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Adult neurogenesis in the brain of Chiroptera

Richard Chawana

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PhD Thesis

Supervisor: Professor Paul Robert Manger

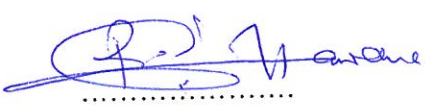
Co-supervisor: Associate Professor Amadi Ogonda Ihunwo

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy.

April, 2016

DECLARATION

This research is my original work, produced with normal supervisory assistance from my supervisor. All the relevant sources of knowledge that I have used during the course of writing this dissertation have been fully credited and acknowledged. Furthermore, this research report has not been submitted for any academic or examination purpose at any other university.



Richard Chawana

28/04/2016

Date

This research paper has been submitted for examination with my approval as the University supervisor(s) for the aforementioned student.



Assoc. Prof. Amadi O. Ihunwo

28/4/2016

Date



Prof. Paul R. Manger

28/4/2016

Date

Matidaishe and Itumeleng

The pursuit of Happyness

To God be the glory

Publications arising from this thesis:

- Chawana, R., Alagaili, A., Patzke, N., Spocter, MA., Mohammed, OB., Kaswera, C., Gilissen, E., Bennett, NC., Ihunwo, AO., Manger, PR. (2014) Microbats appear to have adult hippocampal neurogenesis, but post-capture stress causes a rapid decline in the number of neurons expressing doublecortin. *Neuroscience*, **277**, 724-733.
- Chawana, R., Patzke, N., Kaswera, C., Gilissen, E., Ihunwo, AO., Manger, PR. (2013). Adult neurogenesis in eight species of megachiroptera. *Neuroscience,* **244**, 159-172.
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Abstract

The current thesis, studying adult neurogenesis in the brains of Chiroptera (bats), is a collection of four related studies investigating the occurrence of neurogenesis in the two suborders of adult bats, megachiroptera (megabats) and microchiroptera (microbats), from different environments, including the wild and captive habitats. The studies were carried out in order to understand the dynamics associated with adult neurogenesis in mammals living in their natural habitat given that much of the current understanding is based on experiments done on laboratory bred or captive raised animals. The investigation of megachiropterans and microchiropterans was stimulated by the findings of a previous study which failed to show adult neurogenesis in some microchiropteran species, which is in contrast to the almost universal occurrence of the phenomenon in nearly all mammals. In addition, the use of chiropterans was appealing given their behavioural attributes, which have been previously associated with the occurrence of neurogenesis. These include such behaviours as good spatial abilities, high sociality and complex behaviours such as fusion-fission sociality. In addition, the highly debatable evolutionary history of chiropterans provided a framework in which to evaluate specific neural characters in terms of phylogenetic relationships.

Using immunohistochemical methods, the presence and characteristics of proliferating and newly generated neurons in the brain of eight wild-caught adult megachiropteran species was examined. For the neurogenic patterns observed, direct homologies were evident in other mammalian species. Numerous proliferating cells and immature neurons were identified in the subventricular zone (SVZ) and the dentate gyrus. From the SVZ, these cells migrated to the olfactory bulb through a typically mammalian rostral migratory stream (RMS). Some newly-generated cells were observed emerging from the RMS to the neocortex. Similar to primates,

proliferating cells and immature neurons were identified in the SVZ of the temporal horn of the lateral ventricle of the megachiropterans and were observed to migrate to the rostral and caudal piriform cortex through a primate-like temporal migratory stream.

A similar study using three microchiropteran species revealed almost similar findings. However, distinct differences to the megachiropterans were noted, especially so in the migratory pathway to the piriform cortex, where cells appeared to migrate from the RMS through an insectivore-like ventral migratory stream to populate the entire piriform cortex. In addition microchiropterans had immature axons in the anterior commissure, something which was not observed in megachiropterans but was previously reported in insectivores.

Using immunohistochemical and stereological methods, the effect of animal capture and handling on the occurrence of adult neurogenesis in 10 microchiropterans species was investigated. These animals were euthanized and perfusion fixed at specific time points following capture to investigate the effect of stress as a possible explanation for the negative findings regarding adult hippocampal neurogenesis in microchiropterans reported in a previous study. This investigation revealed that when euthanized and perfused within 15 minutes of capture, abundant putative adult hippocampal neurogenesis could be detected using doublecortin immunohistochemistry, but the ability to readily observe these cells rapidly diminishes if the microchiropterans have not been euthanased within 15 minutes of capture.

Also using immunohistochemical and stereological methods, proliferative and immature cells within the dentate gyrus of adult Egyptian fruit bats from three distinct environments (fifth generation captive bred, wild-caught from the primary rainforest of central Africa and wild-caught from the South African woodlands) was quantified and compared. Four previously

reported methods to assess the effect of the environment on proliferative and immature cells were used. These include: (1) the comparison of raw totals of proliferative and immature cells; and these totals standardized to (2) brain mass, (3) the volume of the granular cell layer (GCL), and (4) the total number of granule cells in the dentate gyrus. For all methods, the numbers of proliferative cells did not differ statistically amongst the three groups. For the immature cells standardizations to brain mass and GCL volume revealed no difference between the three groups studied; however, the raw numbers and standardization to total granule cell numbers indicated that the two groups of wild-caught bats had significantly higher numbers of immature neurons than the captive-bred bats.

In conclusion, the observation of the ventral migratory stream in the microchiropterans and insectivores, in contrast to the temporal migratory stream in megachiropterans and primates adds another neural characteristic supporting the diphyletic origin of Chiroptera, and aligns microchiropterans with insectivores and megachiropterans with primates. In microchiropterans, the presence of doublecortin, revealing adult neurogenesis, in the hippocampus is highly sensitive to capture and handling. Lastly, the interpretation of the effect of the environment on the numbers of immature neurons appears method dependent. It is possible that current methods are not sensitive enough to reveal the effect of different environments on proliferative and immature cells.

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Table of Contents

Item		Page
Declaration		ii
Dedication		iii
Publications arising from the thesis		iv
Presentation arising from the thesis		v
Abstract		vi
Acknowledgements		ix
Table of contents		xi
List of Tables		xviii
List of figures		xix
List of abbreviations		xxiii
CHAPTER ONE:	**Adult neurogenesis: a blind leap into the wild?**	**1**
1.1	Introduction	1
1.2	Adult mammalian neuronal stem cells: characterization and properties	3
1.3	Adult neurogenic microenvironment	5

1.4 Intrinsic and extrinsic factors regulating adult neurogenesis and the cell cycle 8

1.5 Environmental factors modulating adult neurogenesis 12

1.6 What can the wild reveal? 16

1.7 Seeking answers using the Chiroptera 21

1.8 Significant questions to be answered: Aims and Objectives of the study 28

1.8.1 Main aims and studies undertaken 29

CHAPTER TWO: Adult neurogenesis in eight species of megachiroptera 35

2.1 Introduction 35

2.2 Methods 37

2.3 Results 41

2.3.1 The dorsal and ventral proliferative regions of the subventricular zone (SVZ), the rostral migratory stream (RMS) and the olfactory bulb 41

2.3.2 The subgranular zone and the dentate gyrus 43

2.3.3 Neocortex 44

2.3.4 Temporal migratory stream, piriform cortex and the amygdala 44

2.3.5 Tectum, cerebellum and brainstem 45

2.3.6 Counts of Ki-67 Immunopostiive Cells in the Dentate Gyrus 46

2.4 Discussion 67

2.4.1 Subventricular zone and the rostral migratory stream 67

2.4.2 Subgranular zone (SGZ) and dentate gyrus 69

2.4.3 Neocortex 70

2.4.4 Temporal migratory stream 71

2.4.5 Other sites 72

2.4.6 Conclusion 73

CHAPTER THREE: Microbats appear to have adult hippocampal neurogenesis, but post-capture stress causes a rapid decline in the number of neurons expressing doublecortin 74

3.1 Introduction 74

3.2 Methods 76

3.3 Results 80

3.3.1 Doublecortin immunopositive (DCX+) cells in the Microchiropteran hippocampus 80

3.3.2 DCX+ cells in other regions of the Microchiropteran brain 81

3.4 Discussion 95

CHAPTER FOUR: The pattern of adult neurogenesis in three microchiropteran species, *Hipposideros fuliganosis*, *Triaenops persicus* and *Asellia tridens*. 99

4.1 Introduction 99

4.2 Methods 101

4.3 Results 104

4.3.1 Adult hippocampal neurogenesis 104

4.3.2 The subventricular neurogenic zone and the rostral migratory stream (RMS) 105

4.4 Discussion 124

4.4.1 Adult hippocampal neurogenesis across mammalian species 124

4.4.2 The subventricular zone, the rostral migratory stream and terminal fields of adult neurogenesis 126

CHAPTER FIVE: Adult hippocampal neurogenesis in Egyptian fruit bats from differing environments: are differences environmental or methodological? 130

5.1 Introduction 130

5.2 Methods 132

5.2.1 Animals and Treatment 132

5.2.2 Immunohistochemistry 133

5.2.3 Antibody characterization and specificity 135

5.2.4 Quantitative Analyses 136

5.2.5 Statistical Analysis 138

5.2.6 Image Processing 139

5.3 Results 139

5.3.1 The pattern of adult neurogenesis in the Egyptian fruit bat brain 139

5.3.2 Brain mass varied amongst the Egyptian fruit bat groups studied 140

5.3.3 Volume of the granule cell layer of the dentate gyrus of the hippocampus 140

5.3.4 Total number of cells in the granule cell layer of the dentate gyrus of the hippocampus 141

5.3.5 Total numbers of hippocampal Ki-67 and DCX immunopositive cells 142

5.3.6 Ki-67 and DCX immunopositive cell numbers standardized to brain mass 143

5.3.7 Ki-67 and DCX immunopositive cell densities using granule cell layer volume standardization 144

5.3.8 Ki-67 and DCX immunopositive cells as a fraction of total granule cell numbers 145

5.4 Discussion 173

5.4.1 Methodological considerations specific to the current study 174

5.4.2 Method 1 – comparison of raw total numbers of proliferative and immature cells 175

5.4.3 Method 2 – standardizing raw cell counts to brain mass 176

5.4.4 Method 3 – densities of proliferative and immature cells in dentate gyrus 177

5.4.5 Method 4 – proliferative and immature cells as a percentage of total granule cells 178

5.4.6 Does the environment play a role in shaping adult hippocampal neurogenesis? 180

CHAPTER SIX: Adult neurogenesis in the wild: Observations from the Chiroptera **183**

6.1 Adult neurogenesis in the wild: Observations from the Chiroptera 183

6.2 Neurogenesis in the suborders of Chiroptera – potential phylogenetic implications 184

6.3 Hippocampal neurogenesis in captive and wild caught animals 185

6.4 Methodological considerations when using wild caught animals 187

6.5 Significant unanswered questions from the chiroptera: Limitations of the study 188

6.6 Areas for future research 190

6.7 Final Conclusions 192

References 193

Appendices 229

List of Tables **Page**

Table 2.1	Immunopositive regions of Megachiropteran brains.	48
Table 2.2	Hippocampal Ki-67 cell counts of Megachiropteran brains.	50
Table 3.1	Hippocampal DCX counts of the Microchiropteran brains.	84
Table 4.1	Immunopositive regions of Microchiropteran brains.	109
Table 5.1	Stereological data of cell counts and densities of *R. aegyptiacus.*	148
Table 5.2	Level of precision of the stereological estimates.	150

List of Figures **Page**

Figure 1.1	Cytoarchitercture of the SVZ and SGZ microenvironment.	6
Figure 1.2	The cell cycle regulation.	10
Figure 1.3	Potential signal integration in regulation of adult neurogenesis.	16
Figure 1.4	Tree showing phylogenetic relationships among bat families based on traditional classification and molecular classification	24
Figure 2.1	A drawing of Ki-67 immunoreactive regions in the brain of *H. monstrosus*.	52
Figure 2.2	A drawing of DCX immunoreactive regions in the brain of *H. monstrosus*.	54
Figure 2.3	Ki-67 and DCX immunopositive regions in the brain of Megachiropteran species.	56
Figure 2.4	Hippocampal Ki-67 and DCX immunostaining in the brain of Megachiropteran species.	58
Figure 2.5	Neocortical DCX immunostaining in the brain of Megachiropteran species.	60
Figure 2.6	Ki67 and DCX immunostaining of the temporal migratory stream in the brain of Megachiropteran species.	62

Figure 2.7 Ki-67 immunostaining of the colliculi, cerebellum and brainstem of the brain of Megachiropteran species. 64

Figure 2.8 Graph: Ki-67 cell counts against brain mass of Megachiropteran species. 66

Figure 3.1 Hippocampal DCX immunostaining in the brain of Microchiropteran species. 86

Figure 3.2 Hippocampal Nissl staining and DCX immunostaining in the brain of *A. tridens.* 88

Figure 3.3 Bar graphs: hippocampal DCX immunopositive cells in the brain of Microchiropteran species. 90

Figure 3.4 Hippocampal DCX immunostaining in the brain of *A. tridens*. 92

Figure 3.5 DCX immunoreactive regions in the brain of *A. tridens*. 94

Figure 4.1 A drawing of Ki-67 immunoreactive regions in the brain of *T. persicus.* 111

Figure 4.2 A drawing of DCX immunoreactive regions in the brain of *T. persicus.* 113

Figure 4.3 Hippocampal Ki-67 and DCX immunostaining in the brain of Microchiropteran species. 115

Figure 4.4 Ki-67 and DCX immunostaining of SVZ, RMS and OB in the brain of Microchiropteran species. 117

Figure 4.5 DCX immunostaining of the ventral migratory stream in the brain of *H. fuliganosus*. 119

Figure 4.6 Cortical DCX immunostaining in the brain of *H. fuliganosus* and *A. tridens.* 121

Figure 4.7 Nissl staining and DCX immunostaining of the anterior commissure in the brain of *T. persicus.* 123

Figure 5.1 Ki-67 and DCX immunoreactive regions in the brain of *R. aegyptiacus.* 152

Figure 5.2 Boxplots: brain mass, granule cell layer and total granule cell numbers. 154

Figure 5.3 Boxplots: Ki-67 and DCX cell counts. 156

Figure 5.4 Boxplots: Ki-67 and DCX cell counts standardized to brain mass. 158

Figure 5.5 Boxplots: Ki-67 and DCX cell densities per mm^3. 160

Figure 5.6 Boxplots: Ki-67 and DCX cell counts as a fraction of total granule cell numbers. 162

Figure 5.7 Scatterplots: brain weight against granule cell layer volume, granule cell layer volume against total granule cell count, and brain weight against total granule cell count 164

Figure 5.8 Scatterplots: brain weight against Ki-67+ cell count and brain weight against DCX+ cell count 166

Figure 5.9 Scatterplots: Ki-67+ cell density against DCX+ cell density, Ki-67+ cell count against granule cell layer volume, and DCX+ cell count against granule cell layer volume 168

Figure 5.10 Scatterplots: Ki-67+ cell count normalized to total granule cell count against DCX+ cell count normalized to total granule cell count, Ki-67+ cell count against total granule cell count, and DCX+ cell count against total granule cell count 170

Figure 5.11 Scatterplots: Ki-67+ cell density against Ki-67+ cell count normalized to total granule cell count, and DCX+ cell density against DCX+ cell count normalized to total granule cell count 172

LIST OF ABBREVIATIONS

4V – fourth ventricle

7n – facial nerve

III – oculomotor nucleus

Vmot – motor division of trigeminal nerve nucleus

VI – abducens nucleus

VIIv – ventral division of the facial nerve nucleus

X – dorsal motor vagal nucleus

XII – hypoglossal nucleus

ac – anterior commissure

Amyg – amygdala

AON – anterior olfactory nucleus

AP – area postrema

bf – basal forebrain

C – caudate nucleus

CA – cornu ammonis

Cb – cerebellum

cc – corpus callosum

CN – deep cerebellar nuclei

Co – cochlear nuclear complex

C/P – caudate/putamen nucleus

DG – dentate gyrus

DT – dorsal thalamus

EPL – external plexiform layer of olfactory bulb

f – fornix

GC – central grey matter of the midbrain

GCL - granular cell layer of olfactory bulb/cerebellar cortex

GL – glomerular layer of olfactory bulb

GP – globus pallidus

Hb – habenular nuclear complex

Hip - hippocampus

Hyp – hypothalamus

IC – inferior colliculus

ic – internal capsule

icp – inferior cerebellar peduncle

io – inferior olive

IPL – internal plexiform layer of olfactory bulb

LC – locus coeruleus

lot – lateral olfactory nucleus

LV – lateral ventricle

MB – midbrain

MCL – mitral cell layer of olfactory bulb

mcp – middle cerebellar peduncle

ML – molecular layer of cerebellar cortex

mlf – medial longitudinal fasciculus

MO – medulla oblongata

N.Acc – nucleus accumbens

N.Amb – nucleus ambiguus

NEO – neocortex

OB – olfactory bulb

ONL – olfactory nerve layer

OT – optic tract

OV – olfactory ventricle

P – putamen nucleus

PCL – Purkinje cell layer of cerebellar cortex

PIR – piriform cortex

Pons – pons

Pta – pretectal area

py – pyramidal tract

PVG – periventricular grey matter of the pons

R – reticular nucleus of dorsal thalamus

RMS – rostral migratory stream

S – septal nuclear complex

SC – superior colliculus

sc – spinal cord

SCN – suprachiasmatic nucleus

scp – superior cerebellar peduncle

SGZ – subgranular zone

stn – subthalamic nucleus

subC – nucleus subcoeruleus

SVZ – subventricular zone

TMS – temporal migratory stream

TOL – olfactory tubercle

VPO – ventral pontine nucleus

WM – white matter of cerebellar cortex

zi – zona incerta

Chapter 1: *Adult neurogenesis: a blind leap into the wild?*

1.1 Introduction

Adult neurogenesis - the proliferation, maturation, differentiation and integration of newly generated neurons into existing neuronal networks - has been touted to offer a potentially novel therapeutic intervention for neurodegenerative and neuropsychiatric conditions, most of which result from a loss of active neurons (Winner et al., 2011). The concept of potentially using adult neurogenesis as a theurapeutic agent for symptomatic alleviation or reversal of neurodegenerative conditions has been discussed since the findings that the adult mammalian brain continuously generates new neurons throughout its entire life, albeit at a decreasing rate with advancing age (Kuhn et al., 1996, Kempermann et al., 1998, Klempin and Kempermann, 2007). These findings broke a long held dogma that considered the adult mammalian brain as structurally immutable following embryonic development. From the pioneering studies five decades ago, which demonstrated newly generated neuronal cells in adult rats using radioactive nucleosides such as 3H- thymidine which is incorporated into the cells during deoxyribonuncleic acid (DNA) synthesis (Altman and Das, 1965), methodological and technological advancements facilitated the identification and characterisation of neural stem cells and neural progenitor cells in the adult brain (De Marchis and Puche, 2012). These advancements include, among others, the in vitro characterisation of neural progenitor cells through use of cell culture; the in vivo identification of neural progenitor cells using nucleoside analogues such as 5'-bromo-3'-deoxyrudine (BrdU) (a synthetic thymidine analogue); and the manipulation of the phenomenon using virus vectors, genetic manipulation and transgenic animals (Consiglio et al., 2004, Babu et

al., 2011, Dhaliwal and Lagace, 2011, Imayoshi et al., 2011). The use of cell-specific phenotypic antigens, such as Ki-67 (a proliferative cell marker), doublecortin (DCX – an immature neuronal cell marker) and NeuN (a mature neuronal cell marker), has also gained traction in the identification and characterisation of new cells in the past decade. In addition to the identification and characterisation of new cells, the extent of the adult neurogenesis in various animals can be better determined due to improvements in imaging and microscopy, such as magnetic resonance imaging, confocal and two-photon microscopy systems (Couillard-Despres and Aigner, 2011, Vande Velde et al., 2012).

Despite the growing understanding of adult neurogenesis enabled by the methodological and technological improvements outlined above, its functional relevance in mammals remains elusive. The different findings obtained when using different mammals, especially in wild-caught mammals, compounds the challenge of elucidating the relevance of the phenomenon (Patzke et al., 2015). In light of this, here I discuss insights regarding the elements of adult neurogenesis, including the characteristics and properties of adult neuronal stem cells such as self-renewal and multipotency; the adult neurogenic microenvironment; molecular regulation; and the environmental modulation of the phenomenon of adult neurogenesis. This review further interrogates the interplay of these elements at distinct stages of the phenomenon in the context of the observed patterns of adult neurogenesis in various wild-caught mammals and specifically explores how the species belonging to the order Chiroptera could be significant in enhancing the understanding of adult neurogenesis given their unique behavioural and phylogenetic history.

1.2 Adult mammalian neuronal stem cells: characterisation and properties

It is now well established that new neurons are generated in the adult brain of most mammals but are mostly restricted to the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Lois and Alvarez-Buylla, 1993, Gage et al., 1998, Doetsch et al., 1999). The general restriction of the phenomena to the SVZ and SGZ is associated with the presence at these sites of neuronal stem cells (NSCs) and the existence of a microenvironment that allows for the occurrence of neurogenesis under normal conditions (Fuchs et al., 2004, Riquelme et al., 2008). The NSCs are unlimited self-renewing and multipotent cells, that is, they can give rise to multiple cell types of neuroectodermal (neuronal and glial) lineages through symmetric and asymmetric cell division (Reynolds and Weiss, 1992, Doetsch, 2003, Codega et al., 2014). In the SVZ, newly born cells (neuroblasts) are derived through a series of cell divisions from mainly astrocyte-like cells (type B cells) (Doetsch et al., 1997). The morphological and electrophysiological properties of type B cells are similar to that of typical mature astrocytes, but they have smaller soma with an electron-lucent cytoplasm and fewer processes (Alvarez-Buylla and Garcia-Verdugo, 2002, Liu et al., 2006). Like mature astrocytes, type B cells express the intermediate filament protein glial fibrillary acid protein (GFAP) and the glycoprotein CD133 and yet functionally they appear to be an intermediate of astrocytes and radial glia cells (Doetsch et al., 1999, Codega et al., 2014). Cell division of these type B cells gives rise to transit amplifying cells (type C cells) which are highly proliferative spherical cells (Doetsch et al., 1997). Type C cells are intermediate progenitor cells identified by their expression of LeX, Dlx2 and Mash1 (Capela and Temple, 2002, Doetsch et al., 2002a, Parras et al., 2004, Shen et al., 2008). Most of the transit amplifying cells give rise to the bipolar, migrating and DCX, β-tubulin and polysialylated neural adhesion molecule (PSA-

NCAM) expressing neuroblasts (type A cells) (Rousselot et al., 1995, Nacher et al., 2001, Bonfanti and Peretto, 2007). From the SVZ, neuroblasts migrate tangentially to the olfactory bulb through the rostral migratory stream (RMS) (Luskin, 1993). In the olfactory bulb neuroblasts differentiate principally into gamma-aminobutyric acid (GABAergic) granule neurons and a small proportion differentiate into periglomerular interneurons, which express neuronal markers such as βIII tubulin and microtubule associated protein 2 (MAP2) (De Marchis et al., 2001, Carlen et al., 2002, Carleton et al., 2003). The granule neurons eventually integrate with tufted and mitral cells through dendro-dendritic synapses (Petreanu and Alvarez-Buylla, 2002, Belluzzi et al., 2003).

Adult neurogenesis in the hippocampus differs significantly from that of the SVZ as neuroblasts in the SGZ arise from radial-glia-like cells (type 1 cells). These triangular shaped type 1 cells share similar physical and electrophysiological properties with astrocytes, but in addition they have an extensively branched apical process extending to the molecular layer of the dentate gyrus (Filippov et al., 2003, Fukuda et al., 2003, Kronenberg et al., 2003). Type 1 cells are nestin, GFAP, and Sox2 positive (Seri et al., 2001, Filippov et al., 2003, Suh et al., 2007, Kang and Hebert, 2012). Asymmetric division of type 1 cells yields cells with a round soma and thin horizontally oriented processes that entirely lack astrocytic features (type 2 cells) (Fukuda et al., 2003). The highly proliferative nestin-positive but GFAP-negative type 2 cells are classified into DCX-positive type 2a cells and DCX-negative type 2b cells (Seri et al., 2001). Their electrophysiological properties markedly differ from those of astrocytes and they approximate those of immature neurons (Fukuda et al., 2003). Daughter cells of the type 2 cell population are the nestin-negative subpopulation characterised by a round nucleus and expression of DCX and PSA-NCAM (type 3 cells) (Kempermann et al., 2004). The type 3 cells are late progenitors and

give rise to immature neurons. Immature neurons have a triangular nucleus, an apical process and transiently express the mature cell markers calretinin followed by calbindin (Brandt et al., 2003). The immature neurons migrate relatively short distances to the granule cell layer of the dentate gyrus where they differentiate mainly into dentate granule cells which synthesize glutamate, but a small fraction differentiate into GABAergic basket cells (van Praag et al., 2002, Liu et al., 2003, Namba et al., 2005). The granule cells integrate in the dentate gyrus circuitry by extending their dendrites into the molecular layer and their axons into CA3 region (Vivar and van Praag, 2013).

Outside the SVZ and SGZ, potential sites for adult neurogenesis include the striatum, amygdala, substantia nigra, cerebral cortex (piriform and neocortex) and cerebellum (Ortega-Perez et al., 2007), but much less is known about the process of adult neurogenesis in these regions.

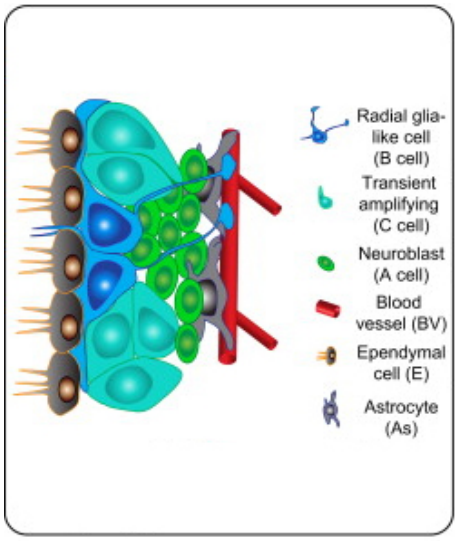
1.3 Adult neurogenic microenvironment

The persistence of neurogenesis in the SVZ and SGZ and lack of it in most other brain regions potentially indicates a fine balance between structural stability and plasticity, which under the right physiological conditions encourages neurogenesis in the SVZ and SGZ and not in other brain regions (Abraham and Robins, 2005, Knobloch and Jessberger, 2011). Regulating this physiological balance is likely to be related to the structural architecture of the SVZ and SGZ as they are structurally and functionally unique (Smart, 1961). It may also be related to the microenvironment in which the NSCs (discussed above) are supported and regulated. The significance of the microenvironment (niche) in adult neurogenesis was highlighted by

transplantation studies in which SVZ- derived NSCs differentiate into glial cells upon grafting into non-neurogenic regions, and spinal cord-derived glial progenitor cells giving rise to granule cells after being grafted into the hippocampus (Shihabuddin et al., 2000, Seidenfaden et al., 2006).

The neurogenic microenvironment of SVZ consists of closely packed cellular elements: the NSCs (discussed above), the astrocytes, ependymal cells and endothelial cells lining blood vessels, in an extracellular matrix rich in diffusible signals (Ma et al., 2009) (see Fig. 1.1a below).

a.



b.

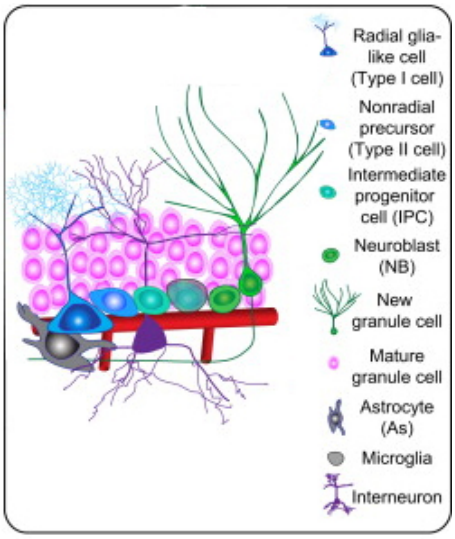


Figure 1.1: *An illustration of the cytoarchitercture and cells constituting the neurogenic microenvironment in the SVZ (a) and SGZ (b).* Adopted and slightly modified from Ming and Song (2011).

The SVZ microenvironment is separated from the cerebrospinal fluid (CSF) of the lateral ventricles by a layer of ependymal cells providing a boundary between the developing neuroblasts and the CSF. These ciliated ependymal cells function to create a gradient between the niche and factors within the CSF which modulate adult neurogenesis such as basic fibroblast growth factors (bFGF) and transforming growth factors (TGF) (Riquelme et al., 2008). In addition, they maintain the quiescent state of NSCs through contact mediated cues and secretion of diffusible molecular cues such as noggin, an endogenous bone morphogenic protein antagonist (see below for adult neurogenesis regulation), and pigment epithelium-derived factor (PEDF) (Lim et al., 2000, Peretto et al., 2004, Andreu-Agullo et al., 2009). Between the ependymal cells are the astrocytic processes whose cell bodies are juxtaposed to the ependymal cells. The astrocytic processes are in contact with all cellular components in the neurogenic microenvironment and can therefore harmonise signals from the niche components (Doetsch et al., 1997). Astrocytes in the SVZ, which are connected to each other through gap junctions, form a functional syncytium that closely support and regulate NSCs (Rouach et al., 2004). Just like ependymal cells, astrocytes modulate adult neurogenesis through cell-cell contact, secretion of diffusible molecular cues such as glutamate, and modulation of the availability of other diffusible signals such as cytokines and growth factors (vascular endothelial growth factor [VEGF]) secreted by endothelial cells lining the vessels in the SVZ (Ming and Song, 2011). In addition to ependymal cells and astrocytes are the blood vessels in the niche. The vasculature of the SVZ, which is surrounded by astrocytic endfeet, serves as channels for conveying systemic signals such as hormones and steroids which are known to modulate adult neurogenesis (Shen et al., 2008). Diffusible signals of the niche include neurotransmitters which are released by the nerve terminals located in the neurogenic microenvironment whose cells bodies are distantly located.

These signals reach the NSCs through the extracellular matrix. The matrix is constituted by collagen, laminins and proteoglycans such as chrondrotin sulphate and heparin sulphate (Riquelme et al., 2008). These components control accessibility of the diffusible signals secreted by the cellular components of the microenvironment to the NSCs.

Unlike the SVZ neurogenic microenvironment which is in close contact with CSF, neurogenesis in the SGZ of the dentate gyrus occurs in close contact with blood vessels (Fig. 1.1b) (Palmer et al., 2000). The SGZ neurogenic microenvironment is found between the granular and plexiform layers of the dentate gyrus. The cellular components of the SGZ niche include mature neurons, nestin-negative horizontal astrocytes (which separate the NSCs from the basal lamina of the blood vessels) and the endothelial cells lining the blood vessels (Kronenberg et al., 2003, Riquelme et al., 2008). Signals modulating neurogenesis in the SGZ are similar to those observed in the SVZ, but there are a few differences. While the local environment of the SVZ and SGZ allows for the generation of new cells under normal physiological conditions, neurogenesis in the potential neurogenic sites can be induced under pathological conditions, such as in Alzheimer disease and Parkinson's disease, underscoring the complex nature of its regulation.

1.4 Intrinsic and extrinsic factors regulating adult neurogenesis and the cell cycle

Regulation of adult neurogenesis by various intrinsic and extrinsic factors affects the different stages of the process, which are proliferation, migration, differentiation and integration. Fundamentally, neuronal stem cells in the principal sites exist in two states: the quiescent state (a non-dividing state, G0, or slowly dividing cells) and the active state (frequently dividing cells)

(Gage, 1998). In adult neurogenesis, quiescent stem cells are activated and proliferation ensues in the active stem cells. There is a fine balance between these two states which ensures both the maintenance of the neuronal stem cell pool by self-renewal and the generation of neuroblasts. The balance between these two states is tightly regulated by intrinsic (intracellular) factors, which include cell cycle regulators, transcription factors and epigenetic factors, and by extrinsic (extracellular) factors, which are signals from niche cells and other systemic signals.

A resting cell (G0 phase), when activated, proceeds to divide into two through progressing from the G1 phase (first growth phase) through the S phase (synthetic phase) and G2 phase (second growth phase) to the M phase (mitotic phase) of the cell cycle. Progression of a cell from the G1 phase to the S phase is initiated by the release of the phosphorylated tumor suppressor protein retinoblastoma (pRb) or the p107 isoform in the SVZ from transcription factor E2F or the E2F1 isoform in the SVZ and SGZ resulting in the eventual transcription of proteins essential for the cell cycle (Nevins, 1992a, b, Beukelaers et al., 2012). The phosphorylation of Rb is dependent on the activity of specific cyclin-dependant kinases (Cdks) which in turn are regulated by cyclins (cyclin D for NSCs) (see figure 1.2).

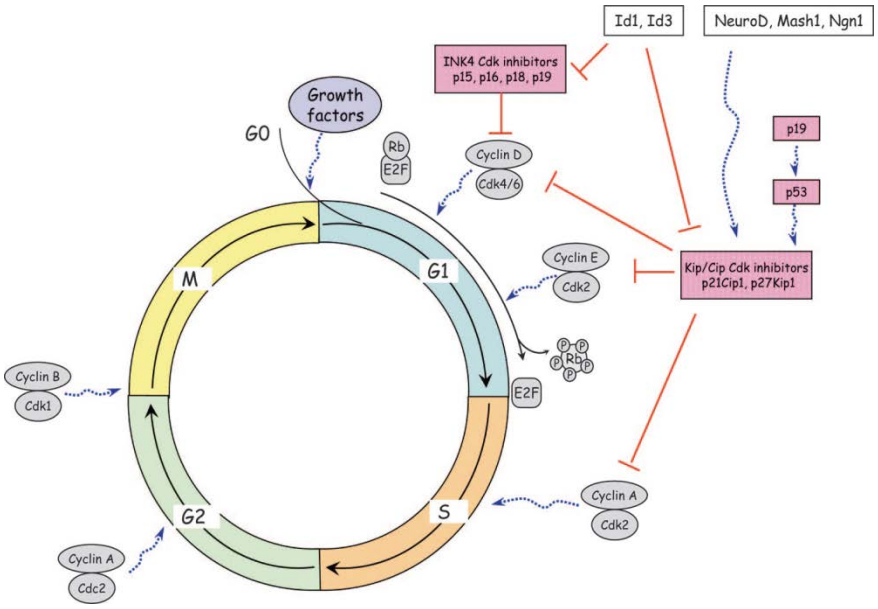


Figure 1.2: A schematic diagram showing how different cyclins influence the different stages of the cell cycle. The cyclin-dependent kinase inhibitors (in the pink box) block the activity of cyclins resulting in the blocking of the phosphorylation of the retinoblastoma-E2F complex to release the transcription factor E2F. Thus the cell is arrested in the quiescent state. ***Source:*** *image from Abrous et al. (2005).*

NSCs are maintained in the quiescent state by cell-cycle regulators such as p21 and p27 (cyclin-dependent kinase inhibitors), which act through regulating the activity of cyclin-dependent kinases (CDK) (Legrier et al., 2001, Doetsch et al., 2002b). The activity of the CDKs (and other intrinsic programs regulating adult neurogenesis) can also be modulated by extrinsic factors (discussed below) contained in the microenvironment (niche) of the NSCs. While cell cycle regulators function in most parts to maintain the NSC pool, other intrinsic factors such as the transcription factors are fundamental in both the maintenance of the NSC pool and the proper progression of the different stages of the process. TLX, an orphan nuclear receptor, and Sox, a notch signalling mediator acting through an extracellular signalling protein sonic hedgehog, are

crucial in maintaining the NSC pool and proliferation of the neuroblasts, while Dlx-2, a homeobox transcription factor, is associated with migrating cells (Ferri et al., 2004, Shi et al., 2004, Brill et al., 2008, Qu et al., 2010). Cell fate specification or differentiation of immature neurons is mediated through Pax 6, a homeobox transcription factor, and Dlx-2 (Hack et al., 2005, Kohwi et al., 2005, Brill et al., 2008). While transcription factors act directly to control transcription in the NSCs, other factors such as those involved in the epigenetic mechanisms act indirectly to control a variety of genes through methylation of DNA, histone modifications and non-coding RNAs regulating gene expression (Sun et al., 2011). It is, however, unclear to what extent the NSCs are intrinsically regulated or influenced by the extrinsic signals or factors.

Extrinsic signals or factors critical in maintaining the balance between the quiescent and active pools of NSCs remain unclear but are generally thought to include neurotransmitters, growth factors and other signalling factors conserved from embryonic neuronal development such as morphogens including Notch signalling, Sonic Hedgehog, Wnt and Bone Morphogenic proteins (BMPs). Neurotransmitters are secreted by projections to the principal neurogenic sites from all over the brain. These include: serotonin which through 5HT1A receptors stimulates proliferation; norepinephrine which through β3-adrenergic receptors activates NSCs and enhances proliferation; dopamine which through D2-like receptors stimulates proliferation of neuroblasts; and glutamate which, acting through N-methyl-D-aspartate (NMDA) receptors, inhibits proliferation (Radley and Jacobs, 2002, Ormerod et al., 2003, Banasr et al., 2004, Hoglinger et al., 2004, Van Kampen et al., 2004, Jhaveri et al., 2010). Unlike neurotransmitters, morphogens are secreted by the cells constituting the neurogenic niche in the principal site and act on transmembrane receptors expressed on the NSCs. Despite the fact that niche cells secreting paracrine BMPs and Shh have not been identified, their importance in maintaining the

NSCs pool was highlighted when self-renewal was blocked on administration of BMP antagonists such as Noggin, Chordin and Neurogesin-1 and when self-renewal was restored in Sox 2 deleted NSCs by administration of exogenous Shh (Piccolo et al., 1996, Lim et al., 2000, Favaro et al., 2009, Mira et al., 2010). While cells secreting Shh and BMPs are unknown, principal neurogenic site resident astrocytes are believed to secret Wnts, which acting through Prox1, have been shown to regulate NSCs' maintenance and granule cell differentiation (Song et al., 2002, Lie et al., 2005). In addition to the discussed intrinsic and extrinsic factors regulating adult neurogenesis under normal conditions, various pathological conditions, physiological and environmental stimuli influence various stages of the process. It is, however, likely that the overall outcome influenced by these factors is effected through an indirect activation and modulation of the neurogenic niche cells (and hence the indirect modulation of the intrinsic and extrinsic signalling pathways – as will be discussed below).

1.5 Environmental factors modulating adult neurogenesis

Changes in the physiological and environmental parameters have been shown to influence the different stages of adult neurogenesis. Such stimuli include environmental enrichment, exercise, environmental complexity (learning tasks), and social environment (early life experience such as maternal deprivation and psychosocial stress). The understanding of the causal-link to adult neurogenesis of any physiological or environmental changes is restricted by the limitations of controlling the confounding factors and interactions. In addition, unlike embryonic neurogenesis where NSCs proliferate, migrate and differentiate as a cluster with a temporal separation between the different clusters, NSCs in the adult brain exists at various

stages of development within their niche, allowing heterogeneous populations of cells at different developmental stage to coexist (Bonfanti and Peretto, 2007). As a result, the observed outcomes of environmental manipulation on adult neurogenesis represent a global outcome of the potential interactions with the various stages of adult neurogenesis.

Exposure to an enriched environment increases hippocampal neurogenesis, synaptic plasticity and dendritic complexity in an adult brain (Mohammed et al., 2002). An enriched environment is an artificial setting in which the animal's habitat is supplemented with toys, tunnels and platforms that might not be usually found in its normal habitat (Mohammed et al., 2002, Mora et al., 2007). The use of these diverse objects in enriched environments is thought to stimulate the animal's sensory and motor abilities and hence their trophic effect on neurogenesis. Given the diverse objects used in enriching the environment, there is a potential interaction of various environmental stimuli resulting in limitations in discriminating cause from effect. Such individual variables which are components or cues of an enriched environment include exercise (running wheels), environmental complexity (spatial memory learning tasks) and stress (social isolation) (Gregoire et al., 2014). Voluntary running in environmental enrichment has been implicated to be the major variable contributing to the observed increase in adult hippocampal neurogenesis (Kobilo et al., 2011, Gregoire et al., 2014). The exercise-associated increase in neurogenesis has been reported to result from salvaging immature neurons and granule cells instead of increased proliferation (Mohammed et al., 2002). It has been shown to be modulated by the Wnt signalling pathway as Wnt-3 expression increases in the niche astrocytes while Wnt-3 inhibitor, the secreted fizzled-related protein 3 - sFRP3, decreases with exercise (Jang et al., 2013, Varela-Nallar and Inestrosa, 2013). It then follows that the increase in astrocytic Wnt-3 in physical activity counters the decline of the same protein associated with aging, thus explaining

how environmental enrichment delays the age-associated decrease of neurogenesis in the dentate gyrus (Varela-Nallar and Inestrosa, 2013). While the Wnt signalling pathway explains the salvage effect of environmental enrichment in aging animals, it remains unclear how the pathway interacts with the cell-cycle regulators, as the observed decline in adult neurogenesis with advancing age has also been associated with increases with age of p16ink4a (a CDK inhibitor) (Molofsky et al., 2006). Although voluntary exercise is widely accepted as an upregulator of adult neurogenesis, its lack of a significant trophic effect using wild-living rodents indicate the extent of the complexity in the regulating mechanisms and the interactions of various environmental stimuli cues in modulating adult neurogenesis (Hauser et al., 2009, Klaus et al., 2009, Klaus et al., 2012).

Physical activity (motor stimulation) is a confounding variable in the association of the observed increase in adult neurogenesis with environmental complexity (spatial learning tasks). However, in studies where physical activity was controlled, mixed results were reported with some recording a positive association between spatial learning tasks and neurogenic rates, and others failing to find a significant association (Olson et al., 2006, Gregoire et al., 2014). In studies where a positive association was reported, the increase in neurogenic rates could possibly be linked to the increase of the vascular endothelial growth factor (VEGF) in animals housed in enriched environment (During and Cao, 2006). VEGF, which is expressed in subgranular astrocytes, acts through the kinase insert domain receptor (KDR) to increase proliferation of the NSCs (Cao et al., 2004). The contrary findings, in which spatial learning tasks or enriched environments were reported not to yield any change in the rate of adult neurogenesis, could be an indication of the limitations in association studies. Such limitations include the difficulties in controlling confounding variables such as the psychosocial stress associated with handling of

animals, training and their separation from their social environment. Social isolation has been shown to suppress the exercise-associated increase in neurogenesis (Stranahan et al., 2006, Leasure and Decker, 2009). This is modulated by social isolation causing a down-regulation of hippocampal seretonergic receptors 5HT1A and thereby counteracting the effect of the exercise-induced increase in serotonin (a positive NSC proliferation stimulant) (Schiller et al., 2003). The up-regulation of 5HT1A receptors associated with social housing could also explain the trophic effect of other social interactions such as adult-adult and adult-offspring interactions that have been shown to modulate adult neurogenesis (Lieberwirth and Wang, 2012).

The multiple factors discussed above have been associated with modulation of adult neurogenesis and yet it remains a challenge to understand the exact nature as to how these factors interact to produce the final observed patterns in adult neurogenesis. It is likely that regulatory pathways converge at a single enhancer site, which acts not as a simple on and off switch, but as an integrator of various transcription factors (as shown in fig. 1.3) (Johnson et al., 2009, Merz et al., 2011, Schwarz et al., 2012). The relevant enhancer working with the other distant enhancers would regulate the gene transcription. In such a case, the rate of neurogenesis observed would be a global outcome of the various variables present in the environment in which the animal is exposed. In addition one would expect differential impacts of a single variable in animals exposed to different environments, such as comparing the effect of running on captive animals when compared to wild animals (Klaus and Amrein, 2012, Klaus et al., 2012). Given this, using wild animals living their natural habitat in studies on adult neurogenesis becomes pivotal to the understanding of the functional relevance of adult neurogenesis.

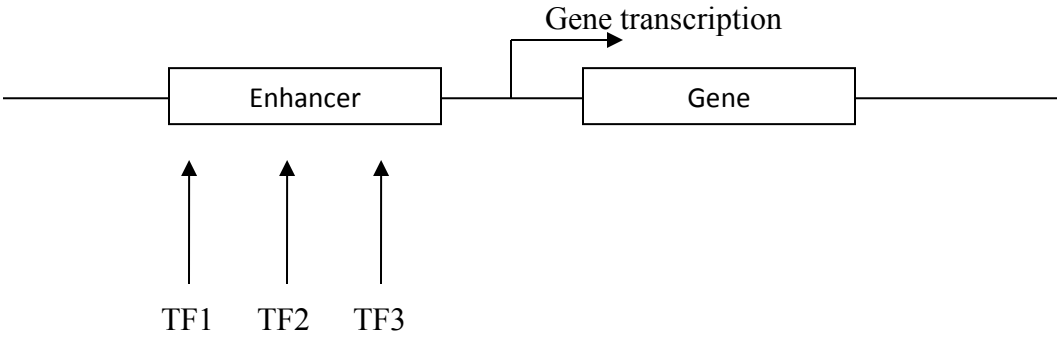


Figure 1.3: A schematic diagram indicating the potential integration of signals on the enhancer site (which could be CREB). In this case, the enhancer integrates various signals and subsequently regulates gene transcription, thus regulating the rate of proliferation. TF = transcription factor.

1.6 What can the wild reveal?

The complex interaction of the multiple factors indicated above is possibly associated with varying rates of adult neurogenesis observed across mammals. The challenge remains to separate and determine how the individual variables underlie the observed changes in patterns and rates of adult neurogenesis. This attempt could be viewed as an appeal to the philosophical argument on nature against nurture. The question in this argument is neither a determination of whether the two interact nor whether only one of the two exists, but it is to determine how much of the phenomenon is predetermined genetically, and how much is determined environmentally. Approaches used to date in studying the biological and molecular roles of genes and therefore the extent of genetic influence on a phenomenon include the knocking in or out of genes and then correlating the findings of the gene underexpression or overexpression to the observed physiological or behavioural changes (Gur et al., 2007). On the other hand, the influence of

nurture on a particular phenomenon is usually assessed by using artificial habitats or environments in the laboratory which mimic the wild habitats or environments while at the same time retaining the ability to control or vary different factors of the environment (Gregoire et al., 2014). While these approaches provide vital information pertaining to gene expression, various environmental factors and the phenomenon of adult neurogenesis, the use of laboratory in-bred and transgenic animal models has its own shortfalls. Unexpected and varying phenotypes or outcomes have been reported in animal models with a similar genetic manipulation (Thyagarajan et al., 2003, Taupin, 2006). If a particular gene is knocked out it is possible for other systems or isoforms which are not usually expressed under normal circumstances to play a significant role in the knock-out animals (Blendy et al., 1996, Sigmund, 2000). Furthermore, other proteins belonging to different systems that could be dependent on the presence or activity of the knocked-out protein could also be affecting the result in different phenotypes (Thyagarajan et al., 2003). Overall, in-bred and genetically manipulated animals are very fragile and not robust given that, when placed in the wild, the animals would not survive as they lack hybrid vigour (Amrein and Lipp, 2009).

While the increasing use of wild animals in studies on adult neurogenesis appears like a blind leap into the wild, it affords opportunities to reveal the natural history of the phenomenon. These studies are aimed at elucidating the occurrence or non-occurrence of the process across a wide range of phylogenetically related and unrelated mammals and understanding the regulatory mechanism underlying the observed rates, patterns and the associated behaviours. Such a screen or survey gives valuable information and makes it possible to ask relevant questions that could point towards the evolution or the biological basis of the phenomenon. As many of the early studies used laboratory-bred rodents in the study of adult neurogenesis, current studies using

wild-living animals are focused on assessing how adult neurogenesis in the wild-living animals conforms to observations made with laboratory-bred animals (Hauser et al., 2009). Given that in laboratory-bred animals nearly all specific or non-specific factors result in changes in the occurrence of adult neurogenesis, it is crucial to assess the effect of similar factors using the wild-living animals. Such factors include physiological stimuli such as longevity, reproductive cycle and exercise, and environmental stimuli such as environmental enrichment, environmental complexity (learning tasks), and social environment (early life experience such as maternal deprivation and psychosocial stress). The measured outcome of the effect of these factors on adult neurogenesis can be physiological, such as changes in proliferation rates and immature neuron survival, or behavioural, such as changes in task performance.

Adult neurogenesis in wild-living animals occurs at a decreasing rate with age in a similar way to that of the laboratory-bred animals (Klempin and Kempermann, 2007). In contrast, the effect of exercise on rate of adult neurogenesis differs in these two groups. Exercise generally increases proliferation rates and immature neuron survival in laboratory-bred rodents (van Praag et al., 1999, van Praag, 2008), but proliferation rates and neuronal survival in wild caught rodents appear to be insensitive to exercise or running (Hauser et al., 2009, Klaus and Amrein, 2012, Schaefers, 2013). Similarly an enriched environment increases adult neurogenesis with impoverished environment having the opposite effect in laboratory-bred rodents (van Praag et al., 2000), while housing wild caught rodents in an impoverished environment does not appear to change the proliferation rate and survival of immature neurons (Schaefers, 2013). In addition to indicating the shortcomings of using laboratory-bred animals in attempting to elucidate the functional relevance of adult neurogenesis, these findings highlight that the factors prevalent in the natural environment associated with positive modulation of adult neurogenesis do not predict

higher levels of adult neurogenesis in these animals. Rather they highlight the complexity of the regulating mechanisms in adult neurogenesis and thereby raise the question as to how much does each of the variables contribute to the observed final outcome (see integration of signals above, figure 1.3). As a result these findings focus attention on the need for wider studies using wild caught animals to look at the prevalent factors and the rates of adult neurogenesis and at the same time attempt to deduce the possible computations taking place to give the observed findings.

Adult hippocampal neurogenesis has been observed in most wild-living mammals, with the notable exception of cetaceans (Patzke et al., 2015). Its near ubiquitous occurrence in wild living mammals is associated with considerable variations across related orders, species and strains (Bonfanti and Peretto, 2011). The observed variations could be attributed to either genetics, where the rates and sites of adult neurogenesis are genetically predetermined and conserved throughout evolution, or to the environment, where rates and sites of adult neurogenesis are determined by the animal's ecological habitat. Support of the former is obtained from varying neurogenic rates on related but different strains of rodents housed in similar experimental conditions (Kempermann et al., 1997a). Different proliferation rates and immature neuronal survival have been reported in related species that inhabit different environments (Cavegn et al., 2013). Despite this, it remains unclear to what extent adult neurogenesis in wild-living animals differs from that reported in the laboratory-bred or captive-bred animals. Similar proliferation rates in the adult hippocampus of wild-living and captive-bred rodents was reported by Epp et al. (2009) and attributed to the balancing effect of spatial memory demands and stress-inducing factors, which differ in the two environments; however, the similar rates could also indicate genetic predetermination in the proliferation rate as both the wild-living and captive-

bred animals were closely related species (Roth et al., 2012). Understanding the effect of the environment on the occurrence of adult neurogenesis mentioned above (Cavegn et al., 2013), Epp's findings raise the question of nature against nurture. In addressing this philosophical argument and in order to understand the effect of habitat complexity on brain (as found in the wild compared to the laboratory setting) and behaviour requires the use of closely related species, quantification of the environmental complexity, and restriction of the confounding variables (e.g. foraging behavior, habitat complexity, spatial task complexity, social complexity). Experiments using same species of relatively the same age obtained from different environments are likely to resolve this challenge and form one of the objectives for the studies in the current thesis.

While there is a strong appeal to using wild-living animals in the investigations of adult neurogenesis, their use is not without its own challenges. Studies undertaken on wild-living animals entail capture and removal from their natural habitat and social environment before the investigations begin. The process of capturing of animals can be a stressful event for the animals, as seen in bats which are agitated by the light in their roost site and the presence of humans (Cardiff et al., 2012). The agitation of the animals is associated with substantial physiological changes which could pose investigational challenges similar in magnitude to those encountered when using in vitro studies of stem cells in order to understand in vivo dynamics (Ming and Song, 2011). The physiological changes prevalent in agitated state of the animals may result in substantial changes of both the neuronal stem cells and the neurogenic niche and hence adult neurogenesis (Mohammed et al., 2002). This challenge can be conceptualised by understanding the complex regulation of adult neurogenesis which entails that neuroblasts in different stages of the process exist in the same niche and are exposed to various niche factors resulting in different

effects for the different stages. In this concept, the regulation at various stages is possibly dependent of differential expression on transmembrane receptors by neuroblasts at different stages of development, leading to selective responses to the stimuli in the niche (Lugert et al., 2010). It could therefore be hypothesised that any rapid change in the physiology, as occurs when animals are agitated, could result in structural changes that might lead to substantial changes in the potential of the NSCs to self-renew or differentiate. Due to these limitations the question that arises then is whether there is a critical temporal frame from the point of capture for studying adult neurogenesis in wild living animals?

The use of wild-living animals to investigate adult neurogenesis raises a number of fundamental questions, as outlined above. It is not without doubt that comparative studies of adult neurogenesis in wild-living animals can provide a basis for further investigations both in the wild and in the laboratory. Appreciating the differences in the wild-living animals in terms of rates, intensity and migration pathways would only be the beginning in the foray to understand the causal-link between adult neurogenesis, behaviour, environment and phylogenetic history. While there are many mammals that can be used in these studies, there are species which possess many attributes that have been previously associated with higher rates of adult neurogenesis. Targeting the use of these animals, such as those belonging to the order Chiroptera, can be a convenient way to investigate the previous associations of behavioural, ecological and evolutionary factors to higher rates of adult neurogenesis and hence possibly the functional relevance of the phenomenon.

1.7 Seeking answers using the Chiroptera

In order to understand the relevance of a particular phenomenon one of the following can be done: (i) abolishing or overexpressing the phenomenon and establish the resulting effects on the organism; or (ii) associating the observed trends of the phenomenon in a variety of organisms with known attributes of the organisms. With adult neurogenesis, the former was performed in mice where CREB-deleted mice exhibited low activity in new environments when compared to the wild-type controls (Hebda-Bauer et al., 2004). Challenges associated with gene deletion experiments have been discussed above. With the latter, adult neurogenesis has been reported in various animals with varying intensities and rates. Higher proliferation rates and increased immature neuron survival have been reported using animals which had a more complex environment (Bartkowska et al., 2010). Using the association studies, it could be hypothesised that highly social, prey organisms, with relatively large home ranges, should present with higher rates of adult hippocampal neurogenesis. In this case, a departure from previously reported trends associating the occurrence of adult neurogenesis to behavioural attributes creates an opportunity to further interrogate previously held assumptions and links. The absent to low rates of adult neurogenesis reported using microchiroptera is one such departure which is not consistent with known evolutionary, ecological and behavioural attributes of the chiroptera and makes the species within this order particularly interesting to the study in terms of adult neurogenesis (Amrein et al., 2007).

Chiropterans are the second most speciose of the nineteen mammalian orders, accounting for approximately twenty percent of the diversity of all mammals (Simmons, 2005); however, the chiropterans do not appear to have any close affinity to other mammalian orders with horses and pangolins being reported as the closest of the distant relatives (Tsagkogeorga et al., 2013). Chiropterans fossils are relatively unknown before the middle Ypresian (54 mya), partly because

of the poor primitive bat fossil records of this period which consist of mainly cranial and dental records. The earliest known complete skeletal remains are from the early Eocene in Wyoming (Simmons et al., 2008). The ancient chiropteran ancestor is believed to have been nocturnal, insectivorous and probably resembled the modern shrews (Speakman, 2001). Being the only order of flying mammals, the primitive bats are thought to have been capable of both flight and echolocation, a characteristic which is believed to have been lost in the most recent common ancestor of the megabats, but retained in the most recent common ancestor of the microbats (Fenton et al., 1995, Teeling et al., 2000). While the phylogenetic origins of the ancestoral bat are hotly debated, the geographic origins of the ancestral bats are generally suggested to have been in the northern hemisphere (present day North America) before dispersing southwards to Godwanaland (present day Africa) giving the present day distribution of bats throughout the world with the exception of the extremes of latitudes, that is the arctic and Antarctica (Dechmann and Safi, 2009).

The order chiroptera is an unusual group when compared to other mammals both morphologically and behaviourally. While morphological attributes, particularly that of the musculoskeletal structure of the flying apparatus, and molecular attributes were used to place the two traditional suborders of microchiroptera (microbats) and megachiroptera (megabats) into the order Chiroptera, the megabat brain more closely resembles that of primates than the microbat brain and thus suggests the possible diphyletic origin of bats (Pettigrew, 1986, Pettigrew et al., 1989). In addition to the neuroanatomical differences, there are other physical and functional differences between the two Chiropteran suborders. Megabats have a body mass between 20 – 1500g and are exclusively plant eating, feeding on flowers and fruits, while microbats have a body mass between 1.5 – 150g and are omnivores, feeding on insects, fruits and are sometimes

carnivorous (Altringham, 1999). In order to locate their food or prey, megabats primarily use their visual system, while the vast majority of microbats use echolocation (Neuweiler, 2000). Megabat species are found only in the Old world (Africa, Asia and Indo-Australasia), while those from the seventeen families of microbats are found in all continents except Antarctica (Altringham, 1999). In addition to the differences between the two suborders, recent molecular findings indicated differences between the microbat families and thus dispute the monophyletic origin of microbats, as they indicate the existence of a close relationship between some microbat families and the sole megabat family (Teeling et al., 2005) (see Fig 1.4 below).

