricular zone has two precise layers of cells: the first is a monolayer of multiciliated cells lining the lateral ventricle called the ependymal layer; and the second layer is a 2-3 cell layer of thick area adjacent to the ependymal layer called the subependymal layer. The subventricular zone neuroblast cells travel a long distance to the olfactory bulb through a network of interconnecting pathways that become confluent at the rostral margin of the lateral ventricular wall to form the rostral migratory stream (RMS) (Watts *et al*., 2005).

The olfactory bulb is continuously being supplied with newly generated neurons from the subventricular zone (Hind, 1968; Altman, 1969; Bayer, 1983; Corotto *et al.*, 1993). Cells in the subventricular zone and rostral migratory stream move rapidly by chain migration (Doetsch *et al.,* 1997; Jankovski *et al.*, 1996; Lois and Alvarez-Buylla, 1993; Kirschenbaum *et al.*, 1994). Studies have shown that SVZ has been a source for cortical and subcortical neurons (Watts *et al.*, 2005; Alvarez-Buylla and Gracia-Verdugo, 2002). The subventricular zone is considered the largest active neurogenic site in the brain (Schauwecker, 2006). There is persistence of neurogenic activities in the DG of the hippocampus which generates neural precursor cells that exhibit stem cell properties (Eriksson *et al.*, 1998; Taupin, 2006a; Ihunwo and Pillay, 2007). The dentate gyrus is characterized by sparse and powerful unidirectional projections to CA3 pyramidal cells; the mossy fibre cells (Treves *et al.*, 2008). In the rodent dentate gyrus as many as 9000 neuronal cells are produced every day, contributing 0.01% of the granule cell population per day. Most of these cells undergo apoptosis with only a restricted number of cells that go on to mature. The matured cells can survive for an extended amount of time, and this may lead to a permanent replacement of cells (Taupin, 2006b). The dentate gyrus in the mammalian lineage is a strikingly well conserved part of the cortex with a trilaminar structure (Stephan, 1975). The outermost layer called the molecular layer is relatively cell free which comprises the dendrites of the dentate principal cells (Treves *et al.*, 2008). Also, it contains axons that originate in a limited number of sources. Hippocampal neurogenesis occurs over the lifetime of a mammal and it appears to maintain normal hippocampal function of brain tissues (Eriksson *et al.*, 1998). The four-striped

mouse and common mole-rat were selected for this study because of their availability.

1.3.2. Other neurogenic sites with neurogenic potential

Adult neurogenesis does not only occur in the subventricular zone and the dentate gyrus but also in other areas of the brain (Reynolds and Weiss, 1992; Ihunwo and Pillay, 2007). Fibroblast growth factor (FGF) was also reported to stimulate proliferation of neuronal progenitors in the septum and striatum in rodents (Palmer *et al.,* 1995). The hippocampus and subventricular zone yield more established colonies and a larger number of progenitors than the septum and striatum. The substantia nigra pars compacta is another region where adult neurogenesis is said to be present as evidenced by the slow turnover of dopaminergic projection neurons in the adult rodent brain (Zhao *et al.,* 2003). However, Frielingdorf and colleagues (2004), found no new dopaminergic neurons in the adult mammalian substantia nigra pars compacta.

Evidence provided by Bernier *et al.,* (2002) indicated that neurogenesis is present in the amygdala and surrounding cortex of adult monkeys with the occurrence of a Temporal Migratory Stream (TMS) which is similar to RMS. From reports of multipotent stem cells in more caudal regions of the neuroaxis such as the spinal cord, it has become clear that stem cells are present in all parts of the central nervous system (Temple and Alvarez, 1999). Prior to this time, Weiss and colleagues (1996) isolated neural stem cells from the thoracic and lumbo-sacral segments of the spinal cord of adult mice.

1.3.3. Non- neurogenic regions with neurogenic potential

Adult neural stem cells reside in regions considered to be non-neurogenic, for example cerebral cortex and olfactory bulb (Gritti *et al.,* 2002; Pagano *et al.,* 2000). Takemura (2005) provided evidence that active neurogenesis persists within the white matter beneath the temporal neocortex when he explored previously overlooked neurogenic region in the adult rat brain and detected the evidence of neuron production within the subcortical white matter. From his results, it was suggested that cell genesis, death and migration persists in a restricted sub-region of the adult white matter. Arsnijevic *et al.,* (2001) demonstrated the existence of multipotent precursor cells in the adult human cerebral cortex and Moyse *et al.,* (2006) confirmed neurogenesis in the dorsal vagal complex of the brain stem (a major centre for autonomic reflexes). Ihunwo and Pillay (2007) provided a detail review of active and potential neurogenic sites in the adult mammalian brain (Table 2).

TABLE 2: Reported sites of neurogenesis in adult mammal brains (Ihunwo and Pillay, 2007).

Site	SVZ	DG	SEP\ STR	SN	III VEN	SC	AMG	CTX	OB\ RMS	DVC
Rodents										
Rats	+	+	+	-	+	-		+	+	+
Mice	+	+	+	+	+	+		-	-	-
Rabbits	+	+	+	-		-		-		-
Human	+	+	-	-		-		+	+	-
Squirrel Monkeys	+	+	-	-		-	+	-	-	-
Macaque monkey	+	+	-	-		-	+	+	-	-

Key: SVZ, subventricular zone; DG, Dentate gyrus; SEP/ STR, Septum and Striatum; SN, Substantia nigra; III VEN, Third Ventricle; SC, Spinal Cord; AMG, Amygdala; CTX, Cerebral cortex; OB/ RMS, Olfactory bulb/ Rostral migratory stream, DVC, Dorsal Vagal Complex (+ indicates presence; - indicates absence).

Kempermann et al., (1997) investigated different strains of laboratory mice and concluded that adult hippocampal neurogenesis is differentially influenced by the genetic background of the species. Amrein et al., (2004) investigated different strains of wild rodents and observed differences in cell proliferation. No significant differences in the relative ratio of neurogenesis and gliogenesis were observed in two strains of laboratory mice. With differences in both laboratory and wild rodents, it becomes imperative to document the pattern of adult neurogenesis in the four-striped mouse and common mole-rat which have not been reported but readily available in the Southern African region.

CHAPTER 2: MATERIALS AND METHOD

2.1. Experimental Animals

Eight adult specimens from each species were used. The four-striped mice were captive reared at the Central Animal Service (CAS) unit of the University of Witwatersrand, Johannesburg, South Africa but had wild caught ancestors. The common mole-rat were wild caught from a golf course in Pretoria, South Africa and transported to the CAS at the medical school of the University of Witwatesrand. All animals used were adults based on their body weight, dentition and sexual maturity. They were kept under standard laboratory conditions with a 14: 10 hourly light-dark cycle with lights on at 6 am for the four-striped mouse and 12 hourly light and dark for the common mole-rat. Room temperature was between 20-24 ºC and 30 % - 60 % relative humidity. A 40 X 12 X 25 cm (length, height and width) lab-o-tec cages (Labotec, Halfway House, South Africa) with saw dust or wood waste shavings as litter and hay as nesting material was used to house this animal. The animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics and Screening Committee (AESC Clearance No: 2007/45/03).

2.1.1. Four-striped mouse (*Rhabdomys pumilio*)

The four-striped mouse *(Rhabdomys pumilio)* belongs to the, Muridae, Rodentia. They are widely distributed in Southern Africa, occurring in different habitats, such as grassland, marsh, forests, semi- deserts and deserts (Skinner and Chimimba, 2005; Figure 2). The four striped mice, *Rhabdomys pumilio*, are small diurnal murid rodent. They do not usually live in colonies. In fact very few populations live as groups, and most are solitary living. They demonstrate bi-

parental care. It is easily identified by the four distinct dark longitudinal stripes running the length of the back (Figure 2.1). Unlike most rodents, the four-striped mouse displays a diurnal bimodal activity pattern with its activities mainly in the mornings and evenings (Schumann *et al.,* 2005). It has a reduced activity in the afternoon or midday period. It is an omnivorous animal. Its diet contains a minimum of 15% water (Wilan and Meester, 1989). It has an extreme plasticity in habitat preference which gives the reason for it widespread distribution throughout Southern Africa (Skinner and Chimimba, 2005). Colour of the stripe varies from dark brown to gray-white. The four-striped mice have a body mass ranging from 40- 80g (Schradin and Pillay, 2004; Maini, 2003) and a small brain with an average mass of about 0.64 g (Bhagwandin *et al.*, 2006).

It breeds seasonally usually from spring to autumn (Schradin and Pillay, 2003). Its gestation period is 22-23 days. Their females, that are free- living, give birth to approximately five pups: captive females have slightly larger litter 6-7 (Pillay, 2000). Their pups begin to consume solid food at ten days after birth and leave their nest from twelfth day after gestation. Weaning starts at around the sixteenth day after birth. Sexual maturity is reached at around fifth to sixth week of life which depends on environmental and social factors as well as its development status (Pillay, 2000).

Four-striped mouse has got a flexible social organization and mating system which is controlled majorly by resource availability and population density. In the arid habitat, they can be described as a territorial, group-living, solitary forager that displays bi-parental care (Schradin and Pillay, 2005a). In grasslands, females maintain intra-sexually exclusive territories, and males'

territories overlap with those of other males and females (Schradin and Pillay, 2005a). Both sexes maintain their territory which overlaps their opponent's (Schradin and Pillay, 2005a). In captivity, males from both mesic and xeric populations display paternal care. Four-striped mouse from the southwestern regions of southern Africa are slightly larger than the northern regions (Yom Tov, 1993).



Figure 2.1: Photograph of the four-striped mouse (*Rhabdomys pumilio*) by Selvakumar. Internet accessed 12 June, 2010.

2.1.2. Common mole-rat (*Cryptomys hottentotus*)

The common mole-rat, *Cryptomys hottentotus*, (Figure 2.2) belongs to the order of the Rodentia. Its family is Bathyergidae and genus is *Cryptomys*. The common mole-rat is a burrowing rodent that is found in Africa, mainly in

southwest Cape Province in South Africa. Also found in other parts of Africa like Lesotho, Malawi, Mozambique, Swaziland, Tanzania, Zambia and Zimbabwe. They have a reduced visual function. They have an average body length of 10.5-16.5 cm with tail length of 1.2-3.8 cm. It has a thick fur with many different colours. Their body shape is cylindrical with short limbs. They have a chisel-like incisor that is used for digging. They have an average body mass of 120.5 g, and a brain mass that on average is 1.26 g (Genelly, 1965). They dwell in small colonies (up to 14 individuals) that are comprised of a breeding female, her consorts, and their non-breeding offspring (the workers). They have a unique characteristic of having one reproductive pair, consisting of the largest female and male in one group. Mating occurs between the months of September and October They breed seasonally (October–January), the gestation period is about 81 days with 2-5 litters. Common mole-rat reaches sexuality at about 450 days. Females maintain reproductive function during non-reproductive months.

Common mole-rats are fossorial mammals that can live in wide range of substrates. They are herbivorous. They are wide spread and they show a sign of localization due to soil requirements. Their pattern of borrowing optimizes their access to food whereas it has a negative economic impact to man in that it damages properties but also improves soil drainage and turnover as a positive view.

In comparison to the naked mole-rat, the common mole-rats have the ability to generate their own heat and keep their body temperature above ambient temperature which gives them an added advantage to survive any weather. They have lower individual body masses in arid environment that helps with energy

conservation. They also have long sensory hairs, vibrissae, which stand out from their fur covering their body.

Common mole-rats are social creatures that live in family units of up to 14 per group. They are widely distributed across the whole of the southern African region which is found in all sorts of habitats. The fur of the common mole-rat is silky, soft and short. Its colour though depends on the colour of the soil it is burrowing in however, they are usually brown in colour and lacks patches on its nose, ear, eye and throat. It has a keen sense of hearing and a very poor eye-sight. Common mole-rats are able to survive underground by exhibiting many thermoregulatory and metabolic adaptations to living in burrows. This is also possible because of their reduced resting metabolic rate and lower core body temperature with a higher thermal conductance (Bennett *et al.,* 1992; Bennett *et al.,* 1994; Bennett *et al.,* 2009). They exhibit specialized behaviour and cooperative care of the young. The younger ones are like to-be workers and older ones may be casual workers. The worker for the most part burrows and forage. The oldest are breeders. The main predator species of common mole-rat are barn owls, eagle owls and mole snakes. If caught out in the open, the cunning leopard will also prey upon the common mole-rat.



Figure 2.2: Photograph of the common mole-rat (*Cryptomys hottenttotus*) Piechl, (2004). Internet accessed on 12 June, 2010.

2.2. Experimental groups

The grouping of the experimental animals was based on two time points; a proliferation time point, 2 hours post BrdU- injection and a survival time point, 4 weeks post BrdU- injection (Lagace *et al.*, 2007). In the proliferation time point, BrdU is administered to the animal 2 hours before sacrifice to check for proliferating cells that are BrdU positive. Likewise in the 4 weeks post BrdU injected animal group, they are injected once and sacrificed after 28 days with animal under observation every day.

TABLE 3: Distribution of animals into groups is as shown below.

	Four-striped mouse	Common mole-rat
2 hrs post BrdU injection to evaluate proliferating cells.	3	3
4 weeks post BrdU injection to evaluate surviving cells.	3	3
Number of controls (No BrdU injection)	2	2
Total	8	8

2.3. Markers of cell Proliferation

There are two main classes of markers used to label proliferating cells: exogenous and endogenous markers. Endogenous markers are molecules that the cell expresses during the progression of the cell cycle, which correlates with the duplication of its DNA or with the mitotic division while exogenous markers are injected then binds to DNA in vivo and may produce DNA mutations. These types of marker have been widely used in the study of adult neurogenesis. There are three markers used in this study, bromodeoxyuridine (BrdU), Ki-67 and doublecortin.

2.3.1. Bromodeoxyuridine (BrdU) Administration

Evidence for neurogenesis is obtained by the use of the thymidine analog bromodeoxyuridine (BrdU) (Cameron et al., 1998), which incorporates into DNA during the S phase of cell cycle (Nowakowski *et al.*, 1989). BrdU labelling is used to reveal neural stem cells and has proven to be a valid marker for studying adult neurogenesis (Cameron *et at*., 1998; Kempermann *et al*., 1997; Taupin, 2007). BrdU crosses the blood- brain barrier (del Rio & Soriano, 1989). Advantages of BrdU to H-thymidine (H-dT) is that its immunohistochemical detection is more

easily combined with that of various cell class-specific markers to determine cell phenotype in small neurons such as granule cells that are difficult to distinguish from astrocytes (Rackic, 2002). It also allows for the analysis of changes in the production of specific cell types under various experimental conditions (Rackic, 2002). With BrdU, it is particularly useful for the detection of newly generated cells that were difficult to identify with the autoradiographic method (Rackic, 2002). However, the immunohistochemical approach can also lead to false conclusion if potential technical problems are ignored (Rackic, 2002; Hayes & Nowakowski, 2002). There are no established criteria for the use of BrdU as a marker of neuronal birth date at the moment, though it is not a marker for cell division, but a marker for DNA synthesis (Rackic, 2002). BrdU immunohistochemistry is not stoichiometric in contrast to H-dT autoradiography (Hayes & Nowakowski, 2002). However, BrdU is also considered to be a mutagen (Morris, 1991). BrdU was dissolved in saline and administered intraperitoneally for the proliferation and survival groups. A dose of 50 mg /kg body weight was administered (Mitra *et al.,* 2006). The disadvantages of BrdU are that it has to be injected into the animal and may require multiple injections.

2.3.2. Ki-67

Ki-67 is an endogenous proliferation marker which reacts with a nuclear antigen. Ki-67 is expressed in all proliferating cells which are in active phases of cell cycle but absent in resting cells. It is a nuclear protein present in all the phases of the cell cycle except in the G0 (Gil-Perotin *et al.,* 2006). In G1, it is predominantly localized in the perinucleolar region and also found in nuclear matrix in the later phases (Gerdes, 1990). It is thought to be involved in the

maintenance of cell proliferation however its exact mechanism for function is unknown. Due to these facts, it is considered an important marker for evaluation of tumour diagnosis and prognosis. It is a nuclear protein present in all the phases of the cell cycle except in the G0 (Gil-Perotin *et al.,* 2006). Ki-67 labels the nuclei of the proliferation cells.

