

The distribution of doublecortin immunopositive cells in the brains of four Afrotherian mammals: hottentot golden mole (*Amblysomus hottentotus*), the rock hyrax (*Procavia capensis*), the eastern rock sengi (*Elephantulus myurus*) and the four-toed sengi (*Petrodromus tetradactylus*).

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Short Title: Afrotherian adult neurogenesis; **Figures:** 8; **Tables:** 0

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ABSTRACT:

Adult neurogenesis in the mammalian brain is now a widely accepted phenomenon, typically occurring in two forebrain structures: the subgranular zone of the hippocampal dentate gyrus and the subventricular zone. Till recently the majority of studies have focused on laboratory rodents, and it is under debate whether the process of adult neurogenesis occurs outside of the subgranular zone and the subventricular zone in other mammalian species. In the present study we investigated potential adult neurogenetic sites in the brains of two elephant

shrews/sengis, a golden mole and a rock hyrax, all members of the superorder Afrotheria. Doublecortin (DCX) immunoreactivity was used as a proxy to visualize adult neurogenesis, which is expressed in neuronal precursor cells and immature neurons. In all four species densely packed DCX positive cells were present in the subventricular zone, from where cells appear to migrate along the rostral migratory stream towards the olfactory bulb. DCX immunopositive cells were present in the granular cell layer and the glomerular layer of the olfactory bulb. In the hippocampus, DCX immunopositive cells were observed in the subgranular zone and in the granular layer of the dentate gyrus, with DCX immunopositive processes extending into the molecular layer. In addition to these well-established adult neurogenic regions, DCX immunopositive cells were also observed in layer II of the neocortex and the piriform cortex. While the present study reveals a similar pattern of adult neurogenesis to that reported previously in other mammals, further studies are needed to clarify if the cortical DCX immunopositive cells are newly generated neurons or are cells undergoing cortical remodelling.

Key words: adult neurogenesis, doublecortin, Afrotheria, habitat diversity, elephant shrew, hyrax, golden mole, mammal, doublecortin, hippocampus, rostral migratory stream.

INTRODUCTION:

The generation of new neurons in the adult brain is a widely accepted phenomenon [Ming and Song, 2005; Lindsey and Tropepe, 2006; Barker et al., 2011], although the function of this evolutionarily conserved neural trait remains elusive. It has been suggested that the newly generated neurons, at least in part, are linked to learning and memory formation [Gould, 1999; Gross, 2000; Shors et al., 2001; Zupanc, 2001; van Praag et al, 2002; Kempermann, 2011]. In mammals adult neurogenesis occurs almost exclusively in two forebrain structures – the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles from where the cells migrate to the olfactory bulb [Ming and Song, 2005; Lindsey and Tropepe, 2006; Gould, 2007; Epp et al., 2009]. To date the majority of studies have focused on laboratory rodents, but it is unknown whether the process of adult neurogenesis occurs outside of the SGZ and SVZ in other mammalian species [Bonfanti and Peretto, 2011; Kempermann, 2012; Patzke et al., 2013a]. There is emerging evidence for adult neurogenesis in other brain areas including the neocortex, striatum, amygdala, substantia nigra, and piriform cortex amongst others [Gould,

2007; Shapiro et al., 2007; Bonfanti and Peretto, 2011; Patzke et al., 2013a], thus a comparative analysis of adult neurogenesis might reveal differences in adult neurogenesis correlated with behavioural specializations or adaptations to specific ecological niches [Bonfanti and Peretto, 2011]. This approach, where ecology can possibly be correlated to the occurrence of neurogenesis, is likely to yield more insight into the function of this neural feature.

The Afrotherian clade contains six mammalian orders: the elephants (Proboscidea), sea cows (Sirenia), hyraxes (Hyracoidea), aardvarks (Tubulidentata), elephant shrews or sengis (Macroscelidea), and golden moles and tenrecs (Afrosoricida). Although species within the Afrotherian superorder are very diverse in their morphology, ranging from the largest terrestrial animal, the African elephant (5,000 kg), to the small lesser long-tailed tenrec (5 g) and occupy a wide range of ecological niches, numerous molecular studies strongly support their close relationships [e.g., van Dijk et al., 2001; Arnason et al., 2008; Hallström and Janke, 2008; Prasad et al., 2008; Asher et al., 2010; Dumbacher et al., 2012; McCormack et al., 2012].

In the current study we used doublecortin (DCX) immunohistochemistry to examine potential adult neurogenesis in four Afrotherian species caught from wild populations: the hottentot golden mole (*Amblysomus hottentotus*), the rock hyrax (*Procavia capensis*), the eastern rock sengi (*Elephantulus myurus*) and the four-toed sengi (*Petrodromus tetradactylus*). The hottentot golden mole is found in the Eastern Cape region of South Africa and inhabits a wide spectrum of sub-terrestrial environments, such as temperate grasslands, savannah woodlands, coastal forests and montane marshlands [Skinner and Chimimba, 2005]. The herbivorous rock hyrax is a medium sized, social mammal that inhabits rocky outcrops in sub-Saharan Africa and the Middle East [Skinner and Chimimba, 2005]. The omnivorous eastern rock sengi is found in the north-western regions of southern Africa, where it inhabits rocky outcrops [Skinner and Chimimba, 2005; Stuart and Stuart, 1997]. The four-toed sengi is one of the most widely distributed elephant-shrew species ranging from Kenya to South Africa [Fitzgibbon, 1995] and is a forest species associated with dense undergrowth, usually in high-rainfall areas [Stuart and Stuart, 1997]. While these four species belong to the same superorder, their habitats differ vastly and may provide an interesting model to analyse the influence of ecology on adult neurogenesis.