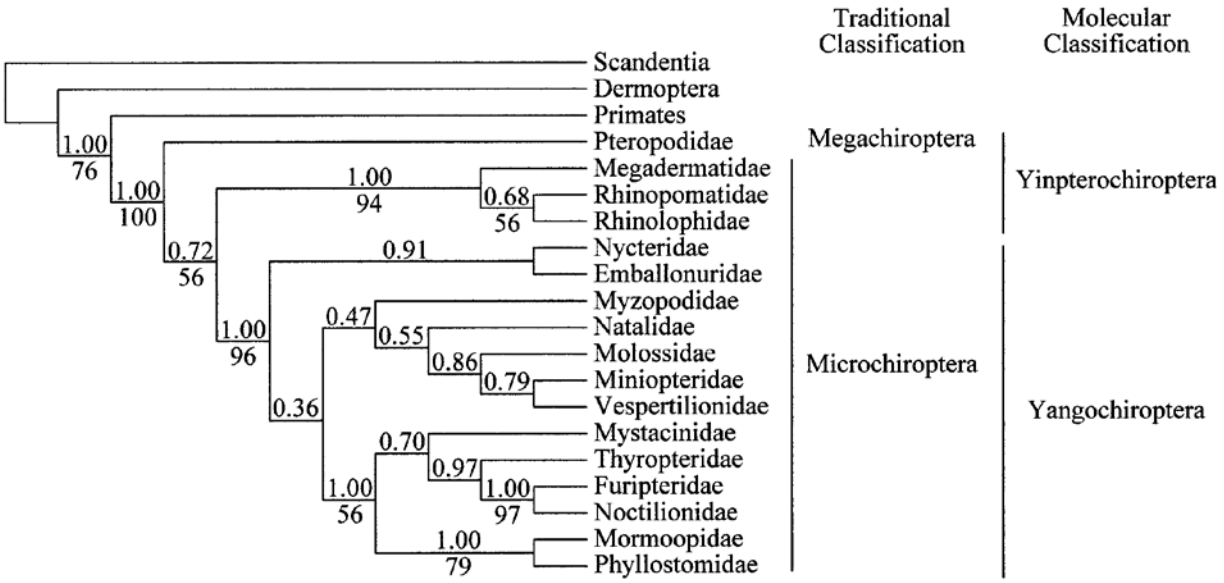


Figure 1.4: Tree showing phylogenetic relationships among bat families based on morphological attributes (traditional classification), particularly that of the musculoskeletal structure of the flying apparatus, and based on molecular attributes (molecular classification). Note that the molecular classification disputes the monophyletic origin of microbats while indicating the existence of a close relationship between some microbat families and the pteropodidae (megabat family). *Source: image from Teeling et al., 2002; 2005*.

While DNA sequencing has led many phylogeneticists to state that the two suborders of the Chiroptera, the megabats and microbats, belong to the same monophyletic mammalian order (e.g., Murphy et al., 2001, Teeling et al., 2002, Teeling et al., 2005), amino acid sequences conflict with the DNA phylogeny and do not support monophyly (e.g., Jaworski, 1995, Li et al., 2007, Haresnape, 2010, Interpro, 2015). The conflict between the DNA and amino-acid phylogenies echoes other studies that question bat monophyly. These began with Linnæus (1758), with more recent analyses of the reproductive organs (Smith and Madkour, 1980), of the retinotectal pathways (Pettigrew, 1986, Pettigrew et al., 2008), and of a suite of other neural and non-neural features (Pettigrew et al., 1989), having led to the development of the concept of a dual and independent phylogenetic origin for megabats and microbats. Most of the similarities between the two kinds of bats, even DNA similarities, can be attributed to the convergent selective pressures of powered flight associated with a very high metabolic rate, (Pettigrew and Kirsch, 1995, Hutcheon et al., 1998, Kirsch and Pettigrew, 1998, Pettigrew and Kirsch, 1998). In the dual phylogenetic origin scenario, the megabats have been proposed to be a sister group to the primates, closely associated with the dermopterans (Pettigrew, 1986, Pettigrew et al., 1989). Unfortunately, the potential phylogenetic relationships of the microbats remain unaccounted for in the dual origin scenario – if the megabats are related to dermopterans and primates, to what group/s are the microbats related? In this sense, the insectivores become interesting, as earlier analyses of the cholinergic, catecholaminergic, serotonergic and orexinergic systems (Maseko et al., 2007, Maseko and Manger, 2007, Dell et al., 2010, Kruger et al., 2010a, Kruger et al., 2010b, Dell et al., 2013) have indicated similarities between the nuclear organization of these systems in the microbat brain with that reported in the brains of the laboratory shrew and the European hedgehog (Maseko et al., 2007, Dell et al., 2010). Thus, chiropteran phylogeny is a truly

unsettled issue in the broader scope of mammalian phylogeny, with the supporters of the monophyletic origin of chiropterans placing them with perissodactyls/carnivores/cetartiodactyls, and the supporters of the diphyletic origin of chiropterans placing the megabats with dermopterans/primates and the microbats with the insectivores, specifically the shrews. The characteristics of adult neurogenesis, especially the pathways and neurogenic zones may provide additional characters of importance to this debate.

Despite the differences between and within the suborders of Chiroptera and the apparent lack of close affinity to other mammalian orders (in the monophyletic scenario), Chiropterans exhibit behaviours and characteristics that are similar within and between the two suborders and to other mammals. In most mammals some of these behaviours, which Chiropterans have in common with them, have been associated with high rates of adult neurogenesis. For example, bats are highly social mammals and have social systems that are suggested to be more diverse than those of other mammalian orders (Kerth, 2008b). Both megabats and microbats live in colonies of thousands in a roost site (Altringham, 1999). The colonies are either maternal conies with female bats and young pup, paternal colonies with male bats, or mixed colonies with both male and female bats (Kerth, 2008b). The root cause of the variety of sociality seen in bats is yet to be determined, but is suggested to include the advantages associated with thermoregulation, social rearing (as in maternal colonies) and information transfer (Kerth and van Schaik, 2012). Social interaction, such as the male-female interaction and mother-pup interaction, may have an influence on the animal's psychology, physiology, and behavior, and could therefore modulate the process of adult neurogenesis. Increased proliferation rates and neuronal survival have been reported in animals that are highly social, and low rates in socially isolated animals as seen with mother-pup separation (Lieberwirth and Wang, 2012). The influence of social separation is

underscored by the fact that it abolishes the salvage effect of running on adult neurogenesis (Stranahan et al., 2006). While bats are highly social animals, the correlation between the social environment and adult neurogenesis appeared questionable in the highly social microbats because of the reported lack of adult neurogenesis (Amrein et al., 2007). Given that adult neurogenesis was reported in the megabats (Gatome et al., 2010), it is only sensible that more studies should be done using different families to further interrogate this association and to establish why the phenomenon may be different in these animals.

While bats are social, their colony size is flexible depending on the season and is associated with a fission-fusion social milieu (Amici et al., 2008, Aureli et al., 2008, Kerth, 2008a, Kerth et al., 2008, Kerth et al., 2011). Fission-fusion sociality is thought to require complex cognitive skills, and therefore may affect the rate of adult neurogenesis (Aureli et al., 2008). The cognitive abilities of bats are not only important for fission-fusion sociality, but also for foraging and migration. Given that they are mostly temperate/tropical animals, seasonal changes in climate and food indicate that they may need to migrate to new roosting sites and foraging habitats on a seasonal basis. For both foraging and migration (sometimes for over thousands of kilometers), bats are dependent on their spatial memory (Richter and Cumming, 2008). While they use a variety of sensory cues in migration, their spatial memory appears to take precedence over external sensory cues, as seen with behavioural studies in which they avoided previously encountered obstacles which have been long removed (Carter et al., 2010, Ross and Holderied, 2013). Given that bats have basal metabolic rates which are higher than most mammals, their spatial memory abilities give them the advantage of being more effective foragers and hence expending less energy (Winter and von_Helversen, 2001). Their apparently well-developed spatial memory could also explain their homing ability and roost fidelity, as they

tend to return to the same roosting and foraging sites each season. In instances where spatial memory fails to reward the bats with profitable feeding sites, other sensory cues such as scent and echoacoustics are used (Carter et al., 2010). In using other sensory cues, the bats go through discriminate learning using target feeding characteristics like flower shape and size, which influence echo reflectance, and associate these characteristics with rewarding feeding sites (Simon et al., 2006). Spatial memory and discrimination learning are hippocampus dependent functions that are associated with increased rate of adult neurogenesis in most mammals. The new granule cells generated during adult neurogenesis are thought to function in pattern separation which assists in spatial separation (Sahay et al., 2011). It is noteworthy that the previous study reporting a lack of adult hippocampal neurogenesis in microbats, which demonstrate such a range of cognitive abilities, underlines the need for a more detailed series of studies on this diverse and speciose order. Such studies become essential given the diversity in foraging habitats and styles observed in the chiropterans. Because of the varying foraging habitats (environmental complexity) and styles, which also mean varying physical activities, there exist variations in the brain size and structure (Safi and Dechmann, 2005, Safi et al., 2005, Dechmann and Safi, 2009). Physical activity has been associated with increased rates of adult neurogenesis. The behavioural diversity of bats presents a great opportunity to examine the influence of behaviour on adult neurogenesis using genetically and phylogenetically related, or perhaps unrelated, animals.

In addition to the phylogenetic history, cognitive abilities and sociality of bats, their longevity makes them ideal, when compared to rodents, for comparison of adult neurogenesis with primates, which also have a longer life span than most other mammals. With a life span between

10 and 40 years, chiropterans have an average life span that is 3.5 times greater than other placental mammals of similar body mass (Wilkinson and South, 2002). Their longer life means a prolonged exposure to the environment and the likely resultant changes to the process of adult neurogenesis, such as the epigenetic changes which could be comparable to those occurring in primates. Thus chiropterans are potentially a good model to provide answers to some of the lingering questions on adult neurogenesis.

1.8 Significant questions to be answered: Aims and Objectives of the study

While much progress has been made towards the understanding of adult neurogenesis, its functional significance, especially in the non-laboratory environment of the real world, remains poorly understood. Compounding the difficulty in understanding the purpose/s of this process are the varying findings observed across a number of mammals and the lack of effect of some variables when tested on wild-living animals. This then raises the question of whether adult neurogenesis is just a rudimentary process of brain development and nothing more. However, given the energy used in the process, it seems unrealistic to maintain an irrelevant, but energy-expensive process, especially in animals with a high metabolic rate such as the bats. A clearer picture on the purpose of adult neurogenesis could emerge from comparative studies across various mammals. Toward this end the use of wild-living animals in the pursuit of the proper understanding of the functional significance of adult neurogenesis could be of value. In using wild animals, a bigger picture than the one derived from the use of laboratory-bred animals could be unearthed given the range of behaviours, complex environments they inhabit and the evolutionary history of wild-living mammals. However, using wild living mammals entails the

capture and handling of these animals, something which they are not used to. This could have an effect on the process of adult neurogenesis and hence our interpretation of the functional significance of adult neurogenesis. In addition, it is difficult to control various confounding factors affecting neurogenesis when using wild living animals, something which can be readily done using captive bred or laboratory bred animals. So how different adult neurogenesis in the wild living animals from the captive bred or laboratory bred animals?

In this thesis I endeavour to add to the understanding of the phenomenon of adult neurogenesis by exploring whether adult neurogenesis varies in the suborders of the Chiropterans due to their evolutionary history, whether the reported occurrence of adult neurogenesis in the wild-living bats could actually be higher than previously reported, and whether the use of wild living species is more beneficial for our developing understanding of adult neurogenesis than the use of laboratory animals.

1.8.1 Main aims and studies undertaken

Chiropterans have both brains and behaviours assumed to be associated with the occurrence of adult neurogenesis and yet a previous study failed to show significant adult neurogenesis in microbats (Amrein et al., 2007), but clear presence in megabats (Gatome et al., 2010). The current study aims to examine a broad range of chiropteran species for adult neurogenesis to determine whether these previous observations are correct, and if not, where these previous studies went wrong. In this sense, the work presented in this current thesis can be divided into four specific studies.

Study 1: Adult neurogenesis in eight megachiropteran species

In this study we evaluated, using immunohistochemical methods, the presence and characteristics of proliferating and newly generated neurons in the brain of eight wild-caught adult Megachiropteran species. For the neurogenic patterns observed, direct homologies are evident in other mammalian species; however, there were several distinctions in the presence or absence of proliferating and immature neurons, and migratory streams that provide important clues regarding the use of the brain in the analysis of Chiropteran phylogenetic affinities. In all eight species studied, numerous Ki-67 and DCX immunopositive cells were identified in the subventricular zone (SVZ). These cells migrated to the olfactory bulb through a Primate-like rostral migratory stream (RMS) that is composed of dorsal and ventral substreams which merge before entering the olfactory bulb. Some cells were observed emerging from the RMS coursing caudally and dorsally to the rostral neocortex. In the dentate gyrus of all species, Ki-67 and DCX-expressing cells were observed in the granular cell layer and hilus. Similar to Primates, proliferating cells and immature neurons were identified in the SVZ of the temporal horn of Megachiropterans. These cells migrated to the rostral and caudal piriform cortex through a Primate-like temporal migratory stream. Sparsely distributed Ki-67 immunopositive, but DCX immunonegative, cells were identified in the tectum, brainstem and cerebellum. The observations from this study add to a number of neural characteristics that phylogenetically align Megachiropterans to Primates.

Study 2: Microbats appear to have adult hippocampal neurogenesis, but post-capture stress causes a rapid decline in the number of neurons expressing doublecortin

A previous study investigating potential adult hippocampal neurogenesis in microchiropteran bats failed to reveal a strong presence of this neural trait. As microchiropterans have a high field metabolic rate and a small body mass, it is possible that capture/handling stress may lead to a decrease in the detectable presence of adult hippocampal neurogenesis. Here we looked for evidence of adult hippocampal neurogenesis using immunohistochemical techniques for the endogenous marker doublecortin in 10 species of microchiropterans euthanized and perfusion fixed at specific time points following capture. Our results revealed that when euthanized and perfused within 15 minutes of capture, abundant putative adult hippocampal neurogenesis could be detected using doublecortin immunhistochemistry. Between 15 and 30 minutes post-capture, the detectable levels of doublecortin dropped dramatically and after 30 minutes post-capture, immunohistochemistry for doublecortin could not reveal any significant evidence of putative adult hippocampal neurogenesis. Thus, as with all other mammals studied to date apart from cetaceans, bats, including both microchiropterans and megachiropterans, appear to exhibit substantial levels of adult hippocampal neurogenesis. This study underscored the concept that, as with laboratory experiments, studies conducted on wild-caught animals need to be cognizant of the fact that acute stress (capture/handling) may induce major changes in the appearance of specific neural traits.

Study 3: The pattern of adult neurogenesis in three microchiropteran species, Hipposideros fuliganosis, Triaenops persicus and Asellia tridens

This study used Ki-67 and doublecortin (DCX) immunohistochemistry to delineate the neurogenic zones, migratory pathways and terminal fields associated with adult neurogenesis in

the brains of three microchiropterans. As with the majority of mammals studied to date, the subgranular and subventricular neurogenic zones were observed. Distinct labelling of newly born cells and immature neurons within the dentate gyrus of the hippocampus was observed in all species. A distinct rostral migratory stream, that appears to split around the medial aspect of the caudate nucleus was observed. These two streams merged at the rostroventral corner of the caudate nucleus to turn and enter the olfactory bulb, where a large terminal field of immature neurons was observed. DCX immunolabelled neurons were observed mostly in the rostral neocortex, but the potential migratory stream to the neocortex was not readily identified. In comparison, a broad swathe of newly born cells and immature neurons appeared to migrate from the caudoventral division of the rostral migratory stream to invest into the piriform cortex. In addition occasional immature neurons were observed in the amygdala and DCX immunopositive axons were observed in the anterior commissure. While the majority of these features have been found in several mammals, the migratory pathway to the piriform cortex and the presence of DCX immunostained axons in the anterior commissure are features only observed in microchiropterans and insectivores to date. In the diphyletic scenario of chiropteran evolution, these observations, along with other observations from unrelated neural systems, align the microchiropterans with the insectivores.

Study 4: Adult hippocampal neurogenesis in Egyptian fruit bats from differing environments: are differences environmental or methodological?

We quantified both proliferative (Ki-67 immunohistochemistry) and immature (doublecortin immunohistochemistry) cells within the dentate gyrus of adult Egyptian fruit bats

from three distinct environments: (1) primary rainforest; (2) subtropical woodland; and (3) fifth-generation captive-bred. We used four different previously reported methods to assess the effect of the environment on proliferative and immature cells: (1) the comparison of raw totals of proliferative and immature cells; (2) these totals standardized to brain mass; (3) these totals expressed as a density using the volume of the granular cell layer (GCL) for standardization; and (4) these totals expressed as a percentage of the total number of granule cells. For all methods, the numbers of proliferative cells did not differ statistically amongst the three groups, indicating that the rate of proliferation, while malleable to experimental manipulation or potentially in response to events of importance in the natural habitat, appears to occur, for the most part, at a predetermined rate within a species. For the immature cells standardizations to brain mass and GCL volume revealed no difference between the three groups studied; however, the raw numbers and standardization to total granule cell numbers indicated that the two groups of wild-caught bats had significantly higher numbers of immature neurons than the captive-bred bats. These contrasting results indicate that the interpretation of the effect of the environment on the numbers of immature neurons appears method dependent. It is possible that current methods are not sensitive enough to reveal the effect of different environments on proliferative and immature cells.

Chapter 2: Adult neurogenesis in eight Megachiropteran species

2.1 Introduction

Adult neurogenesis refers to the process of proliferation of progenitor cells, migration of these newly born neurons, maturation with the development of functional neuronal characteristics, and integration of these neurons into existing neuronal networks (Balu and Lucki, 2009). This trait is assumed to have been inherited by mammals from the common ancestor of extant chordates (Zupanc, 2001, Kempermann, 2012). Considerable differences have been reported in the occurrence of adult neurogenesis in mammals, including animals from closely related orders, species and strains (Bonfanti and Peretto, 2011). Despite the lack of a clear understanding of the functional relevance of adult neurogenesis, it is generally thought that this process is affected by both the animal's ecology and phylogenetic history (Bartkowska et al., 2010). For example, highly social, prey organisms, with large home ranges, show high rates of adult hippocampal neurogenesis (Hutcheon et al., 2002, Amrein and Lipp, 2009, Pravosudov and Smulders, 2010).

Chiropterans, the second largest order of mammals, have diverse behavioural and ecological characteristics (Dechmann and Safi, 2009). With life spans ranging between 10 and 40 years, Chiropterans have an average life span that is 3.5 times greater than other placental mammals of similar body mass (Wilkinson and South, 2002). The flying mammals, Megachiroptera/megabats and Microchiroptera/microbats have been grouped together in the order Chiroptera based on molecular and morphological similarities, particularly that of the musculoskeletal structure of the flying apparatus (Pettigrew et al., 1989, Adkins and Honeycutt,

1991, Baker et al., 1991, Mindell et al., 1991, Ammerman and Hillis, 1992, Bailey, 1992, Stanhope et al., 1992). Despite being placed in the same order, Megachiropterans and Microchiropterans have many contrasting attributes. Megachiropterans are indigenous to the Old World, large bodied and vegetarian (feed on fruit, nectar and flowers), while Microchiropterans are found throughout the world and are mostly insectivorous (Pettigrew et al., 1989). Moreover, the neuroanatomy of Megachiropterans and Microchiropterans differ substantially, with the Megachiropterans displaying brain traits shared with Primates, while the Microchiropterans have brains more similar to Insectivores. These shared neuroanatomical features between megachiropterans and primates include among others a similar apomorphous retinotectal pathway, a laminated lateral geniculate body, a superior colliculus which is larger than the inferior colliculus, presence of the middle temporal area with direct input from area 17, similar arrangement of corticospinal motor neuron fields, large hind limb presentation on the somatosensory cortex, and similar morphology and distribution of the cholinergic and serotonergic neurons (Pettigrew, 1986, Maseko et al., 2007, Maseko and Manger, 2007, Pettigrew et al., 2008, Dell et al., 2010, Kruger et al., 2010a). Microchiropterans and insectivores have in common neuroanatomical features including the morphology and distribution of the cholinergic and serotonergic neurons of the brainstem systems (Pettigrew, 1986, Maseko et al., 2007, Maseko and Manger, 2007, Pettigrew et al., 2008, Dell et al., 2010, Kruger et al., 2010a). This variance in neuroanatomical traits between the two suborders of the Chiroptera has led to the flying Primate hypothesis of Chiropteran evolution, positing Megachiropterans as a branch of the Dermopterans and thus forming a sister group to the Primates (Pettigrew, 1986, Pettigrew et al., 1989).

A previous study revealed absent to low rates of adult hippocampal neurogenesis in Microchiropterans (Amrein et al., 2007), while a similar study examining Epomophorus wahlbergi, a Megachiropteran, yielded contradictory findings (Gatome et al., 2010). Proliferating and immature neurons were observed in the subgranular zone of the dentate gyrus and subventricular zone of the lateral ventricles of the Megachiropteran (Gatome et al., 2010). In the current study we investigated the occurrence of adult neurogenesis in eight wild-caught Megachiropteran species most of which had not been previously studied. We present findings of a qualititative assessment of adult neurogenesis using endogenous markers of proliferating cells (Ki-67) and immature neurons (doublecortin, DCX).

2.2 Materials and Methods

The brains of two individuals of the following Megachiropteran species were used in the current study: *Casinycteris argynnis* (average brain mass = 0.83 g), *Eidolon helvum* (average brain mass = 4.30 g), *Epomops franqueti* (average brain mass = 2.42 g), *Hypsignathus monstrosus* (average brain mass = 3.81 g), *Megaloglossus woermanni* (average brain mass = 0.60 g), *Rousettus aegyptiacus* (average brain mass = 2.01 g), *Scotonycteris zenkeri* (average brain mass = 0.64 g) and *Epomophorus wahlbergi* (average brain mass = 1.81 g). These animals were captured from wild populations near Kisumu, Kenya (*Eidolon helvum* and *Epomophorus wahlbergi*), and the Yoko rainforest, near Kisangani in the Democratic Republic of Congo (the six remaining species). The appropriate permissions were obtained from the Kenya National Museums and the Kenyan Wildlife Services, and the University of Kisangani. All animals were

treated and used in accordance with the University of the Witwatersrand Animal Ethics Committee guidelines (clearance number: 2008/36/1).

To minimize external influences on adult neurogenesis, the animals were anaesthetised (overdose of sodium pentobarbital, 100 mg/kg, i.p.) within one hour of capture and perfused through the left ventricle with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The brain was removed and post-fixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose in 0.1 M PB at 4°C and stored in an antifreeze solution at -20°C until sectioning. Before sectioning, the brains were divided into two halves in the mid-sagittal plane and the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4°C. The specimen was frozen in crushed dry ice and sectioned in the sagittal plane into 50 µm thick sections. A one in three series of sections was stained for Nissl substance (cresyl violet) to reveal cytoarchitectural features, Ki-67 and doublecortin (DCX) immunostaining to reveal proliferation of cells and immature neurons.

In this study we used antibodies to Ki-67, which is present in the nucleus during the G1 to M phases of the cell cycle, and antibodies to DCX, a microtubule-associated protein expressed during the postmitotic periods by migrating and differentiating neurons, as markers of proliferative activity and immature neurons respectively. These antibodies were previously used successfully in studies on Microchiropterans (Amrein et al., 2007) and Megachiropterans (Gatome et al., 2010). The advantage of using these markers to localize adult neurogenesis is that no pre-handling of the animal is needed. These antibodies also provide an average of the rate of expression of new neurons in natural conditions prior to capture of the animal (Bartkowska et al., 2010).

The sections were incubated in a 1.6% H2O2, 49.2% methanol, 49.2% 0.1 M PB solution, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10-minute rinses in 0.1 M PB. To block unspecific binding sites the sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal goat serum – NGS, for the Ki-67 antibody or 3% normal rabbit serum – NRS, for the DCX antibody, plus 2% bovine serum albumin, BSA, and 0.25% Triton X-100 in 0.1 M PB). Thereafter, the sections were incubated for 48 h at 4°C in the primary antibody solution (1:1000, rabbit anti-Ki-67, NCL-Ki-67p DAKO, or 1:300, goat anti-doublecortin, DCX, SC-18 Santa Cruz Biotech) under gentle agitation. The primary antibody incubation was followed by three 10 min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated anti-rabbit IgG, BA1000 for Ki-67, or anti-goat IgG, BA 5000 for DCX, Vector Labs, in 3% NGS/NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-minute rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin-biotin solution (1:125; Vector Labs), followed by three 10-min rinses in 0.1 M PB. Sections were then placed in a solution containing 0.05% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 µl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low power stereomicroscope until the background stain was at a level that would allow for accurate architectonic matching to the Nissl sections without obscuring the immunopositive structures. Development was stopped by placing sections in 0.1 M PB for 10 min, followed by two more 10 min rinses in this solution. Sections were then mounted on 0.5% gelatine coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody,

and sections where we omitted the secondary antibody. In both cases no staining was observed. The observed immunostaining patterns support the specificity of the antibodies as they are compatible with the observations made in another Megachiropteran (Gatome et al., 2010). It was not possible to undertake Western blot control testing, as the equipment and reagents necessary for this procedure was not available in the field.

Staining patterns of Ki-67 and DCX were observed using low power stereomicroscope and architectonic borders were traced according to the Nissl stained sections using a camera lucida. The Ki-67 and DCX staining patterns were then matched to the drawing from the traced Nissl stained sections. Selected drawings were then scanned and redrawn using the Canvas 8 software. Architectonic nomenclature was taken from the previously described neuroanatomical regions of the Megachiropterans (Kramer, 1966). Digital photomicrographs were captured using Zeiss Axioshop and Axiovision software. No pixilation adjustments, or manipulation of the captured images was undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

For the quantitative analysis reported herein, cell counts of proliferating cells (those immunopositive for Ki-67) were done in the left hippocampus, one of the principal constitutive site of adult neurogenesis, of all eight species. Ki-67+ cells numbers were obtained through physical counting using an Axioskop light microscope with a 63X objective. In all the species, immunopositive cells in every third section were quantified and the total cell count obtained through multiplying the cell counts by the inverse of the sampling fraction, a method previously used by Epp et al. 2009 and Gatome et al. 2010.

2.3 Results

In the current study we evaluated the occurrence of adult neurogenesis in eight species of Megachiropterans, seven of which had not been previously examined. In all eight species the pattern of immunostaining for Ki-67 and DCX was almost identical, but we did observe some minor differences in the density of immunopositive structures between species (summarized in Table 2.1). We observed Ki-67 immunopositive cells in two distinct proliferative regions of the subventricular zone that formed dorsal and ventral migratory substreams of the RMS that coursed around the caudate nucleus before they merged to form the consolidated RMS which entered the olfactory bulb. Ki-67 immunopositive cells were also present in the subgranular zone of the hippocampus, the cerebral neocortex, the piriform cortex and amygdala associated with a temporal migratory stream, and in the midbrain tectum, cerebellum and medulla oblongata. DCX immunopositive cells were observed in the subventricular zone and rostral and ventral migratory streams of the lateral ventricle through to the olfactory bulb, dentate gyrus of the hippocampus, cerebral neocortex, amygala and piriform cortex. Although we observed Ki-67 immunopositive cells in the tectum, cerebellum and medulla, no DCX staining was evident in these structures. These results are summarized in diagrammatic form using *Hypsignathus monstrosus* as our example (Figs. 2.1, 2.2).

2.3.1 The dorsal and ventral proliferative regions of the subventricular zone (SVZ), the rostral migratory stream (RMS) and the olfactory bulb

The subventricular zone was localized to the lateral walls of the lateral ventricle in all species studied, forming a thin layer over much of the medial aspect of the corpus striatum. This

region of the adult Megachiropteran brain was that with the greatest amount of adult neurogenesis (Figs. 2.1, 2.2, 2.3). Numerous Ki-67 immunopositive cells were observed in the SVZ, with no significant differences in cell density apparent between species. Many of the cells in this region were also immunopositive for DCX, where we noted immunopositive cells with relatively short unipolar and/or bipolar processes.

The subventricular zone housed two proliferative regions, the dorsal and ventral proliferative regions, which were observed to merge into one migratory stream, the RMS (Fig. 2.3). In the current study, these regions were distinguished on topological grounds and were separated by the medial bulge of the corpus striatum into the lateral ventricle. The dorsal proliferative region of the RMS produced cells that flowed along the dorsal and rostral aspects of the caudate nucleus, between the caudate nucleus and the subcortical white matter, before they turned in a rostral direction to end in the olfactory bulb. In all the species investigated, we observed numerous intensely Ki-67 and DCX immunopositive cells in this stream. The DCX positive cells were fusiform in shape and small in size with short bipolar processes. Most of these cells were obscured by the numerous tangentially oriented fibres in the stream. There was no significant difference between the various species investigated.

The ventral proliferative region of the RMS produced cells that coursed along the ventromedial border of the corpus striatum, flowing between the caudate nucleus and the nucleus accumbens (Figs. 2.1C-G, 2.2C-G). It was observed to merge with the dorsal portion of the RMS at the anterior genu of the RMS, before the RMS entered the olfactory bulb (Figs. 2.1, 2.2, 2.3). Again, numerous Ki-67 and DCX immunopositive cells were observed throughout the ventral portion of the RMS. The density, pattern and appearance of the immunopositive structures in the ventral portion of the RMS were comparable with that seen in the dorsal portion. Again, no

specific species differences were noted. Occassional Ki-67 and DCX immunopositive cells were observed in the corpus striatum, and while these cells had large soma, the dendrites emanating from these cells were not branched.

Ki-67 immunopositive cells were apparent in the olfactory bulb of all species investigated. *S. zeukuri, M. woermanni* and *C. argynnis* had lower densities of proliferating cells compared to the other species, while *H. monstrosus* and *E. franqueti* had higher cell densities. The Ki-67 immunopositive cells were mainly located in the periventricular layer of the olfactory ventricle, with occassional Ki-67 immunopositive cells distributed in the other layers of the olfactory bulb (Fig. 2.3). No species variance was observed with DCX immunoreactivity. In all species, intensely stained and densely packed neurons were observed in the granule cell layer, internal plexiform layer and mitral cell layer of the olfactory bulb (Fig. 2.3). The ovoid DCX immunopositive cells, located mainly in the periventricular layer of the olfactory ventricle, had a relative small to large soma size with unipolar processes radiating towards the glomerular layer. The arborisations of most of the processes terminated at the mitral cell layer with occasional axons reaching the external plexiform layer. Scantly distributed DCX immunopositive cells were observed in the external plexiform and glomerular layers of the olfactory bulb.

2.3.2 The subgranular zone and the dentate gyrus

In all Megachiropteran species a distinct subgranular zone, located between the granular cell layer and the hilus of the dentate gyrus of the hippocampus, was evident displaying both Ki-67 and DCX immunopositive cells. We noted Ki-67 immunopositive cells in the subgranular zone of all species investigated (Fig. 2.4). Of the species examined, *H. monstrosus, E. franqueti*

and *M. woermanni* had the highest densities of Ki-67 immunopositive cells. Moderate Ki-67 immunopositive cell densities were observed in *C. argynnis* and *R. aegypticus*, with fewer cells observed in *S. zeukeri*, *E. helvum*, and *E. wahlbergi* (see below). DCX immunopositive cells, with relatively small cell bodies were also detected in the dentate gyrus. The majority of these cells exhibited bipolar processes that extended into the molecular layer of the dentate gyrus. Occasional cells had short, unipolar processes and some had no obvious processes. No apparent differences in DCX immunopositive structures were observed between species.

2.3.3 Neocortex

A small stream of Ki-67 immunopositive cells were observed to emerge from the genu of the RMS and appear to travel dorsally and caudally within the deeper cortical layers and the white matter deep to the cortex (Figs. 2.1, 2.2). This stream was restricted to the rostral half of the neocortex and throughout this region occassional Ki-67 immunpositive cells were observed appear to migrate superficially through the cerebral cortex to the supragranular layers. In layer II, small clusters of DCX immunopositive neurons were observed in the rostral half of the neocortex. These DCX immunopositive neurons were observed to be multipolar with extensive apical dendrites ramifying into layer I (Fig. 2.5). Horizontal dendritic arbours were also observed emanating from these DCX immunopositive layer II neurons. This neocortical stream of the RMS and DCX immunopositive layer II neurons were observed in all species investigated, with no noticeable species variance.

2.3.4 Temporal migratory stream, piriform cortex and the amygdala

In all species studied we identified a potential neurogenic region surrounding the temporal pole of the lateral ventricle, where a substantial number of Ki-67 immunopositive cells were present (Figs. 2.1, 2.6). The Ki-67 immunopositive cells were either located adjacent to the ventricular ependyma, or were located a short distance from this region in the white matter below the piriform cortex, with one or two cells observed in the amygdala. From this temporal pole neurogenic region what appears to be a significant migratory pathway, coursing both rostral (below the amygdala) and caudal (below the hippocampus), was observed with DCX immunohistochemistry (Figs. 2.2, 2.6). This apparent migration of DCX immunopositive cells was observed to end in the second layer of the piriform cortex, where numerous DCX immunopositive cells with large soma and long, ramified, bipolar processes were observed in clusters reminscent of the cytoarchitecture of this region. It appeared that this temporal migratory stream exclusively supplied the entire piriform cortex with newly generated neurons. In addition, a few DCX immunopositive cells with large soma were observed in the amygdala nuclear complex, but these cells had short unipolar or bipolar processes that exhibited no branching.

2.3.5 Tectum, cerebellum and brainstem

In all the Megachiropterans investigated, we observed randomly and sparsely distributed Ki-67 immunopositive cells in the superior and inferior colliculi, the periventricular grey matter of the brainstem, including area postrema, the cerebellar peduncles and cerebellar cortex (Figs. 2.1, 2.7). These cells often existed in pairs, a possible indication of mitotic activity. The intensity of the immunostaining of the Ki-67 immunopositive cells in this region was similar to that

observed in the dentate gyrus of the hippocampus and the subventricular zone. In contrast, we did not observe any DCX immunopositive cells in these regions.

2.3.6 Counts of Ki-67 Immunopostiive Cells in the Dentate Gyrus

In all species studied, Ki-67 immunpositive cells revealed neurogenesis in the dentate gyrus. In order to test whether reported differences in life-history parameters affected the rate of adult hippocampal neurogenesis, we quantified the numbers of Ki-67 immunopositive cells in the left hippocampus of all eight species studied (Table 2.2). The results of this quantification revealed that the species with the largest number of proliferating cells was *H. monstrosus*, which is also the species with the largest brain. The remaining species with smaller brains had, for the most part, a lesser number of proliferating cells with decreasing brain mass. While it appears that there is a negative allometric relationship to this data, such that for every doubling in brain mass the number of proliferating cells in the left hippocampus increased by 1.8 times, the small dataset developed here did not allow us to find a statistically significant relationship between brain mass and number of proliferating cells (Fig. 2.8). Future studies adding more species to this sort of quantification should reveal a statistically significant relationship between brain mass (as a proxy for hippocampal volume) and the number of proliferating cells.

Table 2.1: Qualitative summary of the density of immunopositive cells in various regions of megachiropteran brains.

Species	SVZ		RMS		OB		SGZ of DG		NEO		TMS		PIR		Amyg		Tectum		Cb		MO	
	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX
Casinycteris argynnis	+++	+++	+++	+++	+	+++	+	++	+	++	++	+++	+	+++	+	+++	+	-	+	-	+	-
Eidolon helvum	+++	+++	+++	+++	++	+++	+	++	+	++	++	+++	+	+++	+	+++	+	-	+	-	+	-
Epomops franqueti	+++	+++	+++	+++	+++	+++	++	+	+	+	++	+++	+	+++	+	+++	+	-	+	-	+	-
Hypsignathus monstrosus	+++	+++	+++	+++	+++	+++	+	++	+	+++	++	+++	+	+++	+	+++	+	-	+	-	+	-
Megaloglossus woermanni	+++	+++	+++	+++	+	+++	++	++	+	++	++	+++	+	+++	+	++	+	-	+	-	+	-
Rousettus aegyptiacus	+++	+++	+++	+++	++	+++	+	++	+	++	++	+++	+	+++	++	++	+	-	+	-	+	-
Scotonycteris zenkeri	+++	+++	+++	+++	+	+++	+	+++	+	+++	++	+++	+	+++	++	++	+	-	+	-	+	-
Epomophorus wahlbergi	++	++	++	++	+	+++	+	+	+	++	+	+++	+	+++	+	+	+	-	+	-	+	-

Amyg – amygdala; **Cb** – cerebellum; **CTX** – cerebral cortex; **DG** – dentate gyrus; **MO** – medulla oblongata; **NEO** – neocortex; **OB** – olfactory bulb; **PIR** – piriform cortex; **RMS** – rostral migratory stream; **SGZ** – subgranular zone; **SVZ** – subventricular zone; **TMS** – temporal migratory stream; **+** – low density; **++** – moderate density; **+++** – high density.

Table 2.2: Quantitative summary of Ki-67 immunopositive cells in the left hippocampus of the megachiropteran brains studied.

Species	**Brain mass (g)**	**Ki-67 count in left hippocampus**
Megaloglossus woermanni	0.6	1383
Scotonycteris zenkeri	0.64	312
Casinycteris argynnis	0.83	1065
Epomophorus wahlbergi	1.81	258
Rousettus aegyptiacus	2.01	1113
Eidolon helvum	2.42	999
Epomops franqueti	2.42	4749
Hypsignathus monstrosus	3.78	6258

Figure 2.1: Diagrammatic reconstruction of parasagittal sections through one half of the brain of *Hypsignathus monstrosus* (a representative animal of the seven Megachiroptera investigated in this study) depicting sites of the observed Ki-67 immunoreactive proliferating cells (each black star representing a single neuron). Each drawing of a parasagittal section is approximately 400 μm apart, with **A** being the most medial section and **T** being the most lateral. See list for abbreviations.

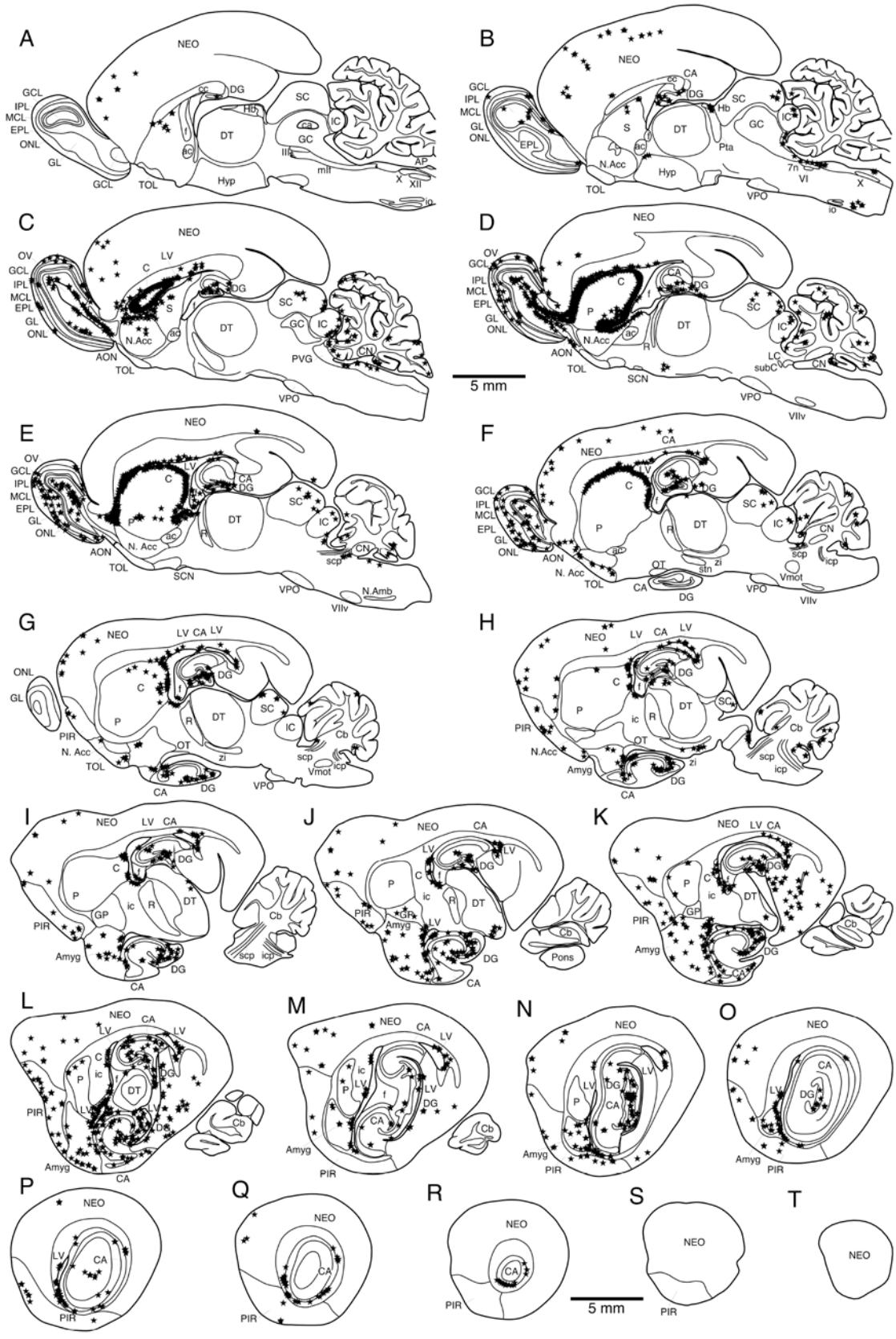

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Figure 2.2: Diagrammatic reconstruction of parasagittal sections through one half of the brain of *Hypsignathus monstrosus* (a representative animal of the seven Megachiroptera investigated in this study) depicting sites of the observed DCX immunoreactive immature neurons and fibres (each black dot representing a single neuron and the shaded areas representing fibres). Each drawing of a parasagittal section is approximately 400 μm apart, with **A** being the most medial section and **T** being the most lateral. See list for abbreviations.

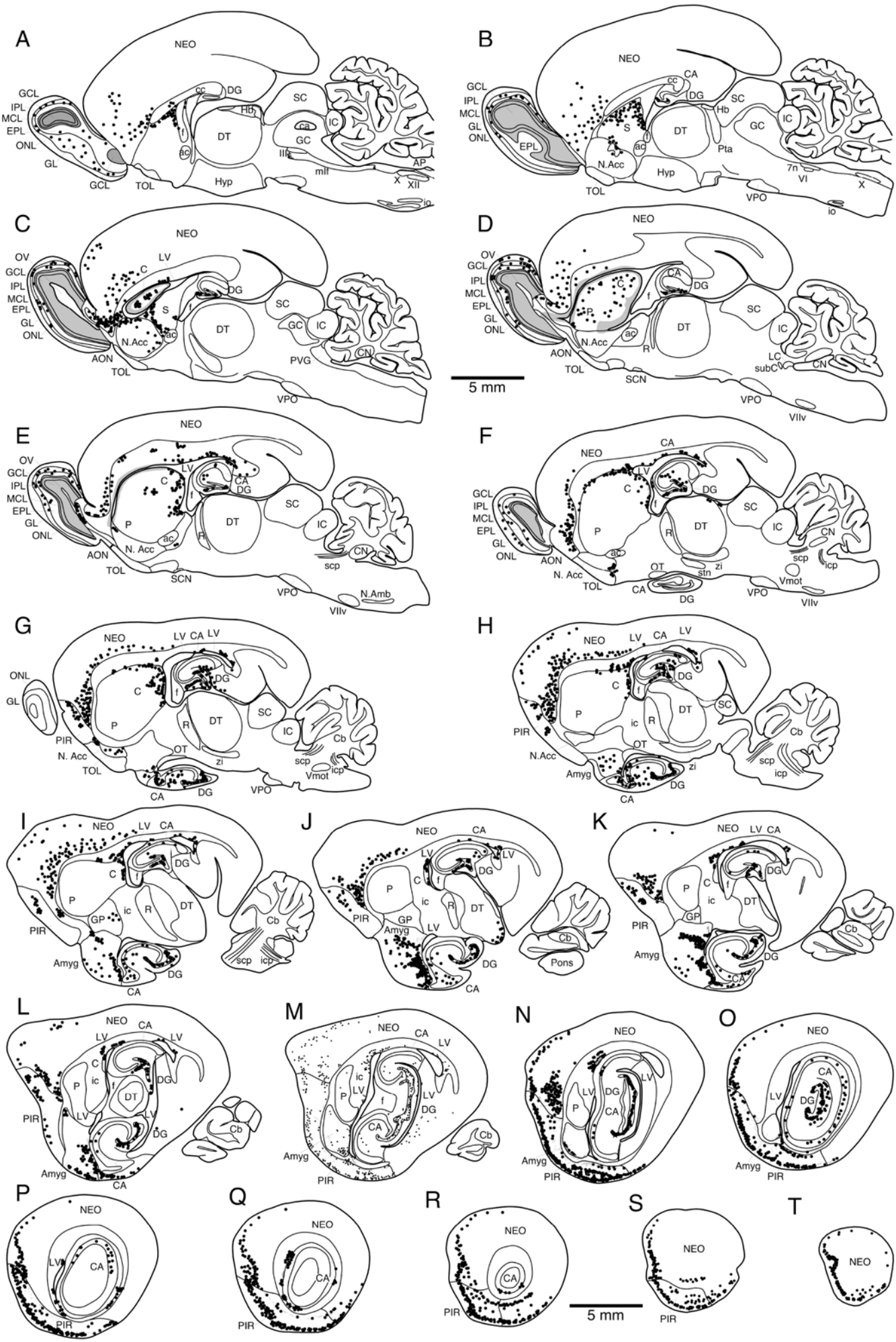
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Figure 2.3: Photomicrographs of Ki-67 (arrowheads) (**A**, **C**, **E**) and DCX (arrows) (**B**, **D**, **F**) immunostained parasagittal sections through the rostral migratory stream in the brain of three Megachiropteran species – **A** and **B** – *Cassinycterus argynnis*, **C** and **D** – *Rousettus aegyptiacus*, **E** and **F** – *Scotonycetris zenkeri*. In all images, rostral is to the left and dorsal to the top. Note the presence of Ki-67 immunostained cells in the subventricular zone of the lateral ventricle and the extensive rostral migration of neurons to the olfactory exposed by DCX staining. Scale bar in **F** = 500 μm and applies to all. See list for abbreviations.

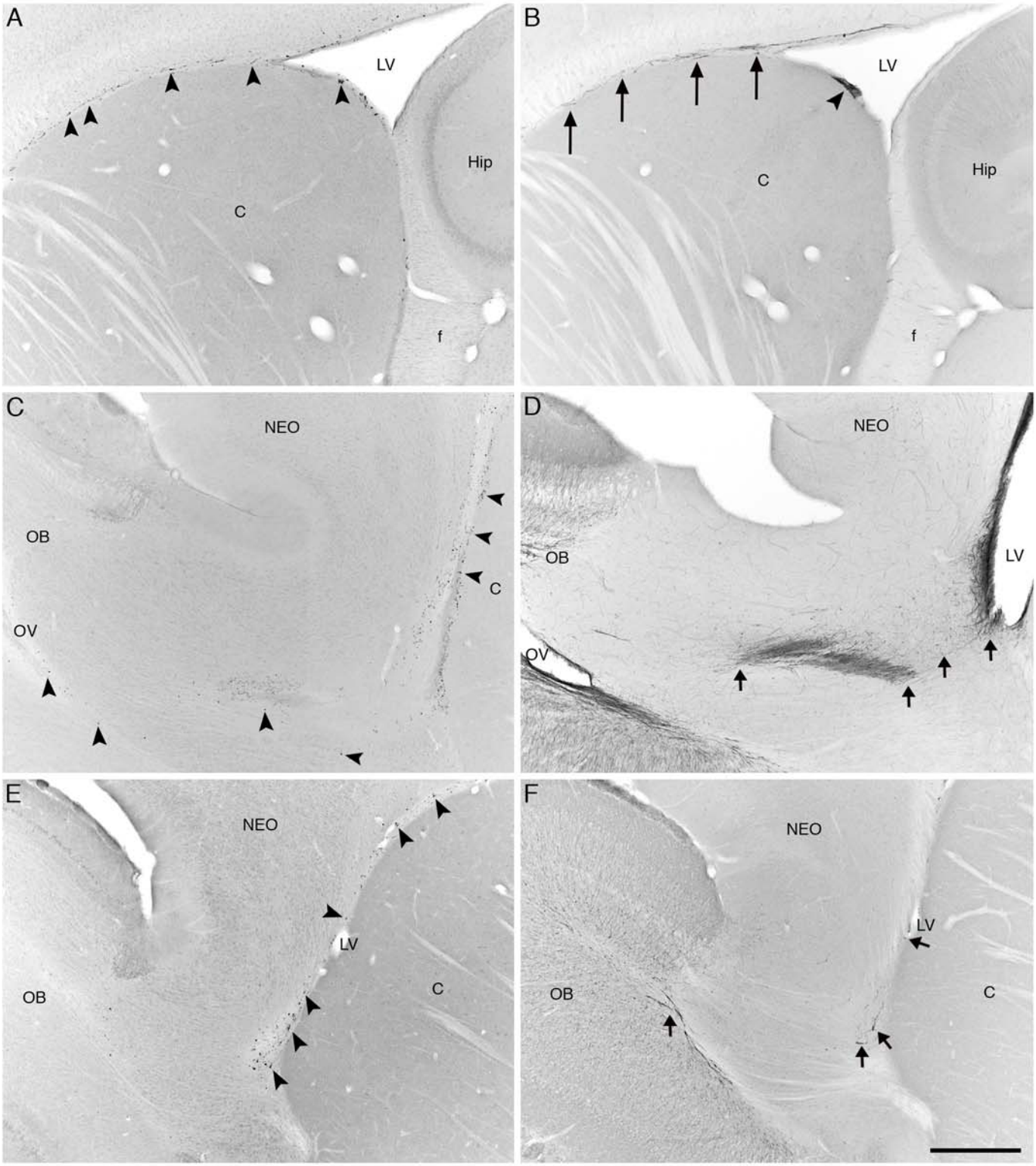
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Figure 2.4: Photomicrographs of Ki-67 (arrowheads) (**A**, **C**, **E**) and DCX (arrows) (**B**, **D**, **F**) immunostained parasagittal sections through the dentate gyrus in the upper portion of the hippocampus proper in the brain of three Megachiropteran species – **A** and **B** – *Epomops franqueti*, **C** and **D** – *Hypsignathus monstrosus*, **E** and **F** – *Megaloglossus woermanni*. In all images, rostral is to the left and dorsal to the top. Note the presence of Ki-67 immunostained cells in the subgranular zone and the maturing DCX immunostained cells in the granular layer of the dentate gyrus. Scale bar in **F** = 500 μm and applies to all.

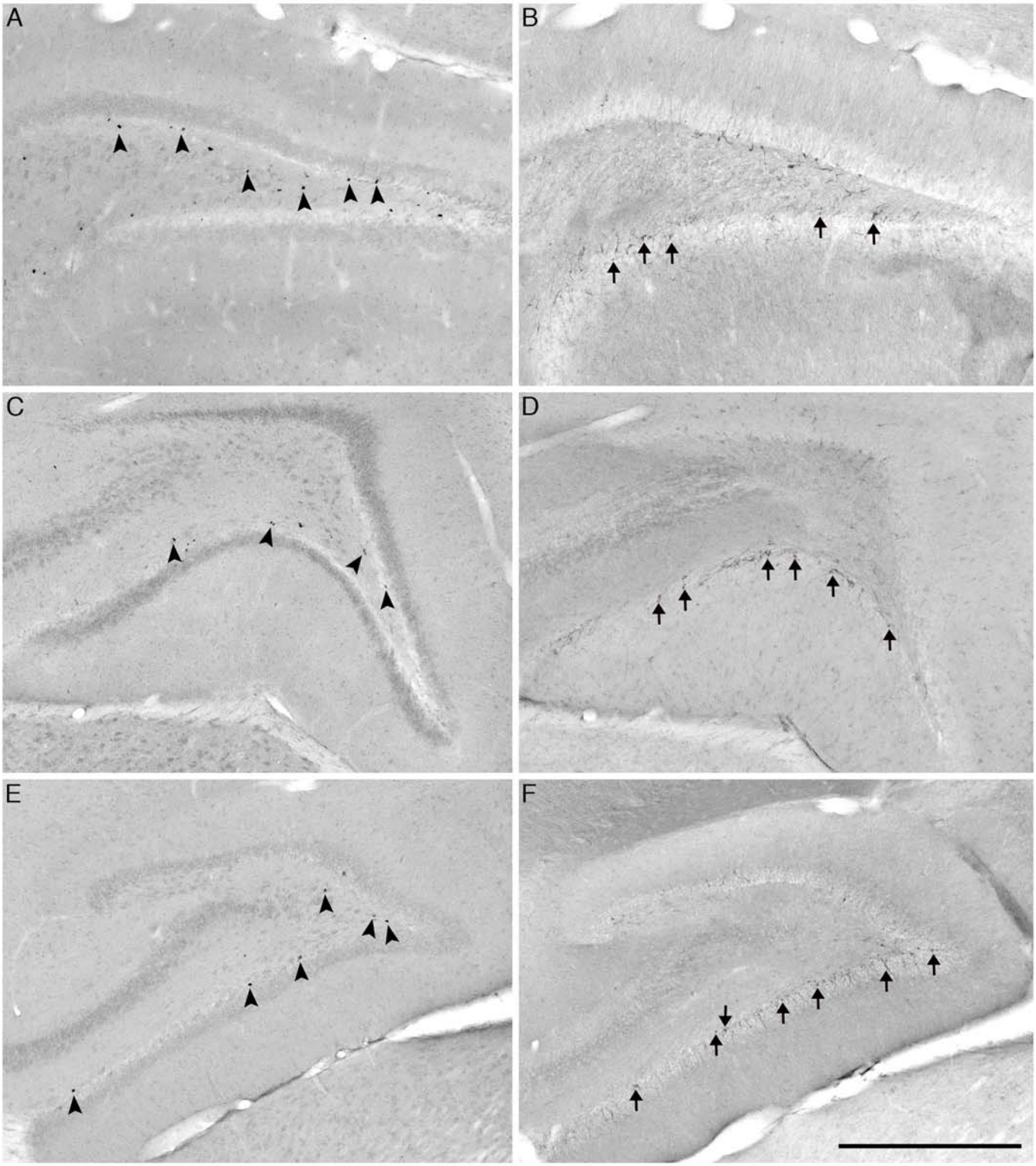
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Figure 2.5: Photomicrographs of DCX immunostained parasagittal sections through the motor region of the neocortex in the brain of four Megachiropteran species – **A** – *Rousettus aegyptiacus*, **B** – *Megaloglossus woermanni*, **C** – *Eidolon helvum*, **D** – *Epomops franqueti*. In all images the pial surface is to the top. Note the presence of DCX immunoreactive cells (arrows) in layer II of the neocortex, with dendrites extending horizontally in the layer and vertically into layer I. Scale bar in **D** = 100 μm and applies to all.

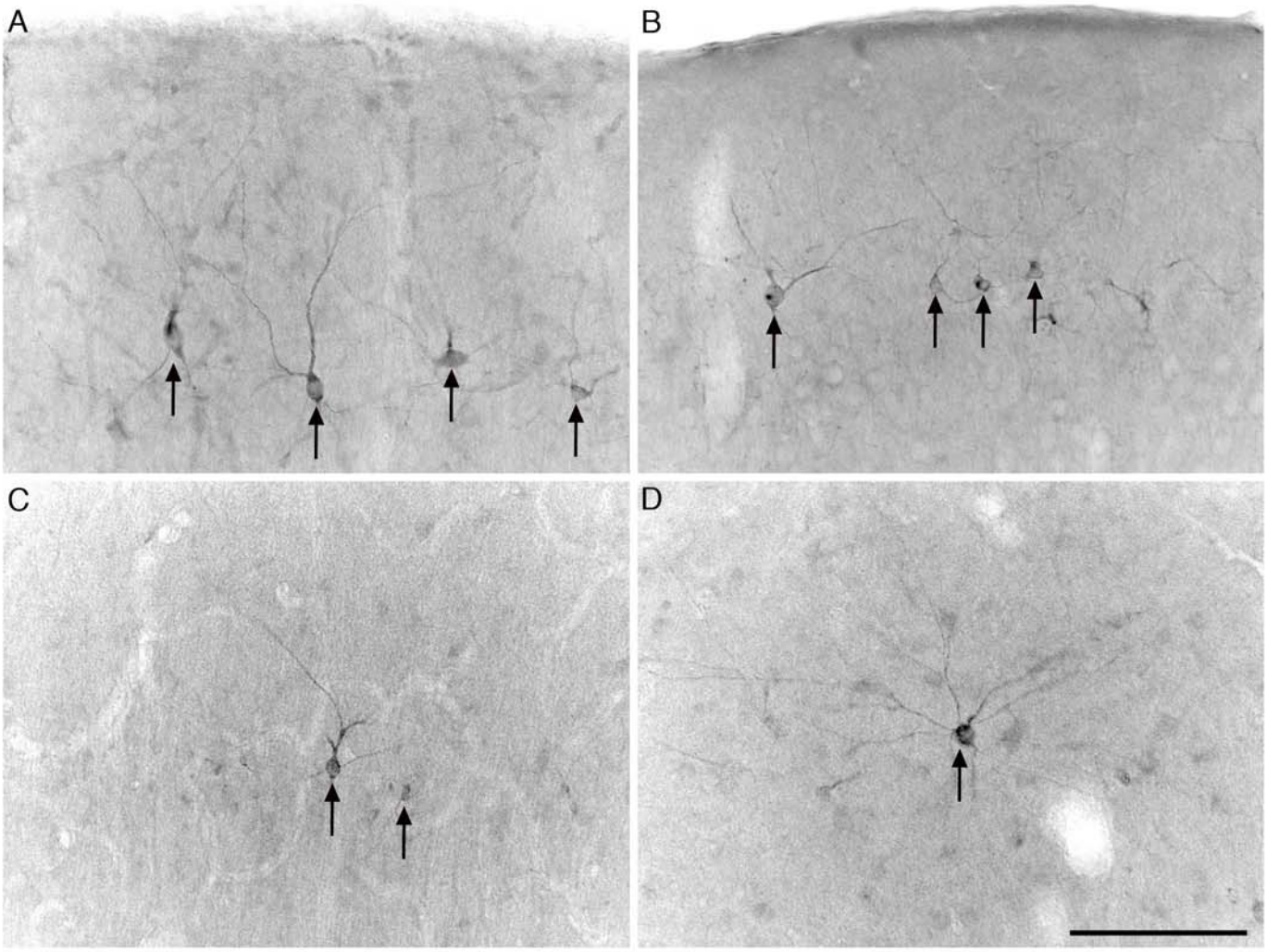
A
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Figure 2.6: Photomicrographs of Ki67 (**A**, **C**, **E**) and DCX (**B**, **D**, **F**) immunostained parasagittal sections through the temporal migratory stream region in the brain of three Megachiropteran species – **A** and **B** – *Hypsignathus monstrosus*, **C** and **D** – *Casinycterus argynnis*, **E** and **F** – *Scotonycteris zenkeri*. In all images, rostral is to the left and dorsal to the top. Note the presence of Ki67 immunostained cells in the temporal pole of the lateral ventricle (arrowheads) and the extensive rostral and caudal migration of neurons to the piriform cortical regions exposed by DCX staining. Scale bar in **F** = 500 μm and applies to all. See list for abbreviations.