2.3.3. Doublecortin (DCX)

DCX is expressed in migrating neurons throughout the central and peripheral nervous system during embryonic and postnatal development (Gleeson *et al.,* 1999). DCX co-assembles with brain microtubules, and recombinant DCX stimulates the polymerization of purified tubulin. Over expression of DCX in heterologous cells leads to a dramatic microtubule phenotype that is resistant to depolymerization. Therefore, DCX likely directs neuronal migration by regulating the organization and stability of microtubules (Gleeson *et al.,* 1999). Doublecortin is a phenotypic marker which stains the cytoplasm of immature cells (Lu *et al.,* 2005).

2.4. Tissue processing

The animals were euthanized with sodium pentobarbital (Euthanaze, i.p. 80 mg/kg) and transcardially perfused with 0. 9% cold saline (4 °C) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brain tissues were then carefully removed from the skull, weighed and post-fixed in 4% paraformaldehyde in 0.1 M PB, then allowed to equilibrate in 30% sucrose in 0.1 M PBS. Brain tissues were then kept frozen in dry ice and sectioned using a

sliding microtome in the saggital plane at 50 µm section thickness covering the complete brain. Subsequently, sections were placed in vials containing PBS.

A one in five series of sections were each stained for BrdU, Ki-67 and doublecortin; the remaining two sections of the series were stored in cryoprotectant solution (CPX) in a freezer at -20 ºC for future experimental purpose. This provided a section of the brain stained at every 250 µm throughout the brain.

2.5. Bromodeoxyuridine immunohistochemistry

2.5.1 Pre- incubation:

Sections were rinsed 3 times for 10 minutes in 0.1M PBS, under gentle shaking at room temperature. Tissues were then pre-incubated, for 2 hours at room temperature, in a solution containing 3 % normal goat serum (NGS; Chemicon Int.), 2 % bovine serum albumin (BSA; Sigma) and 0.25 % Triton X100 (Merck) in 0.1M PB. Triton X100 is a detergent necessary to create micropores in the membranes of the cells, allowing the antibodies and the reactives to penetrate the tissue. It also removes the antibodies from its antigens. The normal serum and BSA, which are proteins, are used to avoid unspecific staining. They help to prevent the primary and secondary antibodies to join to unspecific places that have a similar structure.

2.5.2. Primary Antibody Incubation:

The sections were then placed in 0.25 % Triton X100, 3 % NGS, 2% BSA, and primary mouse anti-BrdU monoclonal antibody (Millipore International, MA,

USA, 1:1000, Mitra *et al.,* 2006) for four-striped mouse and rat anti-BrdU antibody (Millipore International, MA, USA 1:1000) for common mole-rat overnight at 4 °C under a gentle shaking. These antibodies are species specific. Subsequently, tissues were rinsed three times for 10 minutes in 0.1M PB.

2.5.3. Secondary Antibody Incubation:

The tissues were incubated in a secondary antibody solution which contained 1:500 dilution of biotinylated-goat-anti-mouse or goat-anti-rat IgG (Dako, Denmark, 1:100), 3 % normal goat serum, 2 % BSA, in 0.1 M PB, for two hours at room temperature.

2.5.4. Avidin-Biotin-Complex-method:

Sections were rinsed three times for 10 minutes in 0.1 M PB, then incubated for 1 hour in AB solution (the ratio that were used for A and B reactive were 40 µl each in 5000 µl of 0.1 M PB, Vector Labs), and again rinsed three times in 0.1M PB under gentle shaking at room temperature, each for 10 minutes.

2.5.5. 3, 3'-diaminobenzidine tetrahyrochloride (DAB) staining:

Sections were treated in a solution containing DAB in 0.1M PB for 5 minutes. Thereafter, 3 µl of 30 % H_2O_2 per 0.5 ml of solution was added. A low power stereomicroscope was used to follow up the development of the reaction. Once an appropriate level of background staining was observed, the reaction was stopped by placing sections in 0.1 M PBS. The sections were then mounted on 0.5 % gel coated glass slides and air-dried overnight. They were then dehydrated in a graded series of alcohols, cleared in xylene and cover slipped with Entelan. Two controls were used in the immunostainings, one which did not contain primary

antibody and the other which omitted the secondary antibody in selected sections to test whether the anti-bodies worked.

2.6. Ki-67 immunohistochemical staining

Sections were washed 2 times for 10 minutes in PBS and then rinsed with tris buffer saline triton-X (TBST) once for 5 minutes under gentle shaking at room temperature. The sections were then treated for 40 minutes at 94 ºC (in water bath) in citrate buffer pH 6.1 diluted with distilled water (1:10) for anti-gene retrieval. Sections were then allowed to cool down on the bench to room temperature for 20 minutes.

Sections were then washed in TBST 2 times for 5 minutes under gentle shaking and transferred into blocking solution, 5 % NGS in TBST for 30 minutes. Tissues were then transferred into primary antibody, NCL-Ki-67 (Novocastra, Wetzlar, Germany; 1:5000) in TBST supplemented with 2 % BSA and 2 % NGS overnight under gentle shaking at 4º C.

The following day, the tissues were removed from the fridge and allowed for 30 minutes to equilibrate to room temperature under gentle shaking. The tissues were then washed 3 times for ten minutes in TBST under gentle shaking at room temperature. Secondary antibody was then applied, biotinylated goat- anti-rabbit (Vector lab, CA, USA; 1:250) in TBST supplemented with 2 % NGS for 60 minutes at room temperature. Tissues were then washed 3 times for 10 minutes in TBST. ABC reagent (Vector lab, CA, USA; 1:100, A and B) was then applied for 40 minutes at room temperature under gentle shaking. After that, sections were then washed in TBS 2 times for 15 minutes under gentle shaking at room

temperature. Tissues were then washed in TB pH 7.6 two times for 10 minutes under gentle shaking at room temperature. They were then pre-incubated in the dark for 30 minutes with DAB solution, 0.5 mg/ml, in TB 7.6 under gentle shaking at room temperature using 2 ml per vial. 35 µl of 0.5 % H_2O_2 was then added to each vial and mixed very well to develop under visual guidance until a strong nuclear staining was observed. Reaction was then stopped by washing sections TB pH 7.6 3 times for 10 minutes under gentle shaking at room temperature. Tissues were then washed in PB and mounted onto 0.5 % gelatinized slides and air dried overnight. They were then dehydrated in a graded ascending series of alcohols, cleared in xylene, and coverslipped with Entelan.

2.7. Doublecortin (DCX) immunohistochemical staining

Sections were washed 2 times for 10 minutes in PBS and then rinsed with TBST once for 5 minutes under gentle shaking at room temperature. Sections were then treated with blocking solution, 5 % normal rabbit serum (NRbS) in TBST for 30 minutes.

Tissues were then transferred into primary antibody DCX (Santa Cruz biotech, CA, USA; 1:400) in TBST supplemented with 2 % BSA and 2 % NRbS overnight at 4 ºC under gentle shaking.

On the following day, tissues were removed from the fridge and left on the shaker to equilibrate at room temperature followed by a 3 times 10 minutes wash in TBST under gentle shaking at room temperature. Secondary antibody, biotinylated rabbit-anti-goat (Vector lab, CA, USA; 1:250) in TBS supplemented with 2 % NRbS for 60 minutes at room temperature under gentle shaking was

then applied. After that, sections were washed 3 times for 10 minutes each under gentle shaking at room temperature. Thirty minutes prior to the time of use, ABC reagent in TBS (1:100, A plus B) was applied to the tissues for 40 minutes at room temperature under gentle shaking. Sections were then washed in TBS twice at 15 minutes each under gentle shaking at room temperature followed by washing in TB (pH 7.6) two times for 10 minutes each.

Section were then pre-incubated in DAB solution, 0.5 mg/ml in TB (pH 7.6) by using 2 ml per vial for 30 minutes in the dark under gentle shaking at room temperature. 35 µl of 0.5 % H_2O_2 was added to each vial and mixed very well and allowed to develop under visual guidance until strong nuclear staining appears within the rostral migratory stream. The reaction was stopped by washing sections in TB pH 7.6, 3 times for 10 minutes each under gentle shaking at room temperature. Tissues were then washed in PB and mounted onto 0.5 % gelatinized slides and air dried overnight. They were then dehydrated in an ascending graded series of alcohols, cleared in xylene and cover slipped with Entelan.

2.8. Data analysis

Sections of the brains were analysed with a light microscope using the Zeiss Axioskop 2 plus microscope, Germany. The immunostained sections were compared with previous report studies (Lagace *et al.,* 2007; Amrein *et al.,* 2004; Lu et *al.,* 2005; Plumpe *et al.,* 2006). Photomicrographs were taken at different magnification with the aid of the Zeiss Axioskop 2 plus microscope with a fitted camera, model AxioCam HRc Zeiss camera (Germany).

CHAPTER THREE: RESULTS

3.1 General observations

The main objective of this study was to identify proliferative cells in the regions of the adult brain of non captive South African rodents. The distribution of the immunohistochemically reactive brain tissues to the BrdU, Ki-67 and DCX were found to be different between the four-striped mouse and common mole-rat.

BrdU labelled the nuclei of proliferating cells which appeared dark and clustered in the two hours post BrdU injected group. For the four weeks post BrdU injection group the BrdU positive cells appeared more singly with darkly stained nuclei and more rounded in the two active neurogenic sites for both the four-striped mouse and common mole-rat. BrdU positive cells were absent in other potential sites in both the four-striped mouse and common mole-rat.

Ki-69, an intrinsic marker for proliferating cells, labelled the nuclei of the proliferating cells which appeared in clusters in almost all the region that was observed. More new neurons were stained appearing in clusters and in some areas in isolated form. Ki-67 labels proliferating cells in the two active neurogenic sites, subventricular zone and dentate gyrus of the hippocampus of the two experimental animals. In the potential neurogenic sites, Ki-67 positive cells were observed in the substantia nigra, striatum and olfactory bulb of the four-striped mouse while in the common mole-rat, Ki-67 positive cells were present in the substantia nigra and olfactory bulb but absent in the striatum.

DCX is an intrinsic marker for immature neurons, labelled the cytoplasm of the immature cells along with their processes. They appeared in clusters but varied

in shape. The DCX positive cells were categorized according to the shape and presence of apical dendrites as described earlier by Plumpe *et al.*, (2006):

- Category A and B DCX positive cells were with very short or no processes respectively. The processes were less than one nucleus wide in the DCX positive cells of category B.
- Category C and D DCX positive cells had processes of intermediate length and immature morphology. The processes were longer in DCX positive cells in category C compared to processes of DCX positive cells in category B whereby the processes reached the granule cell layer but did not reach the molecular layer.
- In DCX positive cells in category D, the processes reached the molecular layer.
- Category E and F DCX positive cells had a more matured appearance. In the DCX positive cells for category E, they had a one thick dendrite that reached into the molecular layer and displayed a comparatively sparse branching in the molecular layer. The DCX positive cells of category F had a dendritic tree which showed delicate branching and few major branches close to the soma or within the granule cell layer.

DCX positive cells were observed in the two neurogenic sites of the experimental animals. Likewise in the potential neurogenic sites, they were observed in the striatum and olfactory bulb of the four-striped mouse but absent in their substantial nigra. In the common mole-rat, DCX positive cells were present in the striatum, substantia nigra and olfactory bulb. In addition to

these potential neurogenic sites in the common mole-rat, DCX positive cells were observed in the cerebral cortex of the common mole-rat.

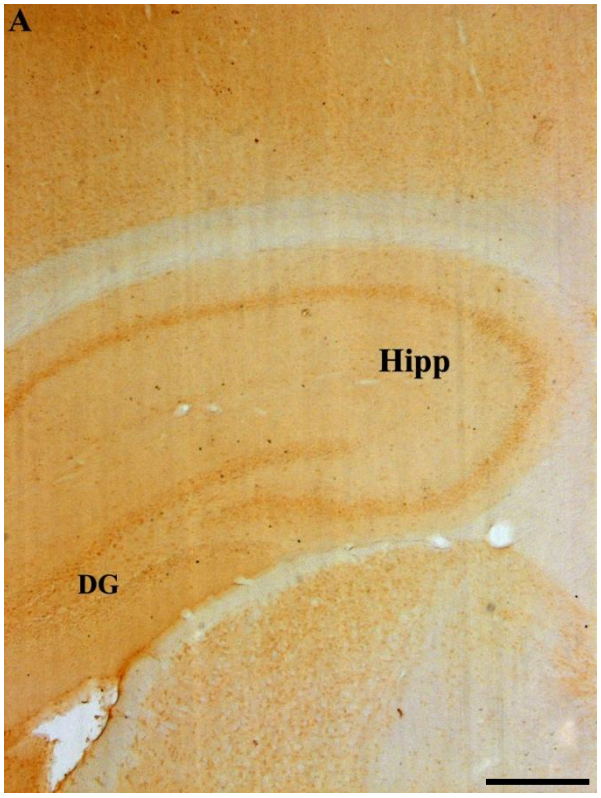
3.2. Immunohistochemical findings in the four-striped mouse

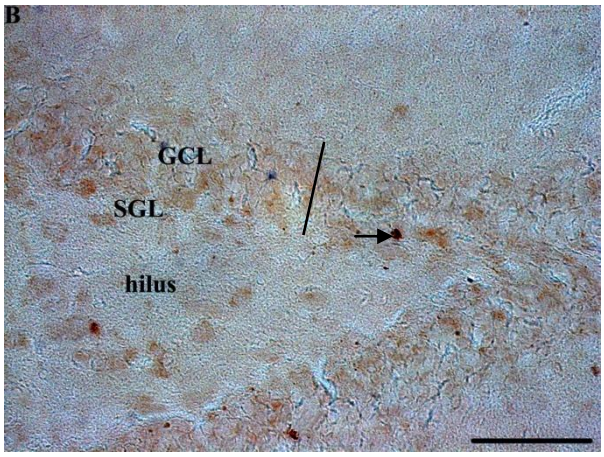
3.2.1. BrdU positive cells in the proliferating and survival group

Proliferative cells were observed in the dentate gyrus of the hippocampus (Figure 3.1). The cells appeared in clusters and irregular in shape in the 2 hours post BrdU injected group (Figure 3.1 B) while the 4 weeks positive cells appeared singly, more rounded with darkly stained nuclei (Figure 3.1 C). These cells were found in the granular cell layer and subgranular layer of the dentate gyrus of the hippocampus and in the hilus.

BrdU positive cells were observed in the subventricular zone of the lateral ventricle. The nuclei were darkly stained appeared in clusters along the wall of the subventricular zone of this region of the brain (Figure 3.2, A-C). Under high magnification, these cells appeared darkly stained, irregular in shape and in clusters in the 2 hours post BrdU injected group (Figure 3.2 B) while in the 4 weeks group, they appeared more regular in shape with a darkly stained nuclei (Figure 3.2 C). BrdU positive cells in the two hours and four weeks post BrdU groups were distributed along the wall subventricular zone area.

Figure 3.1: Representative photomicrograph showing BrdU positive cells in the dentate gyrus of the hippocampus of the four-striped mouse. The two hours proliferating groups (A and B) show the BrdU positive cells at different magnification. Figure 3.1 C, for the four weeks group. The arrows indicate positive BrdU immunostained cells. GCL-granule cell layer and SGL-subgranular layer. Scale bar; A =10 µm, B= 2.5µm and C= 1 µm.