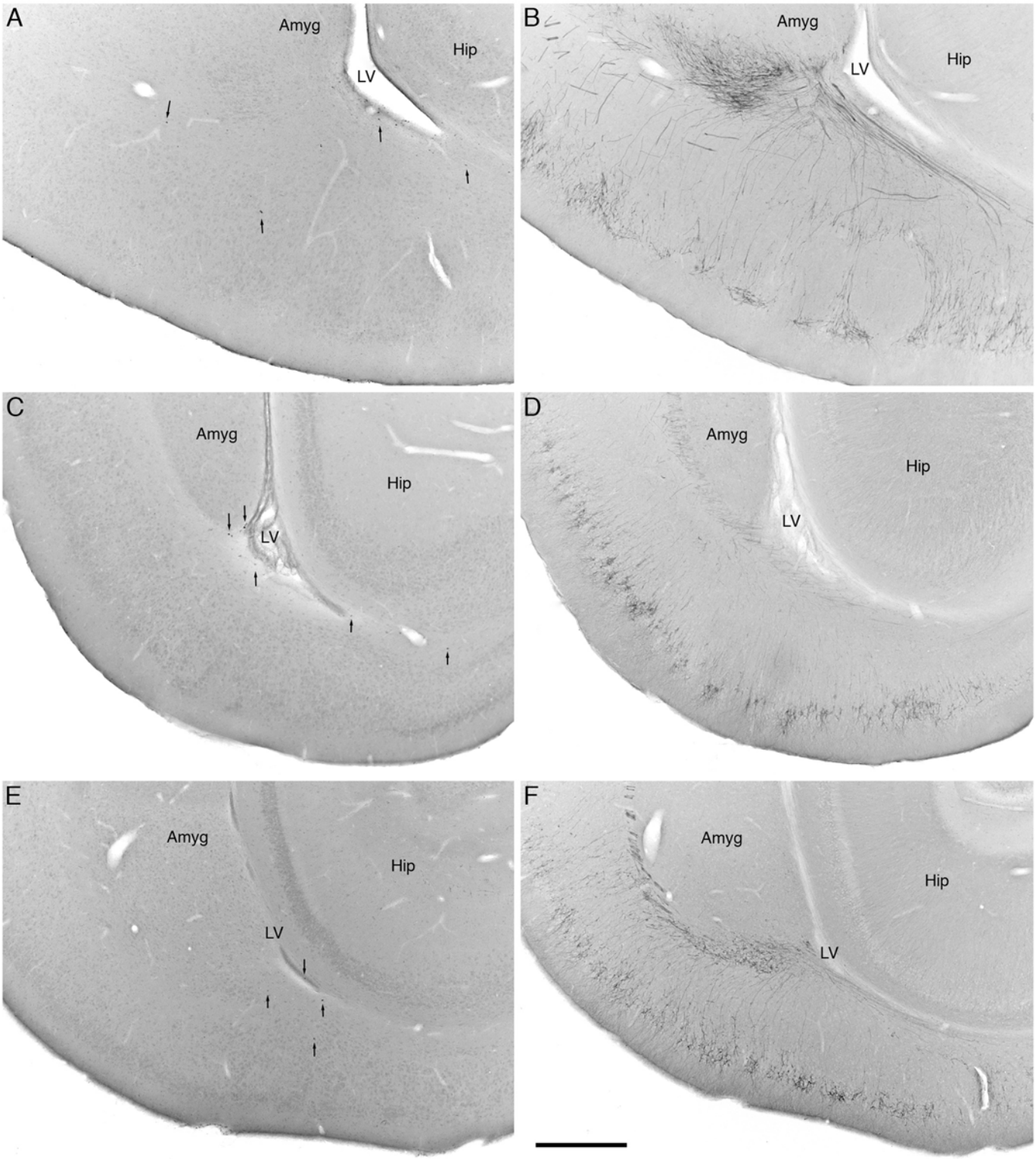
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Figure 2.7: Photomicrographs of Ki-67 immunostained parasaggital sections through the colliculi, cerebellum and brainstem of three species of Megachiropterans. In all images, rostral is to the left and dorsal to the top. **A** – Ki-67 immunostained cells (arrowheads) in the superior colliculus of *Epomops franqueti*. **B** – Ki67 immunostained cells in the superior and inferior colliculi of *Megaloglossus woermanni*. **C** – Ki-67 immunostained cells in the cerebellar cortex of *Epomops franqueti*. **D** – Ki-67 immunostained cells in the cerebellar cortex of *Hypsignathus monstrosus*. **E** – Ki-67 immunostained cells in the cerebellar peduncle and surrounding brainstem of *Megaloglossus woermanni*. **F** – Ki-67 immunostained cells in the dorsal portion of the brainstem caudal to the cerebellar peduncles in *Hypsignathus monstrosus*. Scale bar in **F** = 500 μm and applies to all. See list for abbreviations.

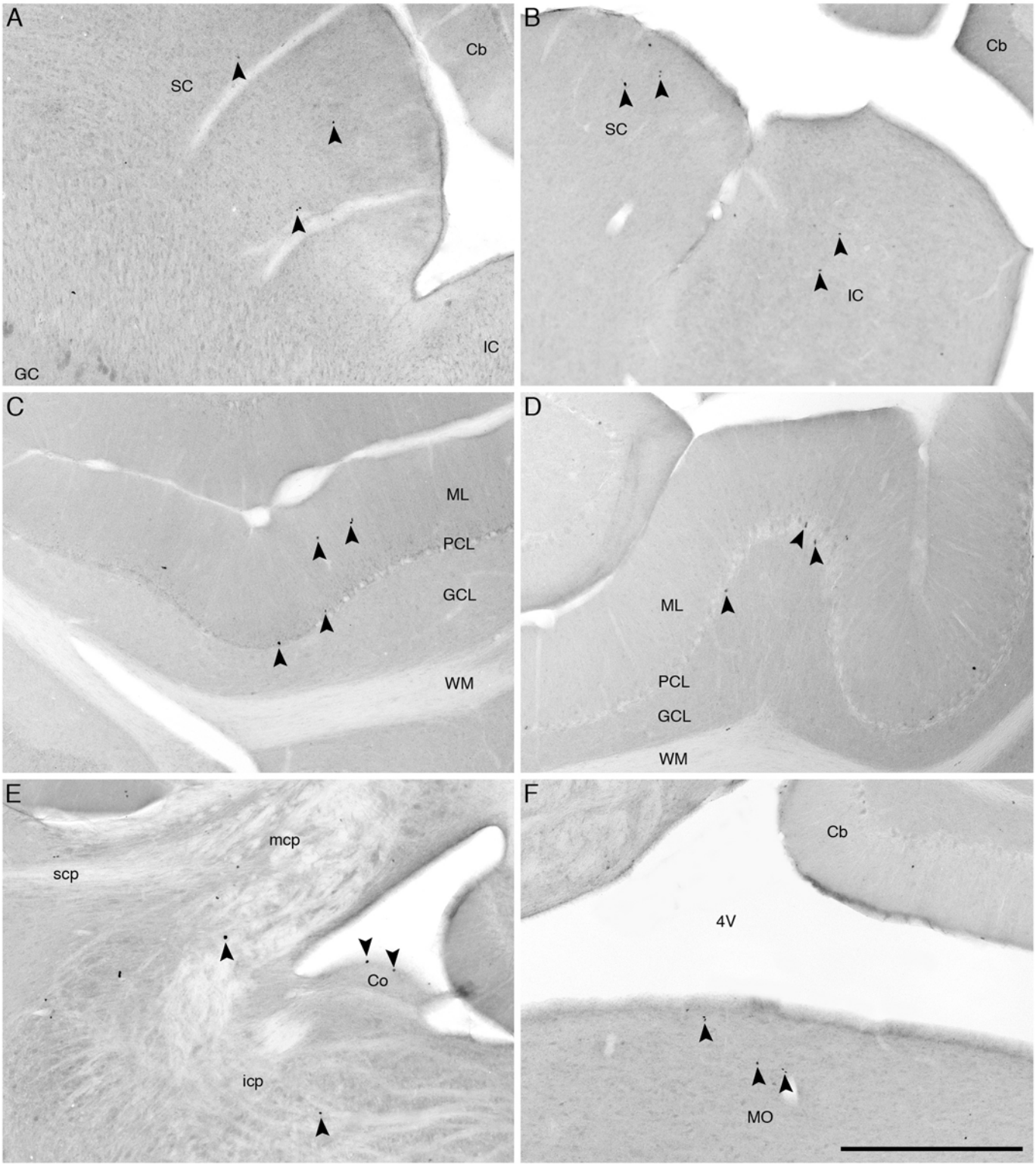
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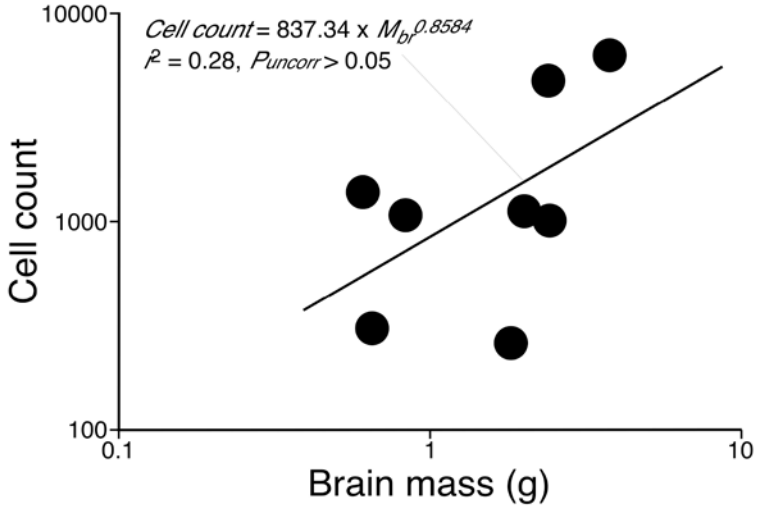
Figure 2.8: Graphical representation of the potential allometric relationship between brain mass and the number of proliferating cells in the left hippocampus of the eight Megachiropteran species studied herein. While the relationship is not statistically significant, it is likely that more data points over a greater range of species with differing brain sizes will reveal a significant relationship. This small dataset indicates that the rate of cell proliferation in the hippocampus may be related to allometric scaling rules rather than being the result of ecological or other life-history parameters.


Cell count = 837.34 x $M_{br}^{0.8584}$
r^2 = 0.28, P_{uncorr} > 0.05
10000
1000
100
Cell count
0.1
1
10
Brain mass (g)

2.4 Discussion

The current study evaluated the presence and characteristics of proliferating and newly generated neurons in the brain of eight wild caught Megachiropteran species. Our observations are congruent with, and extend, a previous report of adult neurogenesis examining the brain of Wahlberg's epaulleted fruit bat, *Epomophorus wahlbergi* (Gatome et al., 2010). Proliferating cells, identified using Ki67, and immature neurons, identified using DCX, were observed in the subventricular zone of the lateral ventricle. These cells were seen to migrate to the olfactory bulb through the RMS. From the genu of the RMS a stream of proliferating cells populating the rostral half of the neocortex was observed in all species studied. Immature neurons were also identified in layer II of the neocortex. As in most other mammals previously investigated, immunopositive proliferating and immature neurons were also observed in the subgranular zone of the dentate gyrus. Unlike most mammals, proliferating cells were observed in the temporal horn of the lateral ventricle. Immature neurons appeared to migrate from this location to form the temporal migratory stream that coursed rostral and caudal to supply the entire piriform cortex with immature neurons. The current study of eight Megachiropteran species, and the previous study of a single Megachiropteran species (Gatome et al., 2010), record similar results, indicating a pattern of adult neurogenesis that can be considered to be specific to the Megachiropterans. Despite this overall similarity in the neurogenic patterns and pathways, soma size, dendritic process length and branching in immature neurons varied from site to site and between species. Such variations can be linked to genetically determined variables that include various brain anatomies, developmental histories and lifespans (Bonfanti and Peretto, 2011).

2.4.1 Subventricular zone and the RMS

The subventricular zone of the lateral ventricle (SVZ) is the principal constitutive site of adult neurogenesis in mammals, and this statement is true of the Megachiropterans. Newly generated cells arising in the mammalian subventricular zone are thought to be destined for the olfactory bulb and the neocortical associative areas (Gould et al., 1999). The extent, organisation and cyto-architecture of the SVZ varies across mammals. In Rodents, proliferating cells originate from the anterior or rostral portion of the lateral ventricle, dorsal to the caudate nucleus (Luskin, 1993). This region of the SVZ in the Rodents has a meshwork of longitudinal astrocytic tubes enveloping proliferating cells, thereby restricting them to migrate tangentially into the rostral migratory stream (RMS) (Bonfanti and Peretto, 2011). In rhesus monkeys, the dorsal portion of the SVZ, capping the caudate nucleus, has a cytoarchitectural structure and exhibits cell densities that are similar to the rostral portion of the SVZ in Rodents (Pencea et al., 2001). The rostrodorsal aspect of the SVZ in Primates is thinner than the ventral SVZ and has a different cytoarchitectural structure, and as a result, proliferating cells are guided to either the dorsal or ventral substreams of the RMS (Gil-Perotin et al., 2009). The ventral part of the rostral migratory stream is also called SVZa-RMS (Bedard et al., 2002) and Vf-RMS (Pencea et al., 2001), and is not found in Rodents. In the rhesus monkey, the dorsal and ventral parts of the RMS merge before turning to course into the olfactory bulb. In the present study of Megachiropterans we observed both dorsal and ventral substreams of the RMS that have a very similar appearance to that observed in the rhesus monkey. The finding of a potential neurogenic pathway/pattern in Megachiropterans that appears to be shared only with Primates provides yet another neural characteristic that suggests a shared phylogenetic history (Pettigrew, 1986; Pettigrew et al., 1989, 2008; Maseko and Manger, 2007; Maseko et al., 2007; Kruger et al., 2010; Dell et al., 2010). It would be of interest to examine the microarchitecture of the two differing regions of the SVZ observed in Megachiropterans to determine how similar they are to that seen in Primates.

2.4.2 Subgranular zone (SGZ) and dentate gyrus

Adult hippocampal neurogenesis (AHN) has been reported across a variety of mammals including mice (Kempermann et al., 1997a, b), hedgehog (Bartkowska et al., 2010), monkeys (Gould et al., 1999, Gould et al., 2001), and humans (Eriksson et al., 1998) (see Kemperman, 2012, for a review of the occurrence of AHN across vertebrates). In the present study we observed a substantial number of Ki-67 and DCX immunopositive neurons, with varying densities, in all Megachiropteran species studied. Similar findings were obtained in Epomophorus wahlbergi using bromodeoxyuridine (BrDU) and proliferating cell nuclear antigen (PCNA) (Gatome et al., 2010). Our quantification of proliferating cells in the dentate gyrus are similar to that reported previously by Gatome et al. (2010) in *Epomophorus wahlbergi*; however, our analysis of seven extra species has revealed that a negative allometry between the rate of cell proliferation and brain mass appears to be likely across mammalian species. Such an allometry would argue against the ecology and other life history parameters of a species being a determinant of the rate of cell proliferation (although it may affect cell survival), rather it argues that phylogenetic history and allometric scaling will be the principal determinants of cell proliferation rates in the mammalian hippocampus. Further studies across a range of species of vastly different brain sizes and phylogenetic relationships will reveal whether the concept proposed above explains cell proliferation rates in the mammalian hippocampus.

The findings in Megachiropterans are in sharp contrast to studies on Microchiropterans, where low to absent hippocampal neurogenesis was reported (Amrein et al., 2007). Given the proposed link of AHN to spatial memory and learning capabilities, of which all Chiropterans appear capable, the low or complete lack of new neurons in

Microchiropterans could be explained by a possible short survival rate of the newly generated neurons in the Microchiropterans (Gould et al., 1999). In this sense, the low rate or absence of AHN in the Microchiroptera, but its clear presence in the Megachiropteran, adds an additional neural feature supporting the diphyletic origin of the Chiropteran lineages (Pettigrew, 1986; Pettigrew et al., 1989, 2008).

2.4.3 Neocortex

Our observation of clusters of DCX positive cells in layer II of the rostral half of the neocortex of Megachiropterans, is similar to that reported in monkeys (Gould et al., 1999, Gould et al., 2001, Zhang et al., 2009) and cats (Cai et al., 2009). The fate of immature neurons in the neocortex remains unclear, but it is thought that some will disappear, while others will form interneurons (Gomez-Climent et al., 2008, Gomez-Climent et al., 2010). The origin of the DCX positive cells in the neocortex has been widely debated. It has been reported that immature neurons arising from the dorsolateral subventricular zone of the lateral ventricle migrate through the overlying white matter to the neocortex (Kakita and Goldman, 1999). Using BrDU in monkeys, Gould et al. (1999) identified BrDU labelled cells in the subcortical white matter which were believed to be a migratory stream from the SVZ; however, Kornack and Rakic (2001) disputed this claim and proposed that the subcortical BrDU labelled cells were endothelial cells lining longitudinally cut capillaries. In the current study of Megachiropterans, we observed Ki-67 positive cells emerging from the genu of the rostral migratory stream that appeared to course dorsally and caudally to populate the neocortex (Fig. 1). This indicates that the DCX positive cells in the neocortex are likely to have migrated along this subcortical path from the RMS. This possibility contradicts the findings that the RMS is a highly precise and exclusive migratory route from the SVZ to the

olfactory bulb (De Marchis et al., 2004). It could, however, be argued that the observed migration of cells from the genu of the RMS to the neocortex is yet another phylogenetic exception, similar to the presence of the ventral proliferative region and substream of the RMS in Megachiropterans and Primates, a trait that is not identified in Rodents (Shapiro et al., 2007).

2.4.4 Temporal migratory stream

Immature neurons were observed in the secondary olfactory structures of the Megachiropterans, the amygdala and piriform cortex. These neurons appeared to be migrating from a neurogenic zone located in the temporal pole of the lateral ventricle, forming the temporal migratory stream (TMS). The TMS appears to supply the entire piriform cortex, with cells coursing both rostrally and caudally from the temporal pole of the lateral ventricle. A TMS was also observed in Wahlberg's epauletted fruit bat (Gatome et al., 2010). A homologous TMS has been observed in rabbits (Bonfanti and Ponti, 2008, Luzzati et al., 2009) and monkeys (Bernier et al., 2002, Zhang et al., 2009); however, in Rodents, because the temporal horn of the lateral ventricle appears to be absent, the DCX positive cells populating the piriform cortex are thought to migrate from the RMS forming the ventrocaudal migratory stream (Shapiro et al., 2007). The TMS has not been reported in the Microchiroptera (Amrein et al., 2007), thus, the presence of the TMS in Megachiropterans, Lagomorphs and Primates, is another neural character supporting the concept of a diphyletic origin of the Chiroptera and aligns the Megachiroptera with the Primates.

It has been proposed that the DCX positive cells in the piriform cortex are not newly generated neurons but are immature neurons generated during a restricted developmental period, similar to neocortical immature neurons. In laboratory rats, Gomez-Climent et al.

(2008, 2010) reported that the immature neurons in the piriform cortex were generated during the embryonic period and maintained their immature status into adulthood. In the present study, we identified Ki-67 immunopositive cells in the neurogenic zone of the temporal pole of the lateral ventricle, with other Ki-67 immunopositive cells having migrated to the piriform cortex and amygdala. These observations of actively dividing cells in the adult piriform cortex indicate that the observed DCX expressing cells in piriform cortex are newly generated neurons.

2.4.5 Other sites

Despite the identification of the SVZ and SGZ as the principal adult neurogenic sites, proliferating cells and immature neurons have also been observed in other brain regions. These include the cortex, amygdala, striatum, substantia nigra, third ventricle, the dorsal vagal complex, and the cerebellum (Ihunwo and Pillay, 2007). In the cerebellum, a germinal matrix similar to the SVZ, the subpial layer, was reported (Bonfanti and Ponti, 2005, Ponti et al., 2006). It is located in the roof of the fourth ventricle and develops postnatally from the granule cell layer. Proliferating cells have been identified in this region (McDermott and Lantos, 1990, Bonfanti and Ponti, 2005, Ponti et al., 2006, Bonfanti and Ponti, 2008); however, no evidence of migration in the cerebellum has been recorded. In the present study, we identified Ki-67 positive cells in the tectum, brainstem and cerebellum in all eight Megachiropteran species investigated. These cells were mainly observed in the superior and inferior colliculus, periventricular grey matter of the brainstem, including the area postrema, the cerebellar peduncles and cerebellar cortex; however, we did not observe any DCX positive cells in these regions. This result indicates a non-neuronal fate for the Ki-67 immunopositive cells in these regions. Dividing DCX positive cells develop into neurons and

dividing GFAP positive cells develop into glial cells (Steiner et al., 2004). Our failure to identify DCX positive cells in these regions possibly points to a glial lineage for these actively dividing cells. A future study using double-immunostaining for Ki67 and GFAP would answer the questions surrounding this observation in the Megachiropterans.

2.4.6 Conclusion

In conclusion, findings from the current study have shown that adult neurogenesis is apparent in megachiropterans. Aspects of adult neurogenesis such as the neurogenic zones, migratory pathways and terminal fields in the megachiropterans are, for the most part, similar to those observed across mammalian orders previously. Of particular note were the migratory pathways from the rostral SVZ to the olfactory bulb (RMS) and temporal horn of the SVZ to the piriform cortex (TMS) which resembled those observed in primates previously but are distinct from those observed in the microchiroptera. These findings have specific relevance to the phylogenetic affinities of the megachiropterans and add to the growing list of neuroanatomical characteristics placing them closer to primates than microchiroptera.

Chapter 3: Microbats appear to have adult hippocampal neurogenesis, but post-capture stress causes a rapid decline in the number of neurons expressing doublecortin

3.1 Introduction

Studies on adult neurogenesis in free-living mammals are becoming more numerous due to the need to understand this biological process in relation to normal life-history parameters (Amrein et al., 2004, 2011; Bartkowska et al., 2008, 2010; Epp et al., 2009; Kempermann, 2012; Patzke et al. 2013, 2015; Chawana et al., 2013; Cavegn et al., 2013). The investigation of free-living mammals may provide a broader understanding of the dynamics and mechanisms influencing adult neurogenesis of species in their natural habitat and ultimately reveal potential reasons for the presence of adult neurogenesis in the mammalian brain. Free living mammals are subject to a number of pressures such as predation, foraging and varying weather patterns, all of which are factors that may influence the process of adult neurogenesis (Kempermann, 2012).

While working on wild-caught mammals has the potential advantage to reveal aspects of interest to a broad understanding of adult neurogenesis, the capture of these animals from their natural environments may be considered to be an acute stressor that is difficult to control and unpredictable. While chemical capture of wild animals (using dart guns) appears to lower blood glucocorticoid levels, physical restraint and translocation leads to significant increases in the stress related release of glucocorticoids (e.g. Widmaier and Kunz, 1993; Morton et al., 1995). In terms of adult neurogenesis, the effect of acute stress has been observed to lead to a reduction in hippocampal neurogenesis in a range of laboratory-kept species (Gould et al., 1998; Tanapat et al., 2001; Falconer and Galea, 2003; Kim et al., 2004;

Dagyte et al., 2009; Hulshof et al., 2012), although in rats the reduction in the number of proliferating cells was observed to occur within 2 hours of the acute stressor and recovery to baseline levels within 24 hours post exposure (Heine et al., 2004).

An earlier study of adult neurogenesis in microchiropterans led to the conclusion that the hippocampus of the species studied had absent to low rates of adult neurogenesis (Amrein et al., 2007). While possible reasons for the absence of adult hippocampal neurogenesis were raised, it appears that no specific conclusion was reached. One issue that was not raised by Amrein et al. (2007) was whether the stress of capture/handling of these small mammals may have had an important role in the lack of detectable adult hippocampal neurogenesis. While Amrein et al. (2007) state the bats were "perfused rapidly after trapping", no estimate of the time that elapsed between trapping and perfusion was provided, thus it is possible that capture stress could pose a serious methodological problem; however, this does not explain the absence of adult hippocampal neurogenesis in the three neotropical bat species obtained from breeding colonies located in Germany, but again, no information regarding the handling of these bats prior to perfusion was provided. Given that microchiropterans have generally lower basal metabolic rates compared to other mammals of similar size (Austad and Fischer, 1991; Neuweiler, 2000), but active or field metabolic rates significantly higher than other mammals and even birds (Neuweiler, 2000), it is possible that even a short period of stress, in the range of minutes, related to capture and handling may have a major effect on the expression of proteins in the microchiropteran brain, and in the case of Amrein et al. (2007) may have led to a false negative report of the absence of adult hippocampal neurogenesis in the bat species studied, a finding that is becoming entrenched in the neurogenesis literature (e.g. Bonfanti and Peretto, 2011; Powers, 2013). Given this potential confound in the study of Amrein et al. (2007), we sought to analyze the relationship between capture stress and adult hippocampal neurogenesis in wild-caught microchiropterans using immunohistochemistry for

the doublecortin protein (DCX), an endogenous marker of putative adult hippocampal neurogenesis (Kempermann, 2012; Patzke et al., 2015).

3.2 Materials and Methods

In the current study we examined 36 brains from 10 microchiropteran species including *Miniopterus schreibersii* (n = 2) captured from a wild population in Gauteng, South Africa, *Cardioderma cor* (n = 2), *Chaerephon pumilis* (n = 2), *Coleura afra* (n = 2), *Hipposideros commersoni* (n = 2), and *Triaenops persicus* (n = 2) captured from wild populations in coastal Kenya, *Hipposideros fuliganosas* (n = 2) and *Nycteris macrotis* (n = 2) captured from wild populations in the Yoko Forest near Kisangani, Democratic Republic of the Congo, and *Pipistrellus kuhlii* (n = 2) and *Asellia tridens* (n = 18) captured from wild populations near Unizah, Saudi Arabia. All animals were adults, as judged from epiphyseal closure of the metacarpophalangeal joints (Anthony, 1988). Appropriate permissions to trap and euthanaze the bats were obtained from the Gauteng Department of Nature Conservation, South Africa, the Kenya National Museums, Kenya, the University of Kisangani, DR Congo, and the Saudi Wildlife Authority, Saudi Arabia. All animals were treated and used in accordance with the University of the Witwatersrand Animal Ethics Committee Guidelines (clearance number 2008/36/1) which parallel those of the NIH for the care and use of animals in scientific experiments. All bats were euthanized (overdose of sodium pentobarbital, 100 mg/kg, i.p.) and perfused through the left ventricle with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) at various times following capture. For *H. fuliganosas* and *T. persicus*, the animals were perfusion fixed within 15 minutes of capture. For *M. schreibersii*, *C. cor* and *C. pumilus*, the specimens were fixed between 15 to 30 minutes of capture, and for *C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*, the

specimens were fixed within an hour of capture. For *A. tridens*, two animals were sacrificed and perfused at each of the following time points (in minutes) post-capture: 10, 15, 20, 30, 60, 120, 180, 240 and 300. Following perfusion, the brains were removed and post-fixed in 4% paraformaldehyde in 0.1 M PB overnight, cryoprotected in 30% sucrose in 0.1 M PB at 4°C and stored in an antifreeze solution at -20°C until sectioning and histological processing. Before sectioning, the brains were divided into two halves along the mid-sagittal fissure and the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4°C. The specimens were cryosectioned in the sagittal plane into 50 µm thick sections. A one in three series of sections was stained for Nissl substance (cresyl violet) to reveal cytoachitectural features, and immunostained at two different dilutions of the primary antibody to doublecortin (DCX, 1:300 and 1:600) to reveal immature neurons.

In the current study we used immunolabelling of doublecortin (DCX), an endogenous marker of putative immature neurons, to ascertain the potential presence or absence of adult neurogenesis. While DCX immunopositive neurons away from the hippocampus may not relate to adult neurogenesis in these regions, such as the piriform cortex (Klempin et al., 2011), it has been established that DCX immunolabelling of granule cells of the dentate gyrus is a good proxy for the presence of adult hippocampal neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005). The presence of DCX is also thought to reflect cumulative adult hippocampal neurogenesis over a period of 2 weeks to 6 months, although this period is species specific (Rao and Shetty, 2004; Kohler et al., 2011). Thus, lack of DCX staining should be a reliable indicator of the absence of adult hippocampal neurogenesis (Patzke et al., 2015) or of a perturbation in the maturation process of newly generated neurons.

Free floating sections were incubated in a 1.6% H_2O_2, 49.2% methanol, 49.2% 0.1 M PB solution, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10-min rinses in 0.1 M PB. To block non-specific binding sites the sections were then

pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal rabbit serum – NRS, 2% bovine serum albumin, BSA, and 0.25% Triton X-100 in 0.1 M PB). Thereafter, the sections were incubated for 48 h at 4°C in the primary antibody solution (1:300 and 1:600, goat anti-doublecortin, DCX, SC-18 Santa Cruz Biotech) under gentle agitation. The primary antibody incubation was followed by three 10 min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated rabbit anti-goat IgG, BA 5000, Vector Labs, in 3% NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin-biotin solution (1:125; Vector Labs), followed by three 10-min rinses in 0.1 M PB. Sections were then placed in 1 ml of a solution containing 0.025% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 µl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low power stereomicroscope. Staining continued until such time that the background stain was at a level that would allow for accurate architectonic matching to the Nissl sections without obscuring the immunopositive structures. Development was arrested by placing sections in 0.1 M PB for 10 min, followed by two more 10 min rinses in this solution. Sections were then mounted on 0.5% gelatine coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody, and sections where we omitted the secondary antibody. In both cases no staining was observed. It was not possible to undertake Western blot control testing due to the nature of the collection of the tissue from wild populations. Staining patterns of DCX were observed using low power stereomicroscope and digital photomicrographs were captured using Zeiss Axioshop and Axiovision software. No

pixilation adjustments or manipulation of the captured images was undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

For quantifying DCX immunopositive cells, a modified unbiased stereological procedure was used as described previously (Segi-Nishida et al., 2008; Malberg et al., 2000; Noori and Fornal, 2011). All sections stained with the 1:300 dilution of DCX were coded to ensure that the analysis was performed by a blind observer (M.A.S) and immunopositive DCX cells were counted at 40 X magnification in the subgranular zone of the left hippocampus of all specimens using an Olympus BX-60 light microscope equipped with a video camera. Cells were included if the cells lay within, or touched, the subgranular zone. The subgranular zone was defined as the area from one cell diameter within the granular cell layer (GCL) from the hilus-GCL border and two cell diameters below the hilus-GCL border (Eriksson et al., 1998). Cells were excluded if the cell was more than two cell diameters from the granular cell layer, focusing through the thickness of the section (optical dissector principle, see Gundersen et al., 1988; West, 1993; Coggeshall & Lekan, 1996) to avoid errors due to oversampling. Every section was counted throughout the hippocampus and the sum was multiplied by 3 (as we used a one in three series, see above) to provide an estimate of the total number of immunopositive DCX cells in the entire left hippocampus (Table 1).

Statistical analysis was done using STATA software package version 13.1 (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP). Given our sample sizes, we performed non-parametric tests (Mann-Whitney tests) to compare hippocampal DCX cell counts of animals from different perfusion delay time groups (those perfused within 15 minutes of capture and those perfused after 15 minutes of capture). In addition we undertook the Spearman's correlation test to measure the strength of correlation of cell counts obtained in animals belonging to a particular time group.

3.3 Results

3.3.1 Doublecortin immunopositive (DCX+) cells in the Microchiropteran hippocampus

Of the 10 microchiropteran species examined in the current study, we found DCX+ cells clearly present in the subgranular zone of the dentate gyrus of 3 species (*A. tridens*, *H. fuliganosas* and *T. persicus*) which were perfused within 15 minutes of capture, partially present in 3 species (*M. schreibersii*, *C. cor*, *C. pumilus*) which were perfused between 15 and 30 minutes of capture, and low to absent in 4 species (*C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*) all of which were perfused between 30 and 60 minutes post-capture (Figs. 3.1, 3.2, 3.3; Table 3.1). The number of DCX-labelled cells in the hippocampus for all animals perfused more than 15 minutes (n = 28) after capture declined nine times (median = 536 cells and range = 345 – 3294 cells) when compared to those perfused within 15 minutes of capture (n = 8; median = 4787 cells and range = 1380 – 6945 cells) (Mann-Whitney test z = 3.08 and p = 0.0001). In addition, in the species where DCX+ cells were observed, the subgranular zone at the base of the granule cell layer was populated by immunopositive cells that had a small soma size with dendritic processes that extended through the granule cell layer to ramify into the molecular layer. Furthermore, the mossy fibres that emanate from these cells were also observed with DCX immunohistochemistry, indicating that they are likely to be in the process of becoming functionally integrated into the hippocampal circuitry (Fig. 3.2B). The morphology of the DCX+ cells, when present, in the denate gyrus of the microchiropterans were similar to that seen in other mammals studied with the same technique.

In the *A. tridens* time series (Table 3.1), DCX+ cells were readily observed at the 10 min post-capture time point (Figs. 3.2B, 3.3B, 3.4A), showing the full range of normal morphology of these immature neurons, including the presence of DCX+ mossy fibres. By 15

min post-capture, the number of DCX+ cells was dramatically reduced (to around 20% of the pre-15 minute specimens, Table 3.1), as were the number of DCX+ dendrites and mossy fibres emanating from these cells (Fig. 3.3B, 3.4B). At 20 min post-capture there was a further reduction in the number of DCX+ cells (to around 6% of the pre-15 minute specimens, Table 3.1), dendrites and mossy fibres (Fig. 3.3B, 3.4C). By 30 min post-capture (Fig. 3.3B, 3.4D; Table 3.1), only a few DCX+ cells remained, and the DCX+ dendrites and mossy fibres were almost absent. The remaining time points examined, up to 300 minutes post-capture, evinced DCX immunostaining similar to that seen in the 30 min post-capture time point, with only a few persistent DCX+ cells, dendrites and mossy fibres (Fig. 3.3B, 3.4; Table 3.1). Similar to the trend observed with all species provided above, our comparison of DCX-labelled cells in *A. tridens* only showed that those animals perfused more than 15 minutes after capture (n =14) was associated with a ten times decline in cell number (median = 401 cells and range = 345 – 513 cells) when compared to those perfused within 15 minutes of capture (n = 4; median = 4078 cells and range = 1380 – 6945 cells) (Mann-Whitney test z = 2.974 and p = 0.0029). A test for correlation between the two specimens in each of the ten time groups which would indicate the repeatability of the results in each groups showed a strong relationship between the counts (r_s = 0.9938).

3.3.2 Doublecortin immunopositive (DCX+) cells in other regions of the Microchiropteran brain

In all the microchiropterans studied, varying densities of DCX+ cells were observed in the subventricular zone of the lateral ventricle (SVZ). From the SVZ, which appeared to occupy the majority of the ventricular wall adjacent to the caudate nucleus, these cells migrated through the rostral migratory stream to the olfactory bulb. We observed a stream of

DCX+ cells arising from the inferior portion of the SVZ that appeared to migrate to the piriform cortex and amygdala. A small stream of DCX+ cells appeared to migrate dorsally from the anterior portion of the rostral migratory stream to populate the cerebral neocortex anterior to the primary somatosensory cortex.

In the *A. tridens* time series, the rostral migratory stream was readily evident in individual animals perfused within 60 min of capture (Fig. 3.5A); however, the strength of labelling of both cells and fibres declined during this first 60 min and the rostral migratory stream was not evident in individuals perfused from 120 min post-capture. DCX+ cells in the frontal neocortex were observed in the individual animals perfused within 10 min of capture (Fig. 3.5B), but after this time point we could find no evidence for these cells. In contrast, DCX+ cells were observed in the piriform cortex in all individual animals at all of the time points examined (Fig. 3.5C), with no significant drop in apparent DCX+ cell number, or expression of DCX in the dendrites emanating from these cells.

Table 3.1: Counts of DCX immunopositive neurons in the left hippocampi of the microchiropterans studied that were sacrificed and perfusion fixed at various time points following capture.

Species	Perfusion delay	DCX cells, specimen 1	DCX cells, specimen 2
Hipposideros fuliganosas	Less than 15 minutes	3762	3963
Triaenops persicus	Less than 15 minutes	5610	5721
Miniopterus schreibersii	Between 15 – 30 minutes	2067	2262
Cardioderma cor	Between 15 – 30 minutes	2718	2568
Chaerephon pumilis	Between 15 – 30 minutes	3294	3072
Coleura afra	Between 30 – 60 minutes	558	603
Hipposideros commersoni	Between 30 – 60 minutes	1059	1026
Nycteris macrotis	Between 30 – 60 minutes	1188	1134
Pipistrellus kuhlii	Between 30 – 60 minutes	1254	1335
Asellia tridens	10 minutes	6621	6945
Asellia tridens	15 minutes	1380	1536
Asellia tridens	20 minutes	438	402
Asellia tridens	30 minutes	399	444
Asellia tridens	60 minutes	384	351
Asellia tridens	120 minutes	393	387
Asellia tridens	180 minutes	360	345
Asellia tridens	240 minutes	414	468
Asellia tridens	300 minutes	495	513

Figure 3.1: Photomicrographs of doublecortin immunoreacted sections of the dentate gyrus of the hippocampus of the various species of microchiropteran examined in the current study. Doublecortin immunoreactive immature neurons (arrows) are clearly present in (**A**) *Hipposideros fuliganosas*, (**B**) *Asellia tridens* and (**C**) *Triaenops persicus*, which were euthanized and perfusion fixed within 15 minutes of capture, partially present in (**D**) *Miniopterus schreibersii*, (**E**) *Cardioderma cor* and (**F**) *Chaerophon pumilus*, which were pefusion fixed between 15 and 30 minutes post-capture, and very low to absent in (**G**) *Coleura afra*, (**H**) *Hipposideros commersoni*, (**I**) *Nycteris macrotis* and (**J**) *Pipistrellus kuhlii*, which were perfusion fixed more than 30 minutes post-capture. The scale bar in **J** = 50 μm and applies to all images.

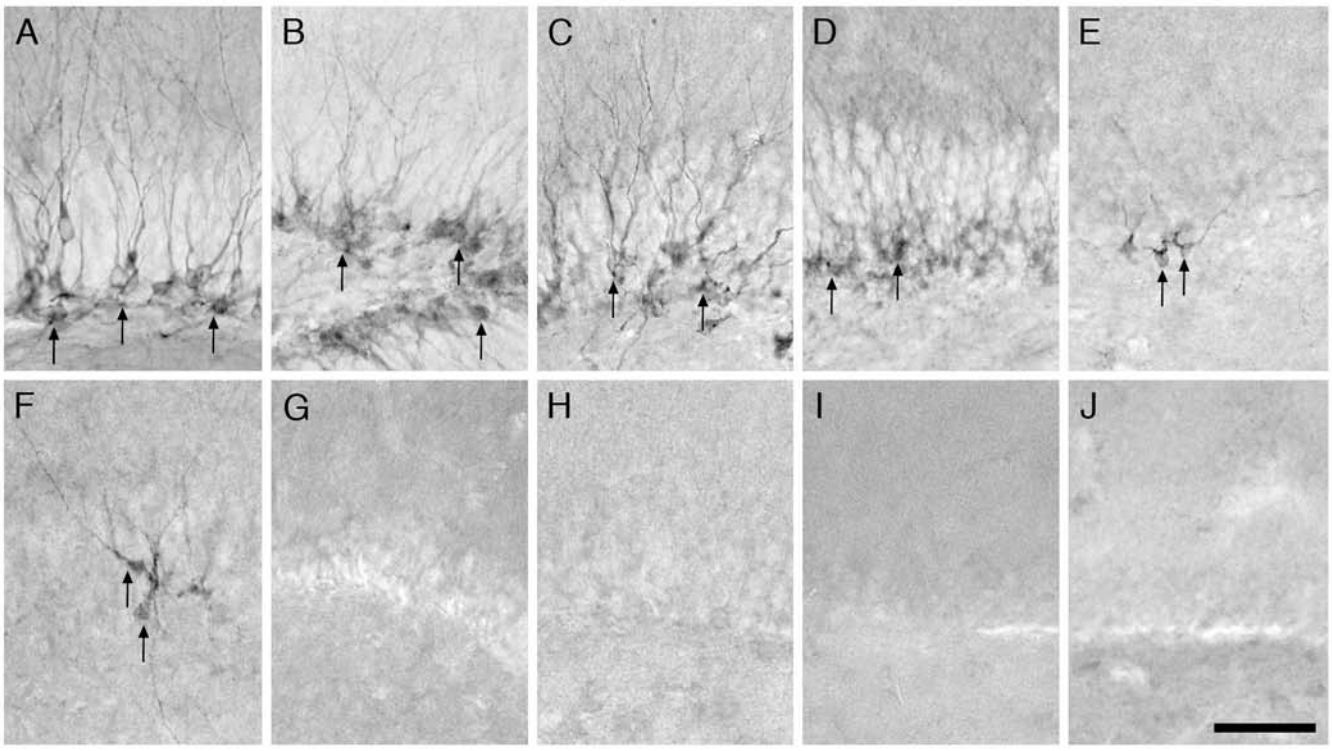

A
B
C
D
E
F
G
H
I
J

Figure 3.2: Photomicrographs of adjacent Nissl stained (**A**) and doublecortin immunoreacted (arrows) (**B**) sections of the dentate gyrus of the hippocampus of *Asellia tridens* at 10 min post-capture. Note the presence of doublecortin immunopositive cells at the base of the granular layer (**GL**), dendrites throughout molecular layer (**ML**) of the entire dentate gyrus, and mossy fibres (**mf**) exiting the dentate gyrus by passing through the polymorphic layer (**PL**). In both images dorsal is to the top and rostral to the left. The scale bar in **B** = 500 μm and applies to both images.

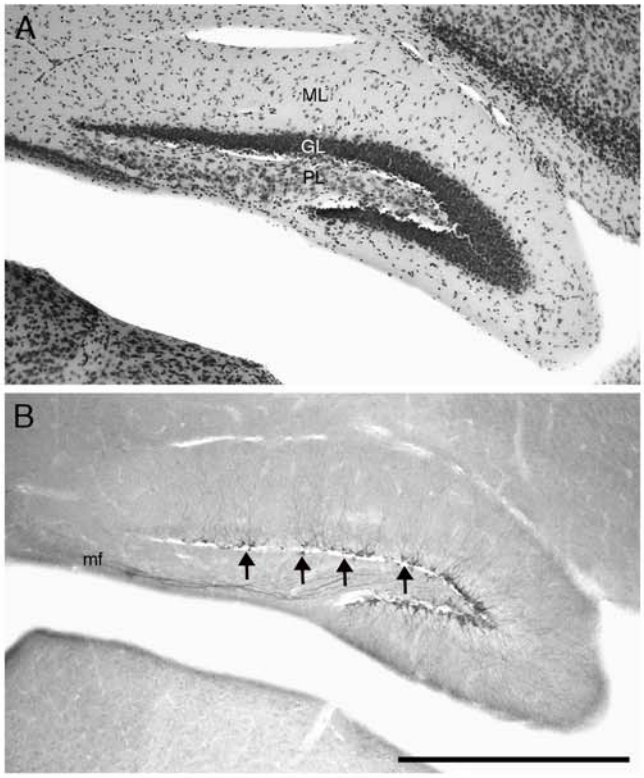

A
ML
GL
PL
B
mf

Figure 3.3: Bar graphs showing the results of our quantitative analysis of the number of doublecortin immunopositive neurons in the left hippocampus of a range of microchiropteran species. **A**. This bar graph shows significant levels of DCX immunoreactive cells in the hippocampus of two species (*H. fuliganosas* and *T. persicus*) that were sacrificed and perfused within 15 minutes of capture from their natural environment. The three species perfused between 15 – 30 minutes of capture (*M. schrebersii*, *C. cor* and *C. pumilis*) showed lower numbers of DCX immunoreactive cells, while those perfused between 30 – 60 minutes after capture (*C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*) all showed very low numbers of DCX immunoreactive cells. **B**. This bar graph shows the results of the quantification of DCX immunopositive neurons in *A. tridens* from specimens that were perfused at a range of time points following capture. Note the significant presence of DCX immunoreactive cells when the animals were perfused 10 minutes following capture, but that this is substantially reduced at 15 minutes following capture and settles at a low level for longer time points. Two individual of each species and at each time point were assessed (specimens 1 and 2).

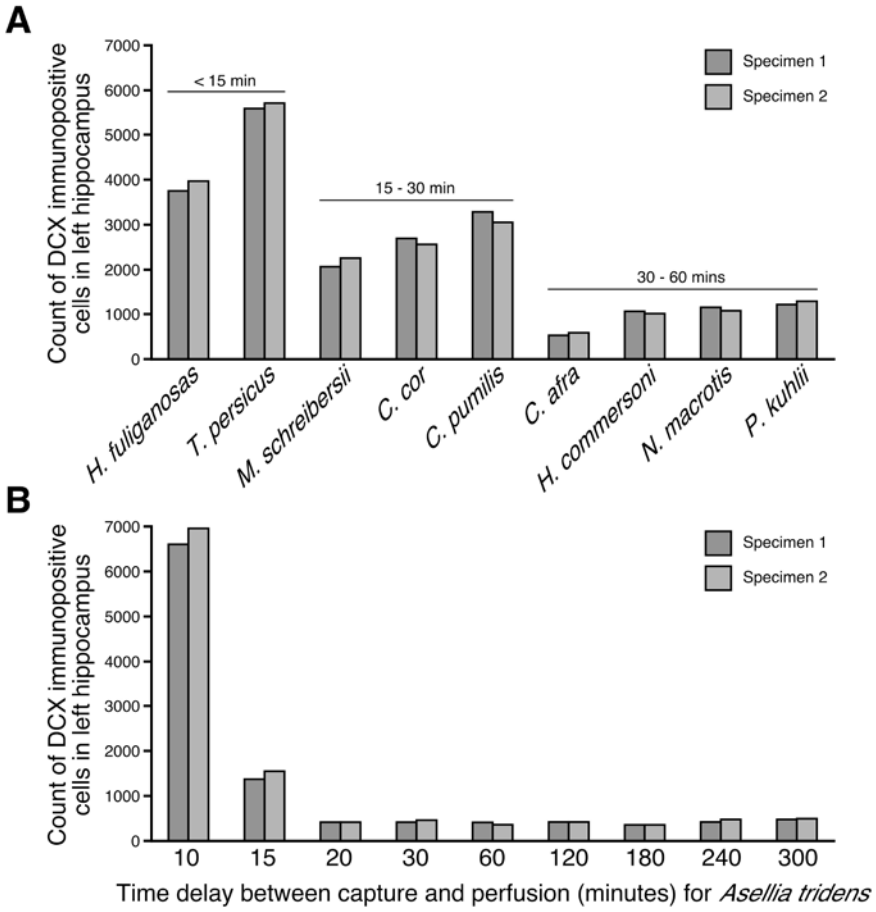

A
Count of DCX immunopositive cells in left hippocampus
7000
6000
5000
4000
3000
2000
1000
0
< 15 min
15 - 30 min
30 - 60 mins
Specimen 1
Specimen 2
H. fuliganosas
T. persicus
M. schreibersii
C. cor
C. pumilis
C. afra
H. commersoni
N. macrotis
P. kuhlii
B
Count of DCX immunopositive cells in left hippocampus
7000
6000
5000
4000
3000
2000
1000
0
Specimen 1
Specimen 2
10
15
20
30
60
120
180
240
300
Time delay between capture and perfusion (minutes) for Asellia tridens

Figure 3.4: Photomicrographs of doublecortin immunoreacted sections of the dentate gyrus of the hippocampus of *Asellia tridens* at different time points post-capture (**pc**). At 10 min post-capture (**A**), numerous cells immunopositive for doublecortin (arrows) are found throughout the entire dentate gyrus. These cells exhibit apical dendrites that ramify into the molecular layer and mossy fibres that exit through the polymorphic layer. At 15 minutes post-capture (**B**), the number of doublecortin immunopositive cells, dendrites and mossy fibres has decreased dramatically, with a further decrease in number of these structures at 20 minutes post-capture (**C**). Later time points (**D**-**F**) show a similar low number of doublecortin immunopositive cells, dendrites and mossy fibres. In all images dorsal is to the top and rostral to the left. The scale bar in **F** = 100 μm and applies to all images.

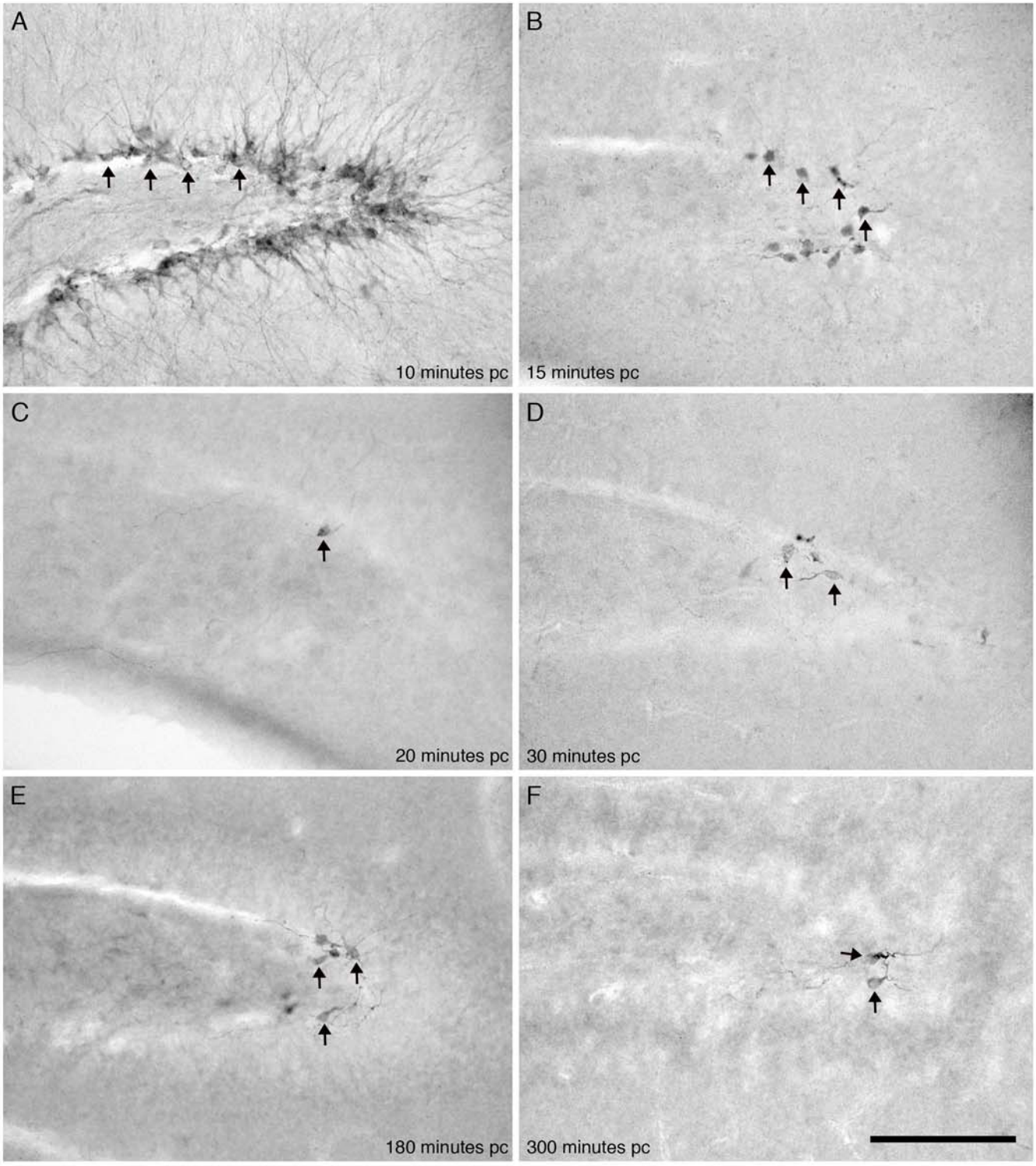
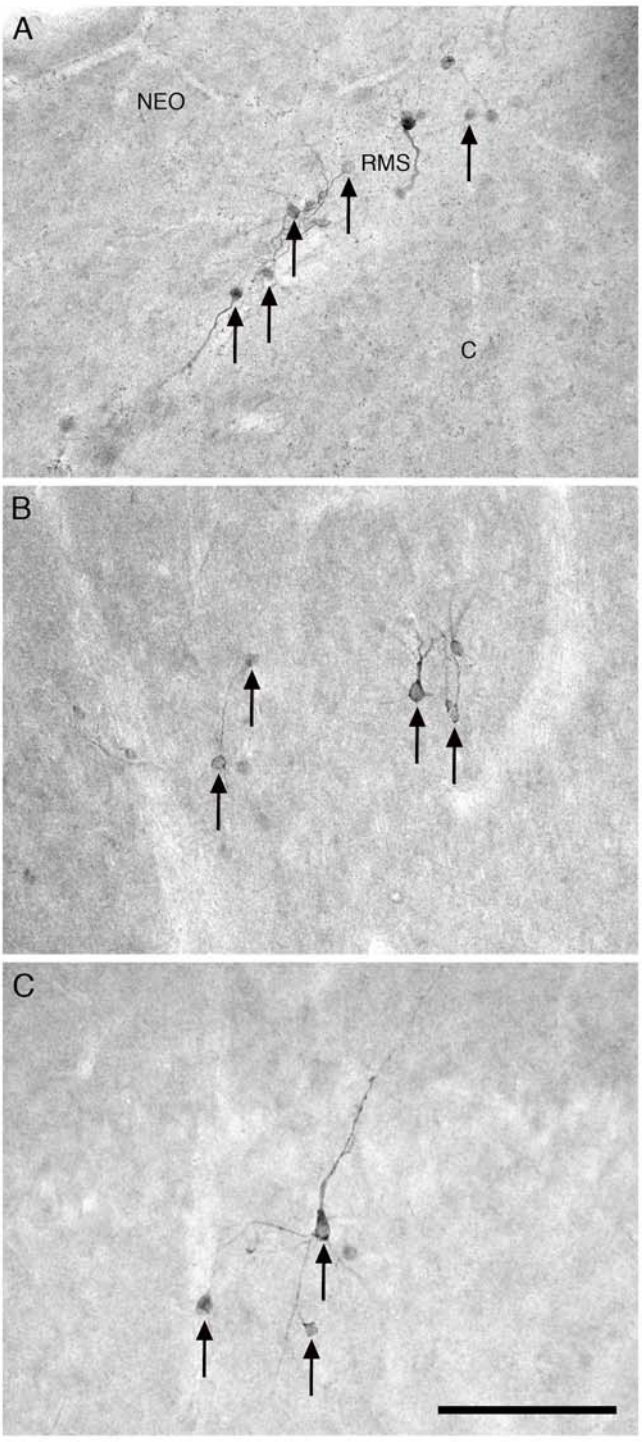

A
10 minutes pc
B
15 minutes pc
C
20 minutes pc
D
30 minutes pc
E
180 minutes pc
F
300 minutes pc

Figure 3.5: Photomicrographs of doublecortin immunoreacted sections in different regions of the brain of *Asellia tridens* at 10 min post-capture. (**A**) Doublecortin immunopositive cells (arrows) and fibres in the rostral migratory stream (**RMS**) located between the caudate nucleus (**C**) and the cerebral neocortex (**NEO**). In this image dorsal is to the top and rostral to the left. (**B**) Doublecortin immunopositive cells showing dendritic ramifications in layer III of the frontal cortex. In this image dorsal is to the top and rostral to the left. (**C**) Doublecortin immunopositive cells showing dendritic ramifications in layer II of the piriform cortex. In this image dorsal is to the bottom and rostral to the right. The scale bar in **C** = 100 μm and applies to all images.


A
NEO
RMS
C
B
C

3.4 Discussion

The present study, demonstrating the likely presence of adult hippocampal neurogenesis in microchiropterans, and that detecting this presence is dependent on the level of post-capture stress/handling to which these animals are exposed, contrasts with a previous report detailing the absence of adult hippocampal neurogenesis in microchiropterans (Amrein et al., 2007). The absence of adult hippocampal neurogenesis in microchiropterans reported by Amrein et al. (2007) has been referred to extensively in the literature (e.g. Bonfanti and Peretto, 2011; Kempermann, 2012) to the point that the idea that chiropterans *in toto*, both microchiroptera and megachiroptera, do not exhibit adult hippocampal neurogenesis is becoming "accepted knowledge" (e.g. Powers, 2013). It should be noted here that Amrein et al. (2007) only studied species from the microchiropteran suborder of bats, and not the megachiropteran suborder, for which two recent reports have detailed the presence of adult hippocampal neurogenesis in a range of megachiropteran species (Gatome et al., 2010; Chawana et al., 2013), making the title and conclusions of the Amrein et al. (2007) paper misleading as they use only the generic term bats.

The present study indicates that the potential problem encountered by Amrein et al (2007), leading to a false-negative report regarding potential adult hippocampal neurogenesis in the microchiropterans, was the length of post-capture stress and handling experienced by the animals prior to euthanasia and fixation of the neural tissue. The four species for which we did not observe significant evidence of adult hippocampal neurogenesis were those that were perfused at a time point greater than 30 minutes post-capture. The three species in which partial evidence for adult hippocampal neurogenesis was observed were perfused between 15 and 30 minutes post-capture, but the three species for which we saw significant evidence for adult hippocampal neurogenesis were all perfused within 15 minutes of capture. The idea that post-capture stress and handling leads to a rapid decline of detectable adult

hippocampal neurogenesis using DCX immunohistochemistry in the microchiropterans is supported by the time series study of *A. tridens* undertaken herein, where at 10 min post-capture DCX immunostaining revealed extensive evidence for adult hippocampal neurogenesis, but that by 15 min post-capture, the extent of staining had decreased dramatically and was very low to near absent in subsequent time points. Similar effects in the decrease in the detectable presence of doublecortin in neurons have been observed in the dentate gyrus (Dagyte et al., 2009; Hulshof et al., 2012) and retrosplenial cortex of the rat after exposure to acute stress (Kutsuna et al., 2012). The observation that post-capture stress rapidly diminishes the detectable presence of adult hippocampal neurogenesis in the microchiropterans may also explain other unusual results in field-caught species, such as the low proliferation rate, but high differentiation rate seen in wild caught South African rodents (Cavegn et al., 2013), where capture stress may have reduced the detectable presence of newly born neurons using Ki-67 immunohistochemistry, but had no specific effect on the differentiating neurons, as the doublecortin immunohistochemistry used to detect differentiating neurons can be present in these neurons over a much longer period.

That the detectable presence of adult hippocampal neurogenesis with DCX immunohistochemistry in the microchiropterans disappeared so rapidly is of interest. As mentioned, the microchiropterans have a very high field metabolic rate in comparison to most other mammals and birds (Neuweiler, 2000), and it is possible that the stress associated with capture or handling of free-living animals, or animals not accustomed to being handled, when combined with a high field metabolic rate, may lead to the rapid non-genomic corticosterone induced proteolysis of proteins associated with cell differentiation/maturation such as doublecortin, but perhaps not cell death or the cessation of cell proliferation (Kutsuna et al., 2012). This is particularly so because, like the cells in CA1 region, granule cells show quick enhancement of miniature excitatory potential post-synaptic currents (mEPSC) and

prolongation of N-methyl-d-aspartate receptor (NMDAR)- mediated influx of calcium ions after exposure to a wave of corticosteroids (Pasricha et al., 2011; Takahashi et al., 2002). The rapid calcium influx activates a calcium dependant enzyme, calpain, which breaks down the cytoskeleton (Andres et al., 2013; Vanderklish et al. 2000). Given that the corticosterone-induced changes in calcium currents occur with 10 minutes of exposure to the steroid (Wiegert et al., 2006) it is possible that in this study, this mechanism could have been activated, resulting in breakdown of the cytoskeleton and DCX, which integrates linkages between the cytoskeleton in neuronal cells and axons (Tint et al., 2009).

The decline in DCX-labelled cells in the hippocampus of the *A. tridens* may be related to age, given that age-related decline of mammalian adult hippocampal neurogenesis is well documented (reviewed by Klempin and Kemperman, 2007). Thus, it could be argued that all the microbats that we caught and perfused within 15 minutes of capture were substantially younger than those perfused more than 15 minutes after capture, with this latter group being made up entirely of microbats in their senility. Though possible, it is highly unlikely for this to be the case in this study despite it being difficult to accurately determine the exact chronological age of the microbats caught from wild populations. Firstly, given that our own observations in this study yielded repetitive results for the two specimens in each of the ten time groups as evidenced by a Spearman's rho = 0.9938, it is more likely that the cell counts are related to the perfusion delay rather than the chance of the two animals at each time point being of the same age. Secondly, our findings are unlikely to include counts of animals from the extremes of ages because we used microbats which had closed epiphyseal plates and very old bats are hardly ever captured (Brunet-Rossinni and Wilkinson, 2009). Given this, it is likely that the changes observed with perfusion delay are due to the effects of corticosterone and stress rather than old age.

Studies on standard laboratory animals often seek to eliminate any potential stressors from the protocol as it is well known that introduced stress can influence the experimental outcome (Balcombe et al., 2004). Similar care should clearly be taken when examining wild-caught species, as capture, handling and removal from a familiar environment may lead to high rates of stress (Morton et al., 1995). In the case of the microchiropterans, it appears that this has led to a false-negative report regarding the possible presence of adult hippocampal neurogenesis (Amrein et al., 2007).

Reports detailing the presence of adult hippocampal neurogenesis across mammalian species are becoming more numerous, and in each case, it would appear that adult hippocampal neurogenesis is present (reviewed in Kempermann, 2012; see also Patzke et al., 2013, 2015; Chawana et al., 2013). Thus, at this stage, with the likely presence of adult hippocampal neurogenesis in the microchiropterans, this neural trait may be a common feature of mammalian brains; however, as mentioned by Kempermann (2012), certain species, such as cetaceans that live in homogeneous environments, do need to be examined to determine whether there is phylogenetic variability in this trait, which appears to be absent in the cetaceans (Patzke et al., 2015). These variations may help to understand whether adult hippocampal neurogenesis relates to either specific aspects of the environment of the species examined (extreme heterogeneity or extreme homogeneity), or whether other explanations may account for this potential variation (Patzke et al., 2015). Thus, at present, adult hippocampal neurogenesis may be thought of as being a likely standard feature of mammalian brains and hippocampal function, but variations as seen for cetaceans (Patzke et al., 2015) may shed more light regarding functional aspects of this interesting neural phenomenon in the adult mammal brain.

Chapter 4: The pattern of adult neurogenesis in three microchiropteran species, *Hipposideros fuliganosis, Triaenops persicus* and *Asellia tridens.*

4.1 Introduction

Adult neurogenesis has been reported to occur, with varying rates, in the subventricular zone (SVZ) and subgranular zone (SGZ) across most mammalian species (Kempermann, 2012; Patzke et al., 2015). Adult neurogenesis in the hippocampus of cetaceans appears to be absent (Patzke et al., 2015) and while its presence in microchiropterans was initially questioned (Amrein et al., 2007), adult hippocampal neurogenesis was shown to be present in microchiropterans, although highly sensitive to capture/handling stress (Chawana et al., 2014). While many studies have focussed on the occurrence, rates and functional significance of adult hippocampal neurogenesis, less attention has been paid to the comparative differences in the topographic distribution of neurogenic zones, migratory pathways and terminal fields across mammals (e.g. Bartkowska et al., 2010; Bonfanti and Peretto, 2011; Ngwenya et al., 2011; Chawana et al., 2013; Patzke et al., 2013, 2014, 2015). Given the variance in the structure of the adult mammalian brain across mammals, it is likely that neurogenic zones, migratory pathways and terminal fields across mammals will also show both consistency and variance. Indeed, aggregates of proliferating and immature neurons, as well as axons, have been reported in different brain regions across mammalian species, and in addition to the generally reported subventricular zone, rostral migratory stream, olfactory bulb and hippocampus, include the septal nuclei, striatopallidal complex, anterior commissure, substantia nigra, walls of the third ventricle, spinal cord, amygdala, neocortex, piriform cortex and dorsal vagal complex (reviewed in

Ihunwo and Pillay, 2007, but see also Bartkowska et al., 2010; Chawana et al., 2013; Patzke et al., 2013, 2014).

Within the neocortex for example, while the presence of immature neurons has been observed, it is less clear whether these cells were derived from resident progenitor cells (Dayer et al., 2005), are remnants of the embryonic neurogenic process which remained immature postnatally (Gomez-Climent et al., 2008), or are migrants from a branch of the rostral migratory stream (Gould et al., 2001; Bernier et al., 2002; Shapiro et al., 2007). Increasing evidence points towards the latter, and various pathways have been described for the migrating proliferating cells and immature neurons to the neocortex (Bernier et al., 2002; Shapiro et al., 2007). One pathway of interest is the temporal migratory stream, where proliferative cells and immature neurons appear to migrate from a neurogenic zone located at the pole of the temporal horn of the lateral ventricle to create a terminal field in the piriform cortex; however, this stream has only been observed in primates (Bernier et al., 2002; Bonfanti and Ponti, 2005) and megachiropterans (Chawana et al., 2013). In rodents and other mammals, the piriform cortex is supplied with new neurons emanating from ventral branches of the rostral migratory stream (Shapiro et al., 2007; Patzke et al., 2013, 2014).

While the presence of adult hippocampal neurogenesis (Chawana et al., 2013) and the rostral migratory stream (Amrein et al., 2007) have been reported in the microchiropteran brain, a full analysis of all potential neurogenic zones, migratory pathways and terminal fields has not been undertaken. In order to fill this gap in our knowledge we use immunhistochemical staining for Ki-67 and doublecortin (DCX) in the brains of three microchiropteran species to provide a full description of adult neurogenesis in representatives of this highly diverse mammalian suborder.

4.2 Materials and Methods

In the present investigation we examined 10 brains from 3 microchiropteran species, *Hipposideros fuliganosus* (n = 3), *Triaenops persicus* (n = 4) and *Asellia tridens* (n = 3). The specimens of H. fuliganosus were captured from wild populations in the Yoko Forest near Kisangani, Democratic Republic of the Congo; *T. persicus* were captured from wild populations near Mombasa, Kenya; and *A. tridens* were captured from wild populations near Unaizah, Saudi Arabia. All animals were adults, as judged from epiphyseal closure of the metacarpophalangeal joints (Anthony, 1988). Appropriate permissions were obtained from the University of Kisangani, DR Congo, the Kenya National Museums, Kenya, and the Saudi Wildlife Authority, Kingdom of Saudi Arabia. All animals were treated and used in accordance with the University of the Witwatersrand Animal Ethics Committee Guidelines (clearance number 2008/36/1), which parallel those of the NIH for the care and use of animals in scientific experiments. All animals were euthanized (overdose of sodium pentobarbital, 100 mg/kg, i.p.) within 20 minutes of capture and perfused through the left ventricle with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). Following perfusion, the brains were removed and post-fixed in 4% paraformaldehyde in 0.1 M PB overnight, cryoprotected in 30% sucrose in 0.1 M PB at 4°C and stored in an antifreeze solution at -20°C until sectioning and histological processing. Before sectioning, the brains were divided into two halves along the mid-sagittal fissure and the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4°C. The specimens were cryosectioned in a sagittal plane (and a coronal plane for one of the specimens of *T. persicus*) into 50 µm thick sections. A one in three series of sections was stained for Nissl substance (cresyl violet) to reveal cytoarchitectural features, as well as Ki-67 and doublecortin (DCX) immunostaining to reveal proliferative cells and immature neurons respectively.

The current investigation made use of an antibody to Ki-67, a protein which is present in the nucleus during the G1 to M phases of cell cycle, and an antibody to DCX, a microtubule-associated protein expressed during the post-mitotic periods by migrating and differentiating neurons. These immunostains provided markers of proliferative cells (Ki-67) and immature neurons (DCX). These antibodies were previously used in studies on microchiropterans (Amrein et al., 2007; Chawana et al., 2014) and megachiropterans (Gatome et al., 2010; Chawana et al., 2013). The advantage of using these intrinsic markers of adult neurogenesis is that no pre-handling of the animal is needed, thereby reducing the potential ill-effects of capture stress and handling on the animals, and the full revelation of neurogenic sites, migratory pathways and terminal fields (Chawana et al., 2014).

For immunohistochemical staining, the sections were incubated in a solution containing 1.6% H2O2, 49.2% methanol, and 49.2% 0.1 M PB, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10-minute rinses in 0.1 M PB. To block non-specific binding sites the sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal goat serum, NGS, for the Ki-67 antibody or 3% normal rabbit serum, NRS, for the DCX antibody, plus 2% bovine serum albumin, BSA, and 0.25% Triton X-100 in 0.1 M PB). Thereafter, the sections were incubated for 48 h at 4°C in the primary antibody solution (1:1000, rabbit anti-Ki67, NCL-Ki67p Novocastra, or 1:300, goat anti-doublecortin, DCX, SC-18 Santa Cruz Biotech in blocking buffer) under gentle agitation. The primary antibody incubation was followed by three 10 min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated anti-rabbit IgG, BA1000 for Ki-67, or anti-goat IgG, BA 5000 for DCX, Vector Labs, in 3% NGS/NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-minute rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin-biotin solution (1:125 in 0.1 M PB, Vector Labs), followed by three 10-min

rinses in 0.1 M PB. Sections were then placed in a solution containing 0.05% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 µl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low power stereomicroscope. Staining continued until such time as the background stain was at a level that would allow for accurate architectonic matching to the Nissl sections without obscuring the immunopositive structures. Development was arrested by placing sections in 0.1 M PB for 10 min, followed by two more 10 min rinses in this solution. Sections were then mounted on 0.5% gelatine coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To test for non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody, and sections where we omitted the secondary antibody. In both cases no staining was observed. The observed immunostaining patterns support the specificity of the antibodies as they are compatible with previous observations made in microchiropterans (Amrein et al., 2007; Chawana et al., 2014).

Using a low-power stereomicroscope with a camera lucida, the architectonic borders based on the Nissl stained sections were drawn. The Ki-67 and DCX staining patterns were then matched to these drawings and cellular as well as migratory pathways added. The drawings were then scanned and redrawn using the Canvas 8 software. Architectonic nomenclature was taken from the previously described neuroanatomical regions of the microchiropterans (Bhatnagar, 2008). Digital photomicrographs were captured using Zeiss Axioshop and Axiovision software. No pixilation adjustments or manipulation of the captured images was undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

4.3 Results

In the present study cells immunoreactive to Ki-67 and DCX were observed within the brain of the three species of microchiropterans examined. The pattern of immunolabelling was consistent across the three species, although the densities of labelled cells varied (Table 4.1). Thus, the following description applies to all three species. In accord with observations made in many mammals previously, we observed the two neurogenic fields of the subgranular and subventricular zones. The proliferative cells of the subgranular zone were seen to migrate to the dentate gyrus of the hippocampus to form a significant terminal field of immature neurons (Figs. 4.1, 4.2, 4.3). The proliferative cells associated with the subventricular zone were observed to enter the rostral migratory stream, and through varied pathways formed terminal fields of immature neurons in the olfactory bulb, neocortex and piriform cortex principally, although immature neurons were observed in low density in the amygdala (Figs. 4.1, 4.2, 4.4, 4.5, 4.6). Lastly, axons immunopositive for DCX, but not cell bodies, were observed in the anterior commissure (Fig. 4.7).

4.3.1 Adult Hippocampal Neurogenesis

Contrary to an earlier report (Amrein et al., 2007), but consistent with a later report (Chawana et al., 2013), adult hippocampal neurogenesis was a feature of the three species investigated herein. Proliferating cells were detected with Ki-67 immunohistochemistry in the subgranular zone of the dentate gyrus (Fig. 4.3A, 4.3C). These cells appeared to migrate the short distance to the subgranular and granular layer of the dentate gyrus as evidenced by the presence of DCX immunopositive immature neurons in these layers (Fig. 4.3B, 4.3D). The DCX immunopositive cells had apical dendritic processes that extended into the molecular layer where they were observed to ramify. DCX immunostaining also revealed a few ovoid-

shaped cells with bipolar horizontally oriented processes in the subgranular layer and plexiform layer, as well as immunopositive fibres, presumably mossy fibres, which appeared to traverse the hippocampus to the CA3 region.

4.3.2 The subventricular neurogenic zone and the rostral migratory stream (RMS)

The subventricular zone of the lateral ventricle was observed to be populated by similar densities of Ki-67 immunopositive cells in the three species. Ki-67 immunopositive cells were not only found in the neurogenic subventricular zone, but marked the RMS through to the olfactory bulb in all three species (Figs. 4.1, 4.4). These proliferating cells were observed to course in two streams, one dorsal (between the dorso-rostral border of the caudate nucleus and the subcortical white matter) and one ventral (between the caudoventral border of the caudate nucleus and the nucleus accumbens), to the medial aspect of the caudate nucleus before merging together to follow the olfactory tract into the deeper layers of the olfactory bulb (Figs. 4.1, 4.4). While not clearly evident with Ki-67 immunostaining, a small dorsal branch of the RMS appeared to approach the frontal pole of the neocortex, and a broad swathe of cells appeared to leave the ventral division of the RMS to approach the piriform cortex (Fig. 4.2). These pathways were also observed with DCX immunostaining, and in some cases, such as the broad stream leading to the piriform cortex, were more clearly delineated with the DCX immunostaining (Fig. 4.5).

Within the olfactory bulb Ki-67 immunopositive cells were limited in their distribution to the deeper layers of the bulb near to where the putative olfactory ventricle would have been located during development (Fig. 4.4C). In contrast, the DCX immunopositive cells were found overlapping the distribution of the Ki-67 immunopositive cells, and were seen to expand radially throughout the layers of the olfactory bulb from the

deep layers of the olfactory bulb (Fig. 4.4D). The DCX immunopositive cells in the periventricular layer were spindle-shaped with horizontally oriented short bipolar processes, while those observed in the granule cell layer were larger, spherical shaped cells with processes extending towards the glomerular layer.

Occasional solitary Ki-67 immunopositive cells were observed in the neocortex of the three species investigated (Fig. 4.1), and while there appeared to be a branch emanating from the RMS at the region of fusion of the dorsal and ventral RMSs, this was not particularly clear in any of the species studied with either immunostain. In contrast to the Ki-67 staining, the DCX staining revealed presumably immature neurons throughout the neocortex, but these were more dense in the rostral neocortex and lessened in density caudally (Figs. 4.2, 4.6). The DCX immunopositive neocortical cells were found mostly in layer II, although occasional neurons were found in layer I (Fig. 4.6A, 4.6B). The majority of these DCX immunopositive neocortical neurons had granular shaped soma and were either bipolar or multipolar, with dendrites extending both horizontally and superficially.

No Ki-67 immunopositive cells were observed in the piriform cortex, although some cells were observed to appear to be in the process of migrating towards this cortical region from the ventral branch of the RMS (Fig. 4.1). In contrast, DCX immunostaining revealed a broad swathe of cells emanating from the ventral branch of the RMS, traversing the lateral portion of the nucleus accumbens to ingress into the piriform cortex (Figs. 4.2, 4.5A). The rostral and caudal aspect appeared to contain the densest aggregation of these putatively migrating cells, but it is possible that this is more a reflection of the sagittal plane of section rather than location, as these rostral and caudal densities were observed throughout the sagittal plane and approached each other in the more lateral aspects (Fig. 4.5B, 4.5C). The immature neurons, those being DCX immunopositive, in the piriform cortex were found mainly in layer II were either bipolar or multipolar, with apically and horizontally oriented

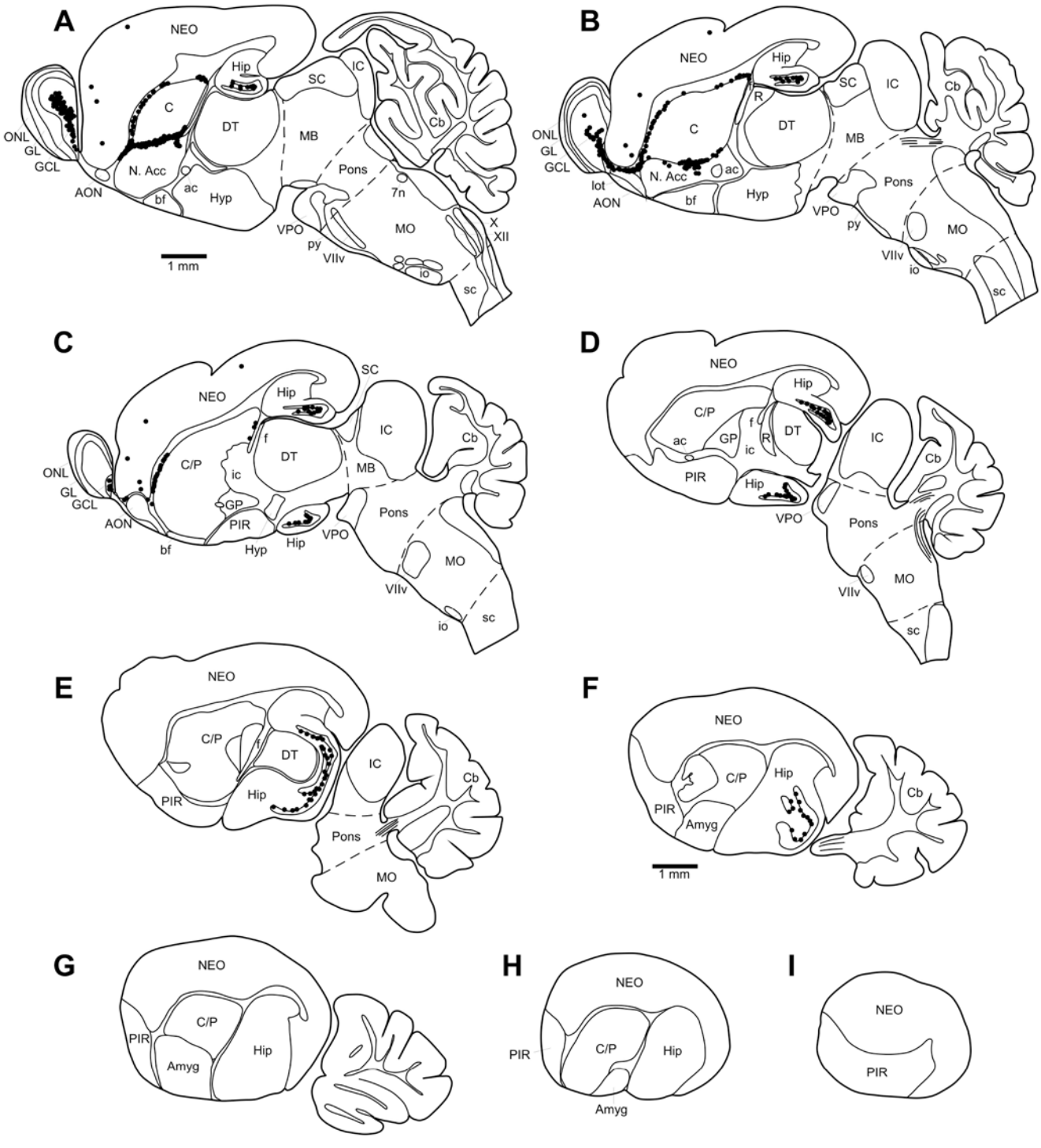
dendritic processes (Fig. 4.6C). The apical processes ramified within layer I of the piriform cortex. The low density of DCX immunopositive cells within the amygdala appears to have also originated from the same stream supplying the piriform cortex (Fig. 4.2).

Lastly, DCX immunopositive axons were observed within the anterior commissure in all species, although this was best seen in the coronally sectioned brain of *T. persicus* (Fig. 4.7). No DCX immunopositive cells, or Ki-67 immunopositive neurons were observed in the anterior commissure of the species studied. The density of DCX immunopositive axons in the anterior commissure was highest in the dorsal aspect of the commissure, but were observed throughout the commissure (Fig. 4.7).

Table 4.1. Qualitative summary of the density of immunopositive cells in the various brain regions of the three microchiropteran species studied. - – absent; + – low density; ++ – moderate density; +++ – high density.

Species	Average Body Mass (g)	Average Brain Mass (g)	Subventricular zone		Rostral migratory stream		Olfactory bulb		Subgranular zone and dentate gyrus		Neocortex		Piriform cortex		Amygdaloid complex	
			Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX
Asellia tridens	14.2	0.35	+	+	+	+	+	++	+	+++	+	+	-	+++	-	++
Hipposideros fuliganosas	15.8	0.32	+	+	++	++	++	++	+	++	+	++	+	+++	+	++
Triaenops persicus	13.7	0.27	++	++	++	++	+++	+++	++	+++	+	+	+	++	+	++

Figure 4.1: Serial drawings of the sagittal sections through the brain of *Triaenops persicus* showing the distribution of cells immunopositive for Ki-67 (circles). Note the presence of Ki-67 immunopositive cells within the subgranular zone of the dentate gyrus and throughout the dorsorostral and caudoventral divisions of the rostral migratory stream that merge to turn and enter the olfactory bulb. Each drawing is approximately 300 μm apart, with **A** being the most medial and **I** the most lateral. See list for abbreviations.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Cb
R
C
DT
MB
ONL
GL
GCL
lot
N. Acc
ac
AON
bf
Hyp
Pons
VPO
py
MO
VIIv
io
sc
C
NEO
Hip
SC
IC
Cb
C/P
f
DT
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GL
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GP
AON
PIR
Pons
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Hyp
Hip
VPO
MO
VIIv
sc
io
D
NEO
Hip
C/P
f
ac
GP
R
DT
IC
Cb
ic
PIR
Hip
MB
VPO
Pons
MO
VIIv
sc
E
NEO
C/P
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DT
IC
Cb
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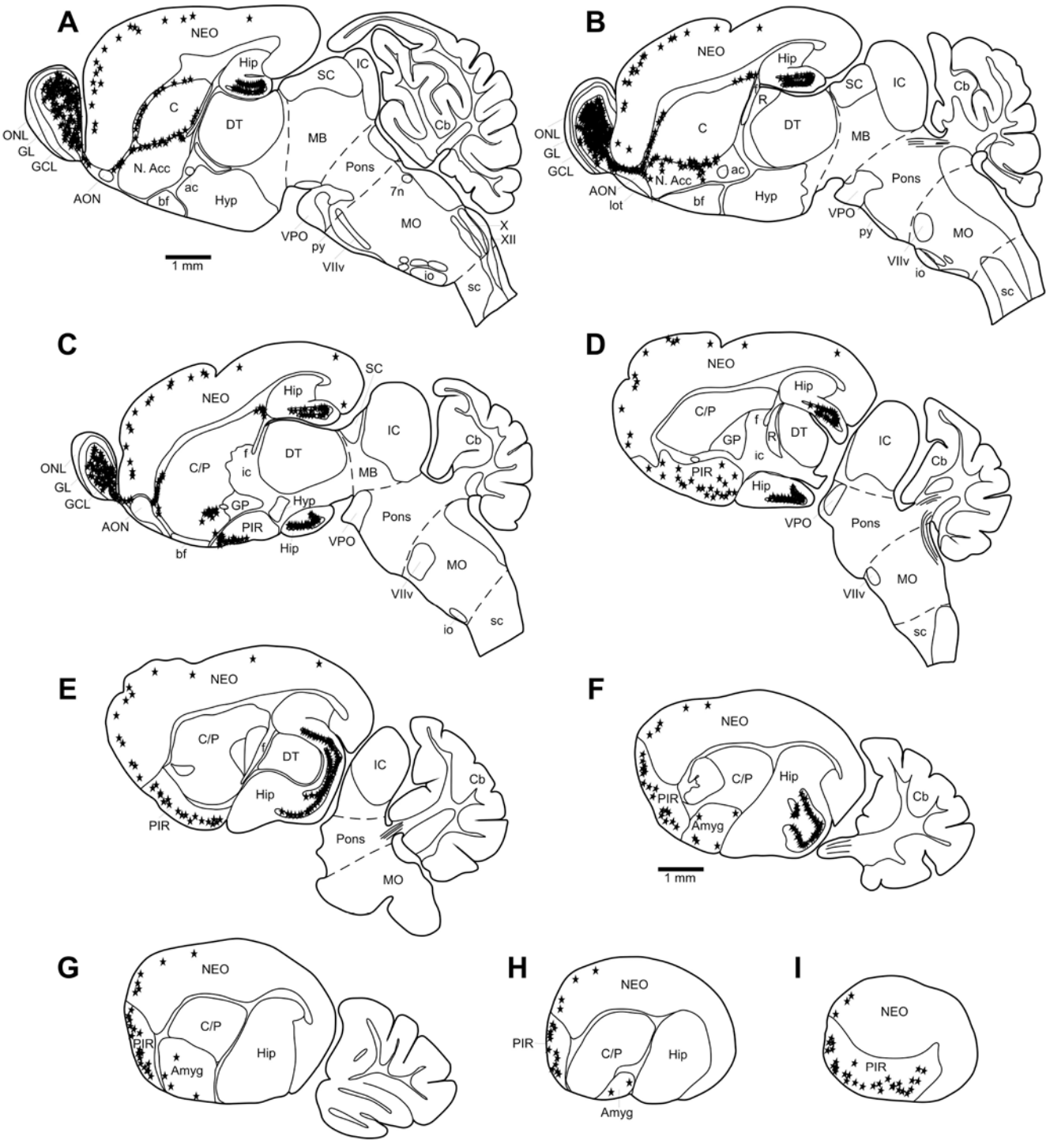
NEO
PIR

Figure 4.2: Serial drawings of the sagittal sections through the brain of *Triaenops persicus* showing the distribution of cells immunopositive for doublecortin (DCX, stars). Note the presence of DCX immunopositive cells within the dentate gyrus and throughout the dorsorostral and caudoventral divisions of the rostral migratory stream, the olfactory bulb, neocortex, piriform cortex and amygdaloid complex. Each drawing is approximately 300 μm apart, with **A** being the most medial and **I** the most lateral. See list for abbreviations.


A
NEO
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N. Acc
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Hip
SC
IC
Cb
R
C
DT
MB
ONL
GL
GCL
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Pons
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lot
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bf
Hyp
VPO
py
MO
VIIv
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C
SC
NEO
Hip
IC
Cb
C/P
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Pons
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DT
IC
Cb
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PIR
Hip
VPO
Pons
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VIIv
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E
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Cb
Hip
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Pons
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NEO
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NEO
C/P
PIR
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NEO
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Amyg
I
NEO
PIR

Figure 4.3: Photomicrographs of sagittal sections through the dorsal region of the hippocampus immunostained for **Ki-67** (arrowheads) (**A**, **C**) and doublecortin, **DCX** (arrows) (**B**, **D**) from *Triaenops persicus* (**A**, **B**) and *Hipposideros fuliganosas* (**C**, **D**). Note the presence of Ki-67 immunostained cells in the subgranular zone of the dentate gyrus, and DCX immunostained cells in the deepest portions of the granular cell layer exhibiting apical dendrites that ramify in the molecular layer. In all images dorsal is to the top and rostral to the left. Scale bar in **D** = 500 μm and applies to all images.

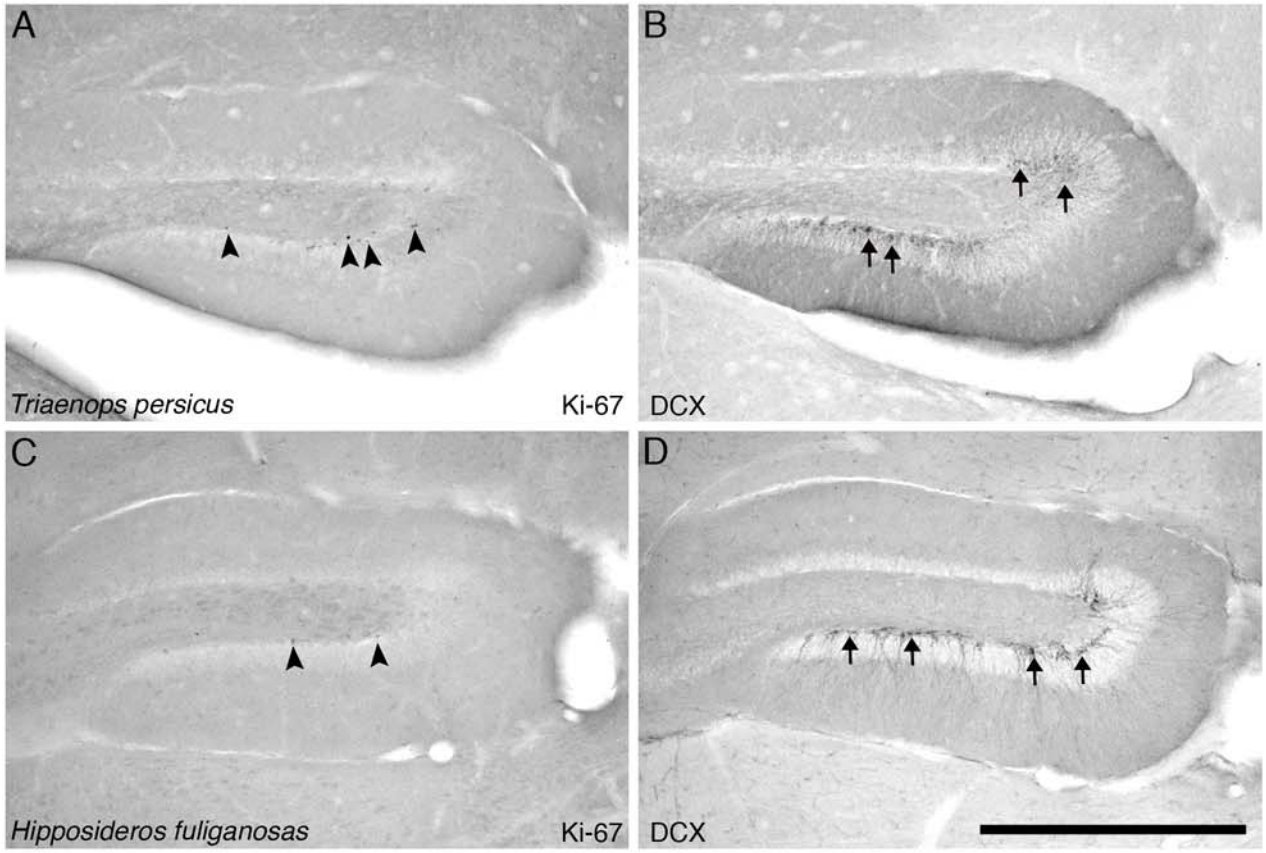

A
Triaenops persicus
Ki-67
B
DCX
C
Hipposideros fuliganosas
Ki-67
D
DCX

Figure 4.4: Photomicrographs of sagittal sections through the rostral migratory stream (**A**, **B**) and olfactory bulb (**C**, **D**), immunostained for Ki-67 (arrowheads) (**A**, **C**) and doublecortin (arrows) (DCX, **B**, **D**), from the brain of *Triaenops persicus*. Note the presence of two divisions of the rostral migratory stream dorsorostral (open arrowheads for Ki-67 in **A** and long arrows for DCX in **B**) and caudoventral (solid arrowheads for Ki-67 in **A** and short arrows for DCX in **B**) to the caudate nucleus (**C**). These two divisions of the rostral migratory stream merge to enter the olfactory bulb. Ki-67 immunopositive cells are only found in the deeper layers of the olfactory bulb (**C**), while DCX immunopositive structures are found throughout most layers of the olfactory bulb (**D**). In all images dorsal is to the top and rostral to the left. Scale bar in **B** = 1000 μm and applies to **A** and **B**, scale bar in **D** = 500 μm and applies to **C** and **D**. **N.Acc** – nucleus accumbens; **NEO** – neocortex; **OT** – olfactory tract.

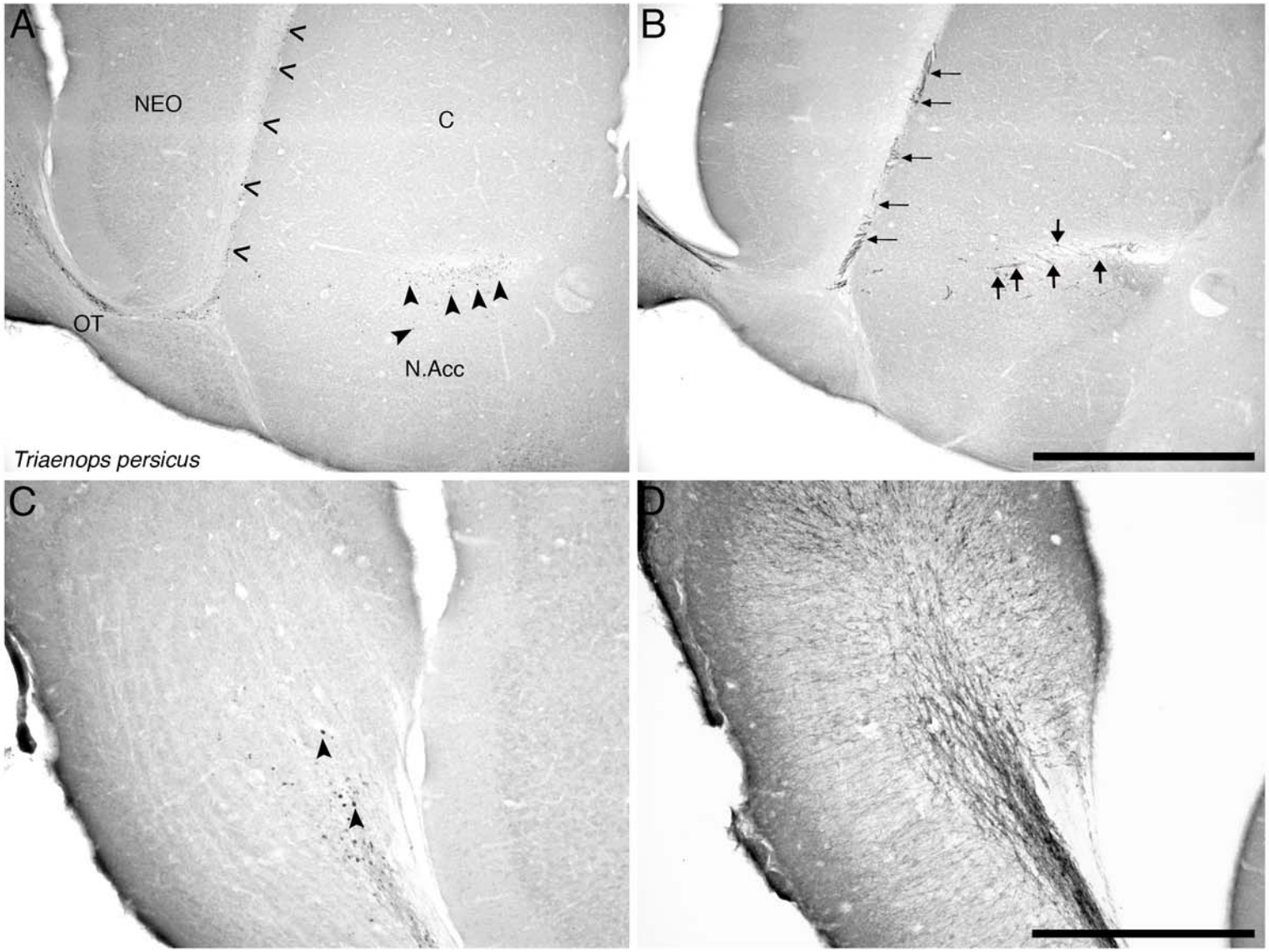

A
NEO
C
OT
N.Acc
Triaenops persicus
B
C
D

Figure 4.5: Photomicrographs of doublecortin (DCX) immunostained sagittal sections from the brain of *Hipposideros fuliganosas* (**A, B and C**). Note the presence of a broad swathe of DCX immunopositive structures (arrows) emanating from the ventral division of the rostral migratory stream, presumably passing through the nucleus accumbens (**N.Acc**) to invest into the piriform cortex (**PIR**) (**A**). While the density appears greater at the rostral (**B**) and caudal ends (**C**) of this stream, this appears to be due to more to the lateral displacement of the section rather than representing two distinct streams to the piriform cortex. In all images dorsal is to the top and rostral to the left. Scale bar in **A** = 500 μm and applies to **A**, scale bar in **C** = 500 μm and applies to **B** and **C**. **C** – caudate nucleus.

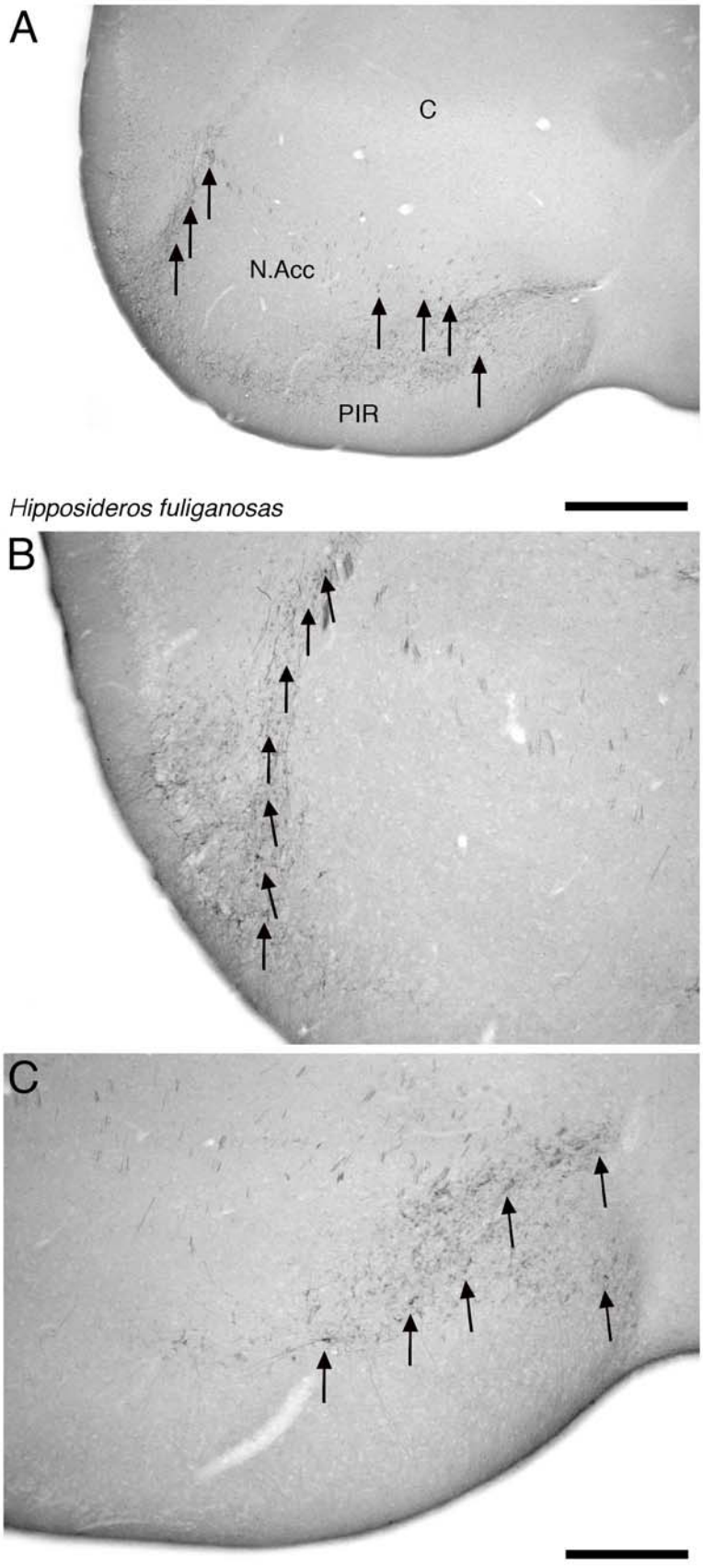

A
C
N.Acc
PIR
Hipposideros fuliganosas
B
C

Figure 4.6: Photomicrographs of doublecortin (DCX) immunostained sagittal sections from the neocortex (**A**, **B**) and piriform cortex (**C**) from the brains of *Hipposideros fuliganosas* (**A**) and *Asellia tridens* (**B**, **C**). Note the presence of DCX immunopositive cells (arrows) in layers I and II of the cortical divisions. Each image has been rotated such that the pial surface is to the top of the image. Scale bar in **C** = 100 μm and applies to all.

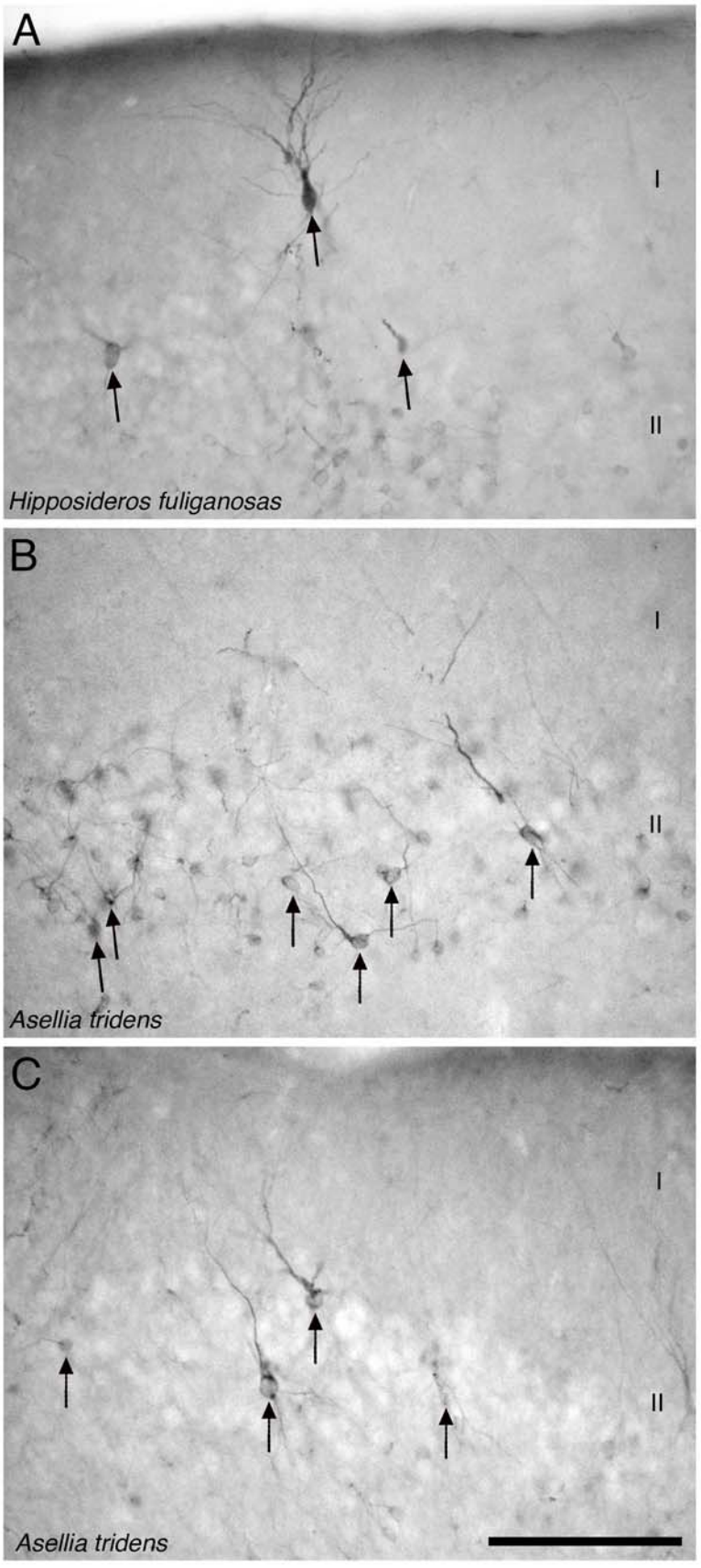
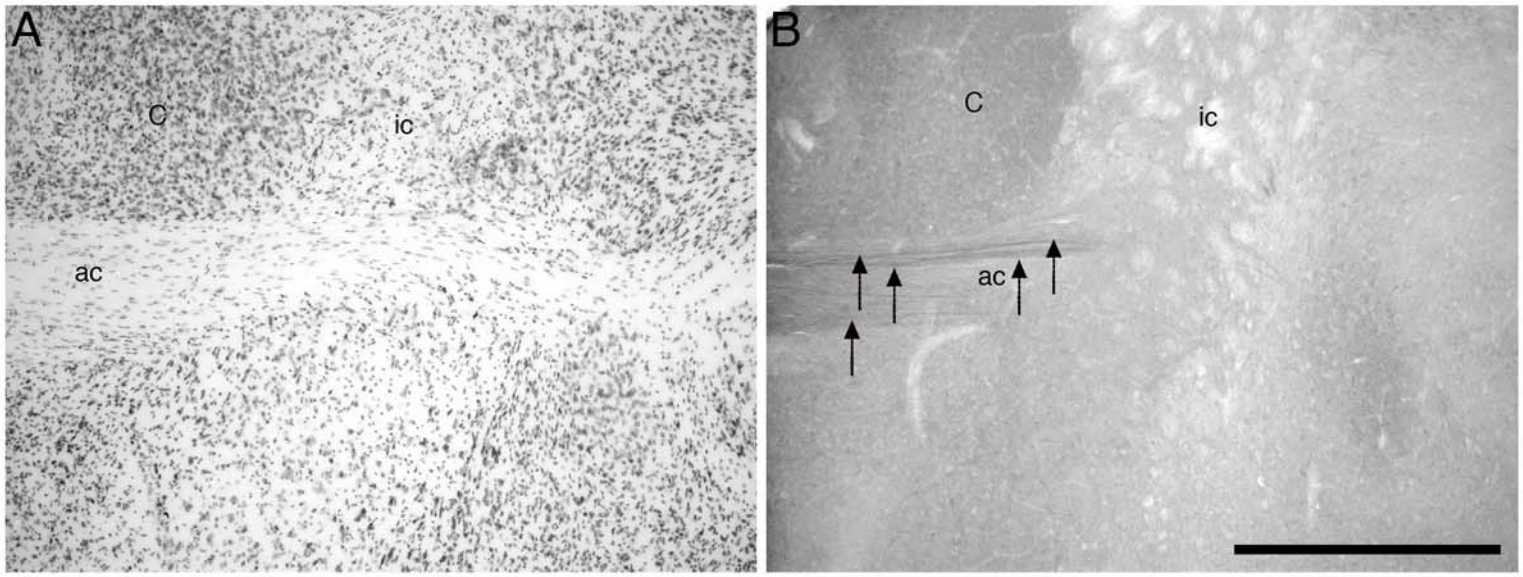

A
I
II
Hipposideros fuliganosas
B
I
II
Asellia tridens
C
I
II
Asellia tridens

Figure 4.7: Photomicrographs of adjacent coronal Nissl stained (**A**) and doublecortin immunostained (DCX, **B**) sections through the anterior commissure (**ac**) and surrounding tissue of the brain of *Triaenops persicus*. Note presence of many DCX immunostained axons (arrows) passing through the anterior commissure (**B**), a feature only seen in microchiropterans and insectivores of the mammals studied to date. In both images dorsal is to the top and medial to the left. Scale bar in **B** = 500 μm and applies to both images. **C** – caudate nucleus, **ic** – internal capsule.


A
C
ic
ac
B
C
ic
ac

4.4 Discussion

In the current study we examined the neurogenic zones, migratory pathways and terminal fields of proliferating cells and immature neurons in the brains of three species of microchiropterans. We observed two neurogenic zones (the subgranular and subventricular zones) each with their own migratory pathways, although the rostral migratory stream is far more complex than the intra-hippocampal migratory pathway, ending in several distinct regions of the brain. Our findings confirm, and extend, earlier reports detailing the presence of adult neurogenesis in the microchiropteran brain (Amrein et al., 2007; Chawana et al., 2014). Despite the overall similarity of the adult neurogenetic zones, pathways and terminal fields in the microchiropteran brain compared to other mammals, specific differences, especially in the pathway to the piriform cortex and presence of DCX immunopositive axons in the anterior commissure, may influence our understanding of the phylogenetic patterns of adult neurogenesis across mammalian species.

4.4.1 Adult hippocampal neurogenesis across mammalian species

While microchiropterans were initially reported to have very low rates or a complete absence of adult hippocampal neurogenesis (Amrein et al., 2007), this conclusion was overturned by subsequent observations that indicated significant susceptibility in the revelation of the doublecortin protein using immunohistochemical procedures in relation to handling or capture stress (Chawana et al., 2014). It was observed that when microchiropterans were held for more than 20 minutes post-capture from wild populations, the number of immature neurons that could be revealed with doublecortin immunohistochemistry decreased dramatically (Chawana et al., 2014). Thus, like the vast majority of other mammals studied (Kempermann, 2012; Patzke et al., 2015), the

microchiropteran bats have clear adult hippocampal neurogenesis, which in the current study has been demonstrated with both Ki-67 and DCX immunohistochemical techniques. The only mammalian group in which adult hippocampal neurogenesis truly appears to be absent is the cetaceans, which also have an unusual hippocampal architecture in comparison to other mammals (Patzke et al., 2015). The microchiropterans, like other non-cetacean mammals that have been studied, have a hippocampal architecture that is consistent in its appearance to other non-cetacean mammals (Chawana et al., 2014).

Thus, with the exception of cetaceans, it appears that adult hippocampal neurogenesis is likely to be a common feature of mammalian brains. The subgranular neurogenic region, the short migration to the base of the granular layer, and the integration into the granular layer of the immature neurons, are all also likely to be features common to adult hippocampal neurogenesis in most mammals. The near ubiquitous presence of this neural trait in mammals, but its absence in cetaceans, is of interest in terms of understanding its function – are there specific aspects of the behaviour of cetaceans in relation to the proposed functions of adult hippocampal neurogenesis that are modified or absent compared to other mammals? The naturally occurring absence of adult hippocampal neurogenesis, especially in species as amenable to classical conditioning paradigms as cetaceans, provides a potentially fruitful area for future research into the understanding of the precise function of adult hippocampal neurogenesis. The microchiropterans might also be a useful group of animals to study in this respect, as it appears that simple handling stress leads to a rapid decline in adult hippocampal neurogenesis (Chawana et al., 2014) that might have a protracted effect. It would be of interest to determine the temporal frame of the stress-related reduction in hippocampal neurogenesis in the microchiropterans to determine whether microchiropterans species might also serve as useful, naturally occurring, models for the understanding of the function of this neural trait.

4.4.2 The subventricular zone, the rostral migratory stream and terminal fields of adult neurogenesis

In the mammals that have been studied, the presence of the subventricular neurogenic zone, giving rise to the rostral migratory stream (RMS), appears to be a consistent feature. The microchiropterans are not different to other species in this regard, as briefly reported previously (Amrein et al., 2007), but described in more detail in the current study. In the microchiropterans we observed that the RMS can be split into two, a rostrodorsal portion that courses around the dorsal and rostral edges of the caudate nucleus, deep to the subcortical white matter, and a ventrocaudal portion that courses caudally to the caudate nucleus initially, before turning rostrally to course between the caudate nucleus and nucleus accumbens. This subdivision of the RMS around the caudate nucleus has been observed in several other mammalian species (Bernier et al., 2002; Bartkowska et al., 2010; Chawana et al., 2013) and thus appears to be a common feature of the initial trajectory of the RMS in mammals. These two divisions of the RMS appear to merge at the anteroventral edge of the caudate nucleus before turning sharply to enter the olfactory bulb, which is generally considered to be the primary destination of the newly born proliferative and immature cells, again a feature common to all mammals that have been studied to date.

In addition to the olfactory bulb, the RMS is thought to supply immature neurons to the neocortex, piriform cortex, amygdala, septal nuclei and striatopallidal complex (reviewed in Ihunwo and Pillay, 2007, but see also Bartkowska et al., 2010; Chawana et al., 2013; Patzke et al., 2013, 2014, 2015). In the current study of the microchiropterans, we observed immature neurons, and sometimes proliferative cells, in the terminal fields of the neocortex, piriform cortex and amygdala, but not the septal nuclei or the striatopallidal complex. We

could not find conclusive evidence that a branch of the RMS supplied the neocortex with immature neurons (or proliferative cells), although our observations do hint at this possibility. A neocortical branch of the RMS, in which immature neurons have been detected, has been reported for rabbits (Luzzati et al., 2003), humans (Sanai et al., 2011) and megachiropterans (Chawana et al., 2013). Thus, it is possible that the microchiropterans have a similar neocortical branch of the RMS that remains to be shown conclusively. Alternatively, the immature neurons observed in the neocortex of the microchiropterans might be neurons that are undergoing modification rather than being newly born. Despite this, cell remodelling and neuritic arborisation in non-newly generated neurons was found to lack doublecortin immunoreactivity (Couillard-Despres et al., 2005). While the doublecortin in the neocortex of the microchiropterans indicates immature neurons, these might be remnants of the embryonic neurogenesis instead of being newly generated, where the embryonic neuroblasts were arrested in the immature state (Gomez-Climent et al., 2008; Cai et al., 2009; Luzzati et al., 2009). Determining whether doublecortin immunolabelled cells within the neocortex of the various mammals in which this feature has been observed are indeed immature neurons or remodelling neurons is a question that needs to be resolved in future studies.