A
Hipp
DG


B
GCL
SGL
hilus

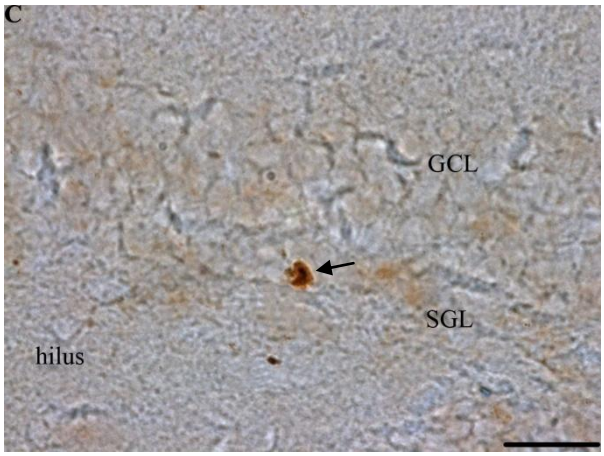
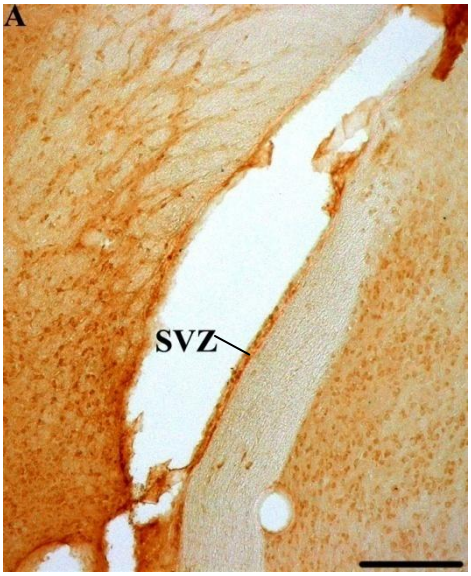
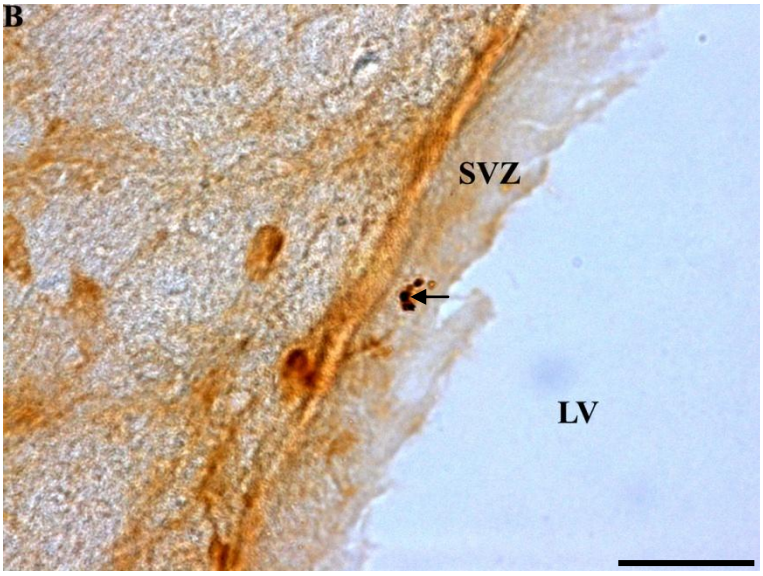
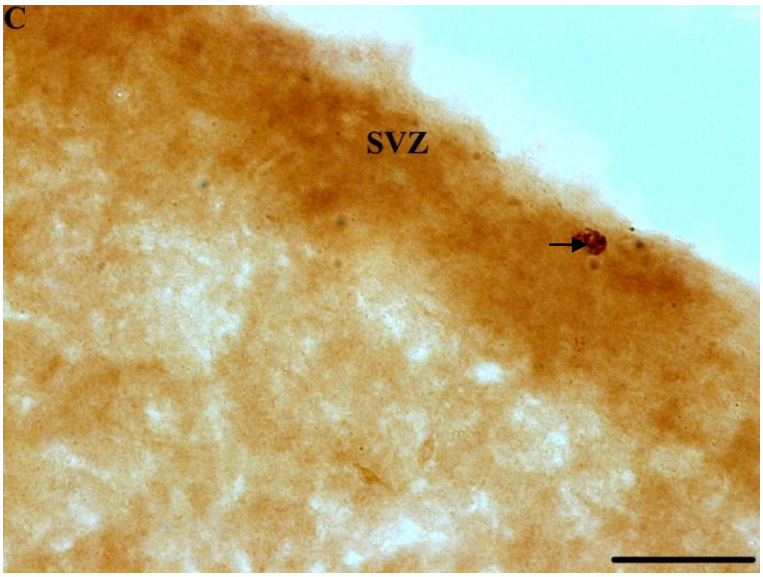

C
GCL
SGL
hilus

Figure 3.2: Representative photomicrograph showing BrdU positive cells in the wall of the subventricular zone of the four-striped mouse. In the two hours group (A and B), the proliferative cells appear in clusters and in the four weeks (C), the immunopositive cell appear singly and more rounded with a darkly stained nucleus. The arrows indicate positive BrdU immunostained cells. SVZ- subventricular zone and LV- lateral ventricle. Scale bar; A= 10 µm, B and C= 1 µm.


A
SVZ


B
SVZ
LV


C
SVZ

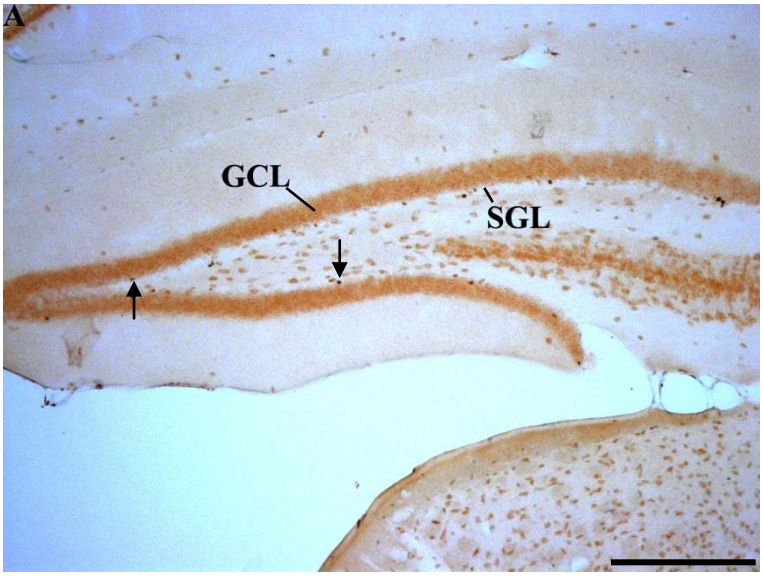
3.2.2. Ki-67 positive cells

The Ki-67 positive cells were observed to be distributed along the length of the granular cell layer of the dentate gyrus of the hippocampus (Figure 3.3, A & B). The nuclei of the Ki-67 positive cells were darkly stained and distributed in the dentate gyrus of the hippocampus (Figure 3.3 B). The Ki-67 positive cells in the subventricular zone appeared in clusters and widely distributed (Figure 3.4, A-C). These immunopositive cells had centrally located darkly stained nuclei (Figure 3.4 C).

Ki-67 positive cells appear darkly stained and in clusters and observed along the rostral migratory stream which is enroute towards the olfactory bulb (Figure 3.5, A & B).

The proliferative Ki-67 positive cells in the substantia nigra appeared darkly stained with a regular shape and in clusters (Figure 3.6, A & B). There were no positive Ki-67 cells in the cerebral cortex in the four-striped mouse.

Figure 3.3: Representative photomicrograph showing Ki-67 positive cells in the dentate gyrus of the hippocampus in the four-striped mouse (A and B). Majority of the cells are located in the subgranular layer (arrows). The cells appear darkly stained and in clusters. GCL-granule cell layer and SGL-subgranular layer. Scale bar; A =10 µm, B=1 µm.


A
GCL
SGL

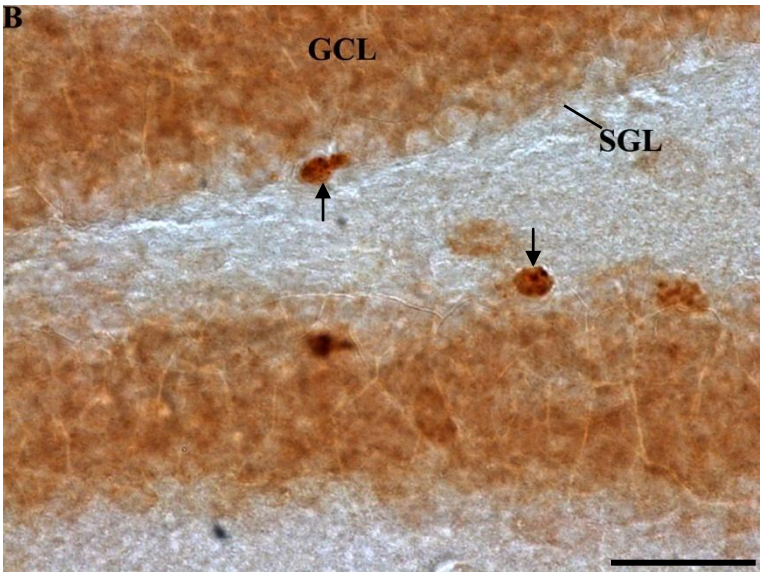
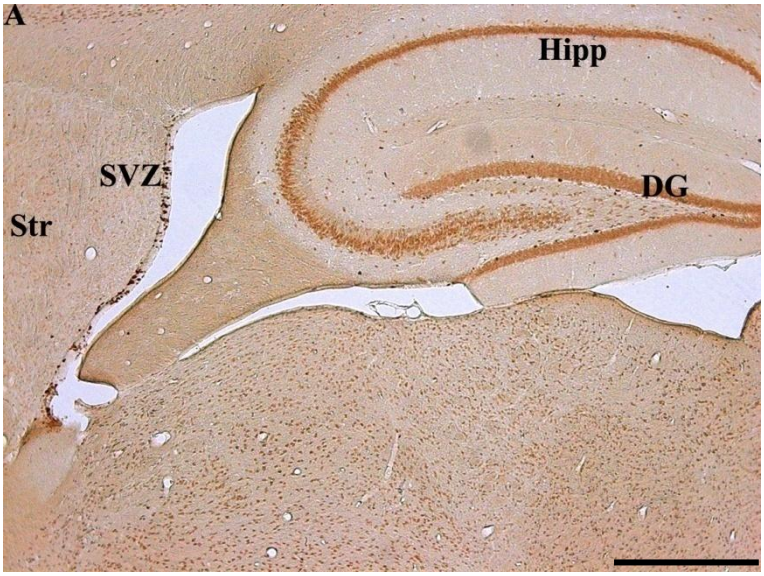
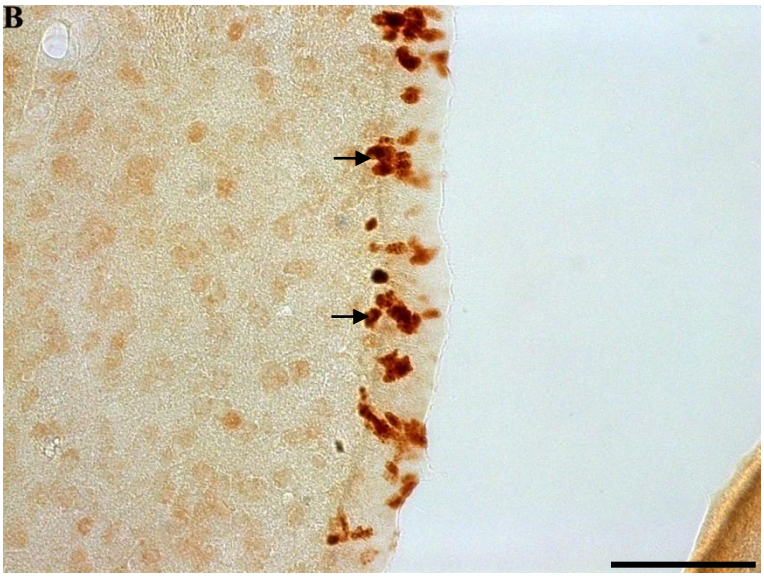

B
GCL
SGL

Figure 3.4: Representative photomicrograph showing Ki-67 positive cells in the subventricular zone of the four-striped mouse. Majority of the cells are located in the subventricular wall at different magnifications (A, B and C). The cells appear darkly stained and in clusters 9 (arrows). DG- dentate gyrus, Hipp- hippocampus, SVZ- subventricular zone and Str- striatum. Scale bar; A= 20 µm, B=2.5 µm, C=1 µm.


A
Hipp
SVZ
DG
Str


B

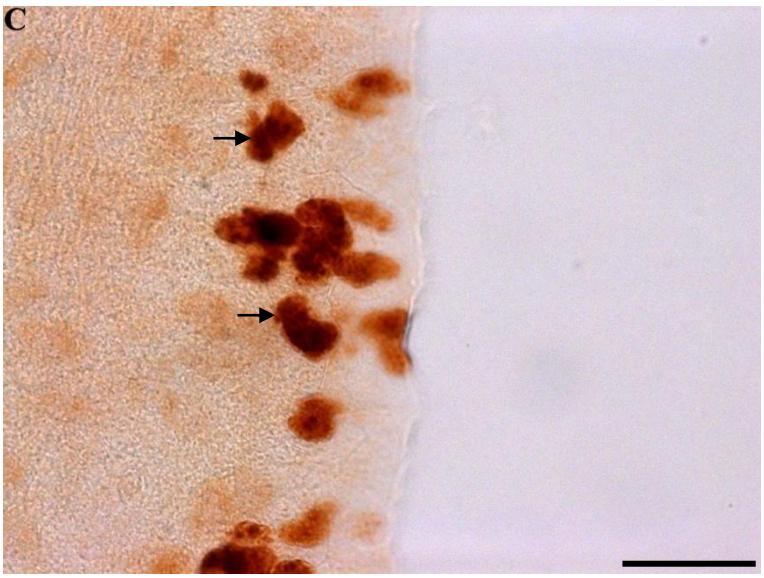

C

Figure 3.5: Representative photomicrograph showing Ki-67 positive cells in the rostral migratory stream/ olfactory bulb of the four-striped mouse. Ki-67 positive cells are migrating towards the olfactory bulb (A). Figure B is a higher magnification of the rostral migratory stream.. OB- olfactory bulb and RMS- rostral migratory stream. Scale bar; A= 20 µm and B=1 µm.

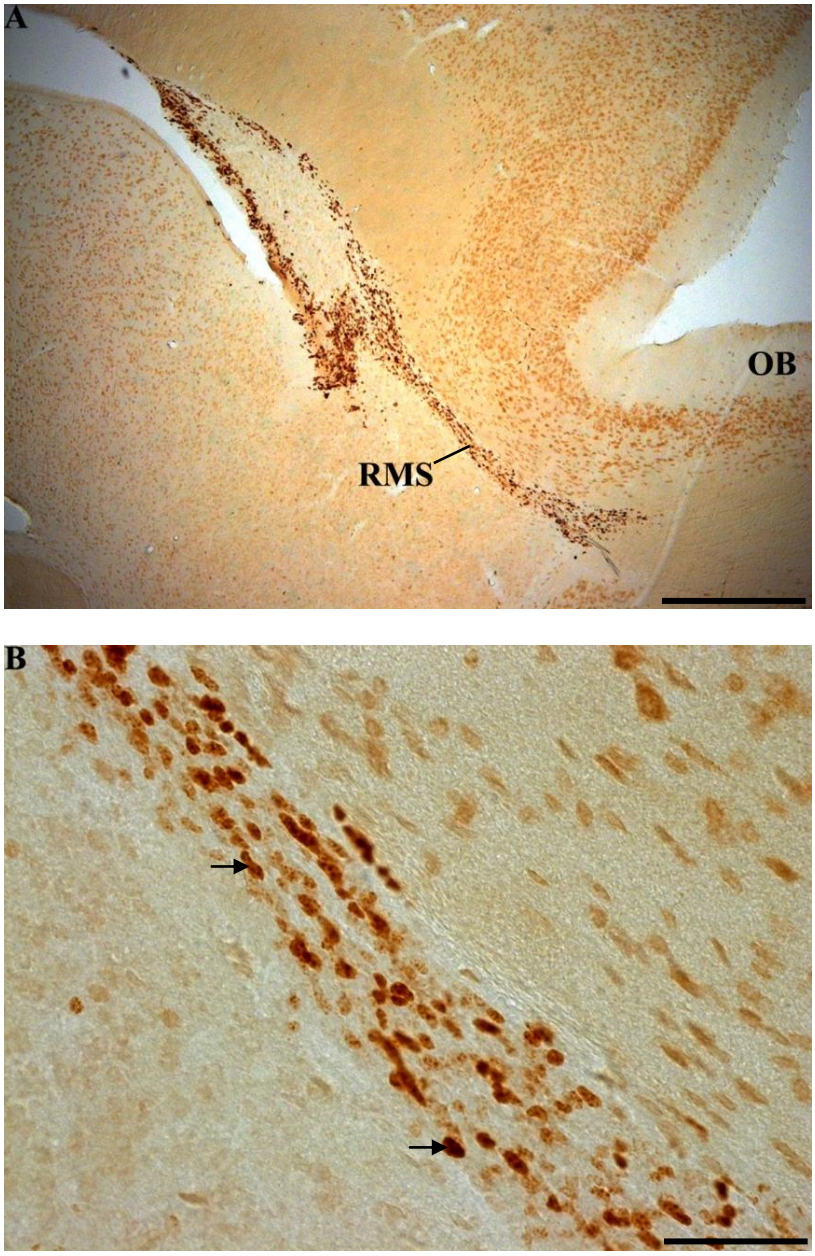
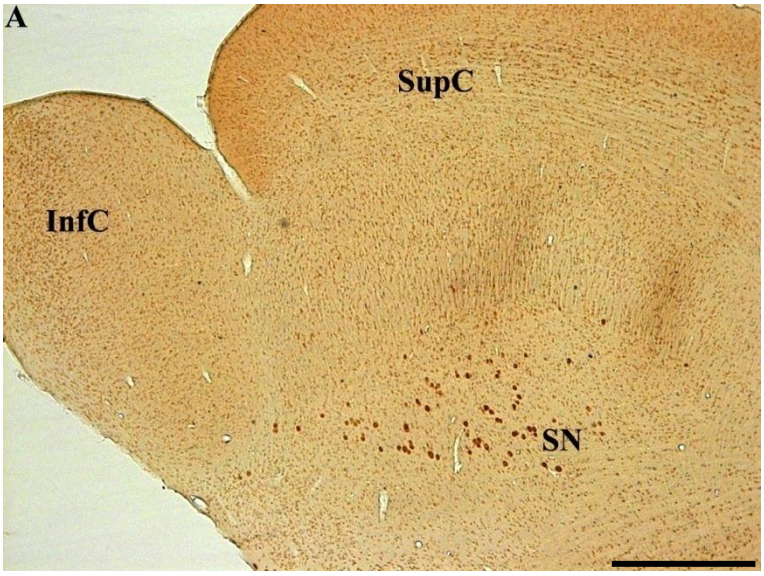
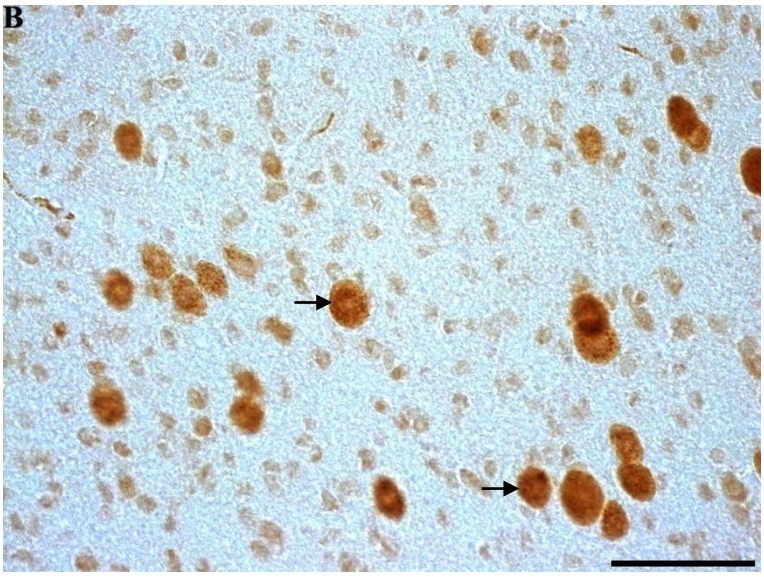