The presence of numerous immature neurons within the piriform cortex, and the low density of immature neurons in the amygdala, of the microchiropterans currently studied is also consistent with several previous observations in other mammalian species (e.g. Bartkowska et al., 2010). As with the immature neurons in the neocortex, it has been argued that the immature neurons within the piriform cortex (and the amygdala) are not newly born, but are undergoing modification (Nacher et al., 2001). The observations made in the current study, as well as in previous studies (e.g. Couillard-Despres et al., 2005, Shapiro et al., 2007, Bartkowska et al., 2010; Chawana et al., 2013; Patzke et al., 2013, 2014), do not support this conclusion, indicating that the immature neurons within the piriform cortex have indeed

migrated from neurogenic zones to the piriform cortex to become integrated into the circuitry. Despite the consistent presence of immature neurons in the piriform cortex across species, even in those species that lack or have a very tiny olfactory bulb such as the cetaceans (Patzke et al., 2015), the actual migratory pathway differs in substantive ways between species. In the current study on microchiropterans we have observed that a broad migratory pathway from the ventral branch of the RMS supplies the piriform cortex with immature neurons. While there do appear to be greater densities of putatively migrating immature neurons at the rostral and caudal end of the ventral branch of the RMS that invest into the piriform cortex, this appears to be a result of the plane of section rather than additional subdivisions of this migratory stream. A similar broad migratory stream leaving the ventral division of the RMS was observed previously in insectivores (Bartkowska et al., 2010), while in rodents the rostral and caudal piriform cortex is populated by immature neurons migrating through the caudoventral and ventrocaudal migratory streams respectively (Shapiro et al., 2007). In contrast, the megachiropteran bats and primates have an additional neurogenic zone in the temporal horn of the lateral ventricle in which proliferative cells and immature neurons are seen to emerge and putatively migrate to the piriform cortex (Bernier et al., 2002; Chawana et al., 2013).

The observation of DCX immunopositive axons within the anterior commissure of the microchiropteran bats is also of interest from a comparative sense. Similar DCX immunopositive axons were observed in the brains of insectivores previously studied (Bartkowska et al., 2010), and DCX immunopositive axons and cells have been observed in the anterior commissure of the giant otter shrew (*Potamogale velox*), an Afrotherian mammal (Patzke et al., 2013). No DCX immunopositive axons or cell bodies have been reported in other mammals. It was suggested by Bartkowska et al. (2010) that these DCX labelled axons

in the anterior commissure may be newly forming connections between the left and right olfactory bulbs.

The observations of the similar broad migratory stream leading to the piriform cortex in the microchiropterans and insectivores, and the presence of DCX immunopositive axons, but not cells, in the anterior commissure of microchiropterans and insectivores appears to provide characters that may be used to phylogenetically align these two mammalian groups. In contrast, the distinct presence of a temporal migratory stream in megachiropterans and primates, to the exclusion of other mammals, adds yet another neural character phylogenetically aligning the megachiropterans with the primates. Several neuroanatomical studies, from widely disparate and non-interacting neural systems, support the diphyletic hypothesis of chiropteran evolution, and align the microchiropterans with insectivores (specifically the shrews), and the megachiropterans with the primates (Pettigrew, 1986; Pettigrew et al., 1989, 2008; Maseko and Manger, 2007; Maseko et al., 2007; Kruger et al., 2010a,b; Dell et al., 2010; Calvey et al., 2013, 2015a,b, 2016). Further neuroanatomical studies are likely to continue to support this diphyletic alignment, however, objections from genetic studies continue to be raised (e.g. Murphy et al., 2001; Teeling et al., 2002, 2005; Arnason et al., 2008; Lee and Camens, 2009; Meredith et al., 2011).

Chapter 5: Adult hippocampal neurogenesis in Egyptian fruit bats from three different environments: are interpretational variations due to the environment or methodology?

5.1 Introduction

The specific role of the hippocampus in learning and memory is still debated, but it is generally regarded to be pivotal in the establishment of episodic and spatial memory (Bird and Burgess, 2008). These functions are mainly processed in the dentate gyrus which receives numerous inputs from various regions of the brain including the entorhinal cortex, subcortical regions, and the neocortex (Amaral et al., 2007). In most mammals, structural plasticity in the dentate gyrus can be achieved through the addition of new neurons, the process of adult neurogenesis, considered crucial for learning and memory functions (Vivar et al., 2012; Vivar and van Praag, 2013). While the maturation of these adult-generated neurons follows a similar path to that of granule cells born during development, physiologically the adult-born immature neurons are highly excitable and have a low firing threshold when compared to those born during development (Wang et al., 2000). These adult-born immature neurons form the basis for the hypothesis proposing that these newly added neurons are crucial for pattern separation, a neural process which disambiguates similar or overlapping inputs into distinct output signals (Clelland et al., 2009; Yassa and Stark, 2011; Tronell et al., 2012, 2015).

The rate of adult hippocampal neurogenesis and the result of specific treatments, environments, diseases or different species have been reported in many different ways depending on the method of standardising the cell counts to account for different variables. These include standardizing the counts in relation to brain mass, granule cell layer length,

total granule cell layer area, granule cell layer volume or total granule cell numbers (e.g. Boekhoorn et al., 2006; Reif et al., 2006; Lucassen et al., 2010; Castilla-Ortega et al., 2011; Low et al., 2011; Chawana et al., 2013; Slomianka et al., 2013). Of these commonly used methods, the total numbers of proliferative cells or immature neurons are consistently reported, especially when comparing results within a single species (e.g. Bartkowska et al., 2010; Trinchero et al., 2015; van der Marel et al., 2015), with this method assuming that brain mass, the volume of the hippocampal dentate granule cell layer, and the number of granule cells remain constant between individuals of the same species. Second, brain mass has been used to standardize the total counts of proliferative cells or immature neurons between species (Chawana et al., 2013), assuming that the volume of the hippocampal dentate granule cell layer, and the number of granule cells are directly proportional to brain mass. Third, the volume of the granule cell layer is used for standardization of cell counts (e.g. Kempermann et al., 1997a; Epp et al., 2009; Castilla-Ortega et al., 2011; Curtis et al., 2012), and assumes that this volume is representative of the dentate gyrus as whole. The Epp et al. (2009) study of wild-caught *Rattus norvegicus* compared with three strains of captive-bred *R. norvegicus*, indicated that the rate of cell proliferation and survival of immature neurons was remarkably similar between the wild-caught and captive-bred rats when using this method. The fourth method uses the number of granule cells in the granule cell layer of the dentate gyrus for standardization (e.g. Gatome et al., 2010; Cavegn et al., 2013; Slomianka et al., 2013; Huang et al., 2015). Cavegn et al. (2013) reported differing proliferating and immature cell densities in various wild-caught rodent species from different environments when using this method. Thus, we have varying methods, sometimes leading to contrasting conclusions, in the literature regarding the effect of manipulations and the natural environment on adult neurogenesis in rodents and other species.

In the current study we explore the potential links between the environment in which an animal is bred and raised and adult neurogenesis by examining the rate of cell proliferation and immature neuron survival in captive-bred and wild-caught Egyptian fruit bats (*Rousettus aegyptiacus*) using four of the methods outlined above: (A) we report total cell numbers; (B) we report cell numbers corrected for brain mass; (C) we report cell numbers corrected for the volume of the granule cell layer of the dentate gyrus; and (D) we report cells number corrected for the total number of dentate gyrus granule cells. We examined adult neurogenesis, using immunohistochemical staining and stereological analyses, within the dentate gyrus of Egyptian fruit bats that were: (1) fifth-generation captive bred, (2) wild-caught from a subtropical woodland environment, and (3) wild-caught from a primary tropical rainforest. This study therefore attempts to elucidate the potential effect of the environment in which an animal is born and raised on the process of adult neurogenesis with four previously utilized methods.

5.2 Methods

5.2.1 Animals and Treatment

In the present study we examined 14 brains from the megachiropteran *Rousettus aegyptiacus*. Five fifth generation captive-bred bats were been obtained from the Copenhagen zoo, Denmark, six bats were caught from a wild population inhabiting a cave near Mokopane in Limpopo, South Africa, and three were captured from wild populations in the Yoko Forest near Kisangani, Democratic Republic of the Congo. All animals were adults, as judged from epiphyseal closure of the metacarpophalangeal joints (Anthony, 1988). Appropriate permissions were obtained from the Copenhagen zoo, the Limpopo Provincial Government Department of Nature Conservation, South Africa, and the University of Kisangani in the

Democratic Republic of the Congo. All animals were treated and used in accordance with the University of the Witwatersrand Animal Ethics Committee Guidelines (clearance number 2008/36/1) which parallel those of the NIH for the care and use of animals in scientific experiments. All bats were euthanized within 20 minutes of capture, or with minimal handling of less than 5 minutes in the captive-bred animals (overdose of sodium pentobarbital, 100 mg/kg, i.p. or i.v.) and perfused through the left ventricle with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). Following perfusion, the brains were removed and post-fixed in 4% paraformaldehyde in 0.1 M PB overnight, cryoprotected in 30% sucrose in 0.1 M PB at 4°C and stored in an antifreeze solution at -20°C until sectioning and histological processing. Before sectioning, the brains were divided into two halves along the mid-sagittal fissure and the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4°C. The specimens were cryosectioned in sagittal plane into 50 μm thick sections. A one in three series of sections was stained for Nissl substance (cresyl violet) to reveal cytoachitectural features, and immunostained for Ki-67 to reveal proliferating cells, and doublecortin (DCX) to reveal immature neurons.

5.2.2 Immunohistochemistry

The current investigation made use of antibodies to Ki-67 and doublecortin (DCX) as markers of proliferative activity and immature neurons respectively. These were previously used successfully in studies on microchiropterans (Amrein et al., 2007; Chawana et al., 2014) and megachiropterans (Gatome et al., 2010; Chawana et al., 2013). The advantage of using these markers to localize adult neurogenesis is that no pre-handling of the animal is needed. The DCX antibody also provides an average of the rate of expression of new neurons in natural conditions prior to capture of the animal (Bartkowska et al., 2010).

The sections were incubated in a 1.6% H_2O_2, 49.2% methanol, 49.2% 0.1 M PB solution, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10-minute rinses in 0.1 M PB. To block non-specific binding sites the sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal goat serum – NGS, for the Ki-67 antibody or 3% normal rabbit serum – NRS, for the DCX antibody, plus 2% bovine serum albumin, BSA, and 0.25% Triton X-100 in 0.1 M PB). Thereafter, the sections were incubated for 48 h at 4°C in the primary antibody solution (1:1000, rabbit anti-Ki67, NCL-Ki67p DAKO, or 1:300, goat anti-doublecortin, DCX, SC-18 Santa Cruz Biotech) under gentle agitation. The primary antibody incubation was followed by three 10 min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated anti-rabbit IgG, BA1000 for Ki-67, or anti-goat IgG, BA 5000 for DCX, Vector Labs, in 3% NGS/NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-minute rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin-biotin solution (1:125; Vector Labs), followed by three 10-min rinses in 0.1 M PB. Sections were then placed in a solution containing 0.05% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 µl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low power stereomicroscope. Staining continued until such time as the background stain was at a level that would allow for accurate architectonic matching to the Nissl sections without obscuring the immunopositive structures. Development was arrested by placing sections in 0.1 M PB for 10 min, followed by two more 10 min rinses in this solution. Sections were then mounted on 0.5% gelatine coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody, and sections where we omitted the secondary

antibody. In both cases no staining was observed. The observed immunostaining patterns support the specificity of the antibodies as they are compatible with the observations made on previous studies using the same species (Chawana et al., 2013).

5.2.3 Antibody characterization and specificity

To reveal proliferating cells we used immunohistochemistry to the endogenous marker Ki-67, a protein expressed in all phases of the cell cycle (G_1, S, G_2 and mitoses) except the resting phase (G_0) (Endl and Gerdes, 2000; Scholzen and Gerdes, 2000). Ki67 is an effective marker of cell proliferation in the initial stages of adult neurogenesis (Kee et al., 2002) and to visualize Ki-67 positive cells in the Egyptian fruit bat brain we used a polyclonal rabbit anti-Ki-67 antibody (Dako, M7240; RRID AB_2142367), the immunogen being a human recombinant peptide corresponding to a 1002 bp Ki-67 cDNA fragment (El Ayachi et al., 2011; Farahani et al., 2012). The pattern of immunostaining obtained in this study is consistent with that reported in previous studies using this antibody (e.g. Gatome et al., 2010; Chawana et al., 2013).

To examine immature/differentiating/migrating neurons in the brain of the Egyptian fruit bats, we used immunohistochemistry to the endogenous marker doublecortin (DCX). DCX is a microtubule associated protein that is expressed in the cytoplasm of immature neurons (Plümpe et al., 2006). It binds, bundles and stabilizes microtubules and may play a role in migration, translocation and maturation of various neural components (Brown et al., 2003). The visualisation of DCX-immunopositive cells also provides an average of the rate of expression of new neurons under natural conditions prior to capture of the animal (Bartkowska et al., 2010). Also, as expression is intrinsic, no pre-handling and injection of markers is required, and accordingly, animals are not exposed to any additional stress.

Accordingly, DCX is a reliable and effective neurogenic marker that enables the detection of modulation in adult neurogenesis during a defined period of time. To visualise DCX we used the goat-anti DCX C-18 primary antibody from Santa Cruz Biotechnology (RRID AB_2088494), since this antibody has been demonstrated to provide a more distinct and intense labelling in rodents (Brown et al., 2003) and other mammals (Liu et al., 2008; Patzke et al., 2015). The pattern of immunostaining obtained in this study is consistent with that reported in previous studies using this antibody (e.g. Brown et al., 2003; Barker et al., 2005; Epp et al., 2009; Gatome et al., 2010; Chawana et al., 2013).

5.2.4 Quantitative Analyses

Brain mass for each individual was measured immediately following extraction of the brain from the skull and removal of the meninges. To estimate the volume of the granule cell layer of the dentate gyrus, we employed the Cavalieri probe of the Microbrightfield Stereo Investigator software. The area of the granule cell layer of the dentate gyrus in every ninth section through the hippocampus was measured (section sampling fraction = 0.111) with 10 to 12 sections analysed per animal. A grid size of 25 x 25 μm was used in order to accommodate the thinnest regions of the granule cell layer and the region was mapped using a 40x objective lens. The obtained volumes were later used to calculate the density of Ki-67 and DCX immunopositive cells per cubic millimetre of the granule cell layer.

To estimate the number of proliferating cells, those that were Ki-67 immunopositive, exhaustive counts using a 63x oil immersion lens were undertaken. Only immunopositive cells in the granule cell layer were included in the counts. In all the individuals, every third section was quantified and the total cell number obtained through multiplying the counts by

the inverse of the sampling fraction. This method was previously used by Epp et al. (2009), Gatome et al. (2010), Bartkowska et al. (2010) and Castilla-Ortega et al. (2011).

To estimate the total number of DCX immunopositive neurons in the granule cell layer of the dentate gyrus, we employed a design-based stereological probe, the optical fractionator (West et al., 1991). The outlines of the granule cell layer (GCL) of each section of the series were drawn using a 5x objective lens of the Zeiss Axioskop Imager Z2 Vario coupled with a computer system running the Stereo Investigator software (MicroBrightField, Inc., Williston, USA). Every twelfth section was used (section sampling fraction = 0.083) leading to between 6 and 10 sections analysed per animal. A counting frame of 70 x 70 μm in a sampling grid size of 90 x 90 μm was used giving an area sampling fraction of 0.605. These counting frame and grid sizes were used in order to achieve an acceptable co-efficient of error (that is CE < 0.1). The optical disector of 10 μm in height was centrally placed in the *z*-axis and the thickness of the tissue was measured by fine focus of the top of the tissue and then the bottom of the tissue before counting. Counting was done using a 63x oil immersion lens (NA = 1.4). The mean weighted estimate of the total number of cells in the granule cell layer of the hippocampus (N) was obtained using the following algorithm which takes into account the section sampling fraction, the area sampling fraction and the thickness sampling factor (the fraction of the disector height to the mean measured thickness):

$$N = Q/(SSF \times ASF \times TSF)$$

Where: N – was the total estimated neuronal number, Q – was the number of neurons counted, SSF – was the section sampling fraction, ASF – is the area sub fraction (this was the ratio of the size of the counting frame to the size of the sampling grid), and TSF – was the thickness sub fraction (this was the ratio of the dissector height relative to cut section thickness) (West et al., 1991).

To estimate the total number of granule cells in the granule cell layer of the dentate gyrus, contours of the GCL were drawn using a 10x objective lens of the Zeiss Axioskop Imager Z2 Vario. Every ninth section was analysed (section sampling fraction = 0.111) which gave rise to between 10 and 12 sections per series from each animal being examined. A counting frame of 25 x 25 μm in a sampling grid size of 210 x 210 μm was used giving an area sampling fraction of 0.014. Counting was done using a 100x oil immersion lens (NA = 1.4). The mean weighted estimate of the total granule cell numbers in the granule cell layer of the hippocampus (N) was obtained using the algorithm provided above.

Given the heterogeneity in the distribution of DCX immunopositive cells in the dentate gyrus, we used the Schaeffer CE as it is most suited for non-homogenously distributed cells (Glaser and Wilson, 1998; Schmitz and Hof, 2000). In all the counts made, our CE was less than 0.1, which is acceptable in stereological counts (Table 1) (Glaser and Wilson, 1998; Slomianka and West, 2005). The variance in the counts between the groups studied was not introduced through the methodology given that our CE^2/CV^2 ratio for all populations was less than 0.5 (Slomianka and West, 2005) (Table 5.2).

5.2.5 Statistical Analysis

Statistical analysis was performed using STATA software package version 13.1 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). We performed non-parametric tests using Kruskal-Wallis equality of populations rank tests to compare significant differences in the dependent variables (brain mass, granule cell layer volume, Ki-67 immunopositive cell density and DCX immunopositive cell density) of the Egyptian fruit bats from different conditions. Pair-wise Wilcoxon rank sum (Mann-Whitney) test was performed on two independent samples (that is tropical rainforest bats against

subtropical woodland bats; tropical rainforest bats against captive-bred bats; and subtropical woodland bats against captive-bred bats) for the dependent variables. To establish any correlations between the granule cell layer volume or total granule cell numbers and cell density (Ki-67 and DCX), and Ki-67 and DCX cell densities, we performed Kendall's rank correlation test with Bonferroni adjustment.

5.2.6 Image Processing

Digital photomicrographs were captured using Zeiss Axioshop and Axiovision software. No pixilation adjustments or manipulation of the captured images was undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop.

5.3 Results

5.3.1 The pattern of adult neurogenesis in the Egyptian fruit bat brain

The location of both proliferative cells and immature neurons, detected with Ki-67 and DCX immunostaining respectively, in the brain of the adult Egyptian fruit bat was identical to that observed previously in the brains of other megachiropterans (Gatome et al., 2010; Chawana et al., 2013). Briefly, both Ki-67 and DCX immunopositive cells were observed in the subventricular zone and the olfactory bulb, with both the dorsal and ventral migratory sub-streams of the rostral migratory pathway being observed (Fig. 5.1A, 5.1B). From the temporal horn of the lateral ventricle, we detected Ki-67 and DCX immunopositive cells, which appeared to migrate to the piriform cortex and the amygdala through the

temporal migratory stream (Fig. 5.1C, 5.1D). In the hippocampus, Ki-67 and DCX immunopositive cells were observed in the subgranular zone and granular layer of the dentate gyrus (Fig. 5.1E, 5.1F). Lastly, Ki-67 and DCX immunopositive cells were also detected in the cerebral neocortex, while Ki-67 immunopositive cells, but not DCX immunopositive cells were detected in the midbrain tectum, cerebellum and medulla oblongata.

5.3.2 Brain mass varied amongst the Egyptian fruit bat groups studied

The Egyptian fruit bats used in this study were obtained from three different environments and showed significant variation in brain masses ($H_{(2)} = 10.568$, $P = 0.005$) (Fig. 5.2A, Table 5.1). The median brain mass of the fifth generation captive bred animals (n = 5) was 1.79 g (Interquartile range (IQR) = 1.7 - 1.9g), while that of the bats from the primary rainforest of central Africa (n = 3) was 2.01 g (IQR = 1.97 - 2.14g), and the largest brains were observed in the bats caught in the subtropical woodland of South Africa (n = 6), having an median mass of 2.4 g (IQR = 2.3 - 2.5g). When the Mann-Whitney test was used to determine whether the brain masses varied between groups, the subtropical woodlands bats had a significantly larger brain masses than the bats from both the tropical rainforest ($P = 0.019$) and the captive-bred bats ($P = 0.0059$); however, no significant difference in the brain masses of the tropical rainforest and captive-bred bats ($P = 0.101$) was observed (Fig. 5.2A).

5.3.3 Volume of the granule cell layer of the dentate gyrus of the hippocampus

The median volume of the granule cell layer (GCL) of the captive-bred animals investigated in this study (n = 5) was 1.7 mm^3 (IQR = 1.68 - 1.84 mm^3). This was smaller than that observed in the animals caught in the primary rainforest of central Africa (n = 3),

which had a median volume of 2.06 mm^3 (IQR = 2.06 - 2.25 mm^3) and those caught in the subtropical woodland (n = 6), which had a median volume of 2.19 mm^3 (IQR = 1.97 - 2.28 mm^3) (Fig. 5.2B; Table 5.1). A comparison of the GCL volume across these three groups indicated that subtropical woodlands bats had a significantly larger GCL volume than the captive-bred bats ($P = 0.045$), but it did not differ significantly from that of the primary rainforest bats ($P = 0.795$). In addition there was no statistically significant difference between the GCL volume of the primary rainforest bats and the captive-bred bats ($P = 0.099$). When the GCL was taken as a fraction of the brain mass (used as a proxy for brain volume) the primary rainforest bats differed significantly from the subtropical woodland bats ($P = 0.039$), but did not differ from the captive-bred bats ($P = 0.8815$). There was also no significant difference between the subtropical woodland bats and the captive-bred bats ($P = 0.082$). There was no correlation between brain mass and granule cell layer volume ($r_\tau = 0.492$, $P = 0.812$) (Fig. 5.2B, Fig. 5.7A).

5.3.4 Total number of cells in the granule cell layer of the dentate gyrus of the hippocampus

The median total number of granule cells in the captive-bred bats (n = 5) was 1 490 003 (IQR = 1 446 470 - 1 513 672). This number was lower than that found in the bats caught in the subtropical woodland (n = 6, median = 1 688 168, IQR = 1 676 402 - 1 780 106) and the primary rainforest (n = 3, median = 1 919 664, IQR = 1 890 560 - 2 363 529) (Table 5.1). Comparing the total granule cell numbers indicated that the bats caught in the primary rainforest had statistically significantly higher total granule cell numbers than the bats caught in the subtropical woodlands ($P = 0.039$) (Fig. 5.2C). The difference in the total number of granule cells was not statistically significant when comparing the primary rainforest bats and

the captive-bred bats (P = 0.101), or when comparing the subtropical woodland bats and the captive-bred bats (P = 0.361). We found no correlation between the volume of the granule cell layer and the total number of granule cells across all animals from the different environments (r_τ = 0.265, P = 1.000) (Fig. 5.7B), nor was there any correlation between brain mass and total granular cell count (r_τ = 0.122, P = 1.000) (Fig. 5.7 C).

5.3.5 Total numbers of hippocampal Ki-67 and DCX immunopositive cells

Ki-67 and DCX immmunopositive cells were observed in the subgranular zone and granule cell layer of all the individual animals investigated in the current study (Fig. 5.1E. 5.1F). The median number of Ki-67 immunopositive cells counted in the captive-bred bats (n = 5) was 417 (IQR = 405 - 612), which was lower than that observed in the bats caught in the subtropical woodland (n = 6, median = 602, IQR = 549 - 624) and those caught in the primary rainforest (n = 3, median = 1 113, IQR = 420 - 1293) (Fig. 5.3A; Table 5.1). A comparison of the Ki-67 immunopositive cell numbers indicated no statistically significant difference between captive-bred and primary rainforest bats (P = 0.101), captive-bred and subtropical woodland bats (P = 0.584), and primary rainforest and subtropical woodland bats (P = 0.302) (Fig. 5.3A). The median number of DCX immunopositive cells counted in the captive-bred bats (n = 5) was 15 087 (IQR = 14 834 – 16 687), which was lower than that observed in the bats caught in the subtropical woodland (n = 6, median = 26 931, IQR = 23 690 – 29 955) and those caught in the primary rainforest (n = 3, median = 33 141, IQR = 30 768 – 37 375) (Fig. 5.3B; Table 5.1). Comparing DCX immunopositive cell numbers showed that captive-bred bats had statistically significant lower numbers than both primary rainforest bats (P = 0.025) and subtropical woodland bats (P = 0.029). The difference in the

DCX numbers was not statistically significant between primary rainforest and subtropical woodland bats ($P = 0.121$) (Fig. 5.3B).

5.3.6 Ki-67 and DCX immunopositive cell numbers standardized to brain mass

The median number of Ki-67 immunopositive cells, expressed as a ratio of the brain mass, within the granule cell layer in the captive bred bats (n = 5) was 319 cells/g (IQR = 219 - 360 cells/g), which was higher than that observed in the subtropical woodland bats (n = 6, median = 242 cells/g, IQR = 229 - 271 cells/g), but lower than that observed in the bats caught in the primary rainforest (n = 3, average = 554 cells/g, IQR= 213 - 604 cells/g) (Fig. 5.4A; Table 5.1). The median number of DCX immunopositive cells, expressed as a ratio of the brain mass, within the granule cell layer in the captive bred bats (n = 5) was 8 726 cells/g (IQR = 7 288 - 13 139 cells/g), which was lower than that observed in the subtropical woodland bats (n = 6, median = 10 974 cells/g, IQR = 9 871 – 13 024 cells/g), and lower than that observed in the bats caught in the primary rainforest (n = 3, median = 16 822 cells/g, IQR = 15 307 - 17 465 cells/g) (Fig. 5.4B, Table 5.1). The differences across all species in the Ki-67 immunopositive cell number to brain mass ratio were statistically insignificant ($H_{(2)} = 1.684$, $P = 0.431$). A comparison of Ki-67 immunopositive cell number to brain mass ratio in captive-bred and primary rainforest bats, captive-bred and subtropical woodland bats, and primary rainforest and subtropical woodland bats were all statistically insignificant ($P = 0.297$, $P = 0.465$ and $P = 0.302$ respectively) (Fig. 5.4A). Comparing the ratio of DCX immunopositive cells to the brain mass indicated that primary rainforest bats had statistically significant higher numbers of DCX immunopositive cells than subtropical woodland bats ($P = 0.039$) but not captive-bred bats ($P = 0.053$). There was no significant difference between the captive-bred and subtropical woodland bats ($P = 0.465$) (Fig. 5.4B).

We did not find any significant correlation between the brain mass and both the Ki-67 immunopositive cell number (r_τ = 0.100, P = 1.000) (Fig. 5.8A) and DCX immunopositive cell number (r_τ = 0.322, P = 1.000) (Fig. 5.8B).

5.3.7 Ki-67 and DCX immunopositive cell densities using granule cell layer volume standardization

The median density of Ki-67 immunopositive cells within the granule cell layer in the captive bred bats (n = 5) was 274 cells/mm^3 (IQR = 187 - 360 cells/mm^3), which was higher than that observed in the subtropical woodland bats (n = 6, median = 257 cells/mm^3, IQR = 234 - 317 cells/mm^3), but lower than that observed in the bats caught in the primary rainforest (n = 3, median = 495 cells/mm^3, IQR = 204 - 628 cells/mm^3) (Fig. 5.5A; Table 5.1). The median density of DCX immunopositive cells within the granule cell layer in the captive bred bats (n = 5) was 8 980 cells/mm^3 (IQR = 8 461 - 11 275 cells/mm^3), which was lower than that observed in the subtropical woodland bats (n = 6, median = 12 802 cells/mm^3, IQR = 10 900 - 15 206 cells/mm^3), and lower than that observed in the bats caught in the primary rainforest (n = 3, median = 16 088 cells/mm^3, IQR = 13 675 – 18 143 cells/mm^3) (Fig. 5.5B; Table 5.1). Despite these differences, the densities of Ki-67 and DCX immunpositive cells, when standardised using granule cell layer volume, did not show any statistically significant differences between the bats from the 3 environments (Ki-67: $H_{(2)}$ = 1.440, P = 0.487; DCX: $H_{(2)}$ = 5.008, P = 0.082); that is between primary rainforest and subtropical woodland bats (Ki-67: P = 0.302; DCX: P = 0.197), primary rainforest and captive-bred bats (Ki-67: P = 0.297; DCX: P = 0.053), and subtropical woodland and captive-bred bats (Ki-67: P = 0.715; DCX: P = 0.144) (Fig. 5.5). Correlation tests revealed that there was no correlation between the Ki-67 and DCX immunpositive cell densities (r_τ = 0.297, P = 1.000) (Fig. 5.9A),

indicating that the density of proliferative cells does not predict of the density of immature neurons. Further tests showed no correlation between the absolute Ki-67 immunopositive cell counts and granule cell layer volume (r_τ = 0.199, P = 1.000) (Fig. 5.9B), and the absolute DCX immunopositive cell counts and granule cell layer volume (r_τ = 0.309 P = 1.000) (Fig. 5.9C). This indicates that the granule cell layer volume was not related to the number of proliferative or immature cells within this species.

5.3.8 Ki-67 and DCX immunopositive cells as a fraction of total granule cell numbers

The median fraction of Ki-67 immunopositive cells in the granule cell layer, expressed as a percentage of total granule cell number, in the captive bred bats (n = 5) was 0.029% (IQR = 0.027 - 0.045 %), which was slightly lower than that observed in the subtropical woodland bats (n = 6, median = 0.033%, IQR = 0.031 – 0.037%), and lower than that observed in the bats caught in the primary rainforest (n = 3, median = 0.055%, IQR = 0.022 - 0.059%) (Fig. 5.6A; Table 5.1). The median fraction of DCX immunopositive cells the granule cell layer, expressed as a percentage of the total granule cell number, in the captive bred bats (n = 5) was 1.11% (IQR = 0.997 - 1.12%), which was lower than that observed in the subtropical woodland bats (n = 6, median = 1.76%, IQR = 1.424 - 1.778%), and lower than that observed in the bats caught in the primary rainforest (n = 3, median = 1.627%, IQR = 1.581 - 1.726%) (Fig. 5.6B; Table 5.1). There were no statistically significant differences in the number of Ki-67 immunopositive cells, expressed as a percentage of total granule cell number, amongst the various bat groups ($H_{(2)}$ = 1.288, P = 0.525). Pair wise tests were insignificant between the primary rainforest and subtropical woodland bats (P = 0.439), primary rainforest and captive-bred bats (P = 0.2967), and subtropical woodland and captive-bred bats (P = 0.584) (Fig. 5.6A). In contrast to these lack of differences, a significant

difference was obtained for the number of DCX immunopositive cells expressed as a percentage of total granule cell numbers ($H_{(2)} = 8.421$, $P = 0.015$), with pair-wise tests indicating that both the wild-caught primary rainforest and subtropical woodland bats having higher percentages of DCX immunopositive cells than the captive-bred bats ($P = 0.025$ and $P = 0.011$ respectively). There was no statistically significant difference between the two groups of wild-caught bats ($P = 0.439$) (Fig. 5.6B). A correlation test between the number of Ki-67 and DCX immunopositive cells showed no relationship between the percentages of Ki-67 and DCX immunopositive cells ($r_\tau = 0.143$, $P = 1.000$) (Fig. 5.10A). In addition, there was no correlation between the absolute Ki-67 immunopositive cell counts and total granular cell counts ($r_\tau = 0.407$, $P = 1.000$) (Fig. 5.10B), and the absolute DCX immunopositive cell counts and total granular cell counts ($r_\tau = 0.604$ $P = 0.140$) (Fig. 5.10C). These two analyses indicate that the numbers of proliferating cells and immature neurons were not related to the number of granule cells in the granule cell layer in this species. Lastly, there was a significant correlation between the density of Ki-67 immunopositive cells (based on granule cell layer volume) and the number of Ki-67 immunopositive cells expressed as percentage of the total granule cell number ($r_\tau = 0.758$, $P = 0.009$) (Fig. 5.11A). In contrast, there was no correlation between the density of DCX immunopositive cells (based on granule cell layer volume) and the number of DCX immunopositive cells expressed as percentage of the total granule cell number ($r_\tau = 0.604$ $P = 0.140$) (Fig. 5.11B).

Table 5.1: Quantitative summary of the density of Ki-67- and DCX- immuno-positive cells in hippocampus of the wild and captive *Rousettus aegyptiacus* brains

1 The coefficient of error in this column is the Gundersen (m=1) for the volume estimates

[2]The coefficient of error in this column is the Schaeffers CE for the DCX+ cell count estimates

[3] The coefficient of error in this column is the Schaeffers CE for the total granule cell count estimates

*Stereological methods using the optical fractionator were not employed for Ki-67+ cell counts. Instead the cells were manually counted exhaustively and the total count was obtained by multiplying by the inverse of the sampling fraction as previously done by Epp et al. (2009)

Rousettus aegyptiacus	Brain Mass (g)	GCL Volume (mm^3)	CE[1]	Ki-67+ Cell Count*	DCX+ Cell Count	CE[2] (Schaeffer)	Ki-67+ cells/g Brain mass	DCX+ cells/g Brain mass	Ki-67+ Cell Density ($cells/mm^3$)	DCX+ Cell Density ($cells/mm^3$)	Total Granule Cells (TGC)	CE[3] (Schaeffer)	Normalised Ki-67+ (% of TGC)	Normalised DCX+ (% of TGC)
Wild-living DRC	2.01	2.25	0.032	1 113	30 768	0.067	554	15 307	495	13 675	1 890 560	0.048	0.059	1.627
	2.14	2.06	0.047	1 293	37 375	0.074	604	17 465	628	18 143	2 363 529	0.051	0.055	1.581
	1.97	2.06	0.057	420	33 141	0.052	213	16 822	204	16 088	1 919 664	0.042	0.022	1.726
Median	2.01	2.06		1 113	33 141		554	16 822	495	16 088	1 919 664		0.055	1.627
IQR	1.97 - 2.14	2.06 - 2.25		420 - 1 293	30 768 - 37 375		213 - 604	15 307 - 17 465	204 - 628	13 675 - 18 143	1 890 560 - 2 363 529		0.022 - 0.059	1.581 - 1.726
Wild-living RSA	2.4	2.21	0.046	549	24 088	0.077	229	10 037	248	10 900	1 691 432	0.037	0.032	1.424
	2.3	1.97	0.077	624	29 955	0.077	271	13 024	317	15 206	1 684 904	0.042	0.037	1.778
	2.6	2.52	0.089	597	40 943	0.069	230	15 747	234	16 247	1 898 956	0.040	0.031	2.156
	2.5	2.17	0.075	804	29 774	0.072	322	11 910	371	13 721	1 676 402	0.047	0.048	1.776
	2.3	1.87	0.068	312	22 221	0.075	136	9 661	170	11 883	1 274 493	0.058	0.024	1.744
	2.4	2.28	0.058	606	23 690	0.090	253	9 871	266	10 390	1 780 106	0.056	0.034	1.331
Median	2.4	2.19		602	26 931		242	10 974	257	12 802	1 688 168		0.033	1.76
IQR	2.3-2.5	1.97 - 2.28		549 - 624	23 690 - 29 955		229 - 271	9 871 - 13 024	234 - 317	10 900 - 15 206	1 676 402 - 1 780 106		0.031 - 0.037	1.424 - 1.778
Captive-bred Denmark	1.70	1.70	0.042	612	14 834	0.066	360	8 726	360	8 461	1 336 796	0.051	0.046	1.110
	1.79	1.84	0.047	945	28 831	0.068	528	16 107	516	15 669	2 098 616	0.051	0.045	1.374
	1.90	2.23	0.041	417	13 535	0.080	219	7 124	187	6 070	1 446 470	0.045	0.029	0.936
	1.27	1.48	0.044	405	16 687	0.081	319	13 139	274	11 275	1 490 003	0.055	0.027	1.120
	2.07	1.68	0.043	288	15 087	0.069	139	7 288	171	8 980	1 513 672	0.054	0.019	0.997
Median	1.79	1.7		417	15 087		319	8 726	274	8 980	1 490 003		0.029	1.11
IQR	1.7-1.9	1.68 – 1.84		405 - 602	14 834 - 16 687		219 - 360	7 288 - 13 139	187 - 360	8 461 - 11 275	1 446 470 – 1 513 672		0.027 – 0.045	0.997 – 1.12

Table 5.2: Summary of the level of precision of the stereological estimates

Rousettus aegyptiacus		Total Granule Cells (TGC)	Coefficient of Error (Schaeffer)	DCX+ Cell Count	Coefficient of Error (Schaeffer)
Tropical Rainforest (Wild DRC)	Mean	2 057 918	0.047	33 761	0.064
	SD	265 067	0.005	3 347	0.011
	CV^2	0.017		0.010	
	CE^2/CV^2	0.133		0.421	
Subtropical Woodland (Wild RSA)	Mean	1 667 716	0.047	28 445	0.077
	SD	210 552	0.009	6 937	0.007
	CV^2	0.016		0.059	
	CE^2/CV^2	0.137		0.099	
Temperate Zoo (Captive Denmark)	Mean	1 577 111	0.051	17 795	0.073
	SD	299 332	0.004	6 270	0.007
	CV^2	0.036		0.124	
	CE^2/CV^2	0.073		0.040	

Figure 5.1: Photomicrographs showing representative staining of Ki-67 (arrowheads) (**A**, **C**, **E**) and doublecortin (arrows) (DCX, **B**, **D**, **F**) immunopositive structures in the brain of an Egyptian fruit bat. In all image rostral is to the left and dorsal to the top of the image. Note the presence of both the dorsal (**dRMS**) (open arrowheads for Ki-67 in **A** and long arrows for DCX in **A**) and ventral (**vRMS**) (solid arrowheads for Ki-67 in **B** and short arrows for DCX in **B**) branches of the rostral migratory stream (**RMS**), running along the borders of the lateral ventricle (**LV**) with the caudate nucleus (**C**). The presence of the temporal migratory stream (**TMS**) with cells migrating towards the piriform cortex (**Pir**), emanating from the temporal horn of the lateral ventricle anterior to the hippocampus (**Hip**), is revealed with both Ki-67 (arrowheads) (**C**) and DCX (arrows) (**D**) immunostaining. Ki-67 (**E**) and DCX (**F**) immunopositive cells are observed in the polymorphic (**pl**) and granule cell (**gcl**) layers of the dentate gyrus, with the dendrites of the DCX immunopositive cells extending into the molecular layer (**ml**). The scale bar in **D** = 1000 μm and applies to **A** – **D**, and the scale bar in **F** = 500 μm and applies to **E** and **F**.

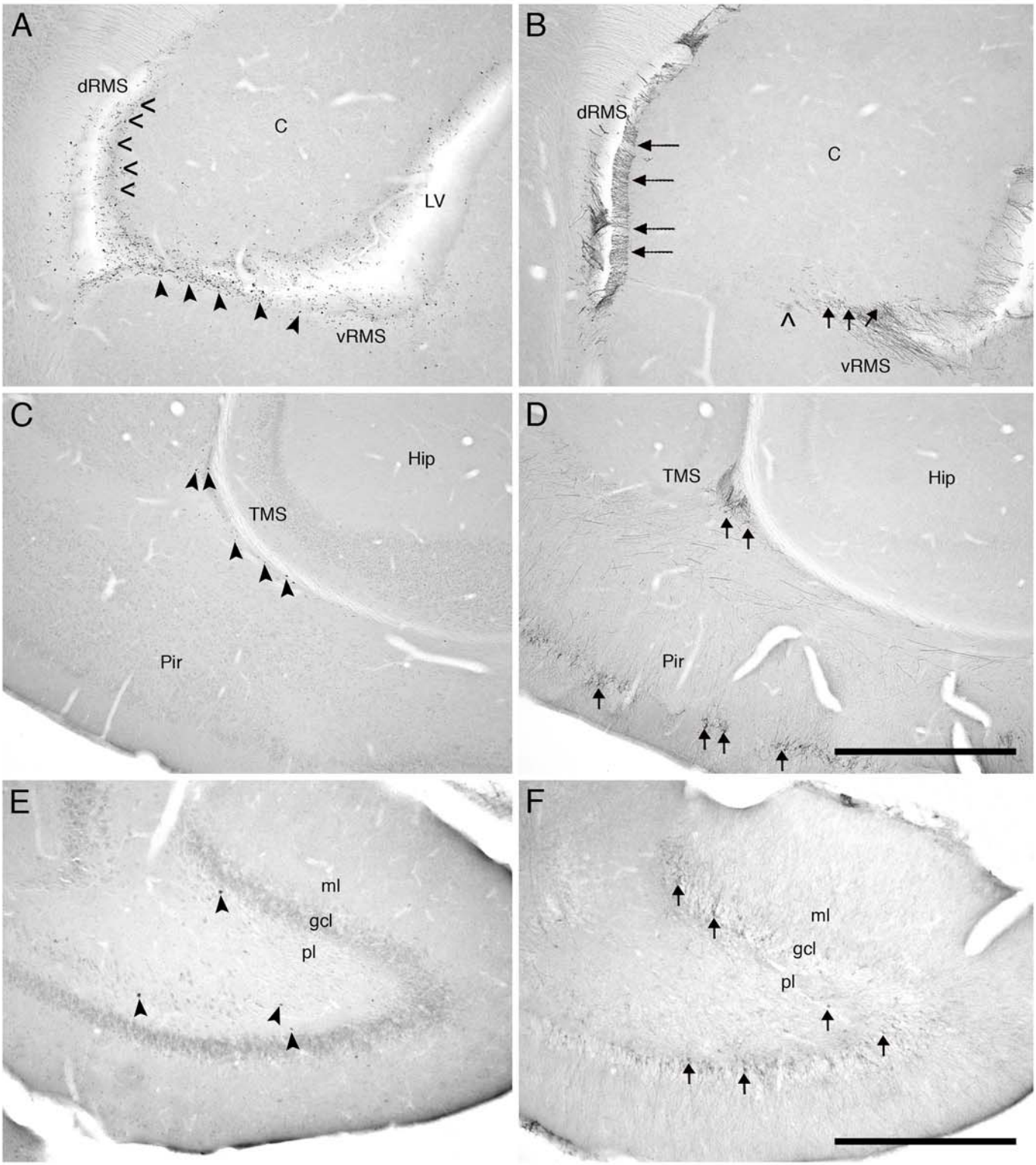

A
dRMS
C
LV
vRMS
B
dRMS
C
vRMS
C
Hip
TMS
Pir
D
TMS
Hip
Pir
E
ml
gcl
pl
F
ml
gcl
pl

Figure 5.2: Boxplots showing the distribution of (**A**) brain mass, (**B**) granule cell layer volume of the dentate gyrus of the hippocampus and (**C**) total granule cell numbers of the dentate gyrus of the hippocampus, in the three groups of Egyptian fruit bats examined in this study. The brain mass of the bats from the subtropical woodlands are statistically significantly (indicated by horizontal bar and asterisk) greater than those from both the primary rainforest and the captive-bred bats. No statistically significant difference was noted in brain mass between the primary rainforest and the captive-bred bats. The volume of the granular cell layer of the subtropical woodland bats was statistically significant greater than that of the captive bred bats, and the total number of granule cells was statistically significantly greater in the primary rainforest bats compared with the subtropical woodland bats. The horizontal line in each box represents the median, upper limit of the box the upper quartile, and the lower limit of the box the lower quartile. T-shaped bars represent the remainder of the normally distributed data, with data points falling outside of the normal distribution, representing outliers, indicated by the black circles.

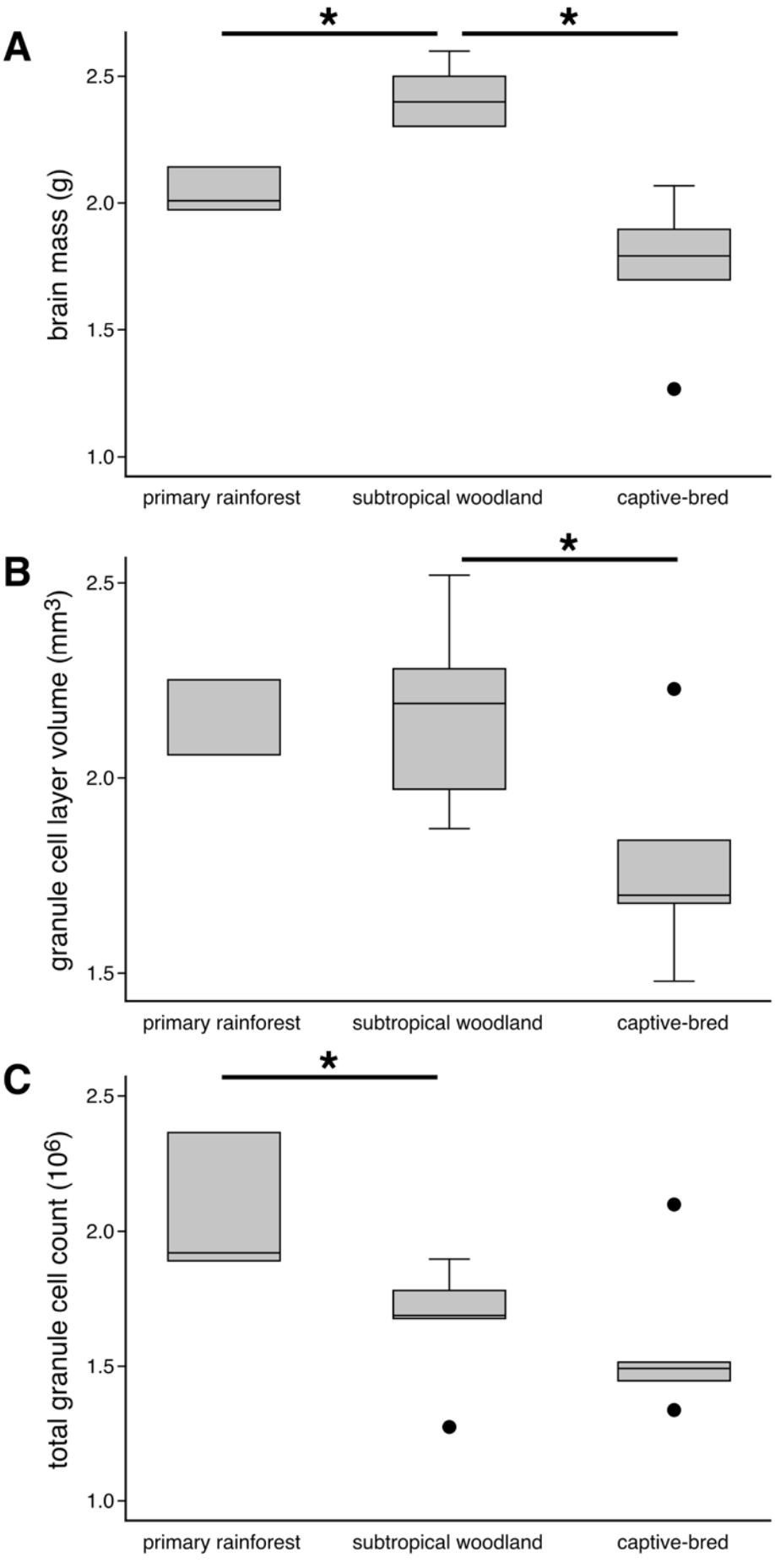

A
brain mass (g)
2.5
2.0
1.5
1.0
*
*
primary rainforest
subtropical woodland
captive-bred
B
granule cell layer volume (mm3)
2.5
2.0
1.5
*
primary rainforest
subtropical woodland
captive-bred
C
total granule cell count (106)
2.5
2.0
1.5
1.0
*
primary rainforest
subtropical woodland
captive-bred

Figure 5.3: Boxplots showing the distribution of (**A**) total number of Ki-67 immunopositive cells and (**B**) total number of doublecortin (DCX) immunopositive cells in the three groups of Egyptian fruit bats examined in this study. Note that while there are no statistically significant differences in the numbers of Ki-67 immunopositive cells across the three groups studied, both the primary rainforest and subtropical woodland bats have statistically significantly higher (indicated by horizontal bar and asterisk) numbers of DCX immunopositive cells than the captive-bred bats. The horizontal line in each box represents the median, upper limit of the box the upper quartile, and the lower limit of the box the lower quartile. T-shaped bars represent the remainder of the normally distributed data, with data points falling outside of the normal distribution, representing outliers, indicated by the black circles.

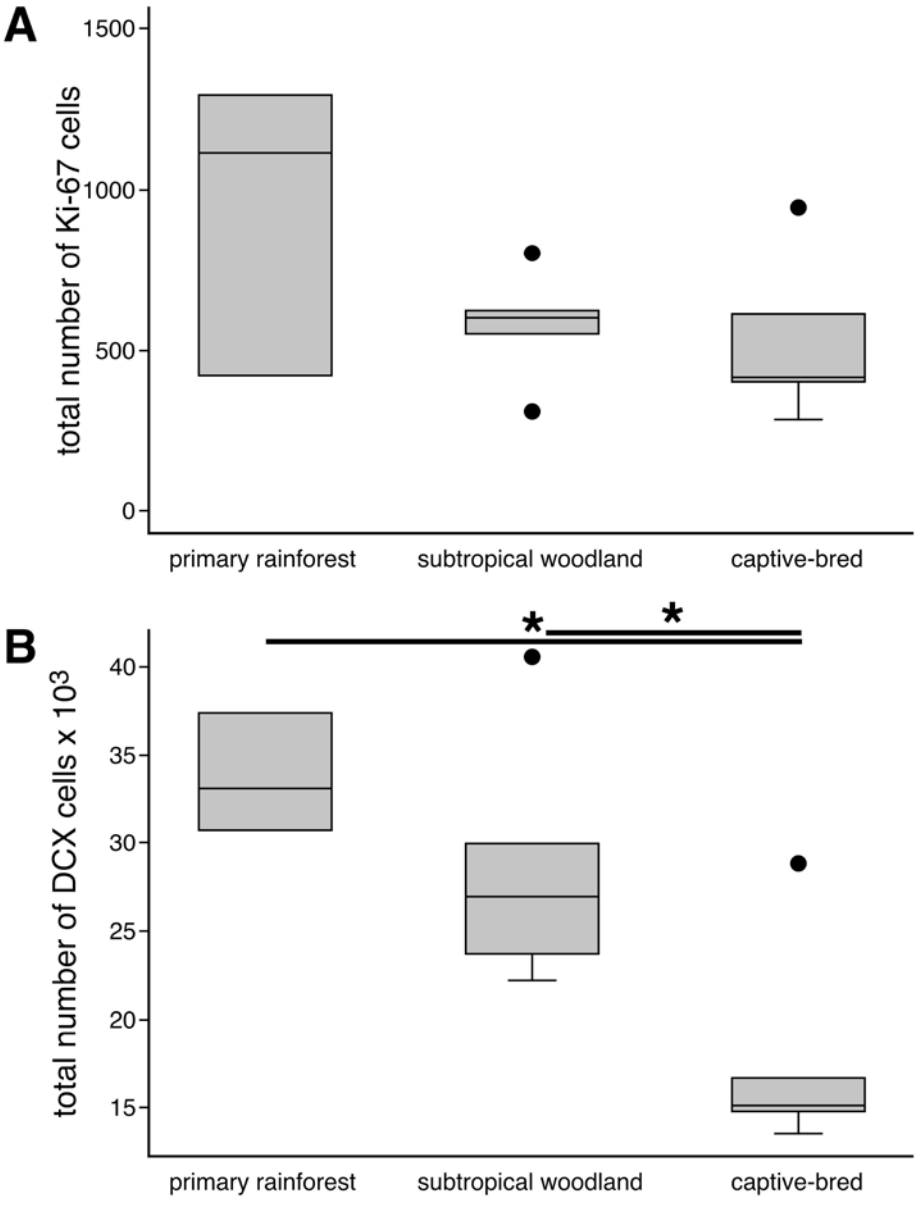

A
1500
1000
500
0
total number of Ki-67 cells
primary rainforest
subtropical woodland
captive-bred
B
40
35
30
25
20
15
total number of DCX cells x 10³
*
*
primary rainforest
subtropical woodland
captive-bred

Figure 5.4: Boxplots showing the distribution of (A) the number of Ki-67 immunopositive cells per gram of brain tissue, and (B) the number of DCX immunopositive cells per gram of brain tissue in the three groups of Egyptian fruit bats examined in this study. No statistically significant differences in the numbers of Ki-67 cells standardized to brain mass were observed, but the numbers of DCX cells standardized to brain mass in the bats from the primary rainforest are statistically signficantly higher (indicated by horizontal bar and asterisk) than that observed in the subtropical woodland bats, although both these groups show no statistically significant difference to the captive-bred bats. The horizontal line in each box represents the median, upper limit of the box the upper quartile, and the lower limit of the box the lower quartile. T-shaped bars represent the remainder of the normally distributed data, with data points falling outside of the normal distribution, representing outliers, indicated by the black circles.

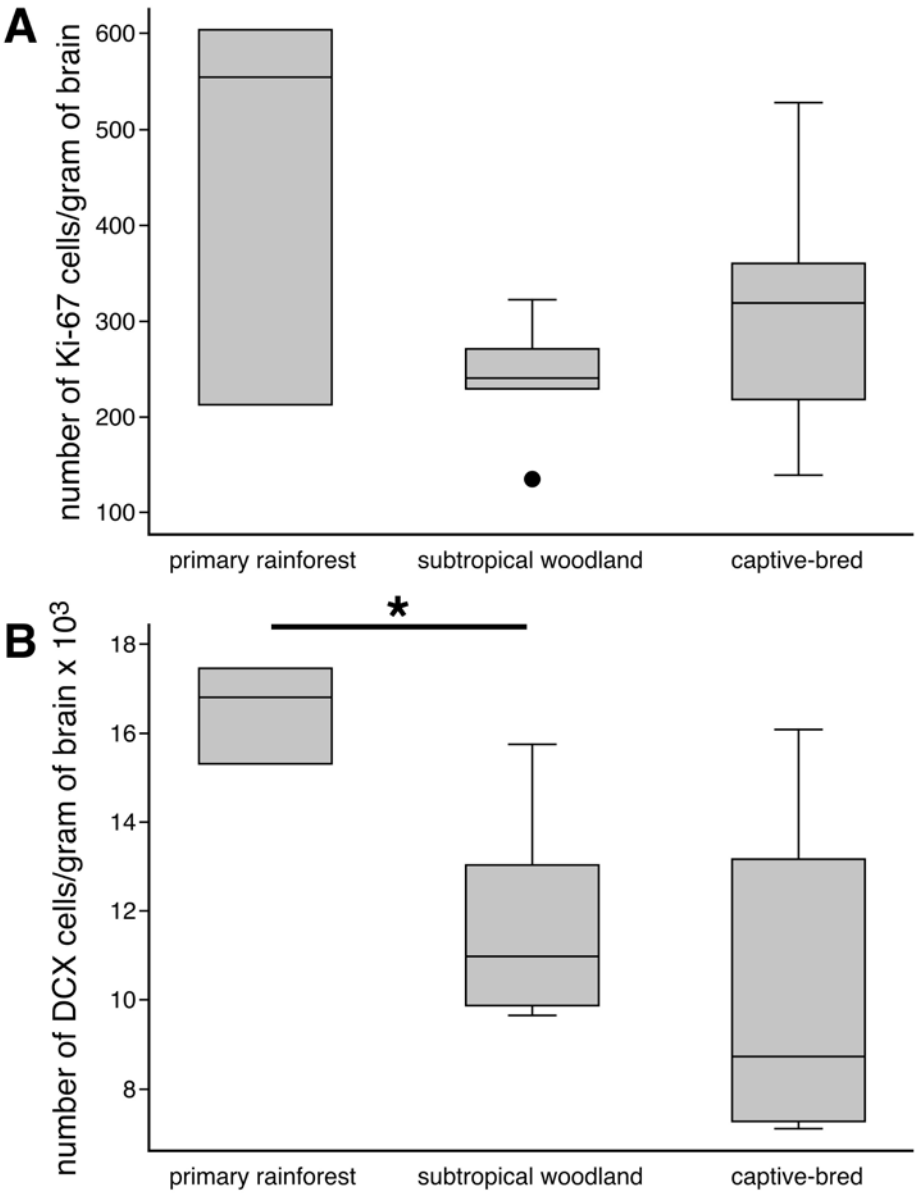

A
number of Ki-67 cells/gram of brain
600
500
400
300
200
100
primary rainforest
subtropical woodland
captive-bred
B
number of DCX cells/gram of brain x 10^3
*
18
16
14
12
10
8
primary rainforest
subtropical woodland
captive-bred

Figure 5.5: Boxplots showing the distribution of (A) the density of Ki-67 immunopositive cells relative to the volume of the granule cell layer of the dentate gyrus, and (B) the density of DCX immunopositive cells relative to the volume of the granule cell layer of the dentate gyrus in the three groups of Egyptian fruit bats examined in this study. No statistically significant differences in the densities of Ki-67 or DCX cells standardized to the volume of the granule cell layer of the dentate gyrus were observed. The horizontal line in each box represents the median, upper limit of the box the upper quartile, and the lower limit of the box the lower quartile. T-shaped bars represent the remainder of the normally distributed data, with data points falling outside of the normal distribution, representing outliers, indicated by the black circles.

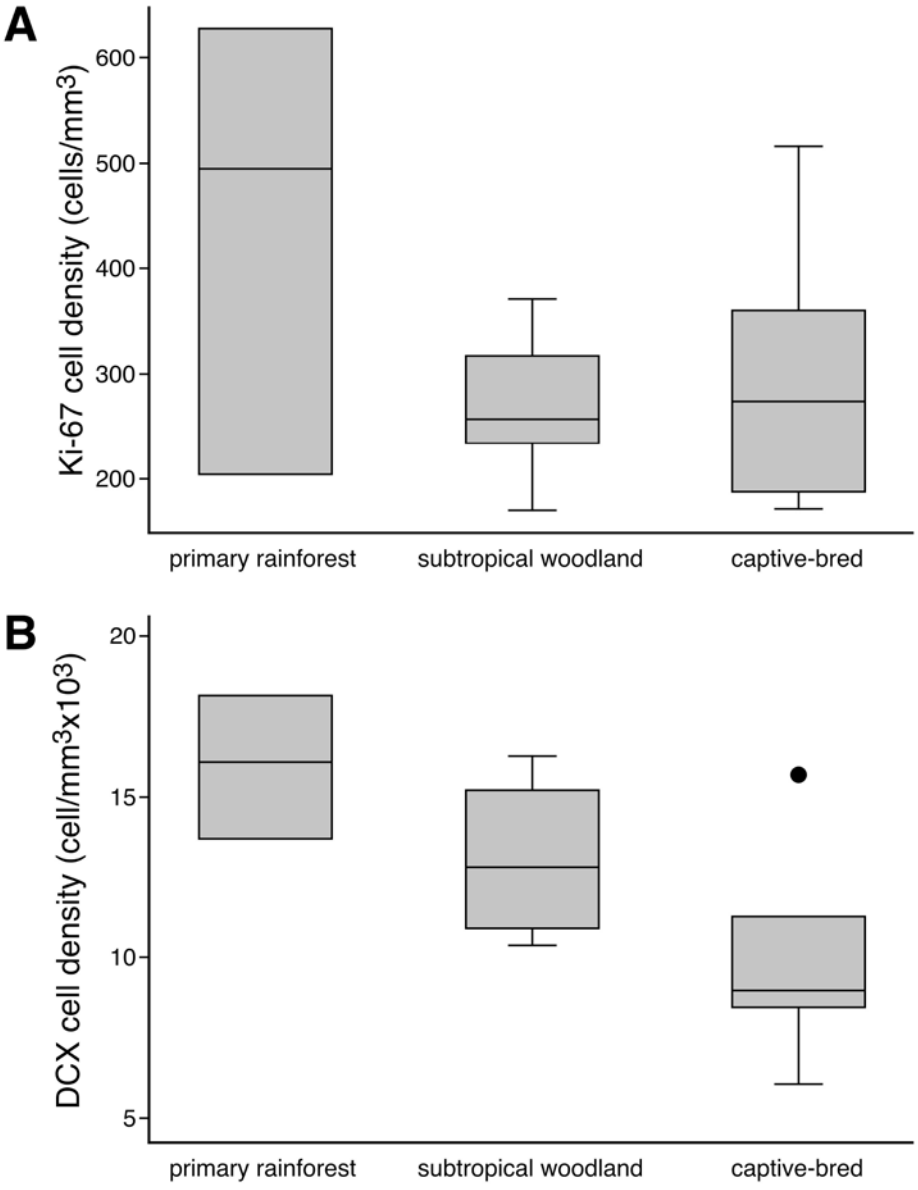

A
Ki-67 cell density (cells/mm3)
600
500
400
300
200
primary rainforest
subtropical woodland
captive-bred
B
DCX cell density (cell/mm3x103)
20
15
10
5
primary rainforest
subtropical woodland
captive-bred

Figure 5.6: Boxplots showing the distribution of (A) the percentage of Ki-67 immunopositive cells relative to the total number of granule cells within the dentate gyrus, and (B) the density of DCX immunopositive cells relative to the total number of granule cells within the dentate gyrus in the three groups of Egyptian fruit bats examined in this study. No statistically significant differences in the percentages of Ki-67 cells standardized to total granule cell number were observed; however, both the bats from the primary rainforest and the subtropcial woodland had statistically significantly higher (indicate by horizontal bar and asterisk) percentages of DCX immunopositive cells than the captive-bred bats, although there was no statistically significant difference between the primary rainforest and subtropical woodland bats. The horizontal line in each box represents the median, upper limit of the box the upper quartile, and the lower limit of the box the lower quartile. T-shaped bars represent the remainder of the normally distributed data, with data points falling outside of the normal distribution, representing outliers, indicated by the black circles.

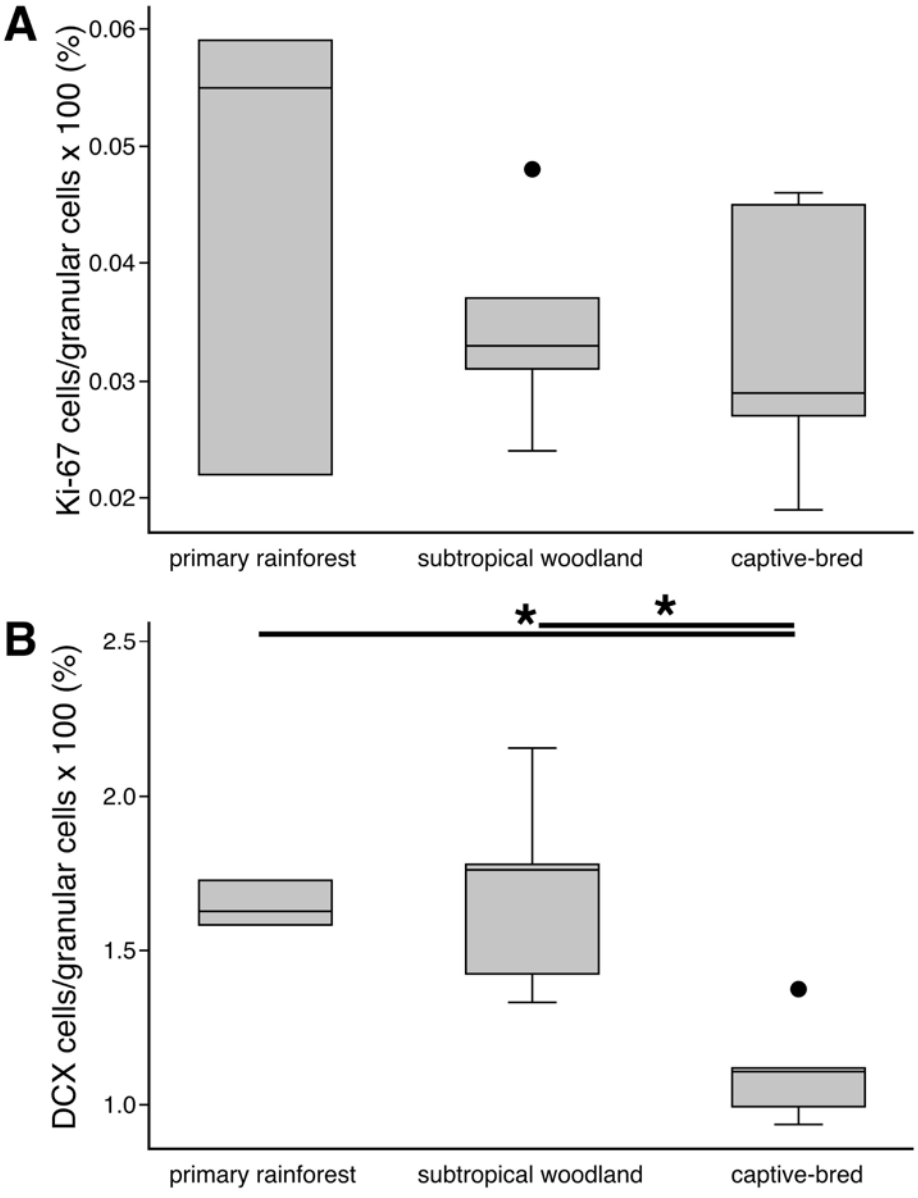
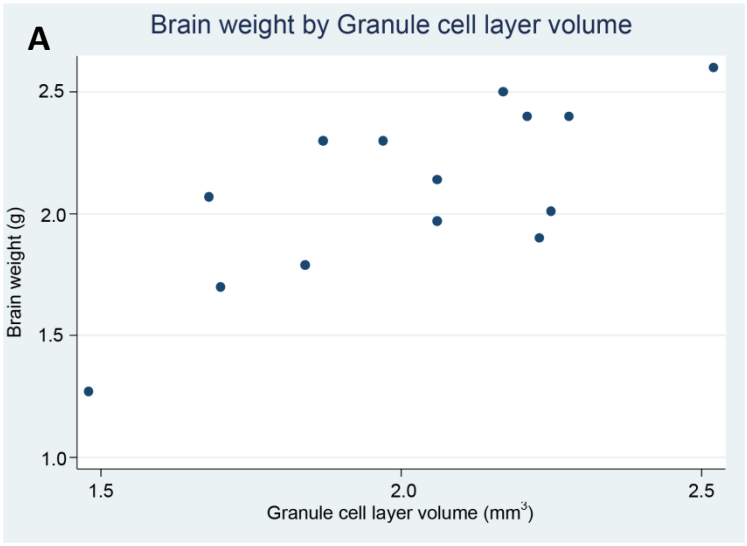
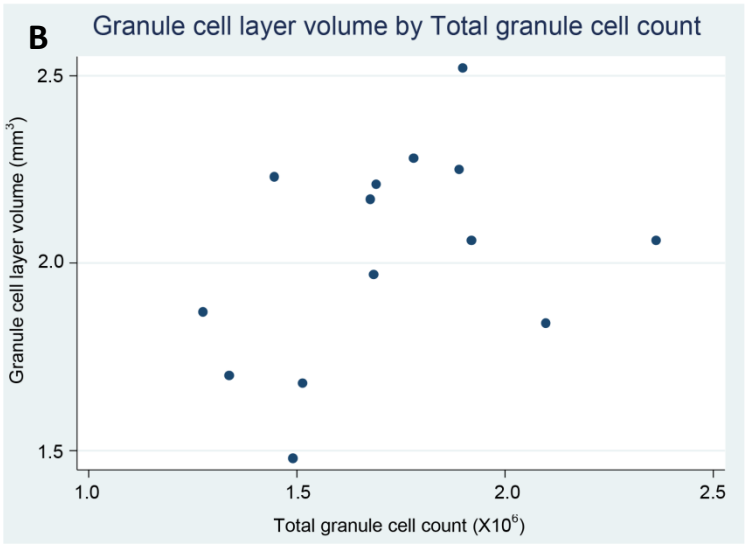

A
Ki-67 cells/granular cells x 100 (%)
0.06
0.05
0.04
0.03
0.02
primary rainforest
subtropical woodland
captive-bred
B
DCX cells/granular cells x 100 (%)
2.5
2.0
1.5
1.0
*
*
primary rainforest
subtropical woodland
captive-bred

Figure 5.7: Scatterplots showing the relationship between the brain weight and the granule cell layer volume (A), the granule cell layer volume and the total granule cell count (B), and the brain weight and the total granule cell count (C). Note that there is no correlation between the two variables in each of these plots.


A
Brain weight by Granule cell layer volume
Brain weight (g)
2.5
2.0
1.5
1.0
1.5
2.0
2.5
Granule cell layer volume (mm3)


B
Granule cell layer volume by Total granule cell count
Granule cell layer volume (mm3)
2.5
2.0
1.5
1.0
1.5
2.0
2.5
Total granule cell count (X10^6)

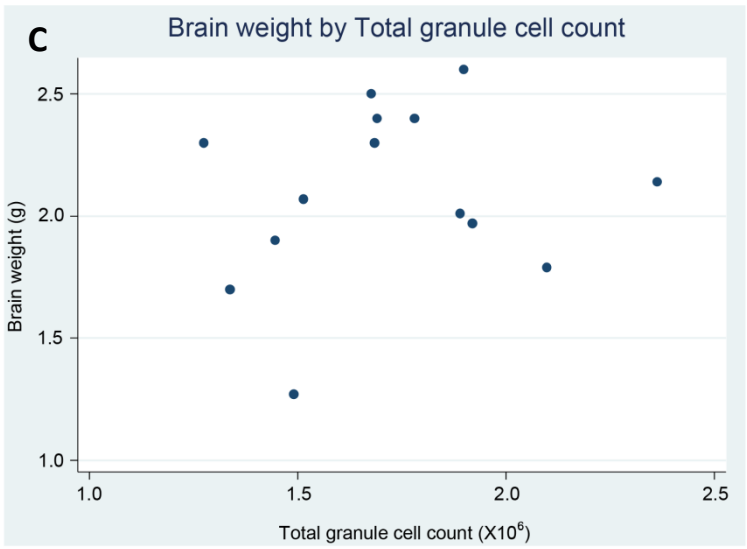
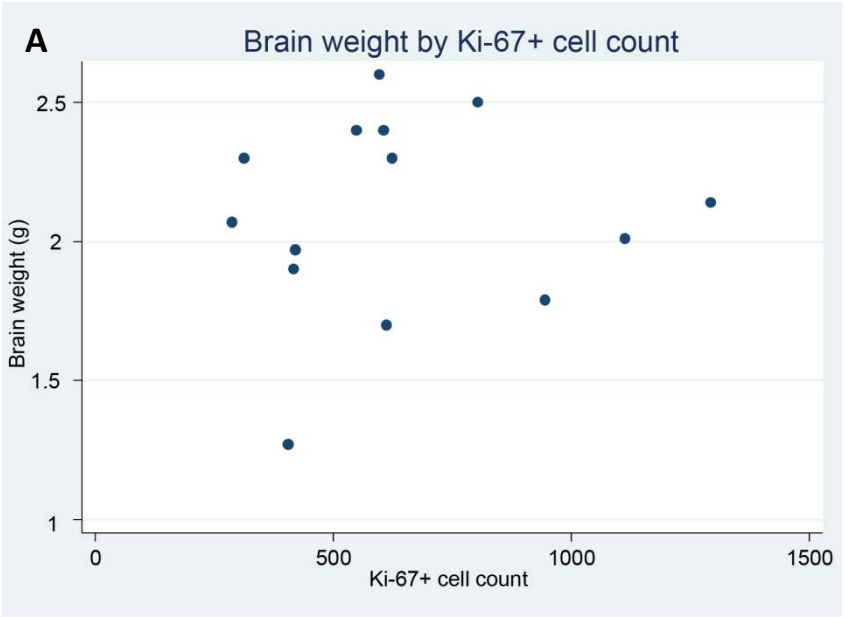

C
Brain weight by Total granule cell count
Brain weight (g)
2.5
2.0
1.5
1.0
1.0
1.5
2.0
2.5
Total granule cell count (X10^6)

Figure 5.8: Scatterplots showing the relationship between brain weight and absolute count of Ki-67 immunopositive cells (A), and brain weight and absolute count of DCX immunopositive cells (B). Note that there is no correlation between the two variables in each of these plots.


A
Brain weight by Ki-67+ cell count
Brain weight (g)
2.5
2
1.5
1
0
500
1000
1500
Ki-67+ cell count

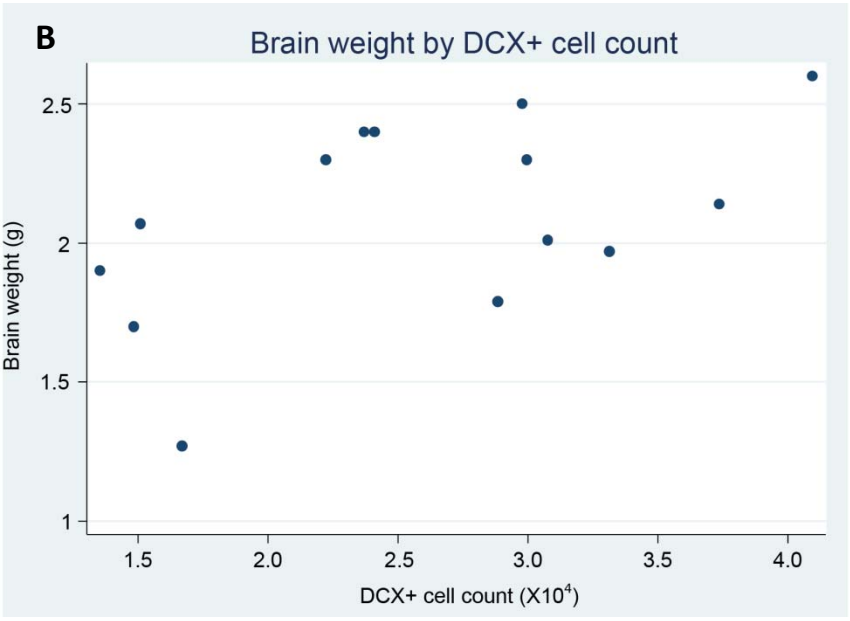
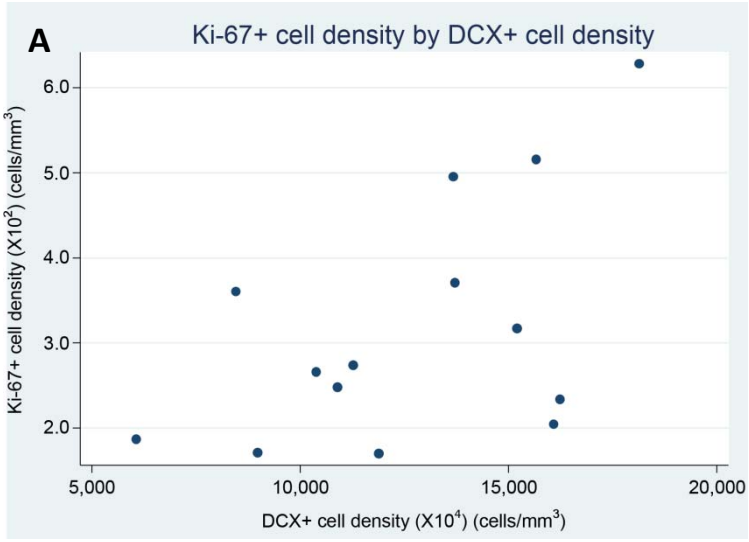
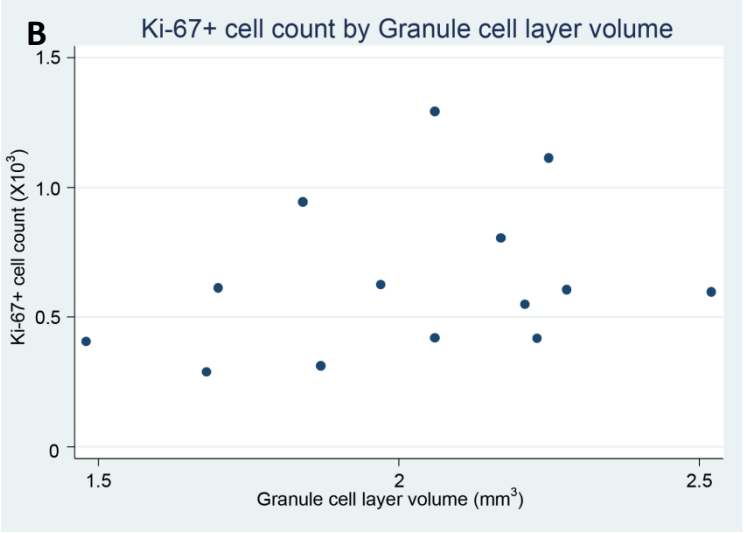

B
Brain weight by DCX+ cell count
Brain weight (g)
2.5
2
1.5
1
1.5
2.0
2.5
3.0
3.5
4.0
DCX+ cell count (X10^4)

Figure 5.9: Scatterplots showing the relationship between Ki-67 immunopositive cell density and DCX immunopositive cell density (A), absolute count of Ki-67 immunopositive cells and the granule cell layer volume (B), and absolute count of DCX immunopositive cells and the granule cell layer volume (C). Note that there is no correlation between the two variables in each of these plots.


A
Ki-67+ cell density by DCX+ cell density
Ki-67+ cell density (X10^2) (cells/mm^3)
6.0
5.0
4.0
3.0
2.0
5,000
10,000
15,000
20,000
DCX+ cell density (X10^4) (cells/mm^3)


B
Ki-67+ cell count by Granule cell layer volume
Ki-67+ cell count (X10^3)
1.5
1.0
0.5
0
1.5
2
2.5
Granule cell layer volume (mm^3)

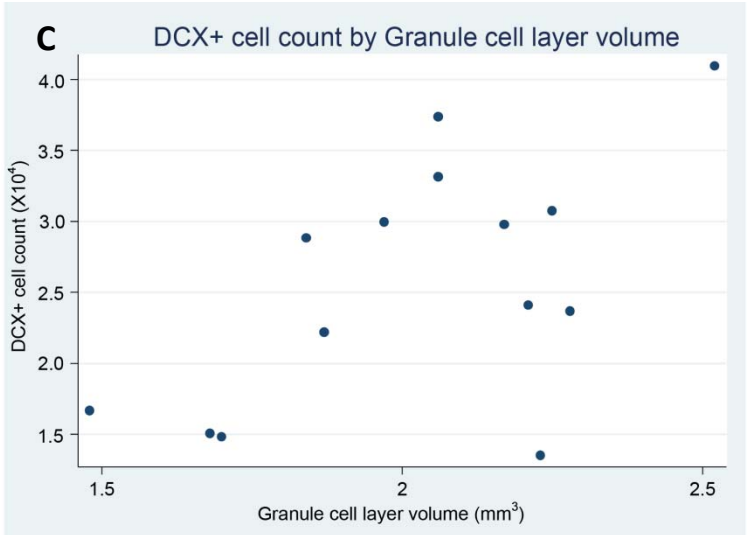
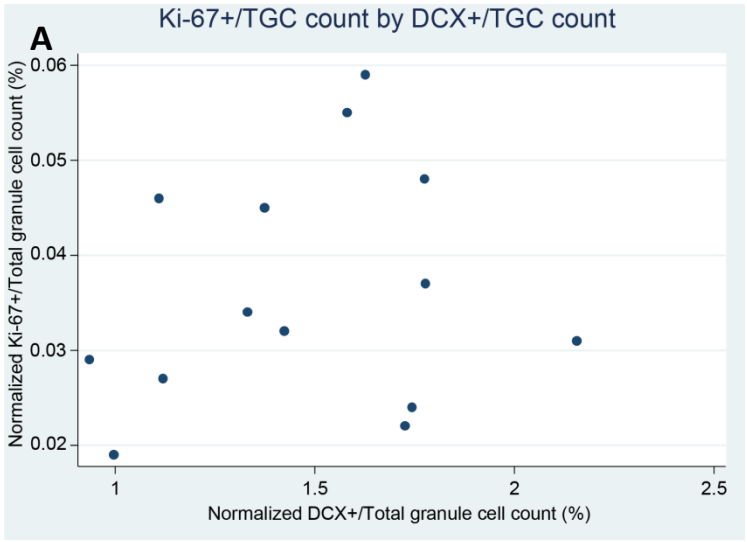
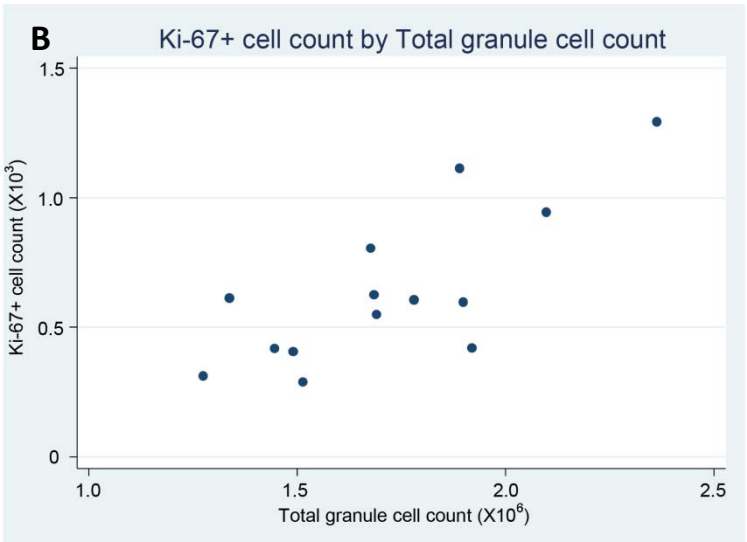

C
DCX+ cell count by Granule cell layer volume
DCX+ cell count (X10^4)
4.0
3.5
3.0
2.5
2.0
1.5
1.5
2
2.5
Granule cell layer volume (mm^3)

Figure 5.10: Scatterplots showing the relationship between the percentage of Ki-67 immunopositive cells as a fraction of the total granule cell count and the percentage of DCX immunopositive cell as a fraction of the total granule cell count (A), the absolute count of Ki-67 immunopositive cells and the total granule cell count (B), and the absolute count of DCX immunopositive cells and the total granule cell count (C). Note that there is no correlation between the two variables in each of these plots.


A
Ki-67+/TGC count by DCX+/TGC count
Normalized Ki-67+/Total granule cell count (%)
0.06
0.05
0.04
0.03
0.02
1
1.5
2
2.5
Normalized DCX+/Total granule cell count (%)


B
Ki-67+ cell count by Total granule cell count
Ki-67+ cell count (X10^3)
1.5
1.0
0.5
0
1.0
1.5
2.0
2.5
Total granule cell count (X10^6)

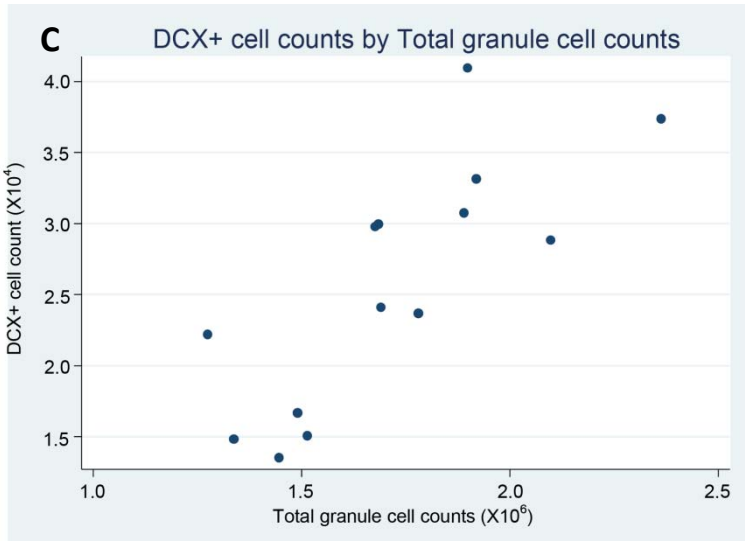
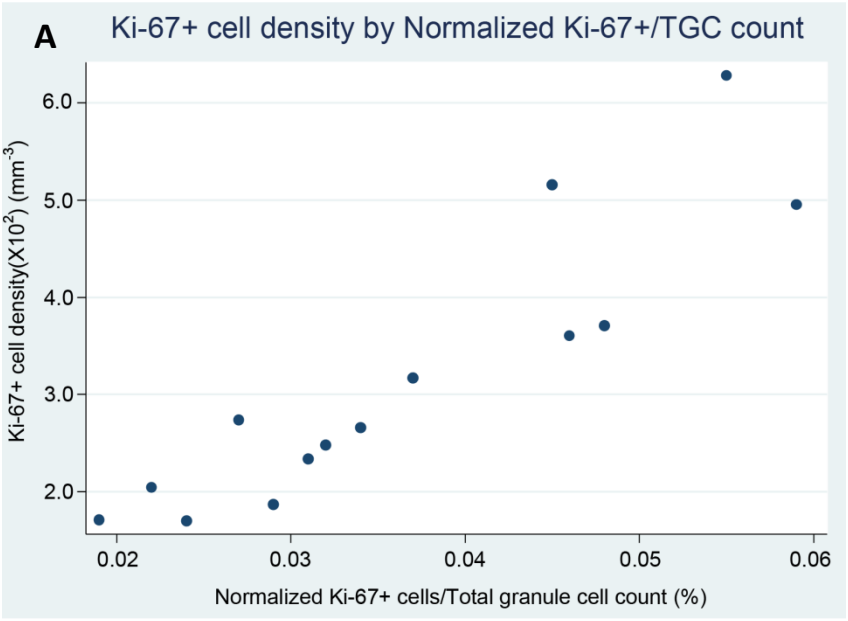
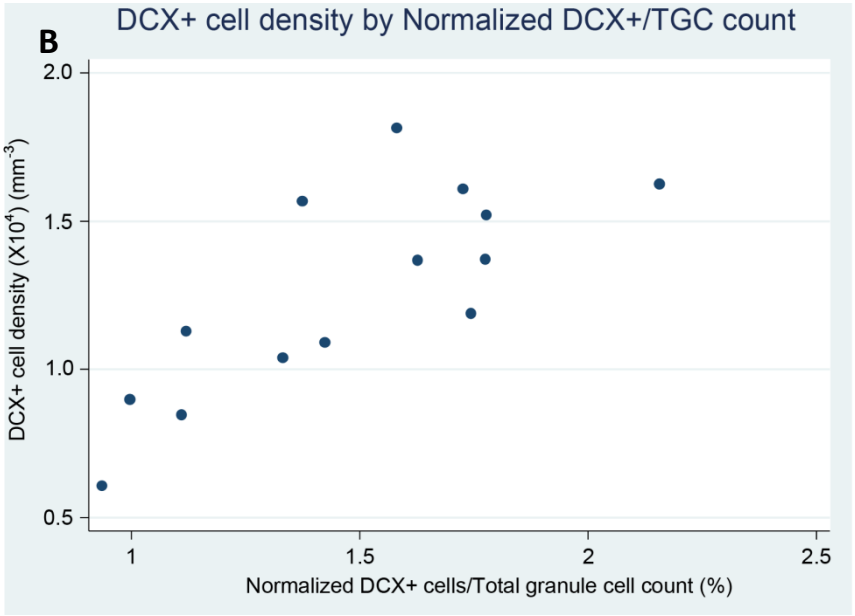

C
DCX+ cell counts by Total granule cell counts
DCX+ cell count (X10^4)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
1.5
2.0
2.5
Total granule cell counts (X10^6)

Figure 5.11: Scatterplots showing the relationship between the Ki-67 immunopositive cell density and the percentage of Ki-67 immunopositive cells as a fraction of the total granule cell count (A), and DCX immunopositive cell density and the percentage of DCX immunopositive cell as a fraction of the total granule cell count (B). Note that there is a positive correlation between the Ki-67 immunopositive cell density and the percentage of Ki-67 immunopositive cells as a fraction of the total granule cell count (A) while there is no correlation between the DCX immunopositive cell density and the percentage of DCX immunopositive cell as a fraction of the total granule cell count (B).


A
Ki-67+ cell density by Normalized Ki-67+/TGC count
Ki-67+ cell density(X10^2) (mm^{-3})
6.0
5.0
4.0
3.0
2.0
0.02
0.03
0.04
0.05
0.06
Normalized Ki-67+ cells/Total granule cell count (%)


B
DCX+ cell density by Normalized DCX+/TGC count
DCX+ cell density (X10^4) (mm^{-3})
2.0
1.5
1.0
0.5
1
1.5
2
2.5
Normalized DCX+ cells/Total granule cell count (%)

5.4 Discussion

Laboratory based studies, mostly of rodents, have led to several theories regarding the function of adult neurogenesis in the mammalian hippocampus, central to which is the hypothesis of pattern-separation (e.g. Clelland et al., 2009; Yassa and Stark, 2011; Tronel et al., 2012, 2015). Laboratory based studies have also indicated that enrichment of the captive environment, by maintaining the animals in groups and adding tunnels, toys, running wheels, nesting materials and altering food locations, leads to higher rates of cell proliferation, differentiation and survival within the dentate gyrus when compared to animals in standard, or impoverished, conditions (e.g. van Praag et al., 2000; Will et al., 2004; Beauquis et al., 2010). These findings lead to the hypothesis that wild-living animals that inhabit complex environments with high rates of variation and unpredictability should have higher rates of adult hippocampal neurogenesis than those inhabiting environments with more predictability and less variation (e.g. Cavegn et al., 2013); however, the results are ambiguous in terms of supporting this concept. For example, when comparing wild-caught Norway rats to captive-bred Norway rats, the wild-caught animals, presumably occupying a more unpredictable environment, Epp et al. (2009) found no clear differences in the rate of adult neurogenesis when standardizing their results against granule cell volume. In contrast, when comparing rates of adult neurogenesis between species from the temperature South African regions to the colder and wetter European region, Cavegn et al. (2013) found that animals from the European region had higher proliferation rates, but lower survival rates of immature neurons compared to the rodents from South Africa when standardizing their results to total granule cell numbers within the hippocampal dentate gyrus. These inconsistent results are perhaps the consequence of methodological differences; thus, in the current study we analyzed the rate of adult neurogenesis (number of proliferative cells revealed with Ki-67 immunohistochemistry and number of immature neurons revealed with DCX immunohistochemistry) with four

different methods to determine in fruit bats from three different environments whether, with different methods, similar or contrasting results would be obtained.

5.4.1 Methodological considerations specific to the current study

One of the central difficulties when studying adult neurogenesis in wild-caught mammals is the potential protracted time between capture and euthanasia. In microchiropteran bats, within 20 minutes of capture evidence of adult neurogenesis, using DCX immunohistochemistry, has all but disappeared (Chawana et al., 2014). In order to overcome this problem all the wild-caught animals used in the current study were euthanized and perfusion fixed within 20 minutes of capture. Handling of the captive-bred animals was kept to a minimum prior to perfusion fixation. By doing this we have reduced the possibility that capture/handling stress will have a major impact on the analysis. In addition, instead of using BrDU to examine proliferative cells, we have used endogenous markers of adult neurogenesis, Ki-67 and DCX, to minimize handling of the animals and extending the period between capture and euthanasia. One specific problem that is difficult to address in wild-caught animals is age. While we have no direct solution for this problem in the current study, we ensured that all the animals used showed full epiphyseal closure of the metacarpophalangeal joints (Anthony, 1988) indicating that they were adults. A final concern in the current study is the sample size, which was smaller in the group of bats from the primary rainforest (n = 3) than the groups from the subtropical woodland (n = 6) and the captive-bred (n = 5) animals. While we would have preferred to have a greater number of animals from the primary rainforest environment, we were unsuccessful in capturing additional animals in the available time. The sample size limited us from performing a regression analysis. As a result if there were any confounders, they were not accounted for

and thus are a potential limitation of the study. However, our handling of the animals being minimized should reduce uncertainty in the numbers of proliferative and immature neurons quantified.

5.4.2 Method 1 – comparisons of raw total numbers of proliferative and immature cells

The reporting and comparisons of absolute numbers of proliferative and immature cells in the dentate gyrus is restricted to animals of the same species, although they have usually been exposed to different conditions (e.g. Bartkowska et al., 2010; Trinchero et al., 2015; van der Marel et al., 2015). This method does not take into account variation within a species of features such as brain mass, volume of the granule cell layer or the total number of granule cells. As such, this method is not suitable for comparisons between species. In the current study we used animals from the same species, but that had been born and raised in three distinct environments. Our raw cell counts indicate that the greatest numbers of proliferative and immature cells were found in the primary rainforest bats, followed by the subtropical woodland bats and lastly the captive bats. This indicates that the more complex and unpredictable environment of the primary rainforest leads to greater rates of adult neurogenesis, while the predictable and stable captive environment requires less adult neurogenesis. Despite this, there were no statistically significant differences between the three groups of bats in terms of the number of proliferative cells, but the captive-bred bats had significantly fewer immature neurons than both the wild-caught groups. Given these results, without further analysis, we could conclude that the rate of proliferation within a species is similar despite differing environments, but that the survival of these proliferative cells into immature neurons is greater in the wild-caught animals living in a natural environment compared to the captive-bred animals where many of the potential stressors of a

natural environment are alleviated. This conclusion, while fitting the concept of an enriched environment leading to greater rates of adult neurogenesis, at least in terms of the survival of proliferative cells to immature neurons, is questionable, as we found significant differences in brain mass, the volume of the granule cell layer and the total number of granule cells between the groups examined in the current study.

5.4.3 Method 2 – standardizing the raw cell counts to brain mass

Although not often used in comparative studies of adult hippocampal neurogenesis (e.g. Chawana et al., 2013), the counting and comparison of cell numbers to brain mass (or other similar brain parameters) is common in the field of comparative neuroscience (e.g. Herculano-Houzel et al., 2006, 2007, 2014) and is used to standardize for differences in brain mass amongst species. In the current study, while no difference was found in the brain mass between the primary rainforest and captive-bred bats, the subtropical woodland bats had statistically significantly higher brain masses than both these groups. When comparing the numbers of proliferative cells as a ratio of the mass of the brain, the primary rainforest bats showed the highest rate of proliferation, while the bats with the largest brains, the subtropical woodland bats, showed the lowest rate of proliferation, although no statistically significant difference was present between the three groups. When comparing the numbers of immature neurons as a ratio of brain mass, the primary rainforest bats had higher numbers of immature neurons than the subtropical woodland bats, which in turn had higher numbers than the captive-bred bats; however, only the difference between the primary rainforest and subtropical woodland bats was statistically significant. If conclusions are drawn from this comparison, as with the raw total numbers of proliferative cells, there appears to be no difference in the rate of cell proliferation between the three groups of animals raised in

different environments. However, in contrast to the raw numbers, the bats from the primary rainforest had greater numbers of immature neurons than the subtropical woodland bats, which might be inferred to suggest the need for greater survival of immature neurons in the primary rainforest. Despite this, the fact that the primary rainforest and captive-bred bats did not show any statistically significant difference in the numbers of immature neurons as a ratio of brain mass indicates that this method of standardization appears to be unsuitable for comparing rates of adult neurogenesis between individuals of the same species from different environments. While not suitable for comparisons of individuals within the same species, this method may prove useful when comparing across a broader range of species when numbers of proliferative and immature cells and brain mass are available and may dissociate scaling laws of form and adaptive features of the rate of adult hippocampal neurogenesis.

5.4.4 Method 3 – densities of proliferative and immature cells in the dentate gyrus

In order to overcome some of the problems mentioned above, and standardize results across individuals and species, the volume of the granular cell layer is often used to provide a density measure of the proliferative and immature cells (e.g. Kempermann et al., 1997b; Epp et al., 2009; Castilla-Ortega et al., 2011; Curtis et al., 2012). In this sense, the rate of proliferation and the numbers of immature neurons are standardized to a relevant measure of the hippocampus and presumably adult hippocampal neurogenesis. In the current study, the volume of the dentate gyrus was largest in the subtropcial woodland bats (which also had the largest brain), followed by the primary rainforest bat and then the captive-bred bats. Despite this, the only statistically significant difference in the volume of the granule cell layer was between the subtropical woodland bats and the captive bred bats. In terms of the density of proliferative neurons, while no statistically significant differences between groups was found,

the bats from the primary rainforest had the highest density, followed by the captive-bred bats and then the subtropical woodland bats. Similarly, the primary rainforest bats had the highest density of immature neurons, but in this case the subtropical woodland bats had the second highest density with the captive-bred bats having the lowest density. Despite this, there were no statistically significant differences in the density of immature neurons between the three groups studied. Thus, using this method, we would conclude that there is no difference in the rate of proliferation, or the survival of immature neurons in the bats from the three different environments. Interestingly, this is exactly the same conclusion arrived at by Epp et al. (2009) using this method to compare wild-caught Norway rats to conspecific laboratory-bred animals. Thus, the current study and that of Epp et al. (2009) both indicate that the environment in which an animal lives does not appear to have any specific effect on the rate of proliferation or the survival of immature neurons. This would then lead to the conclusion that the rate of adult hippocampal neurogenesis, despite several experimental manipulations demonstrating the contrary, is not related to the environment in which an animal lives. This unusual finding, given the results of experimental environmental manipulations, may be correct, or it may indicate that this method of assessing differences in adult hippocampal neurogenesis is not appropriate for wild-caught animals, or that the differences in wild-caught animals are too subtle to be elucidated with this method.

5.4.5 Method 4 – proliferative and immature cells as a percentage of total granule cells

As a proportion of the proliferative and immature cells within the dentate gyrus eventually metamorphose into integrated granule cells, the proportion of proliferative and immature cells expressed as a percentage of total granule cell numbers has been used as a standardization measure for the comparison of the rate of adult hippocampal neurogenesis

both within and amongst mammalian species (e.g. Gatome et al., 2010; Cavegn et al., 2013; Slomianka et al., 2013; Huang et al., 2015). When using this method on the bats investigated in the current study, we found that the percentage of proliferative cells was highest in the bats from the primary rainforest, followed by the subtropical woodland and lastly the captive-bred bats; however, there were no statistically significant differences observed between these groups. When comparing the percentage of immature neurons, the highest percentage was found in the subtropical woodland bats, followed by the primary rainforest bats and lastly the captive-bred bats, and in this case both the subtropical woodland bats and primary rainforest bats had statistically significantly higher percentages than the captive-bred bats (although there was no difference between the subtropical woodland and primary rainforest bats). These findings would lead to the conclusion that while the environment plays no significant role in the rate of proliferation, the survival of immature neurons is enhanced in the wild-living animals compared to the captive-bred animals. This could then be inferred to indicate that the greater uncertainity of the natural environment compared to the captive environment (e.g. variations in food availability and location, environmental conditions, and predation amongst others) necessitates the need for higher numbers of adult-born cells eventually being integrated into the circuitry of the dentate gyrus to enhance both learning and memory. This is very similar to the finding reported by Cavegn et al. (2013) when comparing Murid rodents from Europe and South Africa, with the South African rodents having a greater number of proliferative cells surviving to become immature neurons than the European rodents; however, a direct environmental corollary to explain this difference was not reported. Interestingly, the data from the raw values (Method 1) and those from Method 4 provide very similar results in the present study, and currently appear to be the most sensitive to potential environmental differences affecting adult hippocampal neurogenesis in mammals living in their natural habitat. Despite this, the current study and that of Cavegn et al. (2013) differ in

terms of the rate of proliferation, where we find no difference, Cavegn et al. find significant differences. These differences in the Cavegn et al. (2013) study may be based in the use of closely related species, and as such the differing rate of proliferation may be an indication of species differences, rather than environmental effects, as proliferation rates appear to be genetically pre-determined (Kempermann et al., 1997a). In contrast, the current study used the same species eliminating inter-species genetic variations on the rate of proliferation, as we observed similar rates in both wild caught and captive-bred fruit bats with several different methods,including that used by Cavegn et al. (2013).

5.4.6 Does the environment play a role in shaping adult hippocampal neurogenesis?

The results of the present study, and those of previous studies (e.g. Epp et al., 2009; Cavegn et al., 2013), indicate that the answer to this question can be yes, no or maybe – at present we feel that it is really very unclear whether the environment plays a significant role in determining the outcome of the process of adult hippocampal neurogenesis in animals living in their natural environment. One common feature of the four methods used for analysis in the current study is that the rate of proliferation, measured by the number of Ki-67 immunoreactive cells, appears to be unaffected by the environment. This would then indicate that the rate of proliferation is most likely to be principally a genetically pre-determined aspect of adult hippocampal neurogenesis across species (Kempermann et al., 1997a). While the proliferation rate can be influenced by experimental manipulation, and perhaps by significant experiences encountered in the daily life of animals living in their natural environment, it would appear that the proliferation rate could be a species-specific phenomenon, or may be related to a coarser measure such as average brain mass or

hippocampal volume. This remains to be determined through the examination of a broader range of species that have lived a natural existence.

In contrast, the numbers of immature neurons provides more variable results. When standardized to brain mass (Method 2) or granule cell volume (Method 3), the environment appears to play no role in determining the numbers of immature neurons, indicating similar possibilities regarding adult hippocampal neurogenesis as found for the proliferative neurons (described above). In contrast, when using the raw numbers (Method 1) or when the number of immature neurons is standardized to total granule cell count (Method 4), it appears that the environment has a significant effect with the animals caught from their natural habitats having higher numbers and percentages of immature neurons than those from the captive environment. However, it would be difficult to assert this as an indisputable finding as two of the three standardization methods do not support this conclusion, and the raw numbers suffer from the difficulties in interpretation related to individual variation.

Thus, the current study leads to a rather difficult conclusion. While in general the numbers of proliferative and immature cells across the four methods are highest in the primary rainforest bats, followed by the subtropical bats and then the captive-bred bats, which may at surface value be associated with environmental unpredictability/complexity, these differences are merely trends and for the most part are not supported statistically. This indicates two possibilities, first, that the methods used are not sensitive enough to elucidate the effect of the environment on the numbers of proliferative and immature cells, or second, that there are very subtle, if any, differences in the process of adult hippocampal neurogenesis that are affected by the natural environment. Despite this somewhat negative pair of conclusions, it should be pointed out that only the first two steps in the process of adult hippocampal neurogenesis, proliferation and the survival of proliferative cells to immature neurons, have been examined in this and previous studies of animals from the natural

environment (Epp et al., 2009; Cavegn et al., 2013). The final step in the process of adult hippocampal neurogenesis, integration into the circuitry of the dentate gyrus, has not been examined. Perhaps it is in the examination of this final phase of the neurogenetic process that differences clearly related to different environments will be found.

Chapter 6: Adult neurogenesis in the wild: Observations from the Chiroptera

6.1 Adult neurogenesis in the wild: Observations from the Chiroptera

Adult hippocampal neurogenesis has been shown to occur in a wide range of mammals (Kempermann, 2012; Patzke et al., 2015). Despite the developing understanding of adult hippocampal neurogenesis, its functional relevance in mammals remains uncertain; however, it is thought to function in pattern separation, which is a neural process that disambiguates similar or overlapping inputs into distinct output signals (Clelland et al., 2009). Higher rates of adult hippocampal neurogenesis have been reported in highly social animals, and animals with augmented spatial navigation abilities, larger foraging distances, and those living in complex or enriched environments, as well as in phylogenetically related animals (van Praag et al., 2000, Tronel et al., 2015). A previous study which failed to show adult hippocampal neurogenesis in the microchiroptera challenged the commonly held associations between behavioural attributes and adult hippocampal neurogenesis (Amrein et al., 2007). This negative finding promoted the further interrogation of the interplay of these elements with distinct stages of the process of adult hippocampal neurogenesis. This was particularly relevant given that bats are highly social animals with augmented spatial abilities, and often live and forage in seemingly complex environments (Kerth, 2008a, Kerth, 2008b, Kerth et al., 2011). This background prompted the studies contained in this thesis, which were aimed at investigating the occurrence of all forms of adult neurogenesis in a range of Chiropteran species to provide a solid comparative platform from which to attempt to fully understand the various associations between behavioural attributes and ecological factors with the occurrence and rate of adult neurogenesis. Using immunological and stereological techniques, several insights into adult neurogenesis in the Chiroptera were obtained. These include: (1) how the patterns of adult neurogenesis in the Chiropteran suborders relate to their phylogenetic relationships; (2) that adult neurogenesis is a robust feature of the brain in both

wild-caught and captive bred animals, and that while trends in the rate of adult hippocampal neurogenesis are apparent, these do not appear to be specifically related to environmental complexity; and (3) the revelation of the methodological challenges associated with using wild animals in the study of adult neurogenesis.

6.2 Neurogenesis in the suborders of Chiroptera – potential phylogenetic implications

The order chiroptera is composed of a multitude of diverse species grouped into megachiropteran (megabats) and microchiropteran (microbats) suborders (Altringham, 1999). These two suborders appear to have similar morphological, especially of the flying apparatus, and molecular characteristics, but despite these similarities, the suborders differ in diet, ecological habitat location and neuroanatomy (Pettigrew, 1986, Pettigrew et al., 1989, Altringham, 1999). These differences led to the suggestion of the diphyletic origin of bats (Pettigrew, 1986; Pettigrew et al., 1989), a concept refuted or ignored by many mammalian phylogeneticists. Of the differences, neuroanatomical and habitat differences have been associated with varying findings in the occurrence of adult neurogenesis. At the same time, aspects of adult neurogenesis such as its degree and migratory pathways patterns are thought to be conserved across mammalian phylogeny (Zupanc, 2001). The studies of the two suborders of chiropterans undertaken in the present thesis (Chapters 2 and 4) showed that the neurogenic zones, migratory pathways and terminal fields associated with adult neurogenesis in the megachiropterans and microchiropterans were, for the most part, similar to those observed across mammalian orders previously. Despite this, a marked difference in the migratory pathway leading to the piriform cortex was noted between the two chiropteran suborders that has specific relevance to the phylogenetic affinities of the chiropterans. In the megachiropterans the piriform cortex was supplied with immature neurons from a temporal

migratory stream, originating from a neurogenic zone located in the temporal horn of the lateral ventricle. The only other mammalian species in which this temporal neurogenic zone and stream has been noted are the primates (Bernier et al., 2002). In contrast, the immature neurons migrating into the piriform cortex of the microchiropterans appeared to emanate from the ventral division of the rostral migratory stream, as a broad swathe of potentially migrating cells. A similar pathway of immature neurons leaving the rostral migratory stream to populate the piriform cortex called the ventral migratory stream was noted in insectivores (Bartkowska et al., 2010). Moreover, this broad ventral migratory stream to the piriform cortex in the microchiropterans and insectivores is also different from the more circumscribed streams observed in the rodents (Shapiro et al., 2007). This variance in the migratory stream supplying the piriform cortex in the two suborders of chiropterans, along with numerous other neural characteristics from a range of systems (Pettigrew, 1986; Pettigrew et al., 1989; Calvey et al., 2015a,b, 2016), lends support to the concept of the diphyletic origin of the chiroptera and specifically align the megachiropterans with primates, and the microchiropterans with the insectivores.

6.3 Hippocampal neurogenesis in captive and wild caught animals

The dentate gyrus is central to hippocampal functions associated with learning and memory. It receives afferents from the entorhinal cortex and sends efferents to the CA3 region of the hippocampal circuitry (Amaral et al., 2007). The plasticity of the dentate gyrus is regarded to be crucial for its function and is achieved, at least in part, through the addition of adult born neurons. These immature neurons compete for synaptic connections with established neurons, and those immature neurons forming strong synaptic connectivity survive, while the remainder die through programmed cell death (Kim and Sun, 2011). When

the synaptic connections formed by the adult born neurons become stronger than those of the developmentally born neurons, it triggers death of the developmentally derived neurons (Kim and Sun, 2011). In cases where the adult born neurons fail to form stronger synaptic connections than the developmentally derived neurons, the adult born neurons die. In Chapter 5 the effect of the environment on hippocampal proliferating and immature neurons was investigated by evaluating quantitatively the levels of adult neurogenesis in wild-caught and captive-bred bats. The findings showed a trend indicating that captive-breeding decreases the survival of immature neurons when contrasted with animals caught from their natural environment; however statistical significance of such comparisons were heavily method-dependent. All methods used for comparison and standardization indicated that the rate of proliferation of newly born cells was not affected by the either the complexity of the environment (rainforest vs woodland) or whether the animals were several generations captive-bred. Only with one specific standardization technique (the number of immature neurons as a proportion of the total number of granular cells in the dentate gyrus) was any statistically significant difference noted between different environmental conditions, showing that wild-caught animals had higher numbers of immature neurons than the captive-bred animals. Thus, this study indicates that while the rate of proliferation and survival of immature neurons might vary in response to daily activities, the overall rate of proliferation and survival is likely to be a species specific phenomenon rather than related to the complexity of the environment. However, it should be noted that this is only one of a few studies that have approached this question and substantially more work, in a broader range of species, is required to fully understand whether environment, phylogenetic history, or structural laws of form best explain the rate of production of proliferative cells and immature neurons associated with adult hippocampal neurogenesis across species.

6.4 Methodological considerations when using wild caught animals

While the appeal of using wild caught animals in studies on adult neurogenesis is apparent, their use and the interpretation of the findings require careful considerations. The quantitative and qualitative studies of adult hippocampal neurogenesis in wild-caught microchiropterans (Chapter 3) showed that the microchiropterans were particularly sensitive to the stress associated with capture and handling, leading to massive reductions in the number of hippocampal neurons expressing the doublecortin protein. This occurs to such an extent that it was wrongly concluded previously that microchiropterans lacked adult hippocampal neurogenesis (Amrein et al., 2007). It is possible that the rapid increase in corticosterone levels associated with capture stress induces the non-genomic activation of proteasomes resulting in the proteolysis of the doublecortin protein in the immature neurons (Wiegert et al., 2006). While we suggested that calpain, a calcium-dependant enzyme, could be the enzyme resulting in the observations made, given that its activity is stimulated by rapid rise of corticosterone levels, additional studies are needed to ascertain the signaling pathway involved and to develop ways to block the degradation of the protein (Wiegert et al., 2006), which might prove of clinical importance to humans in the treatment of diseases of the hippocampus. Given that the ways of blocking the degradation of proteins involved in neurogenesis are currently unknown, the findings suggest that there is a critical timeframe of less than ten minutes from capture to sacrifice in the microchiropterans, but this may be longer in other species. Thus, as many previous studies of wild-caught animals have not taken capture stress and its correlates into consideration, it is possible that the rate of adult hippocampal neurogenesis in wild caught animals in actually higher than reported. What is clear is that the handling of the animals, from capture to sacrifice needs to be carefully considered before undertaking qualitative or quantitative analyses of adult neurogenesis, and especially so before reporting false negative results (Amrein et al., 2007).

6.5 Significant unanswered questions from the chiroptera: Limitations of the study

Species belonging to the order chiroptera make an attractive model for use in studies of adult neurogenesis. The order is speciose and the animals inhabitat diverse ecologies. Chiropteran species show a range of social organizations, including the supposedly very complex fission-fusion social system, are thought to possess augmentated spatial abilities, and often have a significantly longer life-span than other similar sized mammals (Kerth et al., 2011, Ross and Holderied, 2013). Thus, the chiropterans may make interesting animal models for the study of epigenetic changes that influence the process of adult neurogenesis. While these chiropteran features may make them a good model for studies of adult neurogenesis, there are other chiropteran features that make it difficult to compare findings on chiropterans to other mammals. For example, currently there are no known methods of precisely and accurately determining the age of wild caught bats, which is important as the inhibitory effect of age on the rate of adult neurogenesis is well known (Klempin and Kempermann, 2007). Despite the claims that very old bats are rarely caught, it is difficult to determine if the variations in the numbers of proliferating cells and immature neurons in the different animals is as a result of true biological variability or a reflection in the different ages of the "adult" bats. Reliable methods to provide estimates of age for wild-caught chiropterans are sorely needed.

Studies using wild caught animals appear important given how robust adult neurogenesis is in these animals; however, one challenge that remains is to determine and separate variables affecting adult neurogenesis within an individual (such as age and recent daily experience) with the extent to which they influence the observed patterns and rates of adult neurogenesis. For example, some wild caught bats may have to forage for long

distances in search of rewarding feeding sites, while others of the same species may not, depending on their immediate environment. Physical activity is known to influence the rate of neurogenesis in adult mammals (van Praag et al., 1999, van Praag, 2008). Given that the distances travelled by each animal are unknown, it is difficult to determine whether the variations in the rates of neurogenesis amongst individuals are a true biological variability or an indication of the varying physical demands placed on the animals living in their natural environment. In addition, there are other factors confounding the observations from the wild-caught animals which include predation risk and environmental complexity which are also known to influence the rate of adult neurogenesis (van Praag et al., 2000; Lieberwirth and Wang, 2012). There are currently no methods allowing the direct determination of the levels of risk of predation for an individual animal and the precise complexity of the environment in which an individual animal may live. Thus, it is difficult to associate the observed patterns and rates of adult neurogenesis within and between species to a single variable. While broad conclusions can be drawn from the global picture of neurogenesis in animals living in their natural environment, there exists a need to understand how the variables shown to affect the rate of adult neurogenesis in the laboratory are influencing this process in natural-living animals by having either a stimulatory or inhibitory effect.

In addition to these potential factors that limit the interpretational strength of the studies presented herein, are both the phylogenetic variability of the chiroptera as well as the phylogenetic relationships of the two chiropteran suborders. First, the microchiropterans in particular, are a very diverse and species rich suborder, that inhabit an enormous geographical and ecological range. In the current study the number of microchiropteran species investigated represents a small proportion of this suborder and also a small proportion of the ecological niches inhabited by this group. While the representation of megachiropteran species was somewhat stronger, these species studied may not be representative of the

suborder as a whole. Therefore, the interpretations provided in this thesis are limited by the range of species examined. Clearly it would be of interest to obtain specimens from as many species as possible, however, given the sheer number or species, being well over 2000, this is a difficult task. Second, the phylogenetic relationships of the two chiropteran suborders, to each other and to the other mammals, is an issue that is far from being settled. It is known that adult neurogenesis, both rates and migratory pathways, is influenced by the phylogenetic history of the species studied (Zupanc, 2001), and this was clearly demonstrated in regard to the differing patterns of migratory pathways observed in the representative species of the two chiropteran suborders studied herein. While the current findings may be used to contribute to the understanding of the phylogenetic origin of species in the chiropteran order, the lack of similar studies using wild caught primates and other potentially phylogenetically relevant mammalian orders under controlled conditions can make comparisons difficult. Thus, the study of the rates and migratory pathways related to adult neurogenesis of a range of further mammalian species is needed to confirm or modify the conclusions reached in the current study.

6.6 Areas for future research

Despite the limitations outlined above, these limitations also create opportunities for further investigations on chiropterans as model animals for the study of adult neurogenesis in wild-living animals. As a first step, a reliable method of determining age in wild caught chiropterans is required. This may need to be undertaken in captive breeding populations where date of birth is known and use techniques such as lens mass to provide reliable estimates of age for the wild-caught animals of the same species; however, this would require the sacrifice of a very large number of animals from a multitude of species. It would be also

of interest to examine a much broader range of chiropteran species, especially those with unusual life histories (such a nectar or blood feeding bats), or that inhabit extreme environments, or undergo processes such as daily torpor. These significant variations from the more generalized life histories of chiropterans would provide compelling arguments in favour of, or against, the effect of certain factors in the process of adult neurogenesis.

A third aspect that requires much more concerted study is that of the species proposed to be phylogenetically related to the chiropterans, such as the primates and Soricid shrews. Understanding the phylogeny of the chiropterans is clearly of importance for the interpretation of the obtained results, and placing these in a broader framework for understanding the phylogenetic history of the adult neurogenetic process and how it relates to humans (and how this process may be exploited in the treatment of human ailments). Moreover, the studies undertaken herein provide further evidence that there could be different regulatory mechanisms involved in the process of adult neurogenesis in different mammalian species, this being specifically highlighted by the rapid decline in doublecortin expression in relation to capture stress in the microchiropterans. It would be of interest to determine the precise mechanism leading to this rapid decline in doublecortin expression in the microchiropterans in relation to stress. Lastly, by using modern tracking methods to follow individual bats from the same species in different environments, the effects of daily experience, such as the level of physical activity, could be quantified in relation to rates of adult neurogenesis. Although some of these suggested investigations may be costly or difficult in a practical sense, their contribution to the understanding of adult neurogenesis and its functional relevance in the natural environment may provide very useful clues to our developing understanding of adult neurogenesis.