A
OB
RMS
B

Figure 3.6: Representative photomicrograph showing Ki-67 positive cells in the substantia nigra of the four-striped mouse. Ki-67 positive cells are more rounded with darkly stained nuclei. Figure B is a higher magnification of the positive cells (arrows). InfC- inferior colliculus, SupC- superior colliculus and SN- substantia nigra. Scale bar; A= 20 µm, B=2.5 µm.


A
SupC
InfC
SN


B

.

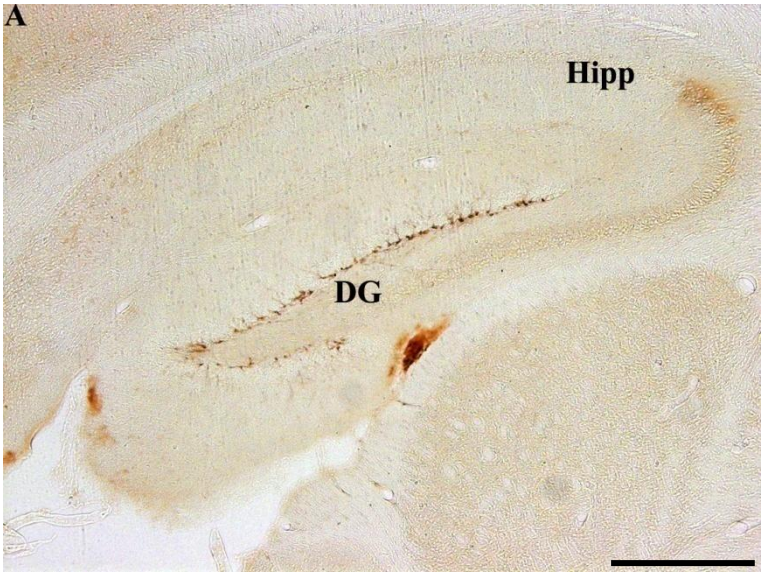
3.2.4. Doublecortin (DCX) positive cells

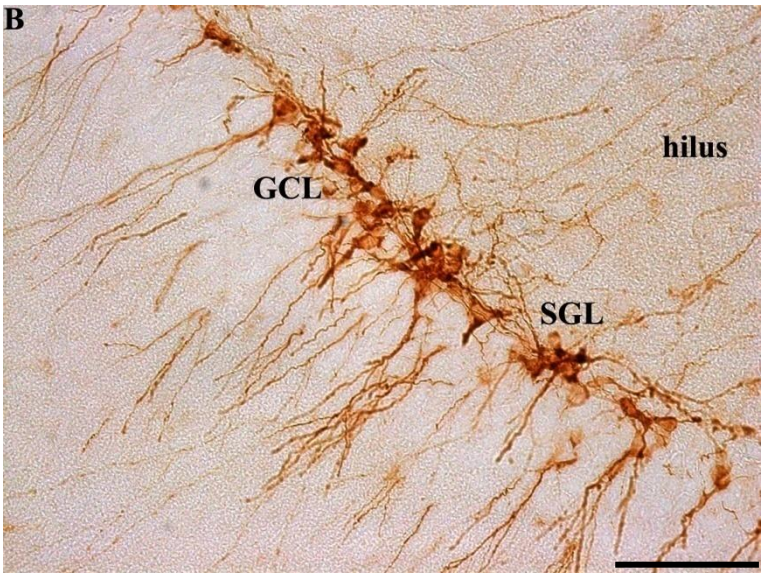
DCX positive cells were observed in the dentate gyrus of the hippocampus as immature neurons along with their processes (Figure 3.7, A-C). The cell bodies lined the subgranular layer of the dentate gyrus of the hippocampus (Figure 3.7 B). Majority of the cells are bipolar with an ovoid soma and fall under category E and F (see section 3.1). Their processes extended into the granular and molecular layers (Figure 3.7, B & C).

The DCX positive cells in the subventricular zone appeared in a web-like fashion in the wall of the subventricular zone at low and high power magnification together with their processes. The cytoplasm of the immature cells stained brown with short or long processes and a well developed cell body (Figure 3.8, A & B). Their soma is ovoid with a prominent nucleus (Figure 3.8 C).

The immature neurons were observed in the olfactory bulb in clusters (Figure 3.9, A-C). The soma of the DCX positive cells are stained brown along with their processes. The soma was ovoid in shape with a more prominent nucleus (Figure 3.9 B) in the olfactory bulb. They migrate from the subventricular zone to the olfactory bulb via the rostral migratory stream with their processes extending into the striatum (Figure 3.9 C).

Figure 3.7: Representative photomicrograph showing DCX positive cells in the dentate gyrus (DG) of the four-striped mouse. DCX positive cells are in cluster (B and C) with their soma and processes (C, arrows). The soma lies in the subgranular layer with their processes projecting as far as the molecular layer. Hipp- hippocampus, DG- dentate gyrus, SGL subgranular layer and GCL- granule cell layer. Scale bar; A= 20 µm, B=2.5 µm, C=1 µm.


A
Hipp
DG


B
hilus
GCL
SGL

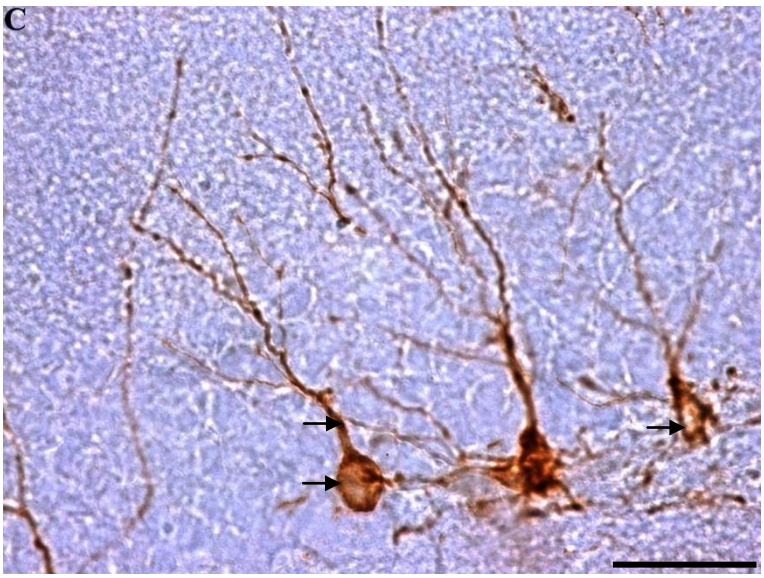

C

Figure 3.8: Representative photomicrograph showing DCX positive cells in the subventricular zone (SVZ) of the four-striped mouse. DCX positive cells are seen in the photomicrograph in cluster (A and B) with their soma and processes. The soma can be seen on the luminar surface of the SVZ with their processes projecting towards the striatum. RMS- rostral migratory stream, SVZ- subventricular zone, LV- laterals ventricle and Str- striatum. Scale bar; A=10 µm and B=1 µm.

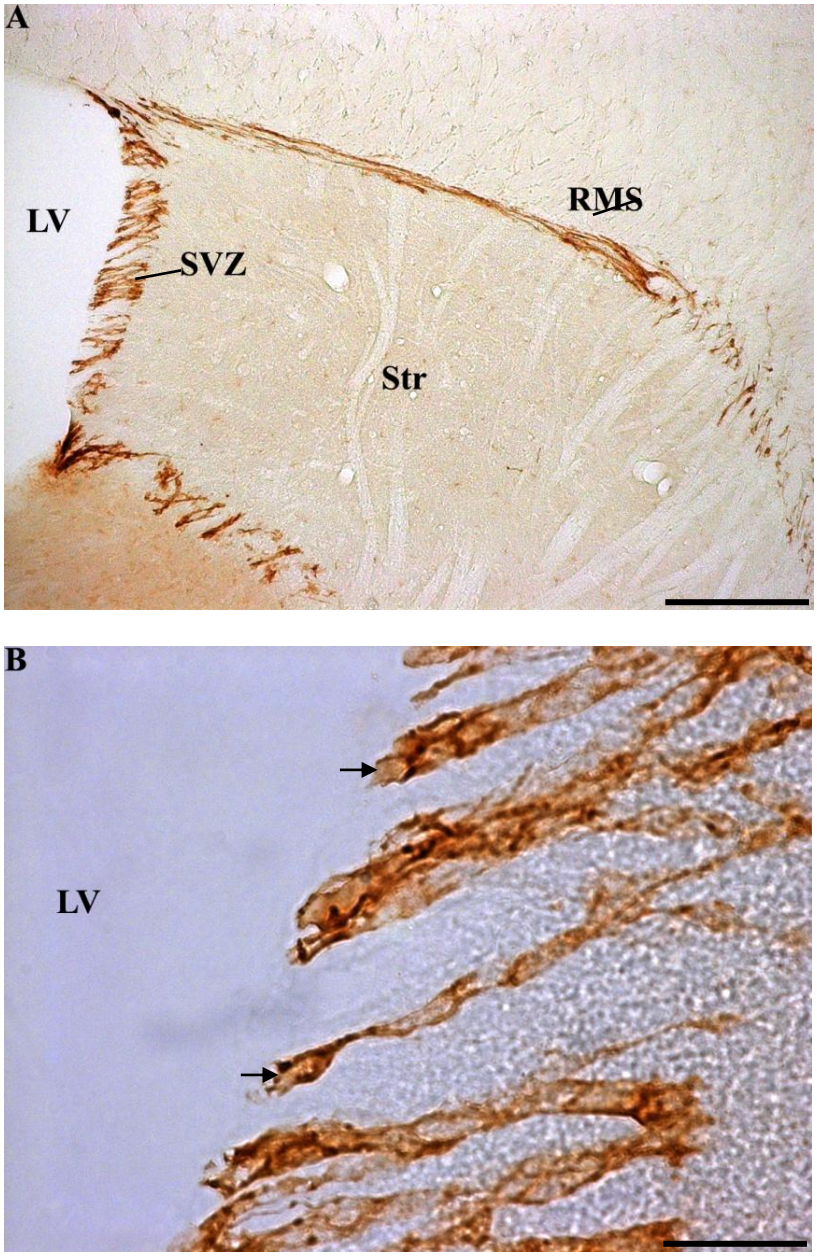

A
LV
SVZ
RMS
Str
B
LV

Figure 3.9: Representative photomicrograph showing DCX immunohistochemistry in the RMS-OB of the four-striped mouse. Figure 3.9 B, shows the six layers of the olfactory bulb as follows; ONL-olfactory nerve layer, GL- glomerular layer, EPL- external plexiform layer, MCL- mitral cell layer, IPL- internal plexiform layer and GCL- granule cell layer. DCX positive cells in cluster (B) with their soma in the granule cell layer and their processes extend as far as the glomerular layer. OB- olfactory bulb and RMS- rostral migratory stream. Scale bar; A= 20 µm, B=10 µm, C and D =2.5 µm.

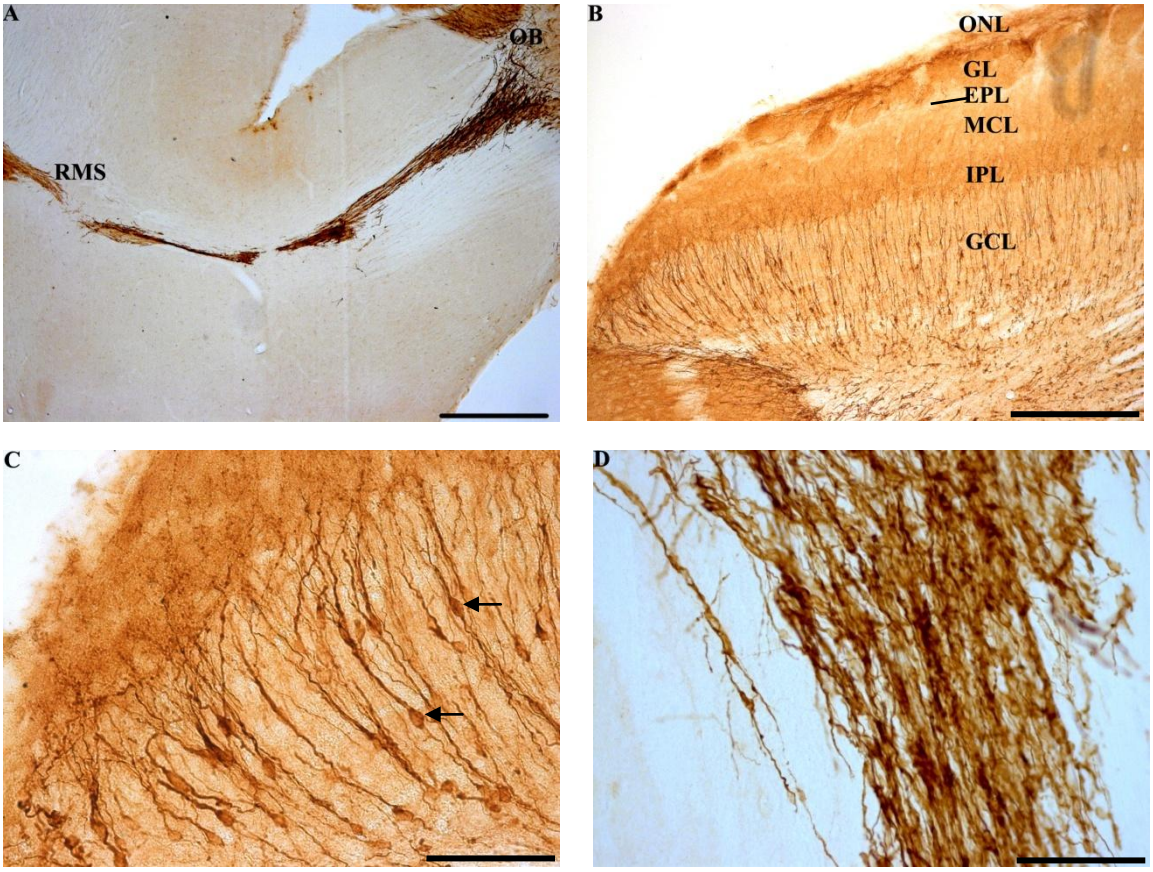

A
OB
RMS
B
ONL
GL
EPL
MCL
IPL
GCL
C
D

Table 4: Summary of active and potential neurogenic sites in the four-striped mouse.