6.7 Final Conclusions

As with all detailed scientific studies, the current studies have limitations, but also open new avenues for future research. The current studies show unequivocally that the full range of adult neurogenesis observed in many other mammalian species are present in both chiropteran suborders, but that particular care in the handling of wild-caught chiropterans, especially the microchiropterans, is needed to reveal these patterns using immunohistochemical techniques for endogenous markers. Moreover, there appears to be a phylogenetic signal in the patterns and pathways associated with adult neurogenesis, especially so in the migratory pathways to the piriform cortex. These pathways provide neural traits of interest in relation to the questions surrounding chiropteran phylogenetic relationships, supporting the diphyletic origin of the two chiropteran suborders, aligning the megachiropterans with primates and the microchiropterans with the insectivores, although further studies are needed. Lastly, these studies demonstrated that the interpretation of data related to the effect of the environment on the rate of adult hippocampal neurogenesis is likely to be more dependent on the analysis than environmental variation. This last finding is of central importance to the study of adult neurogenesis in a broader sense, as it demonstrates that many earlier conclusions related to the epigenetic factors thought to influence the rate of adult neurogenesis may be methodological irregularities rather than real influences. This finding means that potentially more sensitive and accurate methodologies need to be developed for the study of adult neurogenesis, both in laboratory based studies and those examining animals from their varied natural environments. While we are still a long way from understanding why adult neurogenesis occurs, what the function of this neural is, and how it varies phylogenetically and in response to the environment inhabited by a particular animal, it is hoped the current series of studies do provide a substantive step forward in our understanding of this potentially very useful and interesting neural process.

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APPENDICES

Animal Ethics Clearance

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2009/ 35/2B

APPLICANT: Dr R Chawana

SCHOOL: Anatomical Science
DEPARTMENT:
LOCATION: Medical School

PROJECT TITLE: **Adult neurogenesis in the brain of the Egyptian rousette flying fox (Rousettus aegyptiacus)."**

Number and Species

21 adult 120g *rousettus aegyptiacus*

Approval was given for the use of animals for the project described above at an AESC meeting held on 29.09.09. This approval remains valid until 29.09.2011

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:

- **A signature should be provided from the person who will be responsible for the safe keeping, the handling and use of sodium pentobarbital, especially if it will be used in the field.**

Signed: _____ (Chairperson, AESC) Date: 16/11/2009

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: 16/11/2009

cc: Supervisor:
Director: CAS

Works 2000/lain0015/AESCCert.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Animal Ethics Clearance Extension 2014

AESC 2014 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Richard Chawana

b. Department: Anatomical Sciences

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2009	35	2B
Other M&Es :			

d. Project Title:

		No.	Species
e.	Number and species of animals originally approved:	21	*Rousettus aegyptiacus*
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	21	*Rousettus aegyptiacus*
h.	Number of animals used to date:	0	

i. Specific modification / extension requested:

Extension of the AESC clearance to allow us to commence the experiment

j. Motivation for modification / extension:

We applied for and were issued with ethics clearance certificate to carry out a project using the megabats *Rousettus aegyptiacus* in 2009. The bats were supposed to be caught from Limpopo and an application for clearance from the Limpopo provincial government was made back in 2009. Due to some reason the application was never attended to and efforts to follow up the application were futile. It was not until early this year that we managed to get a breakthrough in terms of communication with the wildlife and conversation department from Limpopo thereby resuscitating the application. We eventually received the permit from the Limpopo provincial government on 24th of July 2014. Faced with this we could not ask for extension of the ethics clearance when it expired as we were not sure if we were ever going to get the permit from Limpopo. Given that we have received the permit, we therefore seek an extension of the validity period of our ethics clearance certificate (attached) so as to proceed with the experiments.

Animal Ethics Clearance Extension 2014

AESC 2014 M&E

2014/07/24

Date: Signature:

RECOMMENDATIONS : Approved extension of time to December 2015.
Condition(s): Provide a report on actual numbers captured.

Date: 28 July 2014. Signature:
Chairman, AESC

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/53/01

APPLICANT: **Professor. P Manger**

DEPARTMENT: **Anatomical Sciences**

PROJECT TITLE: **Comparative Neuroanatomy of Vertebrate Species - establishing a world-class brain bank at Wits**

Number and Species

Approved: **Various species of vertebrates, to be detailed in M&Es as they become available**

Approval was given for to the use of animals for the project described above at an AESC meeting held on **27 November 2012**. This approval remains valid until **30 November 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.

Signed: _____ (Chairperson, AESC) Date: 5/12/12

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: 5/12/12

cc: Supervisor:
Director: CAS

LIMPOPO PROVINCIAL GOVERNMENT PERMIT



LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF
ECONOMIC DEVELOPMENT, ENVIRONMENT & TOURISM

PERMIT

DO SCIENTIFIC RESEARCH ON MAMMALS

Permit Holder

Full name	: Prof .P. Manger	**ID No.**	: 6603037196180
Trade Name	: University of Wits	**Reg. No.**	: MKT001-00010
Postal Address	: School of Anatomical Sciences University of Witwatersrand 7 York Road, Parktown	**Physical Address**	: 18 Arundel Road Westdene
PostalSuburb	: Johannesburg	**Suburb**	: Johannesburg
PostalState	: Gauteng	**ResidentialState**	: Gauteng
PostalCountry	:South Africa	**ResidentialCountry**	: South Africa

In terms of and subject to the provisions of the abovementioned legistation and the regulation framed thereunder, the holder of this permit is hereby authorized to catch and/or collect the species and number of mamals specified on the table below for scientific purposes on the property mentioned on this permit.

Permit Details

Permit No	: 0089-MKT001-00001
Date Issued	: 2014/07/15 08:40:00AM
Valid until	: 2015/07/10 08:40:00AM
Paid	: 52.00
Receipt No	: 1059359

Stamp:

CITES & PERMIT MANAGEMENT
ENVIRONMENTAL AFFAIRS
LIMPOPO PROVINCE

Description	Property
Properties	Near Legalameetsi Nature Reserve
District	Mopani
Provice/State	Limpopo
Country	South Africa

Species(Scientific Name)	Qty	Note
Egyptian Rousette fox (Rousettus aegyptiacus)	21	

Issued by: Makgata RJ — **Issue Date** 7/15 08:40: — **Effective Date** 2014/07/15 — **Signature of Permit Holder**

I acknowledge, accept and understand fully the permit conditions as described.

20 Hans Van Rensburg Street / 19 Biccard Street, Polokwane, 0700, Private Bag X 9484, Polokwane, 0700
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LIMPOPO PROVINCIAL GOVERNMENT PERMIT

GENERAL CONDITIONS AND REQUIREMENTS OF PERMIT/LICENCE/CERTIFICATE

1. This permit, licence or certificate shall not be transferable.
2. Any unauthorized alterations to this permit, license or certificate shall invalidate it.
3. This permit, licence or certificate shall be subject to the provisions of any law in force during the period of validity of the permit, licence or certificate, in the area to which the permit, licence or certificate to such person.
4. The holder of this permit, licence or certificate shall, at the request of a person authorised in terms of the relevant legislation so to demand, forthwith produce such permit, licence or certificate to such person.
5. The holder of this permit, licence or certificate shall return this permit, licence or certificate to the Senior Manager: Wildlife Trade and Regulation, Limpopo Province, P.O Box 55464, Polokwane, 0700.
6. This permit, licence or certificate shall be invalid until the signature of the holder thereof has been appended thereto.
7. This permit, licence or certificate shall lapse when it is lost or destroyed and no copy thereof shall be issued.
8. The holder of this permit, licence or certificate who contravenes or fails to comply with any one of the conditions or requirements to which this permit, licence or certificate is subject, shall be guilty of an offence.
9. An officer authorized thereto by the MEC may cancel this permit, licence or certificate at any time.
10. This permit, licence or certificate does not absolve the holder thereof from the necessity of obtaining such other permits and/or documents as may be required by law from the relevant province or country.

Special Conditions

1. THIS PERMIT MUST BE SIGNED BY THE PERMIT HOLDER AND MUST BE IN HIS/HER POSSESSION FOR THE DURATION OF THE PROJECT.

2. THE PROJECT LEADER MUST OBTAIN THE WRITTEN PERMISSION OF THE LANDOWNER ON WHOSE PROPERTY THE ANIMALS WILL BE COLLECTED PRIOR TO THE COLLECTION THEREOF.

3. THIS PERMIT ALSO AUTHORISES THE HOLDER THEREOF TO CONVEY WITHIN THE PROVINCE, OR EXPORT FROM THE PROVINCE THE COLLECTED SPECIES REFER TO ON THIS PERMIT, TO ANY OTHER PROVINCE WITHIN THE COUNTRY. SUCH ACTION WILL BE SUBJECT TO IMPORT PERMITS FROM SUCH PROVINCE.

4. COPIES OF PUBLICATIONS EMANATING FROM THIS RESEARCH PROJECT SHOULD ALSO BE FORWARDED TO THE ADDRESS STATED UNDER POINT 6.

5. A DATASHEET ACCORDING TO THE ATTACHED DATA FORMAT MUST BE COMPLETED FOR EACH SPECIMEN COLLECTED AND BE RETURNED TO THE BIODIVERSITY OFFICE FOR THE PROVINCIAL BIOBASE PROJECT.

6. ALL REQUESTED INFORMATION OR ENQUIRIES MUST BE DIRECTED TO THE LIMPOPO ENVIRONMENTAL MANAGEMENT AUTHORITY, P.O. BOX 55464, POLOKWANE, 0700

7. THE APPLICANT PROFESSOR P. MANGER, HIS CO-WORKERS DR. A IHUNWO AND DR. R. CHAWANA WILL BE CAPTURING 21 EGYPTIAN ROUSETTE FLYING FOX FROM CAVES NEAR LEKGALAMEETSI NATURE RESERVE FOR EXPERIENCIAL PURPOSES.

CITES & PERMIT MANAGEMENT
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paper text:

Adult neurogenesis in the brain of Chiroptera Richard Chawana 331986 PhD Thesis Supervisor: Professor Paul Robert Manger Co-supervisor: Associate Professor Amadi Ogunda Ihunwo A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy. January, 2016 i DECLARATION This research is my original work, produced with normal supervisory assistance from my supervisor. All the relevant sources of knowledge that I have used during the course of writing this dissertation have been fully credited and acknowledged. Furthermore, this research report has not been submitted for any academic or examination purpose at any other university. …… …… Richard Chawana Date This research paper has been submitted for examination with my approval as the University supervisor(s) for the aforementioned student. …… …… Assoc. Prof. Amadi O. Ihunwo Date …… Prof. Paul R. Manger …… Date ii Matidaishe and Itumeleng The pursuit of Happyness To God be the glory iii Publications arising from this thesis: ? Chawana, R., Alagaili, A., Patzke, N., Spocter, MA., Mohammed, OB.,

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RE: AUTHORSHIP OF THE PUBLISHED ARTICLES INCLUDED IN RICHARD CHAWANA'S PHD THESIS

This letter serves to clarify questions regarding the authorship of the two published articles included in Richard Chawana's PhD thesis which are titled:

1. Chawana, R., Alagaili, A., Patzke, N., Spocter, MA., Mohammed, OB., Kaswera, C., Gilissen, E., Bennett, NC., Ihunwo, AO., Manger, PR. (2014) Microbats appear to have adult hippocampal neurogenesis, but post-capture stress causes a rapid decline in the number of neurons expressing doublecortin. Neuroscience, 277, 724-733.
2. Chawana, R., Patzke, N., Kaswera, C., Gilissen, E., Ihunwo, AO., Manger, PR. (2013). Adult neurogenesis in eight species of megachiroptera. Neuroscience, 244, 159-172.

For these papers, Richard did all of the laboratory work, analysis and writing of the first draft of the manuscripts under my direct supervision. In addition, under my guidance, he responded to the reviewers' comments prior to acceptance for publication. The co-other authors included on the papers contributed in identification and capture of the animals in the different regions where they were found, I personally prepared the brains and stored them in my brain bank for student use, and some of the co—authors assisted in some of the benchwork and analysis, as well as all authors reviewing and editing the manuscripts.

If there are any further questions, please do not hesitate to contact me.

Sincerely



Prof. Paul R. Manger

Neuroscience 277 (2014) 724–733

MICROBATS APPEAR TO HAVE ADULT HIPPOCAMPAL NEUROGENESIS, BUT POST-CAPTURE STRESS CAUSES A RAPID DECLINE IN THE NUMBER OF NEURONS EXPRESSING DOUBLECORTIN

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Abstract—A previous study investigating potential adult hippocampal neurogenesis in microchiropteran bats failed to reveal a strong presence of this neural trait. As microchiropterans have a high field metabolic rate and a small body mass, it is possible that capture/handling stress may lead to a decrease in the detectable presence of adult hippocampal neurogenesis. Here we looked for evidence of adult hippocampal neurogenesis using immunohistochemical techniques for the endogenous marker doublecortin (DCX) in 10 species of microchiropterans euthanized and perfusion fixed at specific time points following capture. Our results reveal that when euthanized and perfused within 15 min of capture, abundant putative adult hippocampal neurogenesis could be detected using DCX immunohistochemistry. Between 15 and 30 min post-capture, the detectable levels of DCX dropped dramatically and after 30 min post-capture, immunohistochemistry for DCX could not reveal any significant evidence of putative adult hippocampal neurogenesis. Thus, as with all other mammals studied to date apart from cetaceans, bats, including both microchiropterans and megachiropterans, appear to exhibit substantial levels of adult hippocampal neurogenesis. The present study underscores the concept that, as with laboratory experiments, studies conducted on wild-caught animals need to be cognizant of the fact that acute stress (capture/handling) may induce major changes in the appearance of specific neural traits. **© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: adult neurogenesis, doublecortin, Chiroptera, free-living animals, capture stress, hippocampus.

INTRODUCTION

Studies on adult neurogenesis in free-living mammals are becoming more numerous due to the need to understand this biological process in relation to normal life-history parameters (Amrein et al., 2004, 2011; Bartkowska et al., 2008, 2010; Epp et al., 2009; Kempermann, 2012; Cavegn et al., 2013; Chawana et al., 2013; Patzke et al. 2013a,b). The investigation of free-living mammals may provide a broader understanding of the dynamics and mechanisms influencing adult neurogenesis of species in their natural habitat and ultimately reveal potential reasons for the presence of adult neurogenesis in the mammalian brain. Free living mammals are subject to a number of pressures such as predation, foraging and varying weather patterns, all of which are factors that may influence the process of adult neurogenesis (Kempermann, 2012).

While working on wild-caught mammals has the potential advantage to reveal aspects of interest to a broad understanding of adult neurogenesis, the capture of these animals from their natural environments may be considered to be an acute stressor that is difficult to control and unpredictable. While chemical capture of wild animals (using dart guns) appears to lower blood glucocorticoid levels, physical restraint and translocation leads to significant increases in the stress-related release of glucocorticoids (e.g. Widmaier and Kunz, 1993; Morton et al., 1995). In terms of adult neurogenesis, the effect of acute stress has been observed to lead to a reduction in hippocampal neurogenesis in a range of laboratory-kept species (Gould et al., 1998; Tanapat et al., 2001; Falconer and Galea, 2003; Kim et al., 2004; Dagyte et al., 2009; Hulshof et al., 2012), although in rats the reduction in the number of proliferating cells was

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Abbreviations: BSA, bovine serum albumin; DAB, diaminobenzidine; DCX, doublecortin; DCX+, doublecortin immunopositive; GCL, granular cell layer; NRS, normal rabbit serum; PB, phosphate buffer; SVZ, subventricular zone.

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observed to occur within 2 h of the acute stressor and recovery to baseline levels within 24 h post exposure (Heine et al., 2004).

An earlier study of adult neurogenesis in microchiropterans led to the conclusion that the hippocampus of the species studied had absent to low rates of adult neurogenesis (Amrein et al., 2007). While possible reasons for the absence of adult hippocampal neurogenesis were raised, it appears that no specific conclusion was reached. One issue that was not raised by Amrein et al. (2007) was whether the stress of capture/handling of these small mammals may have had an important role in the lack of detectable adult hippocampal neurogenesis. While Amrein et al. (2007) state the bats were ''perfused rapidly after trapping'', no estimate of the time that elapsed between trapping and perfusion was provided, thus it is possible that capture stress could pose a serious methodological problem; however, this does not explain the absence of adult hippocampal neurogenesis in the three neotropical bat species obtained from breeding colonies located in Germany, but again, no information regarding the handling of these bats prior to perfusion was provided. Given that microchiropterans have generally lower basal metabolic rates compared to other mammals of similar size (Austad and Fischer, 1991; Neuweiler, 2000), but active or field metabolic rates significantly higher than other mammals and even birds (Neuweiler, 2000), it is possible that even a short period of stress, in the range of minutes, related to capture and handling may have a major effect on the expression of proteins in the microchiropteran brain, and in the case of Amrein et al. (2007) may have led to a false-negative report of the absence of adult hippocampal neurogenesis in the bat species studied, a finding that is becoming entrenched in the neurogenesis literature (e.g. Bonfanti and Peretto, 2011; Powers, 2013). Given this potential confound in the study of Amrein et al. (2007), we sought to analyze the relationship between capture stress and adult hippocampal neurogenesis in wild-caught microchiropterans using immunohistochemistry for the doublecortin protein (DCX), an endogenous marker of putative adult hippocampal neurogenesis (Kempermann, 2012; Patzke et al., 2013b).

EXPERIMENTAL PROCEDURES

In the current study we examined 36 brains from 10 microchiropteran species including *Miniopterus schreibersii* ($n = 2$) captured from a wild population in Gauteng, South Africa, *Cardioderma cor* ($n = 2$), *Chaerophon pumilus* ($n = 2$), *Coleura afra* ($n = 2$), *Hipposideros commersoni* ($n = 2$), and *Triaenops persicus* ($n = 2$) captured from wild populations in coastal Kenya, *Hipposideros fuliganosas* ($n = 2$) and *Nycteris macrotis* ($n = 2$) captured from wild populations in the Yoko Forest near Kisangani, Democratic Republic of the Congo, and *Pipistrellus kuhlii* ($n = 2$) and *Asellia tridens* ($n = 18$) captured from wild populations near Unizah, Saudi Arabia. All animals were adults, as judged from epiphyseal closure of the metacarpophalangeal joints (Anthony, 1988). Appropriate permissions to trap and euthanize the bats were obtained from the Gauteng Department of Nature Conservation, South Africa, the Kenya National Museums, Kenya, the University of Kisangani, DR Congo, and the Saudi Wildlife Authority, Saudi Arabia. All animals were treated and used in accordance with the University of the Witwatersrand Animal Ethics Committee Guidelines (clearance number 2008/36/1) which parallel those of the NIH for the care and use of animals in scientific experiments. All bats were euthanized (overdose of sodium pentobarbital, 100 mg/kg, i.p.) and perfused through the left ventricle with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at various times following capture. For *H. fuliganosas* and *T. persicus*, the animals were perfusion fixed within 15 min of capture. For *M. schreibersii*, *C. cor* and *C. pumilus*, the specimens were fixed between 15 and 30 min of capture, and for *C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*, the specimens were fixed within an hour of capture. For *A. tridens*, two animals were sacrificed and perfused at each of the following time points (in minutes) post-capture: 10, 15, 20, 30, 60, 120, 180, 240 and 300. Following perfusion, the brains were removed and post-fixed in 4% paraformaldehyde in 0.1 M PB overnight, cryoprotected in 30% sucrose in 0.1 M PB at 4 °C and stored in an antifreeze solution at −20 °C until sectioning and histological processing. Before sectioning, the brains were divided into two halves along the mid-sagittal fissure and the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4 °C. The specimens were cryosectioned in the sagittal plane into 50-μm-thick sections. A one in three series of sections was stained for Nissl substance (Cresyl Violet) to reveal cytoarchitectural features, and immunostained at two different dilutions of the primary antibody to DCX (1:300 and 1:600) to reveal immature neurons.

In the current study we used immunolabeling of DCX, an endogenous marker of putative immature neurons, to ascertain the potential presence or absence of adult neurogenesis. While DCX immunopositive neurons away from the hippocampus may not relate to adult neurogenesis in these regions, such as the piriform cortex (Klempin et al., 2011), it has been established that DCX immunolabeling of granule cells of the dentate gyrus is a good proxy for the presence of adult hippocampal neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005). The presence of DCX is also thought to reflect cumulative adult hippocampal neurogenesis over a period of 2 weeks to 6 months, although this period is species specific (Rao and Shetty, 2004; Kohler et al., 2011). Thus, lack of DCX staining should be a reliable indicator of the absence of adult hippocampal neurogenesis (Patzke et al., 2013b) or of a perturbation in the maturation process of newly generated neurons.

Free floating sections were incubated in a 1.6% H_2O_2, 49.2% methanol, 49.2% 0.1 M PB solution, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10-min rinses in 0.1 M PB. To block non-specific binding sites the sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal rabbit serum – NRS, 2% bovine serum albumin, BSA, and 0.25% Triton X-100 in

0.1 M PB). Thereafter, the sections were incubated for 48 h at 4 °C in the primary antibody solution (1:300 and 1:600, goat anti-doublecortin, DCX, SC-18 Santa Cruz Biotech, Santa Cruz, California, USA) under gentle agitation. The primary antibody incubation was followed by three 10-min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated rabbit anti-goat IgG, BA 5000, Vector Labs, Burlingame, California, USA, in 3% NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin–biotin solution (1:125; Vector Labs), followed by three 10-min rinses in 0.1 M PB. Sections were then placed in 1 ml of a solution containing 0.025% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 µl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low power stereomicroscope. Staining continued until such time that the background stain was at a level that would allow for accurate architectonic matching to the Nissl sections without obscuring the immunopositive structures. Development was arrested by placing sections in 0.1 M PB for 10 min, followed by two more 10-min rinses in this solution. Sections were then mounted on 0.5% gelatine-coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody, and sections where we omitted the secondary antibody. In both cases no staining was observed. It was not possible to undertake Western blot control testing due to the nature of the collection of the tissue from wild populations. Staining patterns of DCX were observed using low power stereomicroscope and digital photomicrographs were captured using Zeiss Axioshop and Axiovision software (Carl Zeiss Microscopy GmbH, Jena, Germany). No pixilation adjustments or manipulation of the captured images was undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

For quantifying DCX immunopositive cells, a modified unbiased stereological procedure was used as described previously (Malberg et al., 2000; Segi-Nishida et al., 2008; Noori and Fornal, 2011). All sections stained with the 1:300 dilution of DCX were coded to ensure that the analysis was performed by a blinded observer (M.A.S) and immunopositive DCX cells were counted at 40× magnification in the subgranular zone of the left hippocampus of all specimens using an Olympus BX-60 light microscope equipped with a video camera. Cells were included if the cells lay within, or touched, the subgranular zone. The subgranular zone was defined as the area from one cell diameter within the granular cell layer (GCL) from the hilus-GCL border and two cell diameters below the hilus-GCL border (Eriksson et al., 1998). Cells were excluded if the cell was more than two cell diameters from the GCL, focusing through the thickness of the section (optical dissector principle, see Gundersen et al., 1988; West, 1993; Coggeshall and Lekan, 1996) to avoid errors due to oversampling. Every section was counted throughout the hippocampus and the sum was multiplied by 3 (as we used a one in three series, see above) to provide an estimate of the total number of immunopositive DCX cells in the entire left hippocampus (Table 1).

Statistical analysis was done using STATA software package version 13.1 (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP). Given our sample sizes, we performed non-parametric tests (Mann–Whitney tests) to compare hippocampal DCX cell counts of animals from different perfusion delay time groups (those perfused within 15 min of capture and those perfused after 15 min of capture). In addition we undertook Spearman's correlation test to measure the strength of correlation of cell counts obtained in animals belonging to a particular time group.

RESULTS

Doublecortin immunopositive (DCX+) cells in the microchiropteran hippocampus

Of the 10 microchiropteran species examined in the current study, we found DCX+ cells clearly present in the subgranular zone of the dentate gyrus of three species (*A. tridens*, *H. fuliganosas* and *T. persicus*) which were perfused within 15 min of capture, partially present in three species (*M. schreibersii*, *C. cor*, *C. pumilus*) which were perfused between 15 and 30 min of capture, and low to absent in four species (*C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*) all of which were perfused between 30 and 60 min post-capture (Figs. 1–3; Table 1). The number of DCX-labeled cells in the hippocampus for all animals perfused more than 15 min ($n = 28$) after capture declined nine times (median = 536 cells and range = 345–3294 cells) when compared to those perfused within 15 min of capture ($n = 8$; median = 4787 cells and range = 1380–6945 cells) (Mann–Whitney test $z = 3.08$ and $p = 0.0001$). In addition, the species where DCX+ cells were observed, the subgranular zone at the base of the granule cell layer was populated by immunopositive cells that had a small soma size with dendritic processes that extended through the granule cell layer to ramify into the molecular layer. Furthermore, the mossy fibers that emanate from these cells were also observed with DCX immunohistochemistry, indicating that they are likely to be in the process of becoming functionally integrated into the hippocampal circuitry (Fig. 2B). The morphology of the DCX+ cells, when present, in the dentate gyrus of the microchiropterans was similar to that seen in other mammals studied with the same technique.

In the *A. tridens* time series (Table 1), DCX+ cells were readily observed at the 10 min post-capture time point (Figs. 2B, 3B and 4A), showing the full range of normal morphology of these immature neurons, including the presence of DCX+ mossy fibers. By 15 min post-capture, the number of DCX+ cells was dramatically reduced (to around 20% of the pre-15 min

Table 1. Counts of DCX immunopositive neurons in the left hippocampi of the microchiropterans studied that were sacrificed and perfusion fixed at various time points following capture

Species	Perfusion delay	DCX cells, specimen 1	DCX cells, specimen 2
Hipposideros fuliganosas	Less than 15 min	3762	3963
Triaenops persicus	Less than 15 min	5610	5721
Miniopterus schreibersii	Between 15–30 min	2067	2262
Cardioderma cor	Between 15–30 min	2718	2568
Chaerophon pumilus	Between 15–30 min	3294	3072
Coleura afra	Between 30–60 min	558	603
Hipposideros commersoni	Between 30–60 min	1059	1026
Nycteris macrotis	Between 30–60 min	1188	1134
Pipistrellus kuhlii	Between 30–60 min	1254	1335
Asellia tridens	10 min	6621	6945
Asellia tridens	15 min	1380	1536
Asellia tridens	20 min	438	402
Asellia tridens	30 min	399	444
Asellia tridens	60 min	384	351
Asellia tridens	120 min	393	387
Asellia tridens	180 min	360	345
Asellia tridens	240 min	414	468
Asellia tridens	300 min	495	513

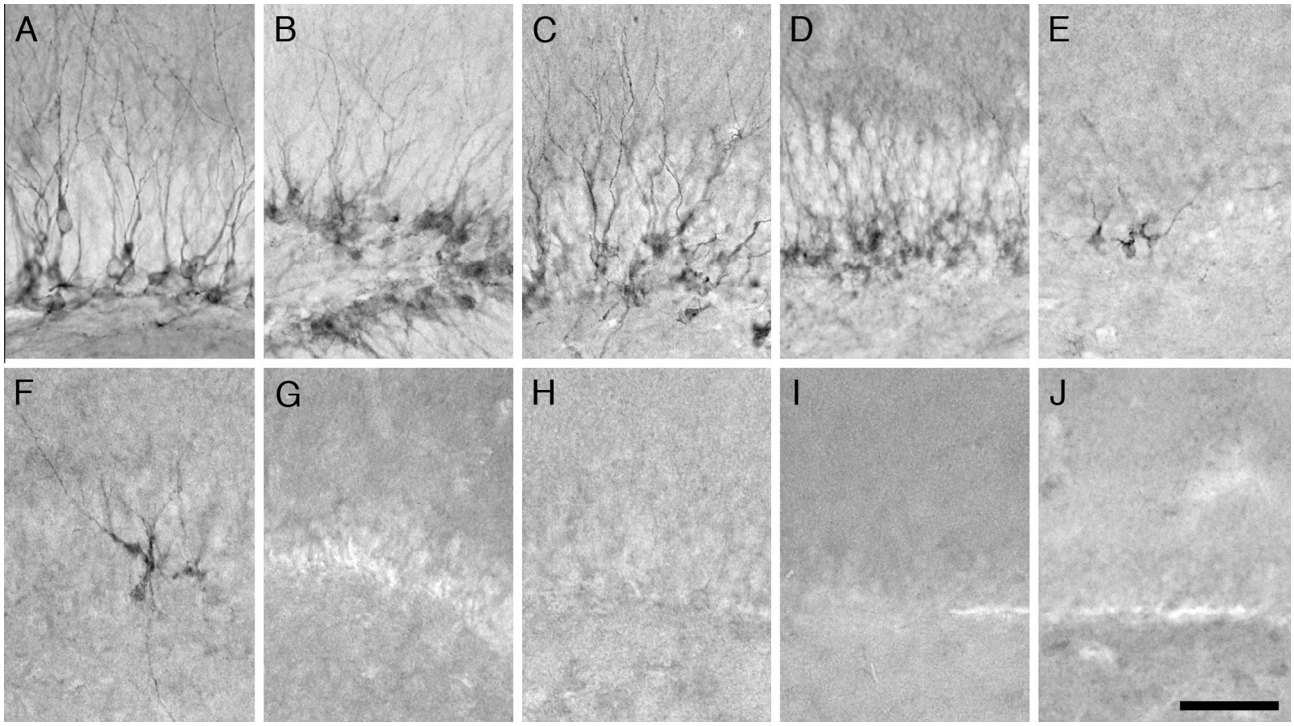

Fig. 1. Photomicrographs of doublecortin immunoreacted sections of the dentate gyrus of the hippocampus of the various species of microchiropteran examined in the current study. Doublecortin immunoreactive immature neurons are clearly present in (A) *Hipposideros fuliganosas*, (B) *Asellia tridens* and (C) *Triaenops persicus*, which were euthanized and perfusion fixed within 15 min of capture, partially present in (D) *Miniopterus schreibersii*, (E) *Cardioderma cor* and (F) *Chaerophon pumilus*, which were perfusion fixed between 15 and 30 min post-capture, and very low to absent in (G) *Coleura afra*, (H) *Hipposideros commersoni*, (I) *Nycteris macrotis* and (J) *Pipistrellus kuhlii*, which were perfusion fixed more than 30 min post-capture. The scale bar in J = 50 μm and applies to all images.

specimens, Table 1), as were the number of DCX+ dendrites and mossy fibers emanating from these cells (Figs. 3B and 4B). At 20 min post-capture there was a further reduction in the number of DCX+ cells (to around 6% of the pre-15 min specimens, Table 1), dendrites and mossy fibers (Figs. 3B and 4C). By 30 min post-capture (Figs. 3B and 4D; Table 1), only a few DCX+ cells remained, and the DCX+ dendrites and mossy fibers were almost absent. The remaining time points examined, up to 300 min post-capture, evinced DCX immunostaining similar to that seen in the 30 min post-capture time point, with only a few persistent DCX+ cells, dendrites and mossy fibers (Figs. 3B and 4; Table 1). Similar to the trend observed

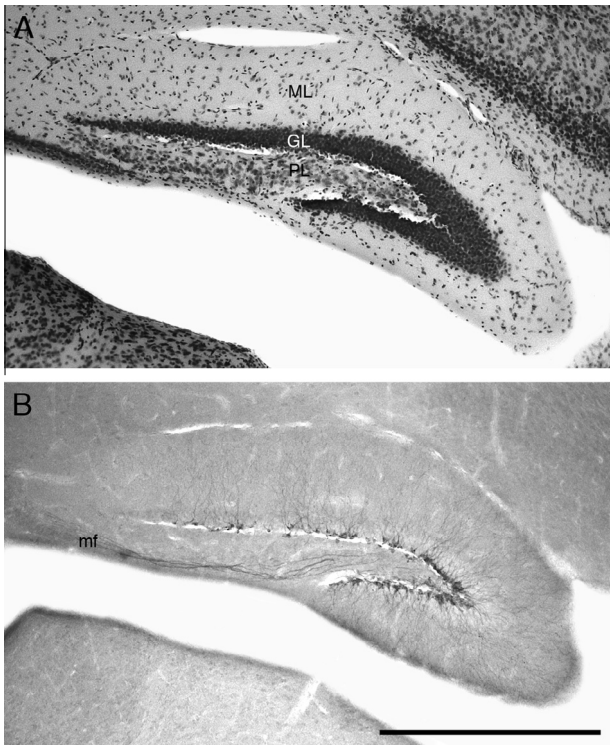

Fig. 2. Photomicrographs of adjacent Nissl stained (A) and doublecortin immunoreacted (B) sections of the dentate gyrus of the hippocampus of *Asellia tridens* at 10 min post-capture. Note the presence of doublecortin immunopositive cells at the base of the granular layer (GL), dendrites throughout molecular layer (ML) of the entire dentate gyrus, and mossy fibers (mf) exiting the dentate gyrus by passing through the polymorphic layer (PL). In both images dorsal is to the top and rostral to the left. The scale bar in B = 500 μm and applies to both images.

with all species provided above, our comparison of DCX-labeled cells in *A. tridens* only showed that those animals perfused more than 15 min after capture (n = 14) was associated with a 10 times decline in cell number (median = 401 cells and range = 345–513 cells) when compared to those perfused within 15 min of capture (n = 4; median = 4078 cells and range = 1380–6945 cells) (Mann–Whitney test z = 2.974 and p = 0.0029).

DCX+ cells in other regions of the microchiropteran brain

In all the microchiropterans studied, varying densities of DCX+ cells were observed in the subventricular zone of the lateral ventricle (SVZ). From the SVZ, which appeared to occupy the majority of the ventricular wall adjacent to the caudate nucleus, these cells migrated through the rostral migratory stream to the olfactory bulb. We observed a stream of DCX+ cells arising from the inferior portion of the SVZ that appeared to migrate to the piriform cortex and amygdala. A small stream of DCX+ cells appeared to migrate dorsally from the anterior portion of the rostral migratory stream to populate the cerebral neocortex anterior to the primary somatosensory cortex.

In the *A. tridens* time series, the rostral migratory stream was readily evident in individual animals perfused within 60 min of capture (Fig. 5A); however, the strength of labeling of both cells and fibers declined during this first 60 min and the rostral migratory stream was not evident in individuals perfused from 120 min post-capture. DCX+ cells in the frontal neocortex were observed in the individual animals perfused within 10 min of capture (Fig. 5B), but after this time point we could find no evidence for these cells. In contrast, DCX+ cells were observed in the piriform cortex in all individual animals at all of the time points examined (Fig. 5C), with no significant drop in apparent DCX+ cell number, or expression of DCX in the dendrites emanating from these cells.

DISCUSSION

The present study, demonstrating the likely presence of adult hippocampal neurogenesis in microchiropterans, and that detecting this presence is dependent on the level of post-capture stress/handling to which these animals are exposed, contrasts with a previous report detailing the absence of adult hippocampal neurogenesis in microchiropterans (Amrein et al., 2007). The absence of adult hippocampal neurogenesis in microchiropterans reported by Amrein et al. (2007) has been referred to extensively in the literature (e.g. Bonfanti and Peretto, 2011; Kempermann, 2012) to the point that the idea that chiropterans in toto, both microchiroptera and megachiroptera, do not exhibit adult hippocampal neurogenesis is becoming ‘‘accepted knowledge’’ (e.g. Powers, 2013). It should be noted here that Amrein et al. (2007) only studied species from the microchiropteran suborder of bats, and not the megachiropteran suborder, for which two recent reports have detailed the presence of adult hippocampal neurogenesis in a range of megachiropteran species (Gatome et al., 2010; Chawana et al., 2013), making the title and conclusions of the Amrein et al. (2007) paper misleading as they use only the generic term bats.

The present study indicates that the potential problem encountered by Amrein et al. (2007), leading to a false-negative report regarding potential adult hippocampal neurogenesis in the microchiropterans, was the length of post-capture stress and handling experienced by the animals prior to euthanasia and fixation of the neural tissue. The four species for which we did not observe significant evidence of adult hippocampal neurogenesis were those that were perfused at a time point greater than 30 min post-capture. The three species in which partial evidence for adult hippocampal neurogenesis was observed were perfused between 15 and 30 min post-capture, but the three species for which we saw significant evidence for adult hippocampal neurogenesis were all perfused within 15 min of capture. The idea that post-capture stress and handling leads to a rapid decline of detectable adult hippocampal neurogenesis using DCX immunohistochemistry in the microchiropterans is supported by the time series study of *A. tridens* undertaken herein, where at 10 min post-capture DCX immunostaining

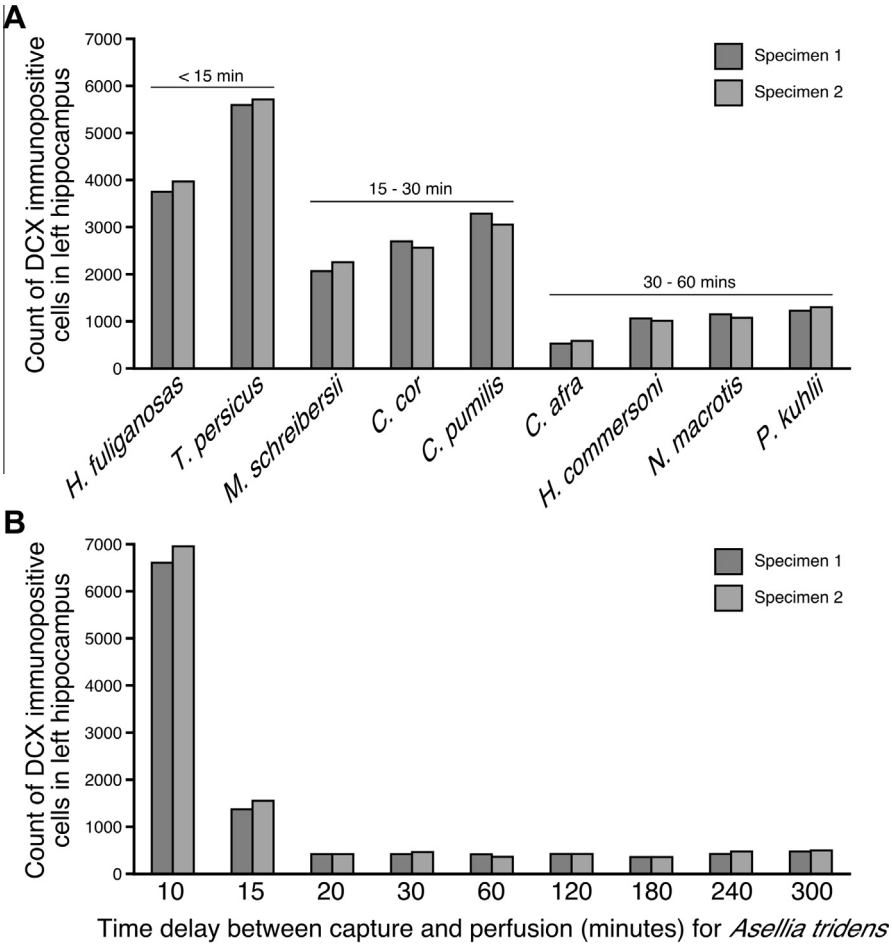

Fig. 3. Bar graphs showing the results of our quantitative analysis of the number of doublecortin immunopositive neurons in the left hippocampus of a range of microchiropteran species. (A) This bar graph shows significant levels of DCX immunoreactive cells in the hippocampus of two species (*H. fuliganosas* and *T. persicus*) that were sacrificed and perfused within 15 min of capture from their natural environment. The three species perfused between 15–30 min of capture (*M. schrebersii*, *C. cor* and *C. pumilus*) showed lower numbers of DCX immunoreactive cells, while those perfused between 30–60 min after capture (*C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*) all showed very low numbers of DCX immunoreactive cells. (B) This bar graph shows the results of the quantification of DCX immunopositive neurons in *A. tridens* from specimens that were perfused at a range of time points following capture. Note the significant presence of DCX immunoreactive cells when the animals were perfused 10 min following capture, but that this is substantially reduced at 15 min following capture and settles at a low level for longer time points. Two individuals of each species and at each time point were assessed (specimens 1 and 2).

revealed extensive evidence for adult hippocampal neurogenesis, but that by 15 min post-capture, the extent of staining had decreased dramatically and was very low to near absent in subsequent time points. Similar effects in the decrease in the detectable presence of DCX in neurons have been observed in the dentate gyrus (Dagyte et al., 2009; Hulshof et al., 2012) and the retrosplenial cortex of the rat after exposure to acute stress (Kutsuna et al., 2012). The observation that post-capture stress rapidly diminishes the detectable presence of adult hippocampal neurogenesis in the microchiropterans may also explain other unusual results in field-caught species, such as the low proliferation rate, but high differentiation rate seen in wild-caught South African rodents (Cavegn et al., 2013), where capture stress may have reduced the detectable presence of newly born neurons using Ki-67 immunohistochemistry, but had no specific effect on the differentiating neurons, as the DCX immunohistochemistry used to detect differentiating neurons can be present in these neurons over a much longer period.

That the detectable presence of adult hippocampal neurogenesis with DCX immunohistochemistry in the microchiropterans disappeared so rapidly is of interest. As mentioned, the microchiropterans have a very high field metabolic rate in comparison to most other mammals and birds (Neuweiler, 2000), and it is possible that the stress associated with capture or handling of free-living animals, or animals not accustomed to being handled, when combined with a high field metabolic rate, may lead to the rapid non-genomic corticosterone-induced proteolysis of proteins associated with cell differentiation/maturation such as DCX, but perhaps not cell death or the cessation of cell proliferation (Kutsuna et al., 2012). This is particularly so because, like the cells in the CA1 region, granule cells show quick enhancement of miniature excitatory potential post-synaptic currents (mEPSC) and prolongation of N-methyl-D-aspartate receptor (NMDAR)-mediated influx of calcium ions after exposure to a wave of corticosteroids (Takahashi et al., 2002; Pasricha et al., 2011). The rapid calcium influx

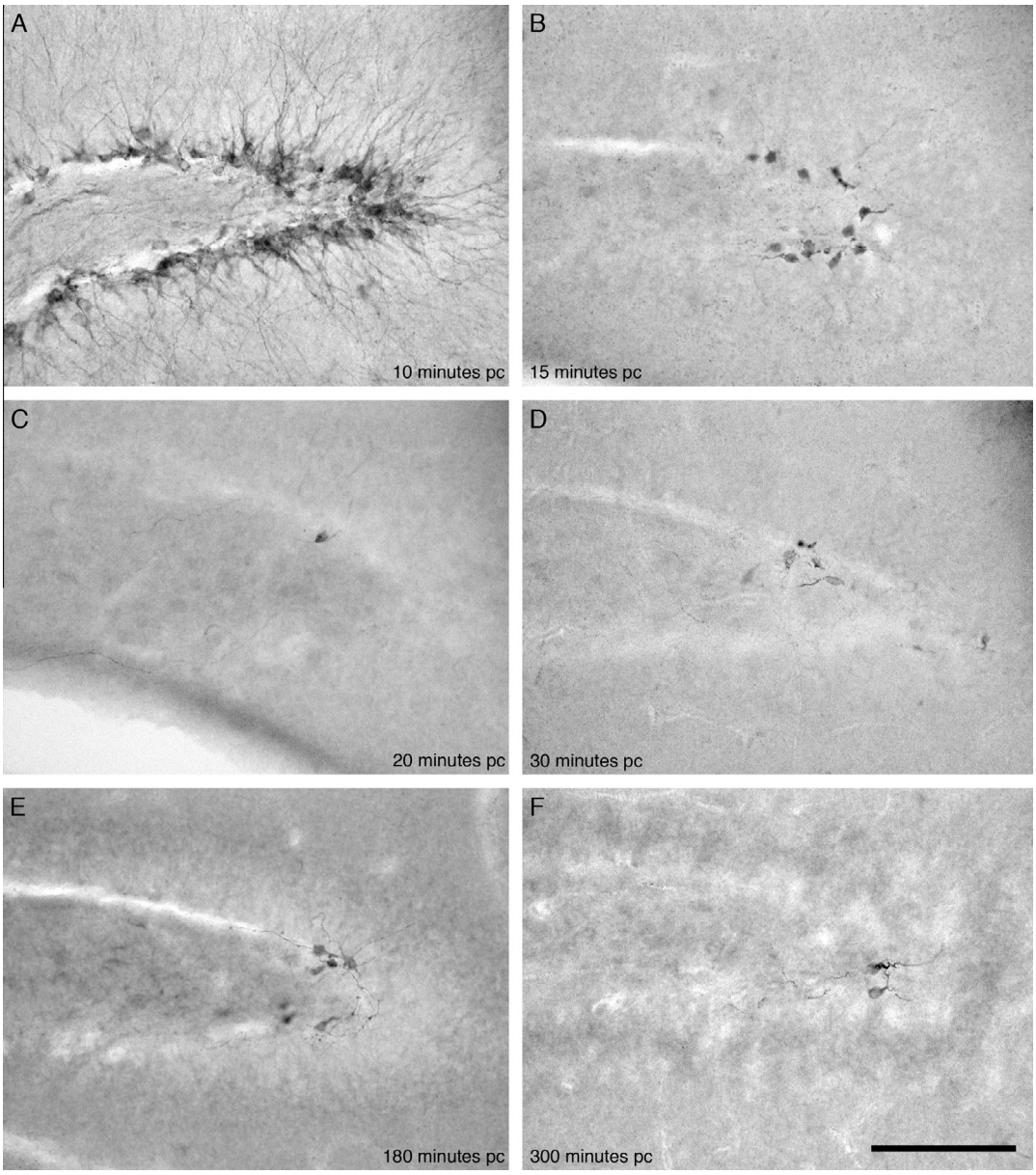

Fig. 4. Photomicrographs of doublecortin immunoreacted sections of the dentate gyrus of the hippocampus of *Asellia tridens* at different time points post-capture (pc). At 10 min post-capture (A), numerous cells immunopositive for doublecortin are found throughout the entire dentate gyrus. These cells exhibit apical dendrites that ramify into the molecular layer and mossy fibers that exit through the polymorphic layer. At 15 min post-capture (B), the number of doublecortin immunopositive cells, dendrites and mossy fibers has decreased dramatically, with a further decrease in number of these structures at 20 min post-capture (C). Later time points (D–F) show a similar low number of doublecortin immunopositive cells, dendrites and mossy fibers. In all images dorsal is to the top and rostral to the left. The scale bar in F = 100 μm and applies to all images.

activates a calcium-dependant enzyme, calpain, which breaks down the cytoskeleton (Vanderklish et al. 2000; Andres et al., 2013). Given that the corticosterone-induced changes in calcium currents occur with 10 min of exposure to the steroid (Wiegert et al., 2006) it is possible that in this study, this mechanism could have been activated, resulting in breakdown of the cytoskeleton and DCX, which integrates linkages between the cytoskeleton in neuronal cells and axons (Tint et al., 2009).

The decline in DCX-labeled cells in the hippocampus of the *A. tridens* may be related to age, given that age-related decline of mammalian adult hippocampal neurogenesis is well documented (reviewed by Klempin and Kempermann, 2007). Thus, it could be argued that all the microbats that we caught and perfused within 15 min of capture were substantially younger than those perfused more than 15 min after capture, with this latter group being made up entirely of microbats in their senility. Though possible, it is highly unlikely for this to be the case in this study despite it being difficult to accurately determine the exact chronological age of the microbats caught from wild populations. Firstly, given that our own observations in this study yielded repetitive results for the two specimens in each of the ten time groups as evidenced

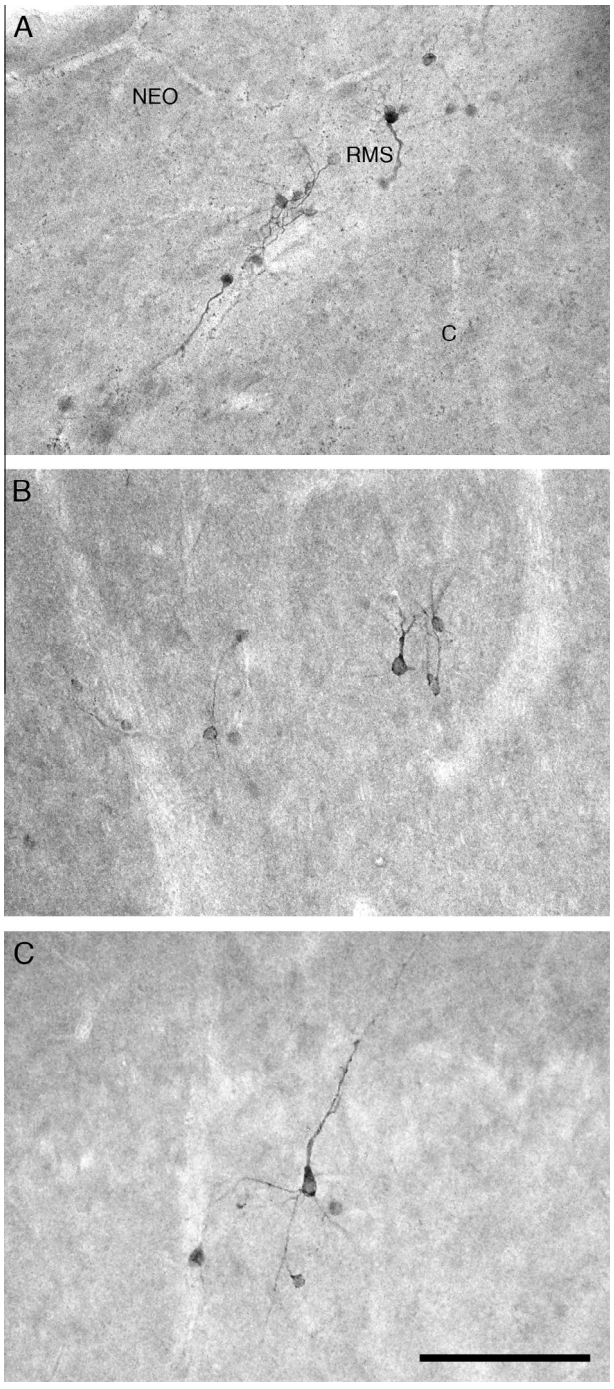

Fig. 5. Photomicrographs of doublecortin immunoreacted sections in different regions of the brain of *Asellia tridens* at 10 min post-capture. (A) Doublecortin immunopositive cells and fibers in the rostral migratory stream (RMS) located between the caudate nucleus (C) and the cerebral neocortex (NEO). In this image dorsal is to the top and rostral to the left. (B) Doublecortin immunopositive cells showing dendritic ramifications in layer III of the frontal cortex. In this image dorsal is to the top and rostral to the left. (C) Doublecortin immunopositive cells showing dendritic ramifications in layer II of the piriform cortex. In this image dorsal is to the bottom and rostral to the right. The scale bar in C = 100 μm and applies to all images.

by Spearman's rho = 0.9938, it is more likely that the cell counts are related to the perfusion delay rather than the chance of the two animals at each time point being of the same age. Secondly, our findings are unlikely to include counts of animals from the extremes of ages because we used microbats which had closed epiphyseal plates and very old bats are hardly ever captured (Brunet-Rossinni and Wilkinson, 2009). Given this, it is likely that the changes observed with perfusion delay are due to the effects of corticosterone and stress rather than old age.

Studies on standard laboratory animals often seek to eliminate any potential stressors from the protocol as it is well known that introduced stress can influence the experimental outcome (Balcombe et al., 2004). Similar care should clearly be taken when examining wild-caught species, as capture, handling and removal from a familiar environment may lead to high rates of stress (Morton et al., 1995). In the case of the microchiropterans, it appears that this has led to a false-negative report regarding the possible presence of adult hippocampal neurogenesis (Amrein et al., 2007).

Reports detailing the presence of adult hippocampal neurogenesis across mammalian species are becoming more numerous, and in each case, it would appear that adult hippocampal neurogenesis is present (reviewed in Kempermann, 2012; see also Chawana et al., 2013; Patzke et al., 2013a,b). Thus, at this stage, with the likely presence of adult hippocampal neurogenesis in the microchiropterans, this neural trait may be a common feature of mammalian brains; however, as mentioned by Kempermann (2012), certain species, such as cetaceans that live in homogeneous environments, do need to be examined to determine whether there is phylogenetic variability in this trait, which appears to be absent in the cetaceans (Patzke et al., 2013b). These variations may help to understand whether adult hippocampal neurogenesis relates to either specific aspects of the environment of the species examined (extreme heterogeneity or extreme homogeneity), or whether other explanations may account for this potential variation (Patzke et al., 2013b). Thus, at present, adult hippocampal neurogenesis may be thought of as being a likely standard feature of mammalian brains and hippocampal function, but variations as seen for cetaceans (Patzke et al., 2013b) may shed more light regarding functional aspects of this interesting neural phenomenon in the adult mammal brain.

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ADULT NEUROGENESIS IN EIGHT MEGACHIROPTERAN SPECIES

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Abstract—The present study evaluated, using immunohistochemical methods, the presence and characteristics of proliferating and newly generated neurons in the brain of eight wild-caught adult Megachiropteran species. For the neurogenic patterns observed, direct homologies are evident in other mammalian species; however, there were several distinctions in the presence or absence of proliferating and immature neurons, and migratory streams that provide important clues regarding the use of the brain in the analysis of Chiropteran phylogenetic affinities. In all eight species studied, numerous Ki-67- and doublecortin (DCX)-immunopositive cells were identified in the subventricular zone (SVZ). These cells migrated to the olfactory bulb through a Primate-like rostral migratory stream (RMS) that is composed of dorsal and ventral substreams which merge before entering the olfactory bulb. Some cells were observed emerging from the RMS coursing caudally and dorsally to the rostral neocortex. In the dentate gyrus of all species, Ki-67- and DCX-expressing cells were observed in the granular cell layer and hilus. Similar to Primates, proliferating cells and immature neurons were identified in the SVZ of the temporal horn of Megachiropterans. These cells migrated to the rostral and caudal piriform cortex through a Primate-like temporal migratory stream. Sparsely distributed Ki-67-immunopositive, but DCX-immunonegative, cells were identified in the tectum, brainstem and cerebellum. The observations from this study add to a number of neural characteristics that phylogenetically align Megachiropterans to Primates. **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: AHN, adult hippocampal neurogenesis; BrDU, bromodeoxyuridine; BSA, bovine serum albumin; DCX, doublecortin; GFAP, glial fibrillary acidic protein; NGS, normal goat serum; NRS, normal rabbit serum; PB, phosphate buffer; SGZ, subgranular zone; TMS, temporal migratory stream.

Key words: adult neurogenesis, doublecortin, Chiroptera, wild-living animals, hippocampus, immunohistochemistry.

INTRODUCTION

Adult neurogenesis refers to the process of proliferation of progenitor cells, migration of these newly born neurons, maturation with the development of functional neuronal characteristics, and integration of these neurons into existing neuronal networks (Balu and Lucki, 2009). This trait is assumed to have been inherited by mammals from the common ancestor of extant chordates (Zupanc, 2001; Kempermann, 2012). Considerable differences have been reported in the occurrence of adult neurogenesis in mammals, including animals from closely related orders, species and strains (Bonfanti and Peretto, 2011). Despite the lack of a clear understanding of the functional relevance of adult neurogenesis, it is generally thought that this process is affected by both the animal's ecology and phylogenetic history (Bartkowska et al., 2010). For example, highly social, prey organisms, with large home ranges, show high rates of adult hippocampal neurogenesis (AHN) (Hutcheon et al., 2002; Amrein and Lipp, 2009; Pravosudov and Smulders, 2010).

Chiropterans, the second largest order of mammals, have diverse behavioral and ecological characteristics (Dechmann and Safi, 2009). With life spans ranging between 10 and 40 years, Chiropterans have an average life span that is 3.5 times greater than other placental mammals of similar body mass (Wilkinson and South, 2002). The flying mammals, Megachiroptera/megabats and Microchiroptera/microbats have been grouped together in the order Chiroptera based on molecular and morphological similarities, particularly that of the musculoskeletal structure of the flying apparatus (Pettigrew et al., 1989; Adkins and Honeycutt, 1991; Baker et al., 1991; Mindell et al., 1991; Ammerman and Hillis, 1992; Bailey et al., 1992; Stanhope et al., 1992). Despite being placed in the same order, Megachiropterans and Microchiropterans have many contrasting attributes. Megachiropterans are indigenous to the Old World, large bodied and vegetarian (feed on fruit, nectar and flowers), while Microchiropterans are found throughout the world and are mostly insectivorous (Pettigrew et al., 1989). Moreover, the neuroanatomy of Megachiropterans and Microchiropterans differ substantially, with the Megachiropterans displaying brain traits shared with Primates, while the Microchiropterans have brains more similar to Insectivores (Pettigrew,

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1986; Pettigrew et al., 1989, 2008; Maseko and Manger, 2007; Maseko et al., 2007; Dell et al., 2010; Kruger et al., 2010). This variance in neuroanatomical traits between the two suborders of the Chiroptera has led to the flying Primate hypothesis of Chiropteran evolution, positing Megachiropterans as a branch of the Dermopterans and thus forming a sister group to the Primates (Pettigrew, 1986; Pettigrew et al., 1989).

A previous study revealed absent-to-low rates of AHN in Microchiropterans (Amrein et al., 2007), while a similar study examining *Epomophorus wahlbergi*, a Megachiropteran, yielded contradictory findings (Gatome et al., 2010). Proliferating and immature neurons were observed in the subgranular zone (SGZ) of the dentate gyrus and subventricular zone (SVZ) of the lateral ventricles of the Megachiropteran (Gatome et al., 2010). In the current study we investigated the occurrence of adult neurogenesis in eight wild-caught Megachiropteran species most of which had not been previously studied. We present findings of a qualitative assessment of adult neurogenesis using endogenous markers of proliferating cells (Ki-67) and immature neurons (doublecortin, DCX).

EXPERIMENTAL PROCEDURES

The brains of two individuals of the following Megachiropteran species were used in the current study: *Casinycteris argynnis* (average brain mass = 0.83 g), *Eidolon helvum* (average brain mass = 4.30 g), *Epomops franqueti* (average brain mass = 2.42 g), *Hypsignathus monstrosus* (average brain mass = 3.81 g), *Megaloglossus woermanni* (average brain mass = 0.60 g), *Rousettus aegyptiacus* (average brain mass = 2.01 g), *Scotonycteris zenkeri* (average brain mass = 0.64 g) and *E. wahlbergi* (average brain mass = 1.81 g). These animals were captured from wild populations near Kisumu, Kenya (*E. helvum* and *E. wahlbergi*), and the Yoko rainforest, near Kisangani in the Democratic Republic of Congo (the six remaining species). The appropriate permissions were obtained from the Kenya National Museums and the Kenyan Wildlife Services, and the University of Kisangani. All animals were treated and used in accordance with the University of the Witwatersrand Animal Ethics Committee guidelines (clearance number: 2008/36/1).

To minimize external influences on adult neurogenesis, the animals were anaesthetized (overdose of sodium pentobarbital, 100 mg/kg, i.p.) within 1 h of capture and perfused through the left ventricle with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was removed and post-fixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose in 0.1 M PB at 4 °C and stored in an antifreeze solution at −20 °C until sectioning. Before sectioning, the brains were divided into two halves in the mid-sagittal plane and the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4 °C. The specimen was frozen in crushed dry ice and sectioned in the sagittal plane into 50-μm thick sections. A one-in-three series of sections was stained for Nissl substance (Cresyl Violet) to reveal cytoarchitectural features, Ki-67 and DCX immunostaining to reveal proliferation of cells and immature neurons.

In this study we used antibodies to Ki-67, which is present in the nucleus during the G1 to M phases of the cell cycle, and antibodies to DCX, a microtubule-associated protein expressed during the postmitotic periods by migrating and differentiating neurons, as markers of proliferative activity and immature neurons respectively. These antibodies were previously used successfully in studies on Microchiropterans (Amrein et al., 2007) and Megachiropterans (Gatome et al., 2010). The advantage of using these markers to localize adult neurogenesis is that no pre-handling of the animal is needed. These antibodies also provide an average of the rate of expression of new neurons in natural conditions prior to capture of the animal (Bartkowska et al., 2010).

The sections were incubated in a 1.6% H_2O_2, 49.2% methanol, 49.2% 0.1 M PB solution, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10-min rinses in 0.1 M PB. To block unspecific binding sites the sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal goat serum – NGS, for the Ki-67 antibody or 3% normal rabbit serum – NRS, for the DCX antibody, plus 2% bovine serum albumin – BSA, and 0.25% Triton X-100 in 0.1 M PB). Thereafter, the sections were incubated for 48 h at 4 °C in the primary antibody solution (1:1000, rabbit anti-Ki-67, NCL-Ki-67p DAKO, Glostrup, Denmark, or 1:300, goat anti-doublecortin, DCX, SC-18 Santa Cruz Biotech, Santa Cruz, California, USA) under gentle agitation. The primary antibody incubation was followed by three 10-min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated anti-rabbit IgG, BA1000 for Ki-67, or anti-goat IgG, BA 5000 for DCX, Vector Labs, Burlingame, California, USA, in 3% NGS/NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin–biotin solution (1:125; Vector Labs), followed by three 10-min rinses in 0.1 M PB. Sections were then placed in a solution containing 0.05% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 μl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low-power stereomicroscope until the background stain was at a level that would allow for accurate architectonic matching to the Nissl sections without obscuring the immunopositive structures. Development was stopped by placing sections in 0.1 M PB for 10 min, followed by two more 10-min rinses in this solution. Sections were then mounted on 0.5% gelatine-coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody, and sections where we omitted the

secondary antibody. In both cases no staining was observed. The observed immunostaining patterns support the specificity of the antibodies as they are compatible with the observations made in another Megachiropteran (Gatome et al., 2010). It was not possible to undertake Western blot control testing, as the equipment and reagents necessary for this procedure was not available in the field.

Staining patterns of Ki-67 and DCX were observed using low-power stereomicroscope and architectonic borders were traced according to the Nissl-stained sections using a camera lucida. The Ki-67 and DCX staining patterns were then matched to the drawing from the traced Nissl-stained sections. Selected drawings were then scanned and redrawn using the Canvas 8 Software (Deneba Software, Miami, Florida, USA). Architectonic nomenclature was taken from the previously described neuroanatomical regions of the Megachiropterans (Maseko et al., 2007; Dell et al., 2010). Digital photomicrographs were captured using Zeiss Axioshop (Carl Zeiss Microscopy GmbH, Jena, Germany) and Axiovision software (Carl Zeiss Microscopy GmbH, Jena, Germany). No pixilation adjustments, or manipulation of the captured images was undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

For the quantitative analysis reported herein, cell counts of proliferating cells (those immunopositive for Ki-67) were done in the left hippocampus, one of the principal constitutive sites of adult neurogenesis, of all eight species. Ki-67+ cells numbers were obtained through physical counting using an Axioskop light microscope with a 63× objective. In all the species, immunopositive cells in every third section were quantified and the total cell count obtained through multiplying the cell counts by the inverse of the sampling fraction, a method previously used by Epp et al. (2009) and Gatome et al. (2010).

RESULTS

In the current study we evaluated the occurrence of adult neurogenesis in eight species of Megachiropterans, seven of which had not been previously examined. In all eight species the pattern of immunostaining for Ki-67 and DCX was almost identical, but we did observe some minor differences in the density of immunopositive structures between species (summarized in Table 1). We observed Ki-67-immunopositive cells in two distinct proliferative regions of the SVZ that formed dorsal and ventral migratory substreams of the RMS that coursed around the caudate nucleus before they merged to form the consolidated RMS which entered the olfactory bulb. Ki-67-immunopositive cells were also present in the SGZ of the hippocampus, the cerebral neocortex, the piriform cortex and amygdala associated with a temporal migratory stream (TMS), and in the midbrain tectum, cerebellum and medulla oblongata. DCX-immunopositive cells were observed in the SVZ and rostral and ventral migratory streams of the lateral ventricle through to the olfactory bulb, dentate gyrus of the hippocampus, cerebral neocortex, amygdala and piriform cortex. Although we observed Ki-67-immunopositive cells in the tectum, cerebellum and medulla, no DCX staining was evident in these structures. These results are summarized in diagrammatic form using *H. monstrosus* as our example (Figs. 1 and 2).

The dorsal and ventral proliferative regions of the SVZ, the rostral migratory stream (RMS) and the olfactory bulb

The SVZ was localized to the lateral walls of the lateral ventricle in all species studied, forming a thin layer over much of the medial aspect of the corpus striatum. This region of the adult Megachiropteran brain was that with the greatest amount of adult neurogenesis (Figs. 1–3). Numerous Ki-67-immunopositive cells were observed in the SVZ, with no significant differences in cell density apparent between species. Many of the cells in this region were also immunopositive for DCX, where we noted immunopositive cells with relatively short unipolar and/or bipolar processes.

The SVZ housed two proliferative regions, the dorsal and ventral proliferative regions, which were observed to merge into one migratory stream, the RMS (Fig. 3). In the current study, these regions were distinguished on topological grounds and were separated by the medial bulge of the corpus striatum into the lateral ventricle. The dorsal proliferative region of the RMS produced cells that flowed along the dorsal and rostral aspects of the caudate nucleus, between the caudate nucleus and the subcortical white matter, before they turned in a rostral direction to end in the olfactory bulb. In all the species investigated, we observed numerous intensely Ki-67- and DCX-immunopositive cells in this stream. The DCX-positive cells were fusiform in shape and small in size with short bipolar processes. Most of these cells were obscured by the numerous tangentially oriented fibers in the stream. There was no significant difference between the various species investigated.

The ventral proliferative region of the RMS produced cells that coursed along the ventromedial border of the corpus striatum, flowing between the caudate nucleus and the nucleus accumbens (Figs. 1C–G and 2C–G). It was observed to merge with the dorsal portion of the RMS at the anterior genu of the RMS, before the RMS entered the olfactory bulb (Figs. 1–3). Again, numerous Ki-67- and DCX-immunopositive cells were observed throughout the ventral portion of the RMS. The density, pattern and appearance of the immunopositive structures in the ventral portion of the RMS were comparable with that seen in the dorsal portion. Again, no specific species differences were noted. Occasional Ki-67- and DCX-immunopositive cells were observed in the corpus striatum, and while these cells had large soma, the dendrites emanating from these cells were not branched.

Ki-67-immunopositive cells were apparent in the olfactory bulb of all species investigated. *S. zeukuri*, *M. woermanni* and *C. argynnis* had lower densities of proliferating cells compared to the other species, while

Table 1. Qualitative summary of the density of immunopositive cells in various regions of Megachiropteran brains

Species	SVZ		RMS		OB		SGZ of DG		NEO		TMS		PIR		Amyg		Tectum		Cb		MO	
	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX	Ki-67	DCX
Casinycteris argynnis	+ + +	+ + +	+ + +	+ + +	+	+ + +	+	+ +	+	+ +	+ +	+ + +	+	+ + +	+	+ + +	+	-	+	-	+	-
Eidolon helvum	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+ +	+ +	+ +	+ +	+ +	+ + +	+	+ + +	+ +	+ + +	+ +	-	+ +	-	+ +	-
Epomops franqueti	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+	+	+ +	+	+ +	+ + +	+	+ + +	+	+ +	+	-	+ +	-	+	-
Hypsignathus monstrosus	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+	+ +	+	+ +	+ +	+ + +	+	+ + +	+	+ +	+	-	+	-	+	-
Megaloglossus woermanni	+ + +	+ + +	+ + +	+ + +	+	+ + +	+ +	+ +	+	+ +	+ +	+ + +	+	+ + +	+	+ +	+	-	+	-	+	-
Rousettus aegyptiacus	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+	+ +	+	+ +	+ +	+ + +	+	+ + +	+ +	+ + +	+	-	+	-	+	-
Scotonycteris zenkeri	+ + +	+ + +	+ + +	+ + +	+	+ + +	+	+ +	+	+ +	+ +	+ + +	+	+ + +	+	+ + +	+	-	+	-	+	-
Epomophorus wahlbergi	+ + +	+ + +	+ + +	+ + +	+	+ + +	+	+ +	+	+ +	+	+ + +	+	+ + +	+	+ +	+	-	+	-	+	-

Amyg, amygdala; Cb, cerebellum; CTX, cerebral cortex; DG, dentate gyrus; MO, medulla oblongata; NEO, neocortex; OB, olfactory bulb; PIR, piriform cortex; RMS, rostral migratory stream; SGZ, subgranular zone; SVZ, subventricular zone; TMS, temporal migratory stream; +, low density; + +, moderate density; + + +, high density; -, no immunopositive cells detected.

H. monstrosus and *E. franqueti* had higher cell densities. The Ki-67-immunopositive cells were mainly located in the periventricular layer of the olfactory ventricle, with occasional Ki-67-immunopositive cells distributed in the other layers of the olfactory bulb (Fig. 3). No species variance was observed with DCX immunoreactivity. In all species, intensely stained and densely packed neurons were observed in the granule cell layer, internal plexiform layer and mitral cell layer of the olfactory bulb (Fig. 3). The ovoid DCX-immunopositive cells, located mainly in the periventricular layer of the olfactory ventricle, had a relative small to large soma size with unipolar processes radiating toward the glomerular layer. The arborizations of most of the processes terminated at the mitral cell layer with occasional axons reaching the external plexiform layer. Scantly distributed DCX-immunopositive cells were observed in the external plexiform and glomerular layers of the olfactory bulb.

The SGZ and the dentate gyrus

In all Megachiropteran species a distinct SGZ, located between the granular cell layer and the hilus of the dentate gyrus of the hippocampus, was evident displaying both Ki-67-and DCX-immunopositive cells. We noted Ki-67-immunopositive cells in the SGZ of all species investigated (Fig. 4). Of the species examined, *H. monstrosus*, *E. franqueti* and *M. woermanni* had the highest densities of Ki-67-immunopositive cells. Moderate Ki-67-immunopositive cell densities were observed in *C. argynnis* and *R. aegypticus*, with fewer cells observed in *S. zeukeri*, *E. helvum*, and *E. wahlbergi* (see below). DCX-immunopositive cells, with relatively small cell bodies were also detected in the dentate gyrus. The majority of these cells exhibited bipolar processes that extended into the molecular layer of the dentate gyrus. Occasional cells had short, unipolar processes and some had no obvious processes. No apparent differences in DCX-immunopositive structures were observed between species.

Neocortex

A small stream of Ki-67-immunopositive cells were observed to emerge from the genu of the RMS and appear to travel dorsally and caudally within the deeper cortical layers and the white matter deep to the cortex (Figs. 1 and 2). This stream was restricted to the rostral half of the neocortex and throughout this region occassional Ki-67-immunopositive cells were observed appear to migrate superficially through the cerebral cortex to the supragranular layers. In layer II, small clusters of DCX-immunopositive neurons were observed in the rostral half of the neocortex. These DCX-immunopositive neurons were observed to be multipolar with extensive apical dendrites ramifying into layer I (Fig. 5). Horizontal dendritic arbours were also observed emanating from these DCX-immunopositive layer II neurons. This neocortical stream of the RMS and DCX-immunopositive layer II neurons were observed in all species investigated, with no noticeable species variance.

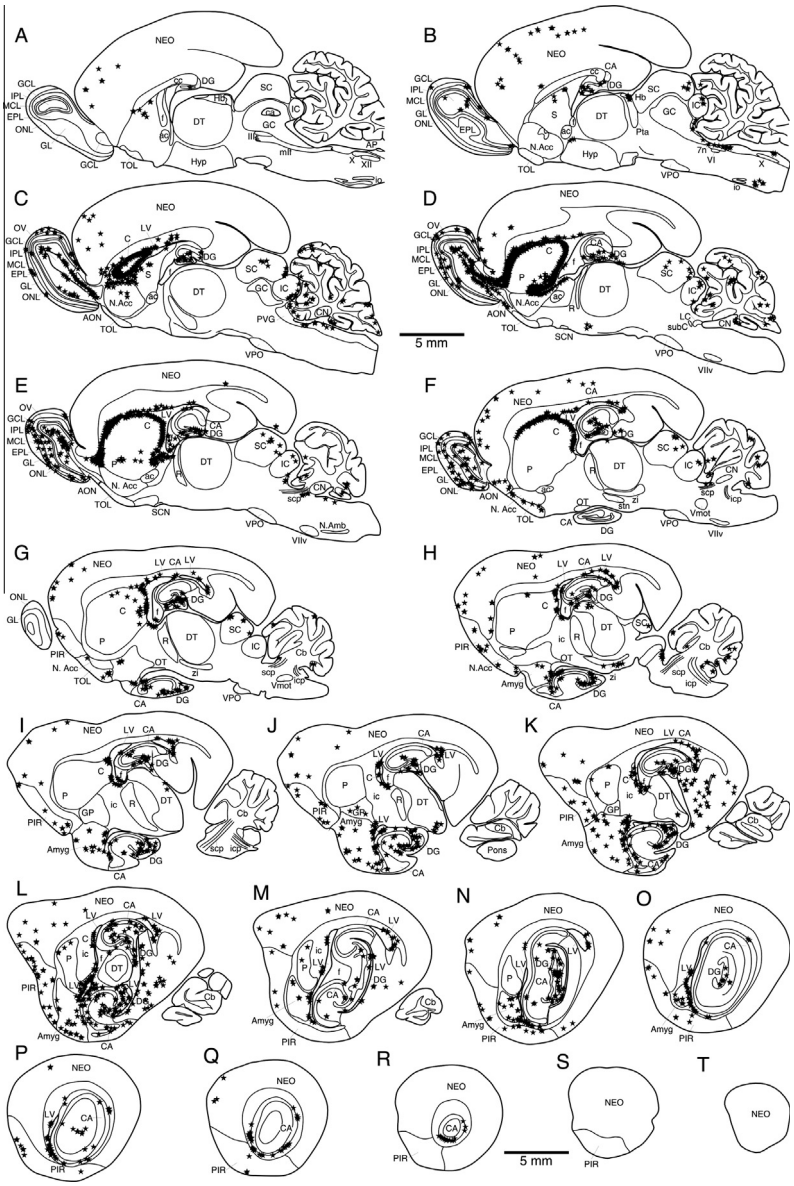

Fig. 1. Diagrammatic reconstruction of parasagittal sections through one half of the brain of *Hypsignathus monstrosus* (a representative animal of the eight Megachiroptera investigated in this study) depicting sites of the observed Ki-67 immunoreactive proliferating cells (each black star representing a single neuron). Each drawing of a parasagittal section is approximately 400 μm apart, with A being the most medial section and S being the most lateral. *Abbreviations used in figures:* 4V, fourth ventricle; 7n, facial nerve; III, oculomotor nucleus; Vmot, motor division of trigeminal nerve nucleus; VI, abducens nucleus; VIIv, ventral division of the facial nerve nucleus; X, dorsal motor vagal nucleus; XII, hypoglossal nucleus; ac, anterior commissure; Amyg, amygdala; AON, anterior olfactory nucleus; AP, area postrema; C, caudate nucleus; CA, cornu ammonis; Cb, cerebellum; cc, corpus callosum; CN, deep cerebellar nuclei; Co, cochlear nuclear complex; DG, dentate gyrus; DT, dorsal thalamus; EPL, external plexiform layer of olfactory bulb; f, fornix; GC, central gray matter of the midbrain; GCL, granular cell layer of olfactory bulb/cerebellar cortex; GL, glomerular layer of olfactory bulb; GP, globus pallidus; Hb, habenular nuclear complex; Hip, hippocampus; Hyp, hypothalamus; IC, inferior colliculus; ic, internal capsule; icp, inferior cerebellar peduncle; io, inferior olive; IPL, internal plexiform layer of olfactory bulb; LC, locus coeruleus; LV, lateral ventricle; MCL, mitral cell layer of olfactory bulb; mcp, middle cerebellar peduncle; ML, molecular layer of cerebellar cortex; mlf, medial longitudinal fasciculus; MO, medulla oblongata; N.Acc, nucleus accumbens; N.Amb, nucleus ambiguus; NEO, neocortex; OB, olfactory bulb; ONL, olfactory nerve layer; OT, optic tract; OV, olfactory ventricle; P, putamen nucleus; PCL, Purkinje cell layer of cerebellar cortex; PIR, piriform cortex; Pta, pretectal area; PVG, periventricular gray matter of the pons; R, reticular nucleus of dorsal thalamus; RMS, rostral migratory stream; S, septal nuclear complex; SC, superior colliculus; SCN, suprachiasmatic nucleus; scp, superior cerebellar peduncle; SGZ, subgranular zone; stn, subthalamic nucleus; subC, nucleus subcoeruleus; SVZ, subventricular zone; TMS, temporal migratory stream; TOL, olfactory tubercle; VPO, ventral pontine nucleus; WM, white matter of cerebellar cortex; zi, zona incerta

TMS, piriform cortex and the amygdala

In all species studied we identified a potential neurogenic region surrounding the temporal pole of the lateral ventricle, where a substantial number of Ki-67-immunopositive cells were present (Figs. 1 and 6). The Ki-67-immunopositive cells were either located adjacent to the ventricular ependyma, or were located a short distance from this region in the white matter below the

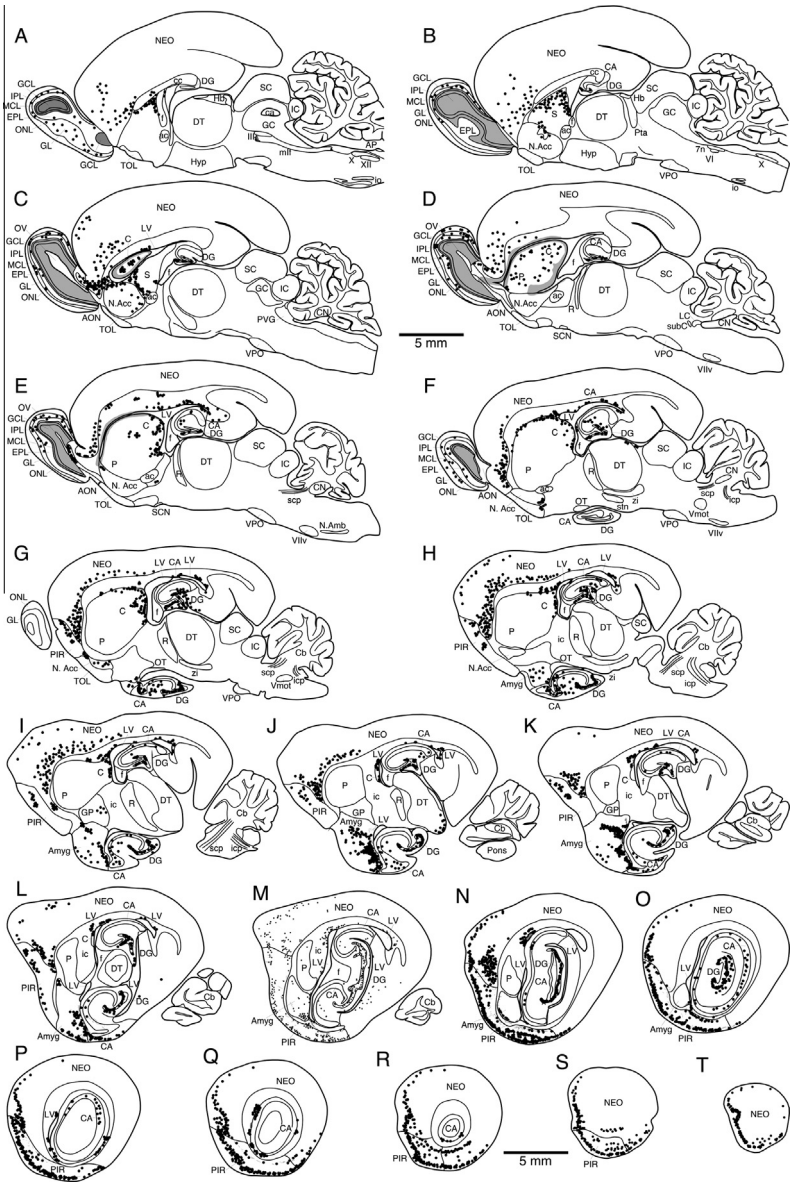

Fig. 2. Diagrammatic reconstruction of parasagittal sections through one half of the brain of *Hypsignathus monstrosus* (a representative animal of the eight Megachiroptera investigated in this study) depicting sites of the observed DCX immunoreactive immature neurons and fibers (each black dot representing a single neuron and the shaded areas representing fibers). Each drawing of a parasagittal section is approximately 400 μm apart, with A being the most medial section and S being the most lateral. See caption of Fig. 1 for abbreviations.

piriform cortex, with one or two cells observed in the amygdala. From this temporal pole neurogenic region what appears to be a significant migratory pathway, coursing both rostral (below the amygdala) and caudal (below the hippocampus), was observed with DCX immunohistochemistry (Figs. 2 and 6). This apparent migration of DCX-immunopositive cells was observed to end in the second layer of the piriform cortex, where numerous DCX-immunopositive cells with large soma and long, ramified, bipolar processes were observed in clusters reminiscent of the cytoarchitecture of this region. It appeared that this TMS exclusively supplied the entire piriform cortex with newly generated neurons. In addition, a few DCX-immunopositive cells with large soma were observed in the amygdala nuclear complex, but these cells had short unipolar or bipolar processes that exhibited no branching.

Tectum, cerebellum and brainstem

In all the Megachiropterans investigated, we observed randomly and sparsely distributed Ki-67-immunopositive cells in the superior and inferior colliculi, the periventricular gray matter of the brainstem, including area postrema, the cerebellar peduncles and cerebellar cortex (Figs. 1 and 7). These cells often existed in pairs, a possible indication of mitotic activity. The intensity of the immunostaining of the Ki-67-immunopositive cells in this region was similar to that observed in the dentate gyrus of the hippocampus and the SVZ. In contrast, we

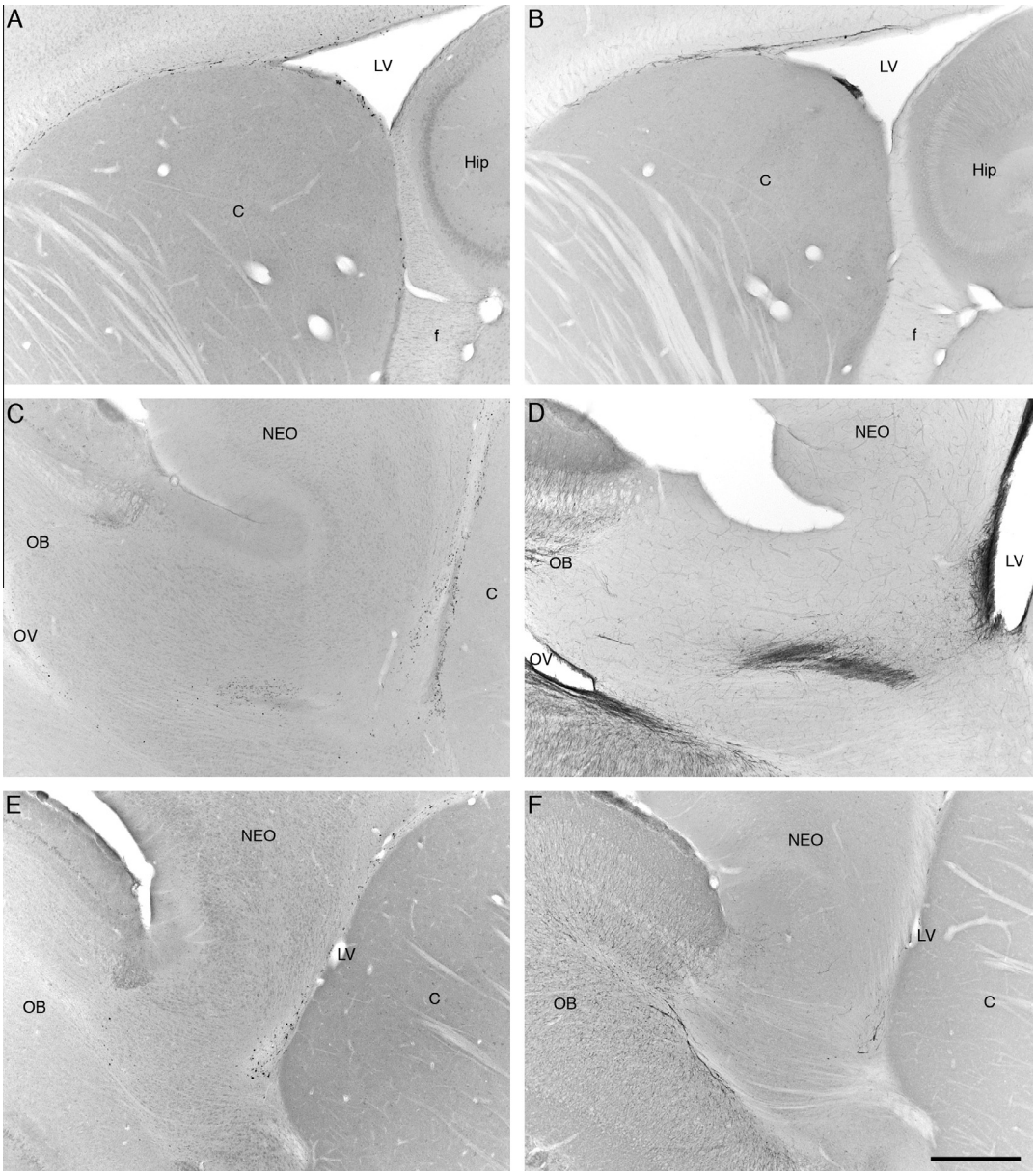

Fig. 3. Photomicrographs of Ki-67- (A, C, and E) and DCX (B, D, and F)-immunostained parasagittal sections through the rostral migratory stream in the brain of three Megachiropteran species – A, B – *Cassinycterus argynnis*, C, D – *Rousettus aegyptiacus*, E, F – *Scotonycetris zenkeri*. In all images, rostral is to the left and dorsal to the top. Note the presence of Ki-67-immunostained cells in the subventricular zone of the lateral ventricle and the extensive rostral migration of neurons to the olfactory exposed by DCX staining. Scale bar in F = 500 μm and applies to all. See caption of Fig. 1 for abbreviations.

did not observe any DCX-immunopositive cells in these regions.

Counts of Ki-67-immunopositive cells in the dentate gyrus

In all species studied, Ki-67-immunpositive cells revealed neurogenesis in the dentate gyrus. In order to test whether reported differences in life-history parameters affected the rate of AHN, we quantified the numbers of Ki-67-immunopositive cells in the left hippocampus of all eight species studied (Table 2). The results of this quantification revealed that the species with the largest number of proliferating cells was *H. monstrosus*, which is also the species with the largest brain. The remaining species with smaller brains had, for the most part, a lesser number of proliferating cells with decreasing brain mass. While it appears that there is a negative allometric relationship to this data, such that for every doubling in brain mass the number of proliferating cells in the left hippocampus increased by 1.8 times, the small dataset developed here did not allow us to find a statistically significant relationship between brain mass and number of proliferating cells (Fig. 8). Future studies adding more species to this sort of quantification should reveal a statistically significant relationship between

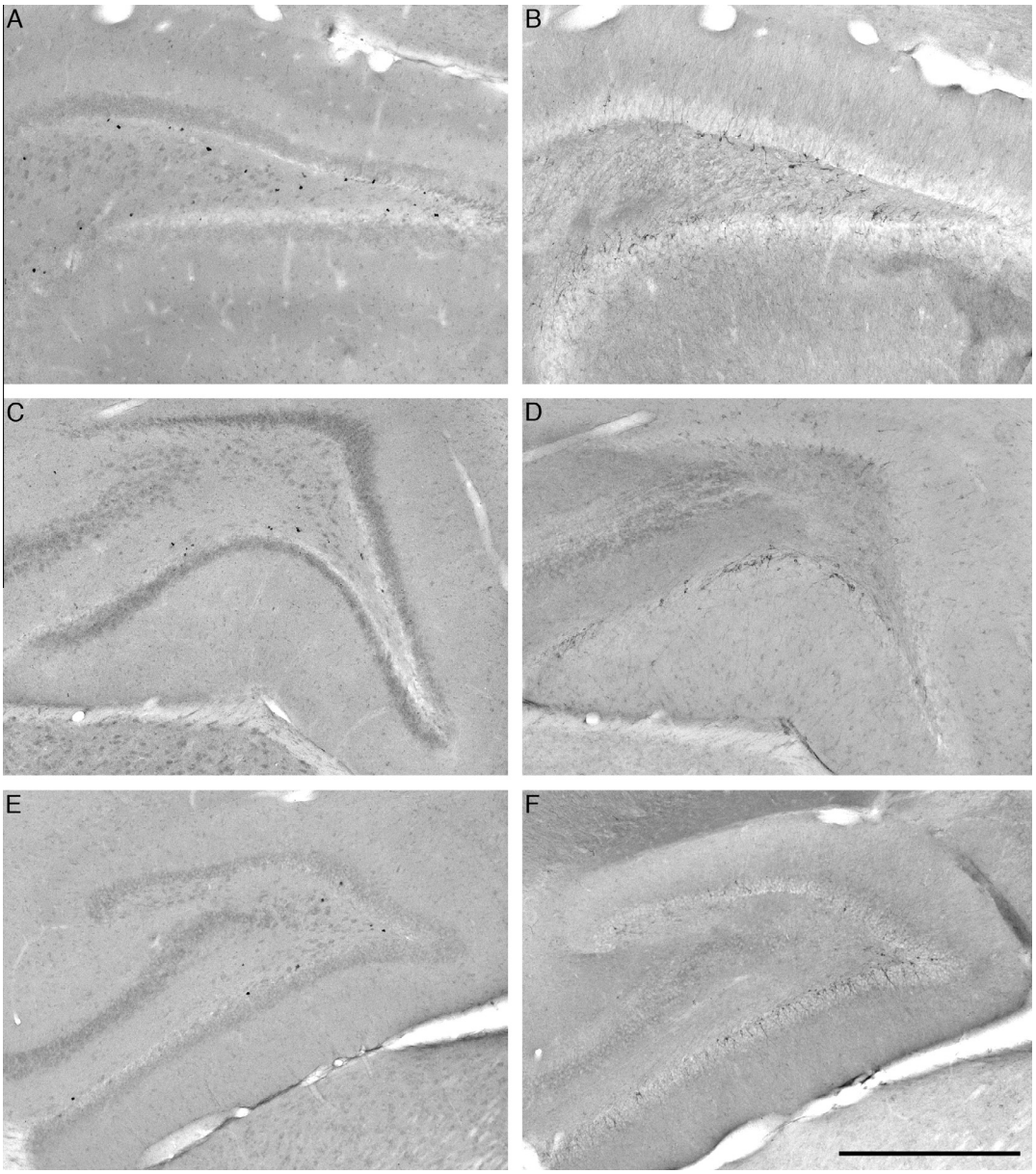

Fig. 4. Photomicrographs of Ki-67- (A, C, and E) and DCX (B, D, and F)-immunostained parasagittal sections through the dentate gyrus in the upper portion of the hippocampus proper in the brain of three Megachiropteran species – A, B – *Epomops franqueti*, C, D – *Hypsignathus monstrosus*, E, F – *Megaloglossus woermanni*. In all images, rostral is to the left and dorsal to the top. Note the presence of Ki-67-immunostained cells in the subgranular zone and the maturing DCX-immunostained cells in the granular layer of the dentate gyrus. Scale bar in F = 500 μm and applies to all.

brain mass (as a proxy for hippocampal volume) and the number of proliferating cells.

DISCUSSION

The current study evaluated the presence and characteristics of proliferating and newly generated neurons in the brain of eight wild-caught Megachiropteran species. Our observations are congruent with, and extend, a previous report of adult neurogenesis examining the brain of Wahlberg's epaulleted fruit bat, *E. wahlbergi* (Gatome et al., 2010). Proliferating cells, identified using Ki67, and immature neurons, identified using DCX, were observed in the SVZ of the lateral ventricle. These cells were seen to migrate to the olfactory bulb through the RMS. From the genu of the RMS a stream of proliferating cells populating the rostral half of the neocortex was observed in all species studied. Immature neurons were also identified in layer II of the neocortex. As in most other mammals previously investigated, immunopositive proliferating and immature neurons were also observed in the SGZ of the dentate gyrus. Unlike most mammals, proliferating cells were observed in the temporal horn of

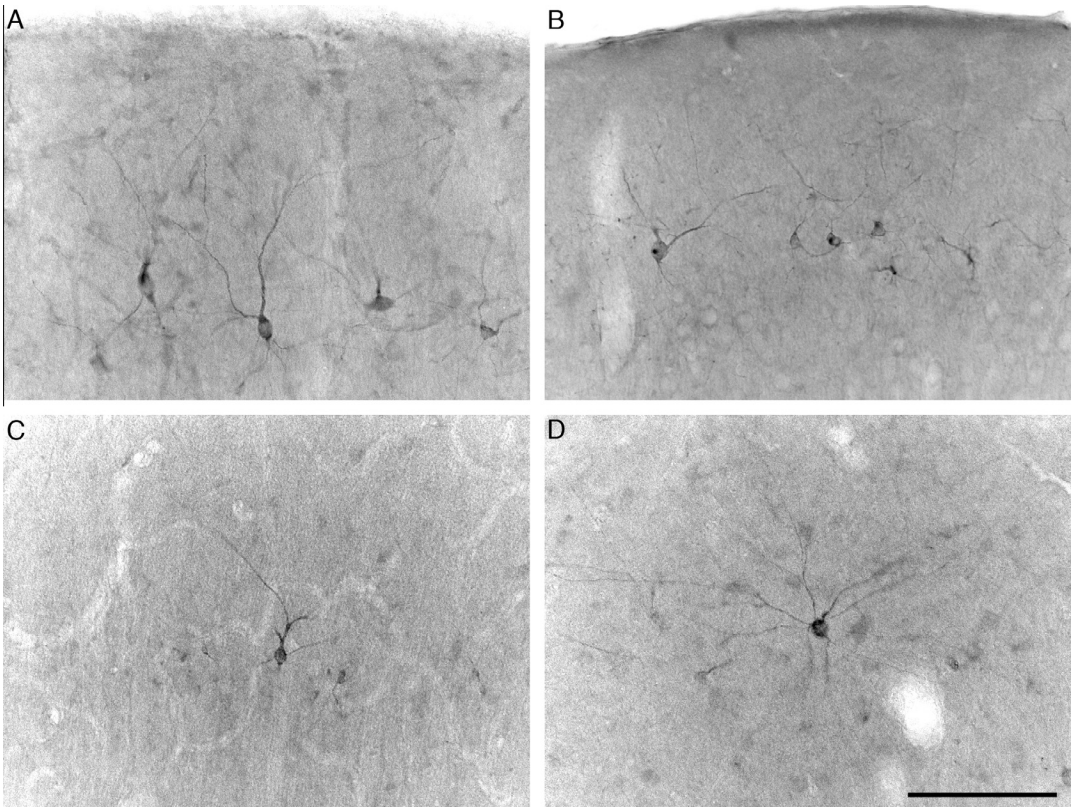

Fig. 5. Photomicrographs of DCX-immunostained parasagittal sections through the motor region of the neocortex in the brain of four Megachiropteran species – A – *Rousettus aegyptiacus*, B – *Megaloglossus woermanni*, C – *Eidolon helvum*, D – *Epomops franqueti*. In all images the pial surface is to the top. Note the presence of DCX immunoreactive cells in layer II of the neocortex, with dendrites extending horizontally in the layer and vertically into layer I. Scale bar in D = 100 μm and applies to all.

the lateral ventricle. Immature neurons appeared to migrate from this location to form the TMS that coursed rostral and caudal to supply the entire piriform cortex with immature neurons. The current study of eight Megachiropteran species, and the previous study of a single Megachiropteran species (Gatome et al., 2010), record similar results, indicating a pattern of adult neurogenesis that can be considered to be specific to the Megachiropterans. Despite this overall similarity in the neurogenic patterns and pathways, soma size, dendritic process length and branching in immature neurons varied from site to site and between species. Such variations can be linked to genetically determined variables that include various brain anatomies, developmental histories and lifespans (Bonfanti and Peretto, 2011).

SVZ and the RMS

The SVZ of the lateral ventricle (SVZ) is the principal constitutive site of adult neurogenesis in mammals, and this statement is true of the Megachiropterans. Newly generated cells arising in the mammalian SVZ are thought to be destined for the olfactory bulb and the neocortical associative areas (Gould et al., 1999). The extent, organization and cyto-architecture of the SVZ varies across mammals. In Rodents, proliferating cells originate from the anterior or rostral portion of the lateral ventricle, dorsal to the caudate nucleus (Luskin, 1993). This region of the SVZ in the Rodents has a meshwork of longitudinal astrocytic tubes enveloping proliferating cells, thereby restricting them to migrate tangentially into the RMS (Bonfanti and Peretto, 2011). In rhesus monkeys, the dorsal portion of the SVZ, capping the caudate nucleus, has a cytoarchitectural structure and exhibits cell densities that are similar to the rostral portion of the SVZ in Rodents (Pencea et al., 2001). The rostrodorsal aspect of the SVZ in Primates is thinner than the ventral SVZ and has a different cytoarchitectural structure, and as a result, proliferating cells are guided to either the dorsal or ventral substreams of the RMS (Gil-Perotin et al., 2009). The ventral part of the RMS is also called SVZa-RMS (Bedard et al., 2002) and Vf-RMS (Pencea et al., 2001), and is not found in Rodents. In the rhesus monkey, the dorsal and ventral parts of the RMS merge before turning to course into the olfactory bulb. In the present study of Megachiropterans we observed both dorsal and ventral substreams of the RMS that have a very similar appearance to that observed in the rhesus monkey. The finding of a potential neurogenic pathway/pattern in Megachiropterans that appears to be shared only with Primates provides yet another neural characteristic that suggests a shared phylogenetic history (Pettigrew, 1986; Pettigrew et al., 1989, 2008; Maseko and Manger, 2007; Maseko et al., 2007; Dell et al., 2010; Kruger

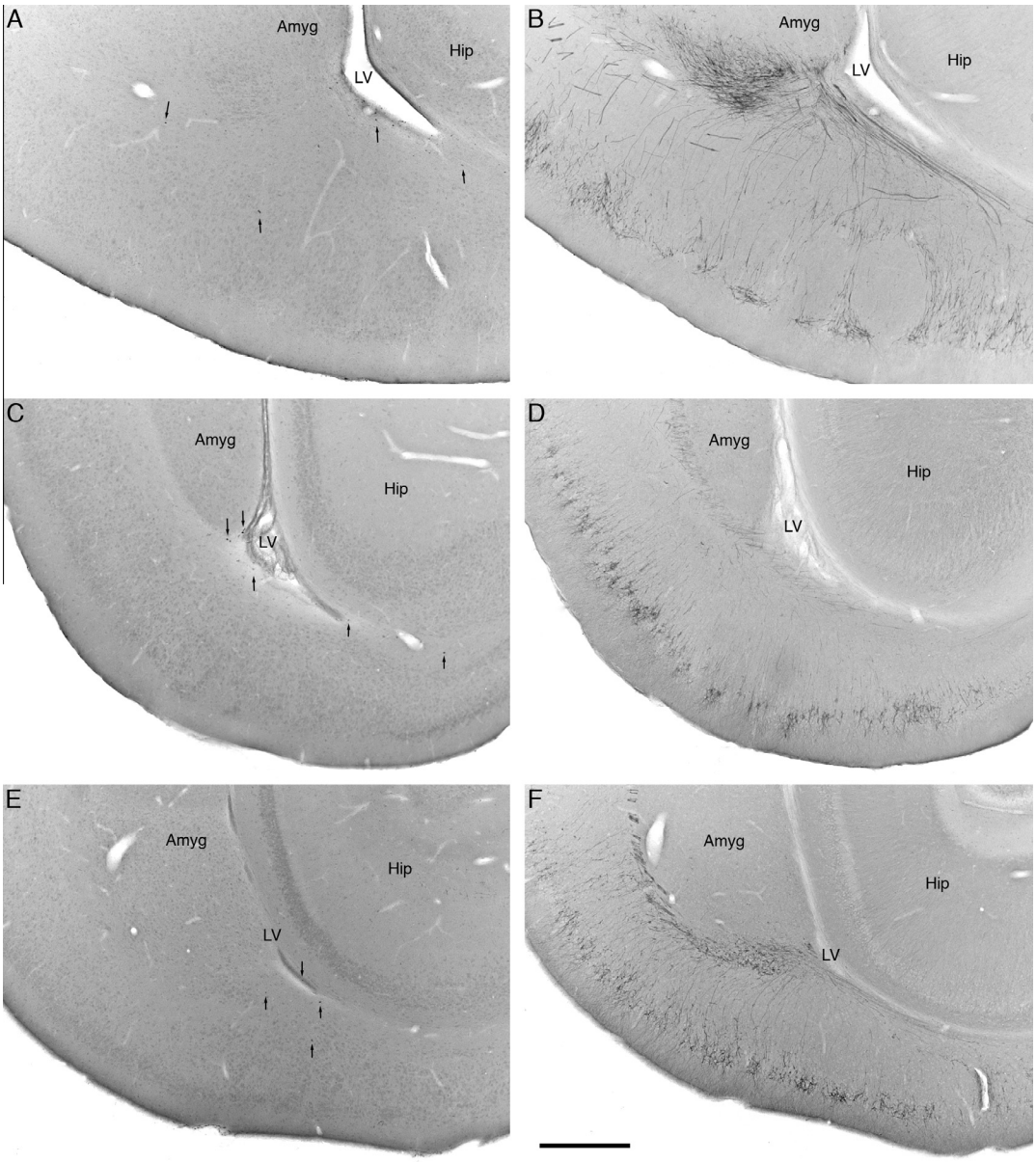

Fig. 6. Photomicrographs of Ki67- (A, C, and E) and DCX (B, D, and F)-immunostained parasagittal sections through the temporal migratory stream region in the brain of three Megachiropteran species – A, B – *Hypsignathus monstrosus*, C, D – *Casinycterus argynnis*, E, F – *Scotonycteris zenkeri*. In all images, rostral is to the left and dorsal to the top. Note the presence of Ki67-immunostained cells in the temporal pole of the lateral ventricle (arrowheads) and the extensive rostral and caudal migration of neurons to the piriform cortical regions exposed by DCX staining. Scale bar in F = 500 μm and applies to all. See list for abbreviations.

et al., 2010). It would be of interest to examine the microarchitecture of the two differing regions of the SVZ observed in Megachiropterans to determine how similar they are to that seen in Primates.

SGZ and dentate gyrus

AHN has been reported across a variety of mammals including mice (Kempermann et al., 1997), hedgehog (Bartkowska et al., 2010), monkeys (Gould et al., 1999, 2001), and humans (Eriksson et al., 1998) (see Kempermann, 2012, for a review of the occurrence of AHN across vertebrates). In the present study we observed a substantial number of Ki-67- and DCX-immunopositive neurons, with varying densities, in all Megachiropteran species studied. Similar findings were obtained in *E. wahlbergi* using bromodeoxyuridine (BrDU) and proliferating cell nuclear antigen (PCNA) (Gatome et al., 2010). Our quantification of proliferating cells in the dentate gyrus is similar to that reported previously by Gatome et al. (2010) in *E. wahlbergi*; however, our analysis of seven extra species has revealed that a negative allometry between the rate of cell proliferation and brain mass appears to be likely across mammalian species. Such an allometry would argue against the ecology and other life history

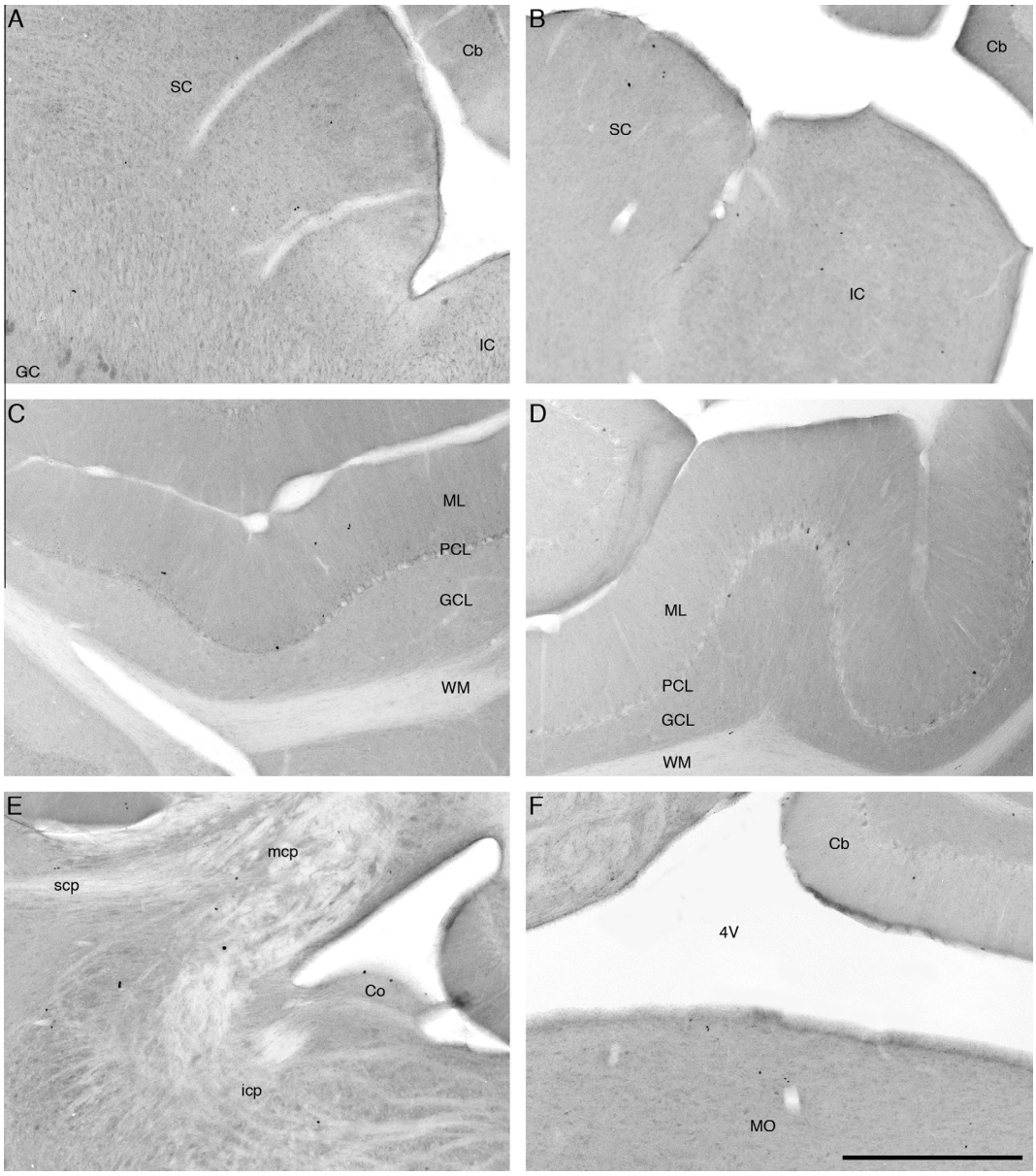

Fig. 7. Photomicrographs of Ki-67-immunostained parasaggital sections through the colliculi, cerebellum and brainstem of three species of Megachiropterans. In all images, rostral is to the left and dorsal to the top. A – Ki-67-immunostained cells in the superior colliculus of *Epomops franqueti*. B – Ki67-immunostained cells in the superior and inferior colliculi of *Megaloglossus woermanni*. C – Ki-67-immunostained cells in the cerebellar cortex of *E. franqueti*. D – Ki-67-immunostained cells in the cerebellar cortex of *Hypsignathus monstrosus*. E – Ki-67-immunostained cells in the cerebellar peduncle and surrounding brainstem of *M. woermanni*. F – Ki-67-immunostained cells in the dorsal portion of the brainstem caudal to the cerebellar peduncles in *H. monstrosus*. Scale bar in F = 500 μm and applies to all. See list for abbreviations.

parameters of a species being a determinant of the rate of cell proliferation (although it may affect cell survival), rather it argues that phylogenetic history and allometric scaling will be the principal determinants of cell proliferation rates in the mammalian hippocampus. Further studies across a range of species of vastly different brain sizes and phylogenetic relationships will reveal whether the concept proposed above explains cell proliferation rates in the mammalian hippocampus.

The findings in Megachiropterans are in sharp contrast to studies on Microchiropterans, where low to absent hippocampal neurogenesis was reported (Amrein et al., 2007). Given the proposed link of AHN to spatial memory and learning capabilities, of which all Chiropterans appear capable, the low or complete lack of new neurons in Microchiropterans could be explained by a possible short survival rate of the newly generated neurons in the Microchiropterans (Gould et al., 1999). In this sense, the low rate or absence of AHN in the Microchiroptera, but its clear presence in the Megachiropteran, adds an additional neural feature supporting the diphyletic origin of the Chiropteran lineages (Pettigrew, 1986; Pettigrew et al., 1989, 2008).

Neocortex

Our observation of clusters of DCX-positive cells in layer II of the rostral half of the neocortex of Megachiropterans,

Table 2. Quantitative summary of Ki-67-immunopositive cells in the left hippocampus of the Megachiropteran brains studied

Species	Brain mass (g)	Ki-67 count in left hippocampus
Megaloglossus woermanni	0.6	1383
Scotonycteris zenkeri	0.64	312
Casinycteris argynnis	0.83	1065
Epomophorus wahlbergi	1.81	258
Rousettus aegyptiacus	2.01	1113
Eidolon helvum	2.42	999
Epomops franqueti	2.42	4749
Hypsignathus monstrosus	3.78	6258

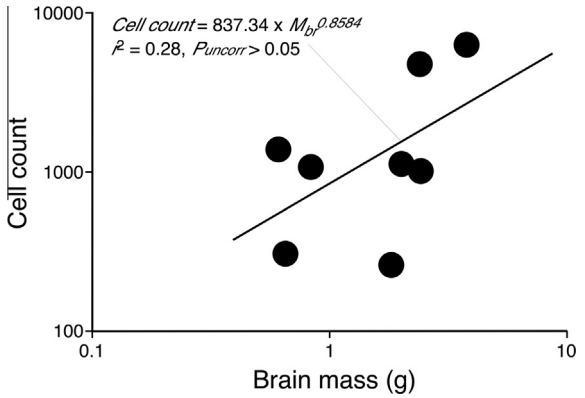

Fig. 8. Graphical representation of the potential allometric relationship between brain mass and the number of proliferating cells in the left hippocampus of the eight Megachiropteran species studied herein. While the relationship is not statistically significant, it is likely that more data points over a greater range of species with differing brain sizes will reveal a significant relationship. This small dataset indicates that the rate of cell proliferation in the hippocampus may be related to allometric scaling rules rather than being the result of ecological or other life-history parameters.

is similar to that reported in monkeys (Gould et al., 1999, 2001; Zhang et al., 2009) and cats (Cai et al., 2009). The fate of immature neurons in the neocortex remains unclear, but it is thought that some will disappear, while others will form interneurons (Gomez-Climent et al., 2008, 2010). The origin of the DCX-positive cells in the neocortex has been widely debated. It has been reported that immature neurons arising from the dorsolateral SVZ of the lateral ventricle migrate through the overlying white matter to the neocortex (Kakita and Goldman, 1999). Using BrDU in monkeys, Gould et al. (1999) identified BrDU labeled cells in the subcortical white matter which were believed to be a migratory stream from the SVZ; however, Kornack and Rakic (2001) disputed this claim and proposed that the subcortical BrDU labeled cells were endothelial cells lining longitudinally cut capillaries. In the current study of Megachiropterans, we observed Ki-67-positive cells emerging from the genu of the RMS that appeared to course dorsally and caudally to populate the neocortex (Fig. 1). This indicates that the DCX-positive cells in the neocortex are likely to have migrated along this subcortical path from the RMS. This possibility contradicts the findings that the RMS is a highly precise and exclusive migratory route from the SVZ to the olfactory bulb (De Marchis et al., 2004). It could, however, be argued that the observed migration of cells from the genu of the RMS to the neocortex is yet another phylogenetic exception, similar to the presence of the ventral proliferative region and substream of the RMS in Megachiropterans and Primates, a trait that is not identified in Rodents (Shapiro et al., 2007).

TMS

Immature neurons were observed in the secondary olfactory structures of the Megachiropterans, the amygdala and piriform cortex. These neurons appeared to be migrating from a neurogenic zone located in the temporal pole of the lateral ventricle, forming the TMS. The TMS appears to supply the entire piriform cortex, with cells coursing both rostrally and caudally from the temporal pole of the lateral ventricle. A TMS was also observed in Wahlberg's epauletted fruit bat (Gatome et al., 2010). A homologous TMS has been observed in rabbits (Bonfanti and Ponti, 2008; Luzzati et al., 2009) and monkeys (Bernier et al., 2002; Zhang et al., 2009); however, in Rodents, because the temporal horn of the lateral ventricle appears to be absent, the DCX-positive cells populating the piriform cortex are thought to migrate from the RMS forming the ventrocaudal migratory stream (Shapiro et al., 2007). The TMS has not been reported in the Microchiroptera (Amrein et al., 2007), thus, the presence of the TMS in Megachiropterans, Lagomorphs and Primates, is another neural character supporting the concept of a diphyletic origin of the Chiroptera and aligns the Megachiroptera with the Primates.

It has been proposed that the DCX-positive cells in the piriform cortex are not newly generated neurons but are immature neurons generated during a restricted developmental period, similar to neocortical immature neurons. In laboratory rats, Gomez-Climent et al. (2008, 2010) reported that the immature neurons in the piriform cortex were generated during the embryonic period and maintained their immature status into adulthood. In the present study, we identified Ki-67-immunopositive cells in the neurogenic zone of the temporal pole of the lateral ventricle, with other Ki-67-immunopositive cells having migrated to the piriform cortex and amygdala. These observations of actively dividing cells in the adult piriform cortex indicate that the observed DCX expressing cells in piriform cortex are newly generated neurons.

Other sites

Despite the identification of the SVZ and SGZ as the principal adult neurogenic sites, proliferating cells and immature neurons have also been observed in other

brain regions. These include the cortex, amygdala, striatum, substantia nigra, third ventricle, the dorsal vagal complex, and the cerebellum (Ihunwo and Pillay, 2007). In the cerebellum, a germinal matrix similar to the SVZ, the subpial layer, was reported (Bonfanti and Ponti, 2005; Ponti et al., 2006). It is located in the roof of the fourth ventricle and develops postnatally from the granule cell layer. Proliferating cells have been identified in this region (McDermott and Lantos, 1990; Bonfanti and Ponti, 2005, 2008; Ponti et al., 2006); however, no evidence of migration in the cerebellum has been recorded. In the present study, we identified Ki-67-positive cells in the tectum, brainstem and cerebellum in all eight Megachiropteran species investigated. These cells were mainly observed in the superior and inferior colliculus, periventricular gray matter of the brainstem, including the area postrema, the cerebellar peduncles and cerebellar cortex; however, we did not observe any DCX-positive cells in these regions. This result indicates a non-neuronal fate for the Ki-67-immunopositive cells in these regions. Dividing DCX-positive cells develop into neurons and dividing glial fibrillary acidic protein (GFAP)-positive cells develop into glial cells (Steiner et al., 2004). Our failure to identify DCX-positive cells in these regions possibly points to a glial lineage for these actively dividing cells. A future study using double-immunostaining for Ki67 and GFAP would answer the questions surrounding this observation in the Megachiropterans.

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