Site	SVZ	DG	SEP\ STR	SN	CTX	OB\ RMS
Cell proliferation (2hrs)	+	+	-	-	-	+/-
Cell Survival (4weeks)	+	+	-	-	-	+/-
Ki-67	++	++	+/-	+/-	-	+++
DCX	+++	+++	+/-	-	-	++++

Key: SVZ, subventricular zone; DG, Dentate gyrus; SEP/ STR, Septum and Striatum; SN, Substantia nigra; CTX, Cerebral cortex; OB/ RMS, Olfactory bulb/ Rostral migratory stream, (+ indicates presence; - indicates absence, +/- indicates indecisive and more than one "+" indicates increasing level of immunopositive staining).

3.3. Immunohistochemical findings in the common mole-rat

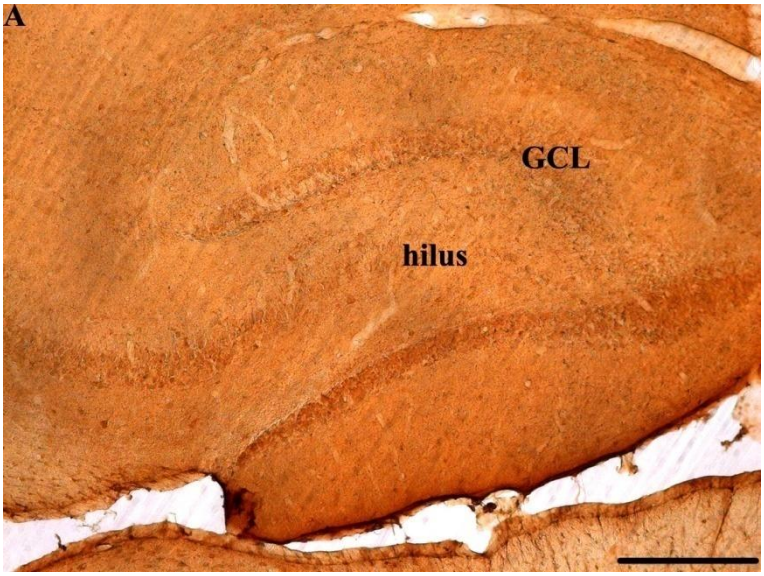
3.3.1. BrdU positive cells in proliferative and survival groups

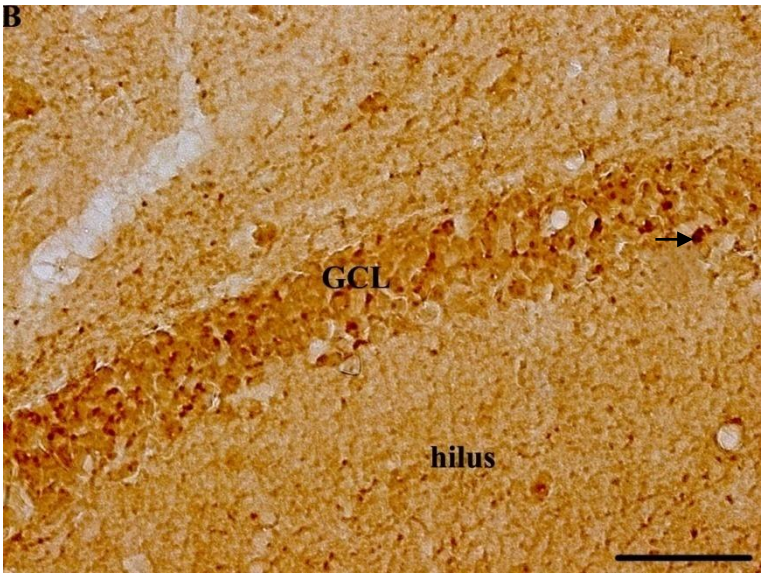
BrdU positive cells were observed in the dentate gyrus of the hippocampus of common mole-rat. In the 2 hours post BrdU injected group, the cells appeared in cluster and irregular in shape with darkly stained nuclei (Figure 3.10, A-C). In the 4 weeks post BrdU injected group, BrdU positive cells appeared isolated and more rounded with darkly stained nuclei (Figure 3.10 C).

In the subventricular zone, BrdU positive cells in the 2 hours group appeared in clusters and irregular in shape with darkly stained nuclei (Figure 3.11). While in the 4 weeks group the BrdU positive cells appeared singly and more rounded in shape with darkly stained nucleus (Figure 3.11 B).

In the olfactory bulb and rostral migratory stream, BrdU positive cells in the 2 hours group appeared in cluster and irregular in shape with darkly stained nuclei (Figure 3.12, A & B). While in the 4 weeks group the BrdU positive cells appeared singly and more rounded in shape with darkly stained nuclei (Figure 3.12B).

Figure 3.10: Representative photomicrograph showing BrdU positive cells in the dentate gyrus of the hippocampus of the common mole-rat in the two time points. In the two hours group, the proliferative cells are darkly stained nuclei in clusters (Figure 3.10 B, arrow) while in the 4 weeks group (Figure 3.10 C), the matured cells appear more rounded and singly. The arrows indicate positive BrdU immunostained cells. SGL- subgranular layer and GCL- granule cell layer. Scale bar; A= 10 µm, B= 2.5 µm, C 1 µm.


A
GCL
hilus


B
GCL
hilus

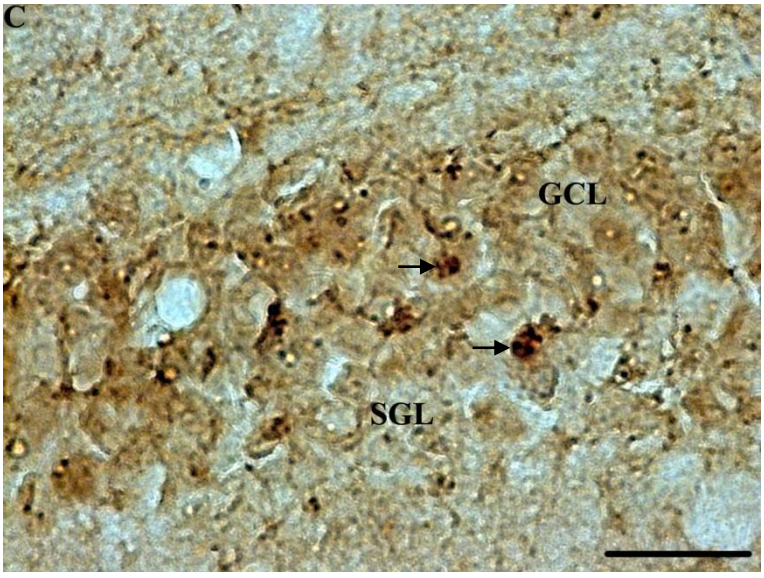
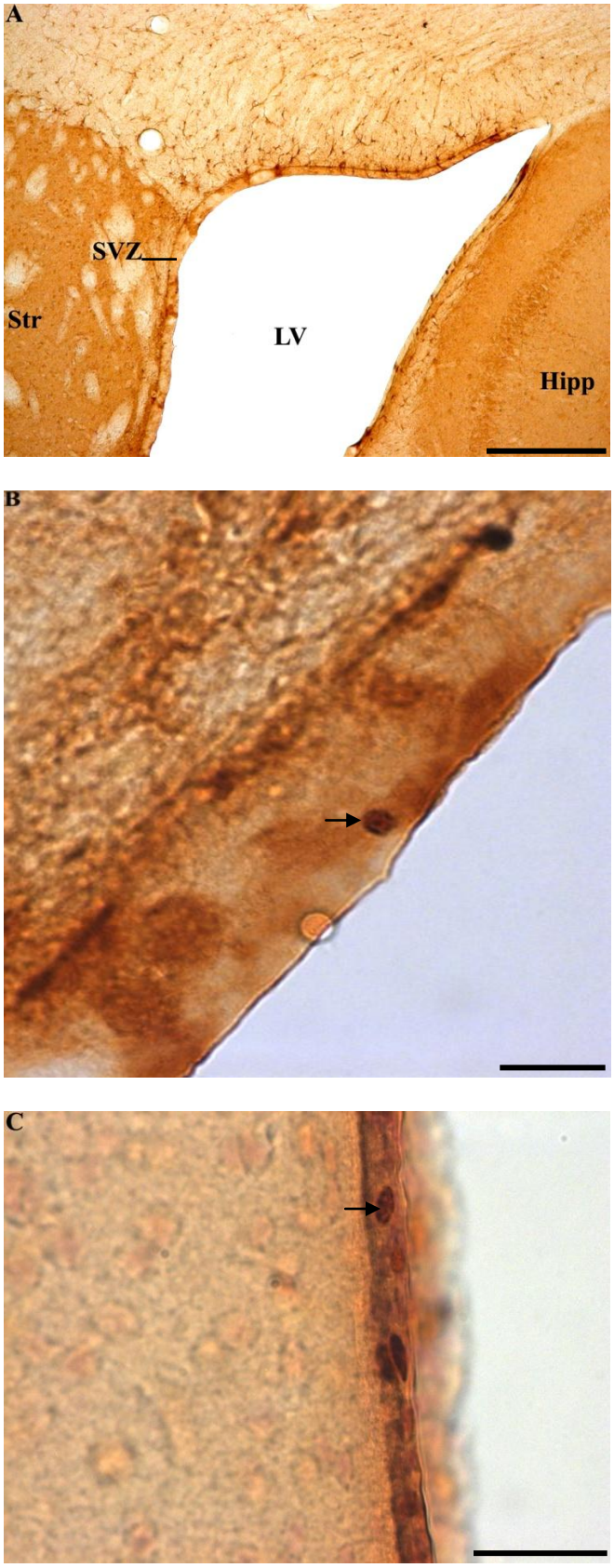

C
GCL
SGL

Figure 3.11: Representative photomicrograph showing BrdU positive cells in the common mole-rat (Figure 3.11, A-B) while in the four weeks (C), they appear more rounded with darkly stained nuclei. The arrows indicate positive BrdU immunostained cells. SVZ- subventricular zone, LV- lateral ventricle, Str- striatum, Hipp- hippocampus and OB- olfactory bulb. Scale bar; A=10 µm, B, C = 1 µm.


A
SVZ
Str
LV
Hipp
B
C

3.3.2. Ki-67 positive cells

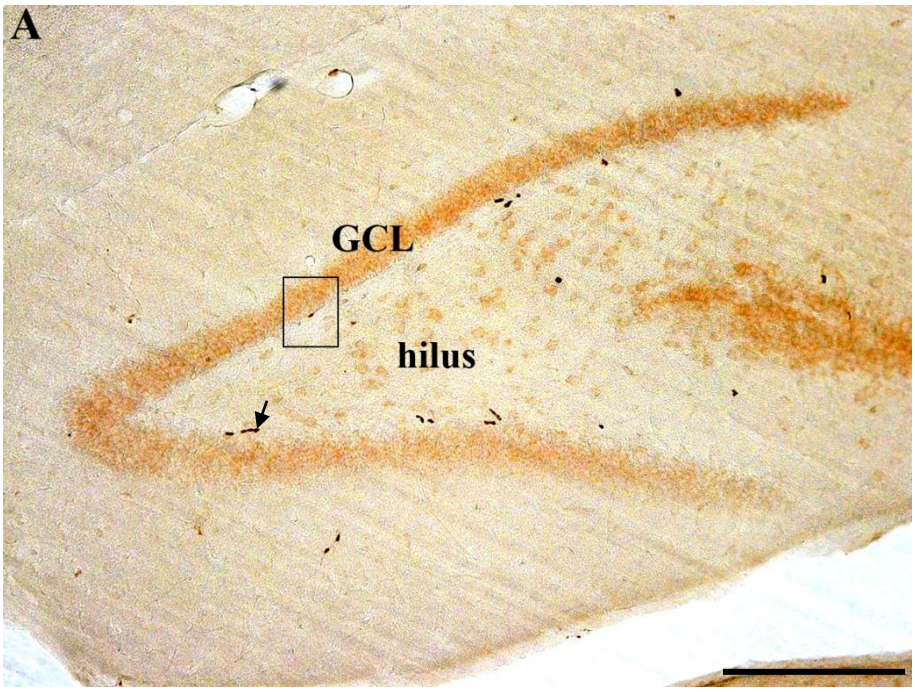
Ki-67 positive cells were present in the subgranular layer of the dentate gyrus of the hippocampus (Figure 3.12, A & B). The Ki-67 positive cells were distributed along the subgranular layer of the dentate gyrus of the hippocampus with darkly stained nuclei (Figure 3.12 B).

The new neurons were found in the subventricular zone, more of which are on the lateral ventricle connecting to the rostral migratory stream (Figure 3.13, A & B). They appeared in clusters with darkly stained nucleus (Figure 3.13 B).

The Ki-67 positive cells in the olfactory bulb and rostral migratory stream appeared in clusters (Figure 3.14, A-C). The ki-67 positive cells were observed from the rostral migratory stream enroute the olfactory bulb (Figure 3.14 A). The Ki-67 positive cells were also observed lining the rostral migratory stream coming from the subventricular zone (Figures 3.14 C).

In the substantia nigra, the Ki-67 positive cells that were observed appeared in clusters and irregular in shape with less prominent nuclei (Figure 3. 15, A & B). Ki-67 positive cells were absent in the striatum and cerebral cortex.

Figure 3.12: Representative photomicrograph showing Ki-67 positive cells in the dentate gyrus of the common mole-rat. Ki-67 positive cells are in cluster and more rounded with darkly stained nuclei (Figure 3.12 B). SGL- subgranular layer and GCL- granule cell layer. Scale bar; A= 10 µm and B=1 µm.


A
GCL
hilus

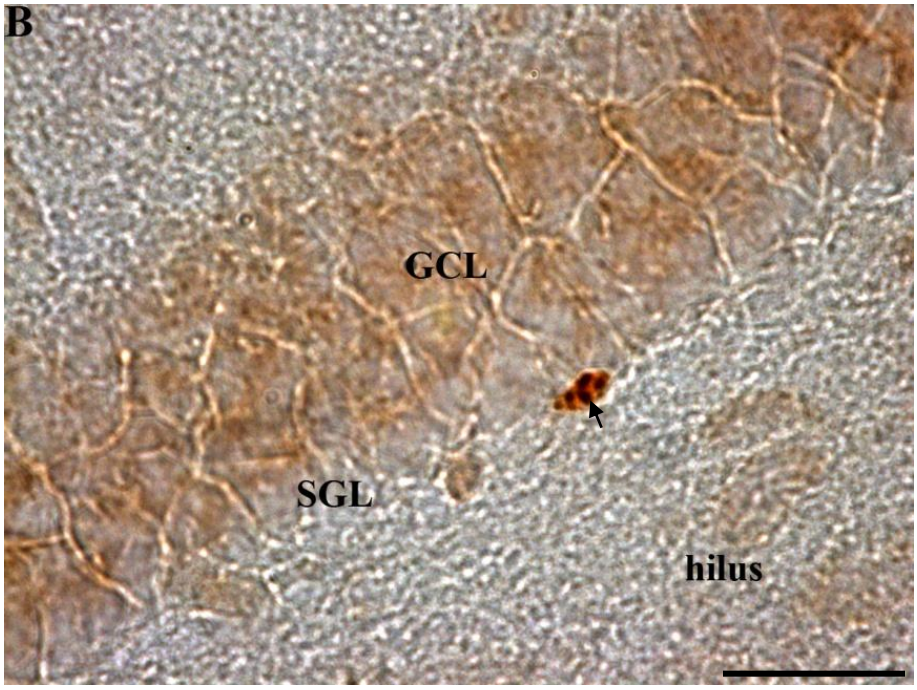

B
GCL
SGL
hilus

Figure 3.13: Representative photomicrograph showing Ki-67 positive cells in the subventricular zone of the common mole-rat. Ki-67 positive cells are in cluster and more rounded with darkly stained nuclei (B). SVZ- subventricular zone, Str- striatum, LV lateral ventricle and RMS- rostral migratory stream. Scale bar; A= 10 µm, B=1 µm.

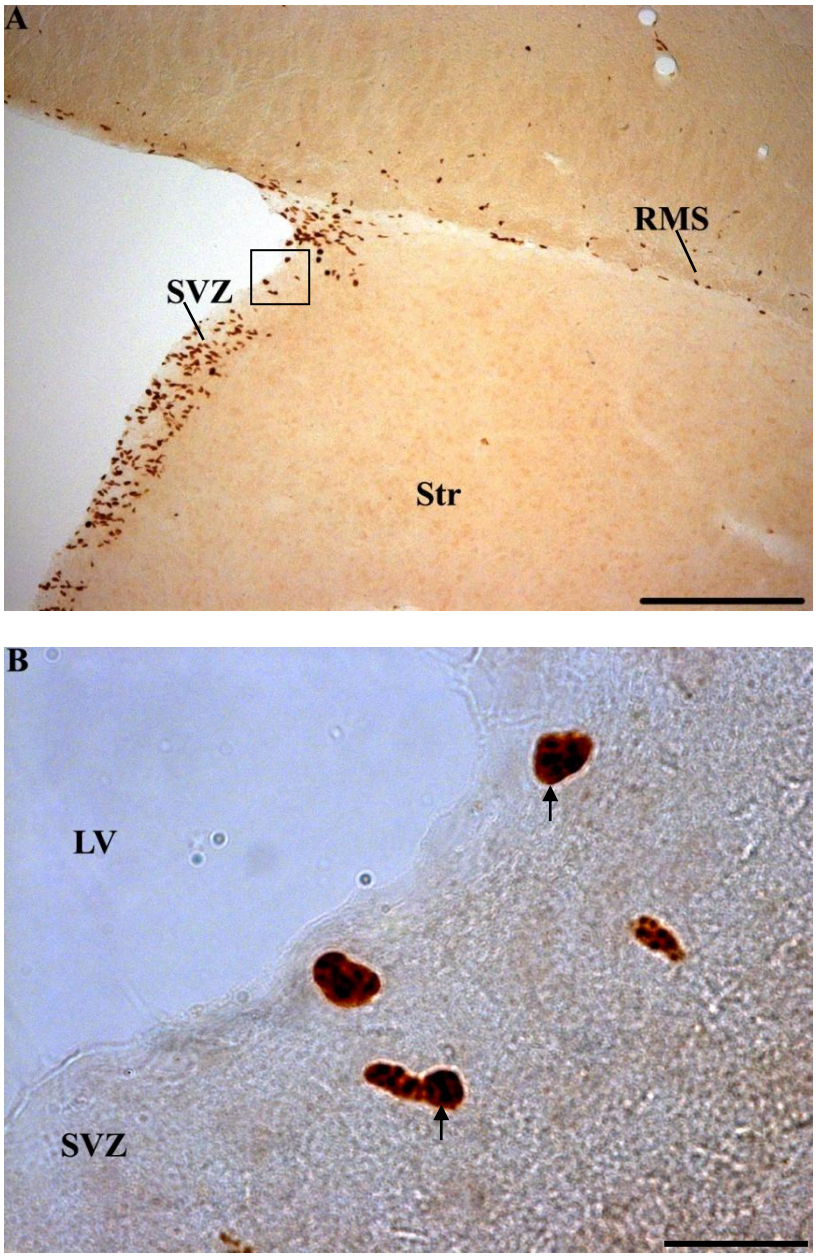
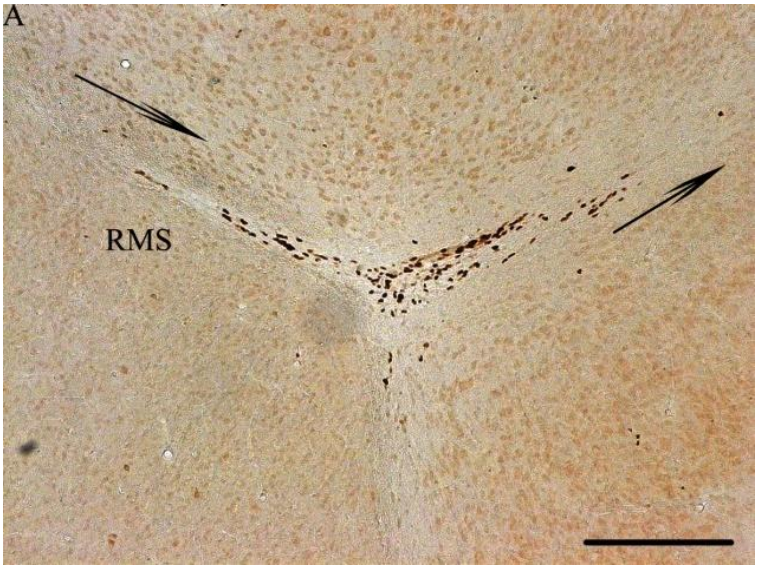
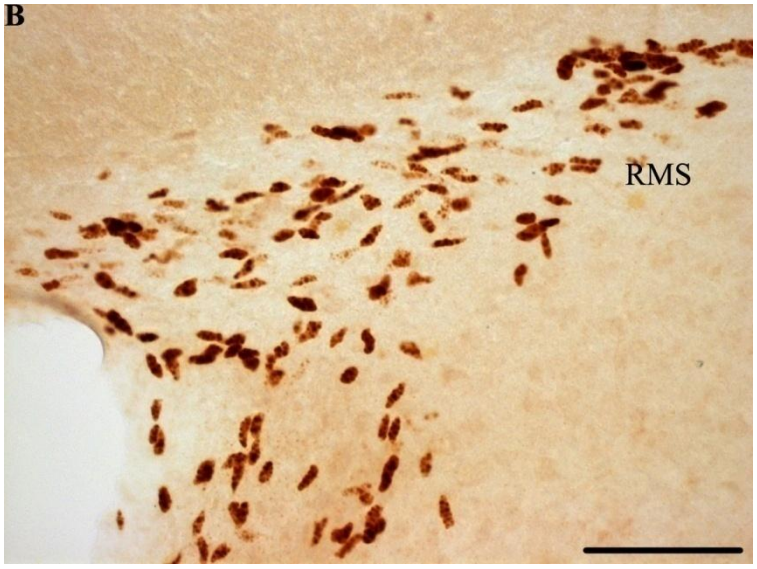

A
RMS
SVZ
Str
B
LV
SVZ

Figure 3.14: Representative photomicrograph showing Ki-67 positive cells in the common mole-rat. Ki-67 positive cells in the rostral migratory stream (A, arrows indicate the direction of migration).Figure B is a higher magnification of A. Figure C shows positive Ki-67 cells in the olfactory bulb. RMS- rostral migratory stream and OB- olfactory bulb. Scale bar; A= 10 µm, B=2.5 µm, C=1 µm.


A
RMS


B
RMS

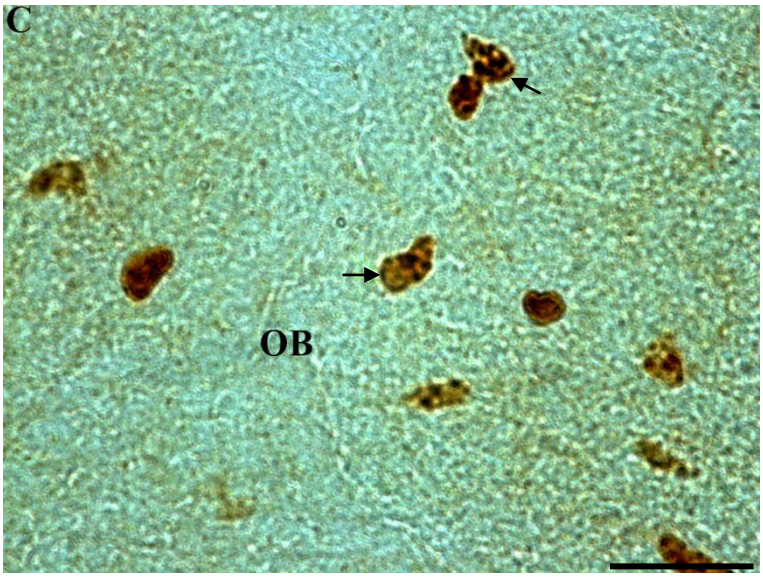
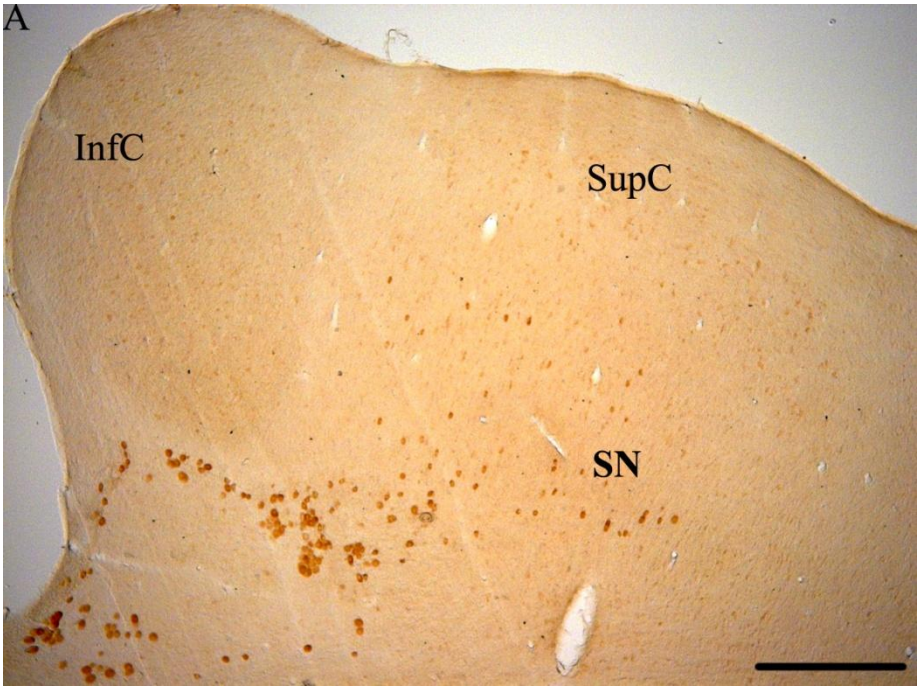
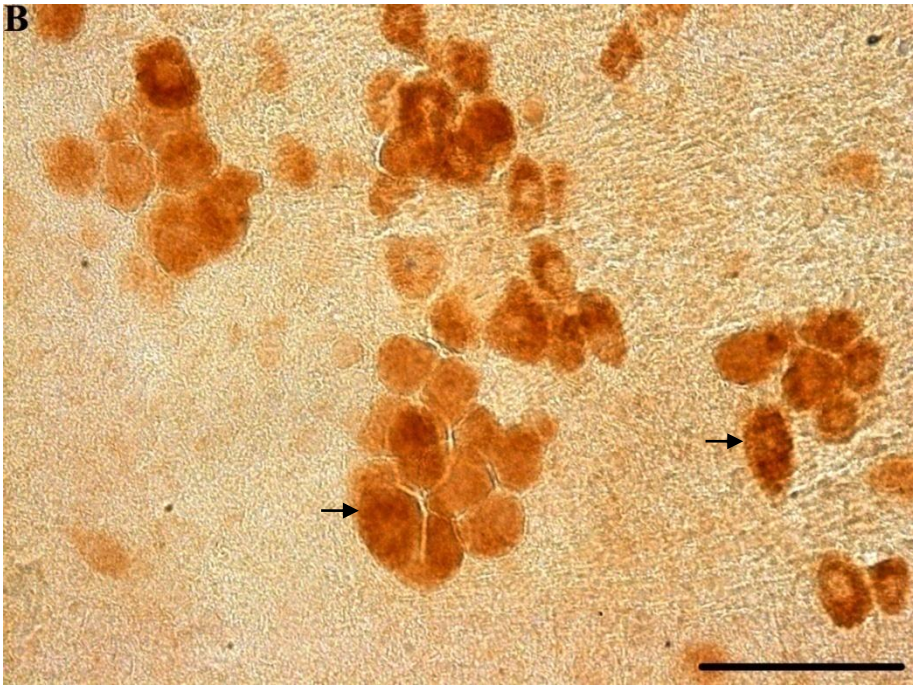

C
OB

Figure 3.15: Representative photomicrograph showing Ki-67 positive cells in the substantia nigra of the common mole-rat. Ki-67 positive cells appear in cluster (arrows) and more rounded (A, and B at higher magnification). InfC- inferior colliculus, SupC- superior colliculus and SN- substantia nigra. Scale bar; A= 10 µm and B =1 µm


A
InfC
SupC
SN


B

3.3.3. Doublecortin (DCX) positive cells

The DCX positive cells in the dentate gyrus of the hippocampus were observed in the subgranular layer of the dentate gyrus of the hippocampus (Figure 3.116, A-C). The soma of these cells appeared ovoid in shape with their dendrites reaching as far as the molecular layer of the dentate gyrus of the hippocampus and some dendrites only ending in the granular layer and could be classified as E and F categories of DCX positive cells (Figure 3.16, B & C).

The DCX positive cells in the subventricular zone appeared in a web-like fashion with their processes. At high magnification, the DCX positive cells presented ovoid shaped soma and branched processes in multi-directional way (Figure 17 B).

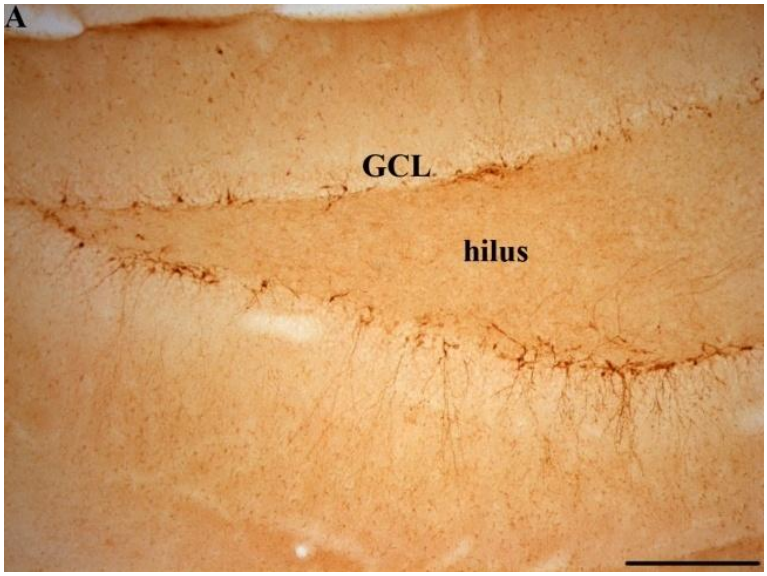
DCX positive cells were observed in the olfactory bulb-rostral migratory stream (Figure 3.18, A-C). These cells appeared in clusters in a uniformed direction. The soma of the DCX positive cells were meshed within processes and located in the granule cell layer of the olfactory bulb with their processes extending as far as the external plexiform layer of the olfactory bulb (Figure 3.18 B). They continued from the subventricular zone via the rostral migratory stream enroute the olfactory bulb (Figure 3.18).

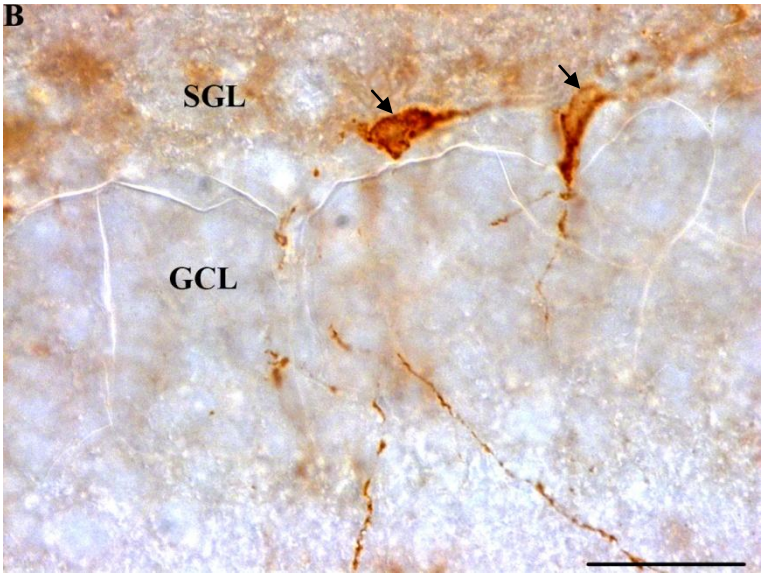
DCX positive cells were observed in the striatum (Figure 3.19, A). These cells appeared isolated with an ovoid soma (Figure 3.19, C & D). The processes are bi-directional with branched dendrites (Figure 3.19, C & D).

There were DCX positive cells present in the cerebral cortex (Figure 3.20, A-C). The majority of the DCX positive cells were observed in the second layer of the somatosensory, entorhinal and piriform cortices. The DCX positive neuron in

layer II had bipolar and ovoid shaped soma (Figure 3.20 C). Small number of the DCX positive cells has an ovoid shaped soma with apical dendrites which bifurcates (Figure 3.20, B & C). Some DCX positive cells were observed in layer III with bipolar and some pyramidal in shape (Figure 3.20 A, red arrow).

Figure 3.16: Representative photomicrograph showing DCX positive cells in the dentate gyrus of the common mole-rat. DCX positive cells appear in cluster (A) with their soma and definite processes which is category B can C respectively (B and C). The soma can be seen in the SGL with their processes projecting as far as into the molecular layer. SGL- subgranular layer and GCL- granule cell layer. Scale bar; A=10 µm, B and C=1 µm.


A
GCL
hilus


B
SGL
GCL

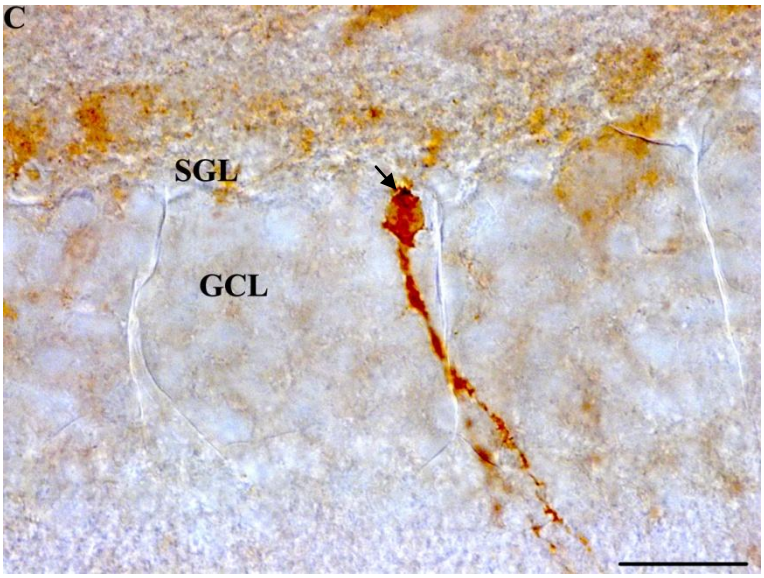
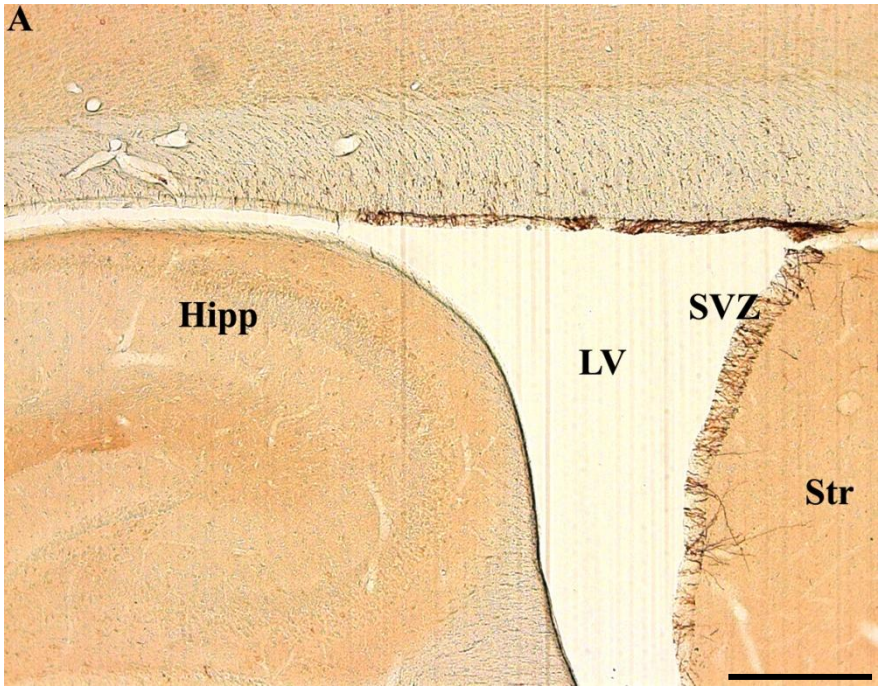

C
SGL
GCL

Figure 3.17: Representative photomicrograph showing DCX positive cells in the subventricular zone of the common mole-rat. DCX positive cells are in cluster (B) with their soma in the wall of the SVZ and processes projecting into the striatum. The soma can be seen on the wall of the SVZ with their processes projecting as far as into the Striatum. LV- lateral ventricle, SVZ- subventricular zone, Hipp- hippocampus and Str- striatum. Scale bar; A=20 µm, B=1 µm.


A
Hipp
LV
SVZ
Str

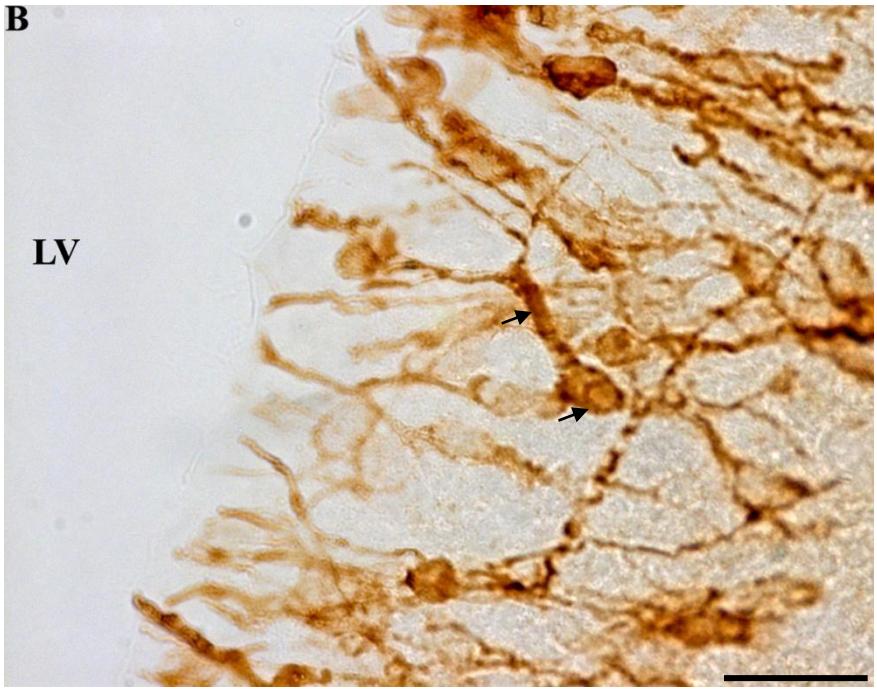

B
LV

Figure 3.18: Representative photomicrograph showing DCX positive cells in the rostral migratory stream/ olfactory bulb of the common mole-rat. DCX positive cells are in cluster (B and C) with their soma and processes (C). Figure 3.18 A, shows the six layers of the olfactory bulb as follows; ONL-olfactory nerve layer, GL- glomerular layer, EPL- external plexiform layer, MCL- mitral cell layer, IPL- internal plexiform layer and GCL- granule cell layer. The soma can be seen in the granule cell layer with their processes projecting into the glomerular layer. OB- olfactory bulb and RMS- rostral migratory stream. Scale bar; A=20 µm, B and C=2.5 µm.

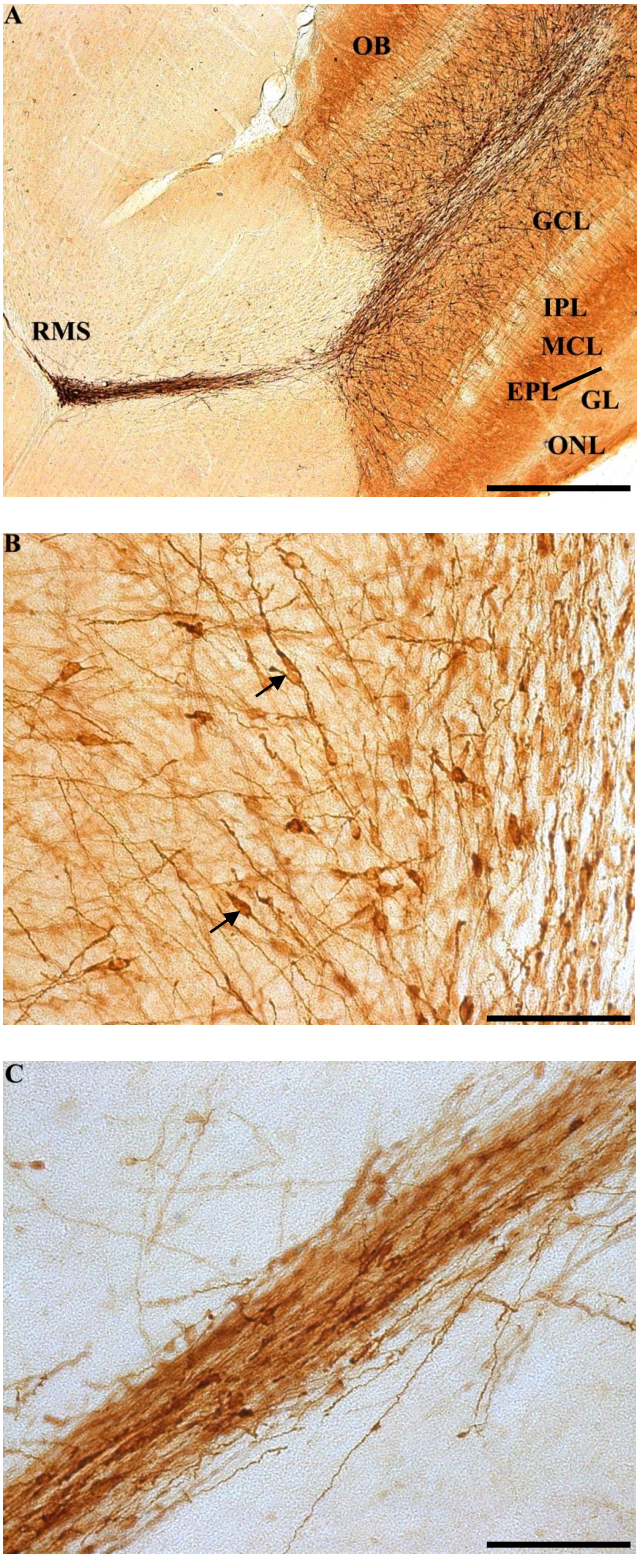
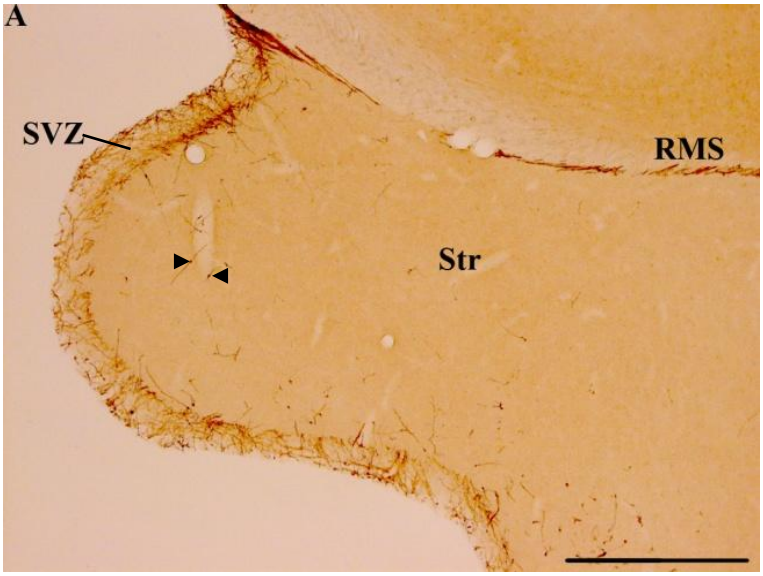
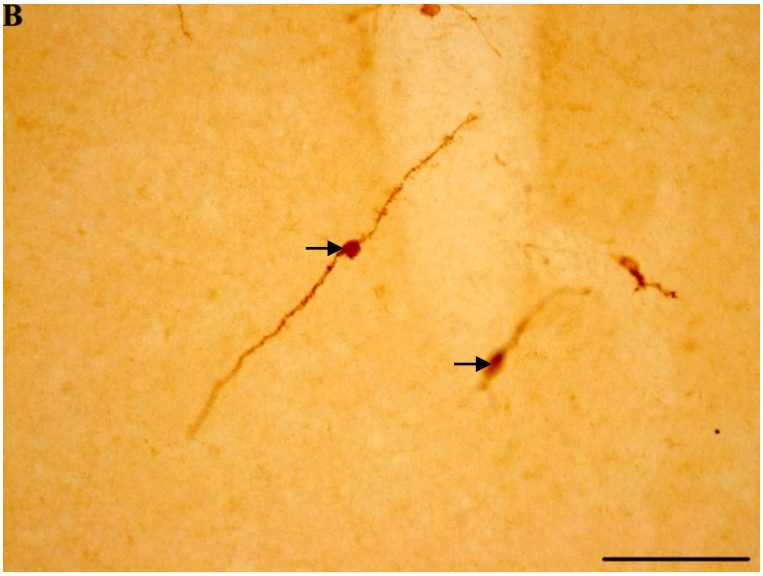

A
OB
GCL
IPL
MCL
EPL
GL
ONL
RMS
B
C

Figure 3.19: Representative photomicrograph showing DCX positive cells in the striatum of the common mole-rat. DCX positive cells appearing more singly (B-D) with their soma and processes (C and D). The soma can be seen with bifurcated processes projecting out. SVZ- subventricular zone, RMS- rostral migratory stream and Str- striatum. Scale bar; A =20 µm, B=2.5 µm, C and D=1 µm.


A
SVZ
RMS
Str


B

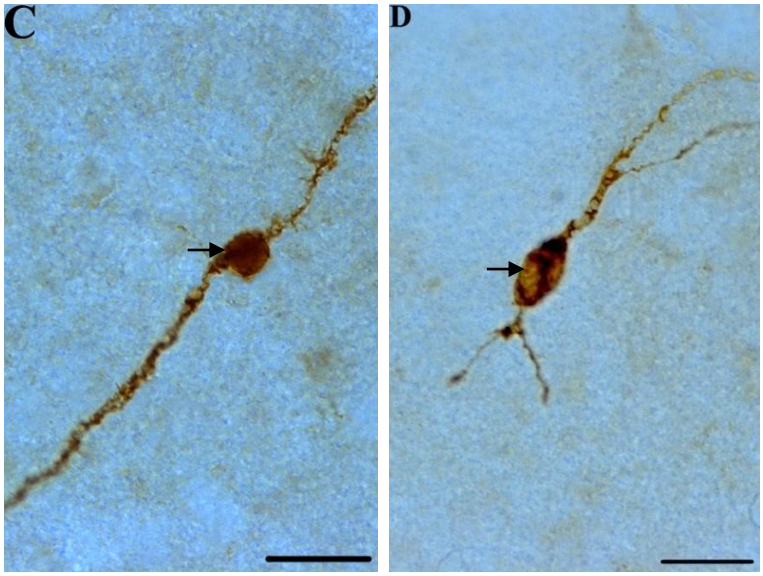
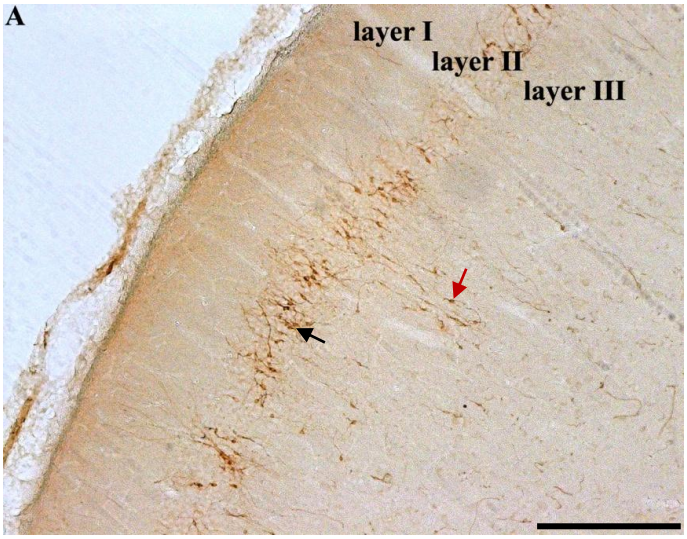
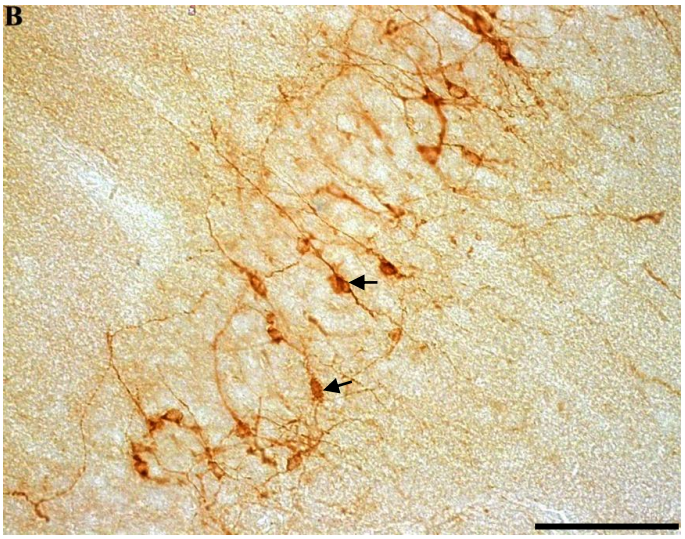

C
D

Figure 3.20: Representative photomicrograph showing DCX positive cells in the cerebral cortex of the common mole-rat. DCX positive cells appear in clusters (C) with their soma and processes (C). The DCX positive cells are more numerous in layer II (B) and in layer III (A, red arrows). Scale bar; A =10 µm, B=2.5 µm, C=1 µm.


A
layer I
layer II
layer III


B

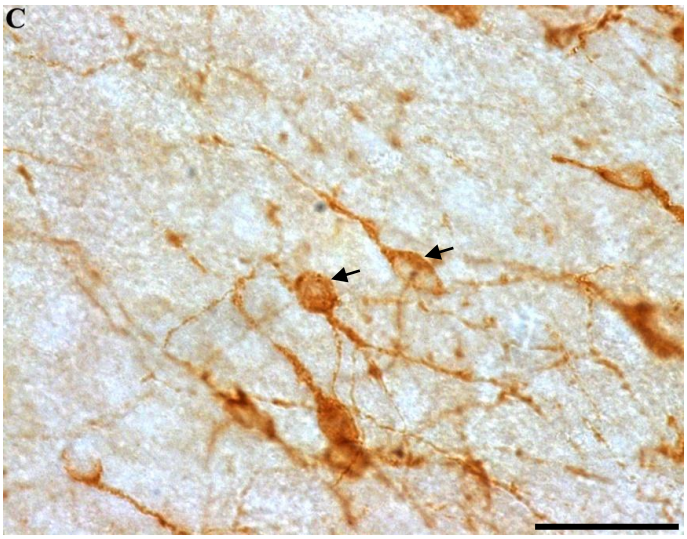

C

Table 5: Summary of active and potential neurogenic sites in the common mole-rat.

Site	SVZ	DG	SEP\ STR	SN	CTX	OB\ RMS
Cell proliferation (2hrs)	+	+	-	-	-	+/-
Cell Survival (4weeks)	+	+	-	-	-	+/-
Ki-67	++	++	-	+/-	-	+++
DCX	+++	+++	+/-	+	++++	++++

Key: SVZ, subventricular zone; DG, Dentate gyrus; SEP/ STR, Septum and Striatum; SN, Substantia nigra; CTX, Cerebral cortex; OB/ RMS, Olfactory bulb/ Rostral migratory stream, (+ indicates presence; - indicates absence, +/- indicates indecisive and more than one "+" indicates increasing level of immunopositive staining).

CHAPTER FOUR: DISCUSSION

This study was to provide a descriptive evidence of the existence of adult neurogenesis in the brain of two South African rodents; the four-striped mouse (*Rhabdomys pumilio*), a captive bred animal originating from wild caught parents and the common mole-rat (*Cryptomys hottentotus*), wild caught animals compared to inbred, laboratory strains of rodents that were studied previously (Nacher *et al.*, 2001; Kee *et al.*, 2002; McDonald and Wojtwicz, 2005; Plumpe *et al.*, 2006; Zhang *et al.*, 2009).

A combination of BrdU, Ki-67 and DCX immunohistochemistry has provided evidence of adult neurogenesis in the active sites and in some potential sites in the four-striped mouse and common mole-rat. There were variations based on the different markers and animal species.

4.1. Adult neurogenesis in the established active neurogenic sites

In the adult brain, the subventricular zone and dentate gyrus of the hippocampus remain the active neurogenic sites in adult neurogenesis; these have been assessed by different cell markers for cell proliferation (Eriksson *et al.*, 1998; Nacher *et al.*, 2001; Pham *et al.*, 2003; Schauwecker, 2006; Plumpe *et al.*, 2006; Lagace *et al.*, 2007; Kim *et al.*, 2009; Hauser *et al.*, 2009). BrdU is cleared from the rat brain within a short phase of 2 hours (Cameron and McKay, 2007), endogenous Ki-67 is altered during active cell cycle (Hayes and Nowakowski, 2002) and DCX stains immature neurons (Lu et al., 2005). The subventricular zone has two precise layers of cells: the first is a monolayer of multiciliated cells lining the lateral ventricle called the ependymal layer; and the second layer is a 2-

3 cell layer thick area adjacent to the ependymal layer called the subependymal layer (Alvarez-Buylla and Garcia-Verdugo, 2002). Although the exact identity of the cell lineage in the subventricular zone was not part of this study, it is evident that adult neurogenesis do occur at this site in the four-striped mouse and common mole-rat. The subventricular zone neuroblast cells travel a long distance to the olfactory bulb through a network of interconnecting pathways that become confluent at the rostral margin of the lateral ventricular wall to form the rostral migratory stream (Watts *et al.,* 2005). This was also present in the four-striped mouse and common mole-rat.

In the hippocampus, few BrdU positive cells were also observed compared to previous work in which the same type of markers were used in rats (Lagace *et al.,* 2007). This could have been as a result of the limitation in the cell cycle stage at which the BrdU stains the proliferating cells. BrdU staining is limited to the S-phase of the cell cycle while Ki-67 stains all the phases except the resting (G_o) phase (Alvarez-Buylla and Garcia-Verdugo, 2002). There are reports of the use of repeated doses of BrdU to enhance cell proliferation (Nacher *et al.,* 2001; Kee *et al.,* 2002) with the associated toxicity (Taupin, 2007). Consequently, a single dose of BrdU was administered in this study and may have contributed to the sparse distribution and identification of the positive cells but with less toxicity in the four-striped mouse and common mole-rat.

In addition to the question of dose and toxicity of BrdU, the procedure of its injection has been proposed to induce an element of stress which could also decrease neurogenesis. Although Gould et al., (1997) reported a decrease in adult neurogenesis in the tree shrew following psychosocial stress, the reduced staining

of BrdU positive cells in this study cannot be linked to stress as this was minimal during the injection of BrdU. The blood-brain barrier that limits the penetration of BrdU and its non specificity for labelling dividing cells (Taupin, 2007) may also be associated with less staining. Apoptosis (Biebl *et al.,* 2000) may also contribute to the low turn-out of BrdU positive cells in these sites but this was not tested in this investigation. A similar experiment was performed on different strains of adult laboratory animals, BALB/ c, C3H/ H3J, DBA/ 2J, 129/ SVJ and CD1, by Kempermann *et al.,* (1997) showed some BrdU positive cells were identified although in varying amounts. This led Kempermann *et al.,* (1997) to conclude that this might be as a result of strain differences which may reflect genetic differences of the laboratory animal that were used.

Ki-67 immunostaining was used as a confirmatory study to the BrdU staining and the results indicated adult neurogenesis in the four-striped mouse and common mole-rat. Hauser *et al.*, (2009), confirmed in the wood mouse that the subventricular zone was a site of cell proliferation not only in laboratory animals but also in wild non captive animals. The report correlated with the result from this study that adult neurogenesis is present in the subventricular zone and dentate gyrus of the hippocampus captive reared four-striped mouse and wild caught common mole-rat using the Ki-67 marker.

Ki-67 positive cells are numerous in the subventricular zone and they can be seen throughout the wall of the subventricular zone forming a kind of confluence which then continues as the rostral migratory stream. Reports have indicated a low rate of BrdU positive cells in the dentate gyrus of the hippocampus in Sprague-Dawley rats (Nacher *et al.,* 2001). Likewise in

previously studied adult animals like laboratory rodents, dogs and monkeys, it is suggested that the migration of newly generated cells in the hippocampus might be hindered in these experimental animals (Siwak-Tapp *et al.,* 2007; Zhang *et al.,* 2009). However in this study, DCX immunohistochemistry showed DCX positive cells in the dentate gyrus of the hippocampus with processes projecting from the subgranular layer to the molecular layer as confirmed by previous studies (Plumpe *et al.,* 2006; Hauser *et al.,* 2009; Kim *et al.,* 2009; Zhang *et al.,* 2009). The cells were observed in the subgranular layer of the dentate gyrus of the hippocampus and the processes extending as far as the molecular layer where synaptic contacts are expected to be established. With this outcome, it confirms that the subventricular zone and the dentate gyrus of the hippocampus is a continuous adult neurogenic site as reported (Amrein *et al.,* 2004; Ihunwo and Pillay, 2007; Hauser *et al.,* 2009). From the result of Ki-67 and DCX immunohistochemistry, adult neurogenesis does occur in the four-striped mouse and common mole-rat. However, results of the BrdU do indicate some technical shortcomings.

4.2. Adult neurogenesis in the potential neurogenic sites

Other regions of the brain investigated for adult neurogenesis were the olfactory bulb, rostral migratory stream, substantia nigra, cortex and striatum. The result showed that cells from the subventricular zone migrated to the olfactory bulb via the rostral migratory stream in the four-striped mouse and common mole-rat. However, there were no BrdU positive cells in the substantia nigra (Zhao *et al.,* 2003), cortex (Takemura, 2005), striatum (Luzzati *et al.,* 2007) and amygdala (Bernier *et al.,* 2002) as against the findings in the laboratory rodents.

Although, it was reported that adult neurogenesis may be restricted to a particular region of the brain (Palmer *et al.*, 1995), potential adult neurogenic sites were confirmed using additional markers namely, Ki-67 and DCX markers for immature cells, that stained more cell stages since the BrdU marker used in this experiment though did not stain other neurogenic sites in the brains of the four-striped mouse and common mole-rat. In the four-striped mouse, Ki-67 positive cells were observed in striatum as darkly stained cells which could have been as a result of migrating cells from the rostral migrating stream or they even deviated and incorporated into the striatum. The converse was true for the common mole-rat as no Ki-67 positive cells were observed in the striatum.

Most of the DCX positive cells in the subventricular zone are believed to migrate to the olfactory bulb and incorporate there (Bartkowska *et al.,* 2010) while some may deviate from this track and move inferiorly towards the striatum or superiorly towards the frontal cortex between layers II and III. These cells on reaching the striatum are incorporated depending on the state of the cell or might undergo apoptosis (Biebl *et al.* 2000). Though absent in the cerebral cortex of the four-striped mouse, DCX positive cells were observed in the somatosensory cortex of the brain of the common mole-rat. Luzzati *et al.,* (2007) proposed that neurogenesis in these potential neurogenic regions can be due to progenitors derived from the subventricular germinal zone and/ or local parenchyma progenitor. However, the presence of immature neurons in the somatosensory and entorhinal cortices of the common mole-rat may be associated with the social lifestyle of the common mole-rat with its continuous burrowing which indicate a motor function of the brain. Mole rats have keen burrowing habits especially at

nights and produce different compartments with tunnels of about one kilometre and 15 centimetres to 20 centimetres below the ground surface. Eloff (1952) cited from Jarvis and Sale, (1971) observed that the foot vibration of *Cryptomys* during digging last about one second and the vibrations are between 25 to 30 times. The presence of cell proliferation in the entorhinal and piriform cortices associated with memory may provide the explanation for the common mole-rat navigation of each compartment with the burrows.

Adult neurogenesis remains a complex process involving the proliferation, survival, differentiation and functional integration of new cells in the brain. The cellular and molecular mechanisms that regulate adult neurogenesis remain unclear (Schauwecker, 2006) but can be modulated by a variety of factors including glutamate receptor activation (Cameron et al., 1995; Gould *et al.*, 1997; Bernabeu and Sharp, 2000), dietary restriction (Lee *et al.*, 2002a, b), growth factors (Scharfman *et al.*, 2005; Palmer *et al.*, 1995), stress (Brunson *et al.*, 2005; Nichols, *et al.*, 2005) and neuronal injury (Parent, 2003; Cooper-Kuhn *et al.*, 2004). Enriched environments (Kempermann *et al.*, 1997), running wheel exercise (van Praag *et al.*, 1999; Hauser *et al.*, 2009), hippocampal-dependent learning (Gould *et al.*, 1996) and dietary restriction (Lee *et al.*, 2002a), all these increase adult neurogenesis in the adult hippocampus. It was reported that estradiol alter adult hippocampal neurogenesis in female rodents (Galea *et al.*, 2006) but decreases in hippocampal neurogenesis do not always correlate with the development of learned helplessness in male rats (Vollmayr *et al.*, 2003).

With the different environment from which the four-striped mouse and common mole-rat were obtained, it might be of interest to further investigate the

possibility that these might influence the rate of cell proliferation in these animal models in terms of an enriched and survival instinct of these animals in the wild.

CHAPTER FIVE: CONCLUSION AND FURTHER STUDIES

5.1. Conclusion

This study has provided evidence of adult neurogenesis in the four-striped mouse (*Rhabdomys pumilio*) and common mole-rat (*Cryptomys hottentotus*) using the BrdU, Ki-67 and DCX markers for cell proliferation. The results showed adult neurogenesis in the two active sites; subventricular zone of the lateral ventricle and the dentate gyrus of the hippocampus.

BrdU labelled the nuclei of proliferating cells which appeared dark and clustered in the two hours post BrdU injected group. For the four weeks post BrdU injection (survival) group the BrdU positive cells appeared more singly with darkly stained nuclei and more rounded in the two active neurogenic sites for both the four-striped mouse and common mole-rat. BrdU positive cells were absent in other potential sites in both the four-striped mouse and common mole-rat.

Ki-69, an intrinsic marker for proliferating cells, labelled the nuclei of the proliferating cells which appeared in clusters in almost all the region that was observed. More new neurons were stained appearing in clusters and in some areas in isolated forms. Ki-67 labels proliferating cells in the two active neurogenic sites, subventricular zone and dentate gyrus of the hippocampus of the two experimental animals. In the potential neurogenic sites, Ki-67 positive cells were observed in the substantia nigra, striatum and olfactory bulb of the four-striped mouse while in the common mole-rat, Ki-67 positive cells were present in the substantia nigra and olfactory bulb but absent in the striatum.

DCX, an intrinsic marker for immature neurons, labelled the cytoplasm of the immature cells along with their processes with most falling under the category D, processes reaching molecular layer of the dentate gyrus of the hippocampus. They appeared in clusters but varied in shape. DCX positive cells were observed in the two neurogenic sites of the experimental animals. In the potential neurogenic sites, they were observed in the striatum and olfactory bulb of the four-striped mouse but absent in their substantial nigra. In the common mole-rat, DCX positive cells were present in the striatum, substantia nigra and olfactory bulb. In addition to these potential neurogenic sites in the common mole-rat, DCX positive cells were observed in the cerebral in the somatosensory and entorhinal cortices of the cerebral cortex but absent in the four-striped mouse.

Such cell proliferation in the cerebral cortex opens up the potential sites for further investigation in the light of reports on cerebral cortex as a reactive site. The substantial nigra, in both the four-striped mouse and common mole-rat indicate it is a potential site which must be further investigated for adult neurogenesis.

5.2. Further studies

With the descriptive qualitative results obtained so far, it becomes imperative for further investigation to be conducted in the area of quantification of newly formed cells and the cell numbers of the dentate gyrus of the hippocampus. Further research will therefore focus on:

1. Cell counting of the Ki-67 positive cells in the dentate gyrus of the hippocampus for possible correlation with pyknotic cells.

2. Plastic embedding of the second half of the cerebral hemispheres followed by sectioning at 20 µm and staining with Giemsa staining for identification of pyknotic cells and correlation of dentate gyrus for quantification.
3. Cell counting of pyknotic cells in the dentate gyrus of the hippocampus for correlation with Ki-67 proliferating cells.
4. Total granule cell count of the dentate gyrus of the hippocampus using the optical fractionator stereology which will be performed at the University of Zurich, Switzerland.

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