While the presence of DCX in neurons outside of the hippocampus may or 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 also reflects cumulative adult neurogenesis over a period of 2 weeks to 6 months, although this period is species specific [Rao and Shetty, 2004; Kohler et al., 2011]. In this sense DCX immunolabelling is particularly useful when studying field-caught mammalian species, as no specific intervention is required to reveal potential sites and streams associated with adult neurogenesis.

MATERIALS AND METHODS:

Specimen and tissue preparation

In the present study brains from two *Amblysomus hottentotus* (brain mass = 1.3 and 1.2 g), two *Procavia capensis* (brain mass = 20.4 and 20.8 g), two *Elephantulus myurus* (brain mass = 1.3 and 1.19 g), and three *Petrodromus tetradactylus* (brain mass = 3.05, 2.80 and 2.95 g) were analyzed. The *E. myurus* and *P. capensis* were caught in Limpopo Province, South Africa. The *P. tetradactylus* were caught in the Yoko forest, near Kisangani, Democratic Republic of the Congo, and the *A. hottentotus* were caught in the Eastern Cape Province, South Africa. All animals were caught under appropriate governmental permissions and were used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee, which parallel those of the NIH for the care and use of animals in scientific experimentation (Clearance no. 2008/36/1).

As the specimens were caught from the wild it is difficult to assess their ages precisely; however, as we are interested in adult neurogenesis, it was important to know if the animals were adults. In order to assess the developmental status of the individuals, we compared the body mass of our specimens with data obtained from previously published literature. According to the data from the literature it would appear that our specimens are clearly adult animals. For the specimens used in the current study, the two *A. hottentotus* had body masses of 72 and 86 g, the two *P. capensis* weighed 4300 and 4500 g, the two *E. myurus* weighed 50.6 and 51.1 g, and the three *P. tetradactylus* weighed 150, 138 and 124 g. According to Skinner and Chimimba [2005] adult body mass for *A. hottentotus* ranges

between 37 – 85 g, for *P. capensis* 1500 – 4300 g, for *E. myurus* 41 – 98 g, and for *P. tetradactylus* 160 – 280 g. These body mass ranges are all from the Southern African subregion, where all but the *P. tetradactylus* were caught. Based on this body mass data, it appears that the specimens of *A. hottentotus*, *P. capensis* and *E. myurus* are clearly adults, but that the *P. tetradactylus* specimens appear to be a little small to be considered adults. In contrast to Skinner and Chimimba [2005], Silva and Downing [1995] list average male and female body masses of *P. tetradactylus* as 118.9 and 203.6 g respectively. This data would then indicate that the *P. tetradactylus* used in the current study are indeed adult. The difference in adult body masses for *P. tetradactylus* may be due to regional differences in the populations of this species.

To minimize external influences, such as handling stress, on adult neurogenesis, the animals were captured in their natural habitat and euthanized within 30 minutes of capture with a weight appropriate overdose of sodium pentobarbital (200 mg sodium pentobarbital/kg, i.p.) and perfused transcardially, first with 0.9% saline and then with 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The brains were extracted immediately after perfusion, post fixed overnight in 4% paraformaldehyde and cryoprotected in 30% sucrose in 0.1 M PB at 4°C. The specimens were subsequently stored at -20°C in an antifreeze solution until processing.

Tissue staining and immunohistochemistry

The brains were examined immunohistochemically using antibodies directed against the intrinsic marker doublecortin (DCX). DCX is a microtubule-associated phosphoprotein that is expressed for up to 2-3 weeks in actively dividing neuronal precursor cells and their neuronal daughter cells [Brown et al., 2003]. The use of DCX as a marker is advantageous in that it minimises pre-handling of animals while providing an average rate of expression of new neurons in natural conditions prior to capture of the animal [Bartowska et al., 2010]. Goat anti-DCX (C-18, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.) was used to visualise DCX, as this antibody has been previously demonstrated to provide distinct labelling in rodents, humans and other mammals [Brown et al., 2003; Liu et al., 2008; Ngwenya et al., 2011; Patzke et al., 2013a,b].

All immunolabelling procedures were performed on free-floating sections. Prior to sectioning, the brains were equilibrated in 30% sucrose in 0.1M PB at 4°C for 72 h and then

frozen in crushed dry ice. The specimens were cryosectioned on a sliding microtome into 50 μm thick sections in the sagittal plane. Alternate sections were stained for Nissl substance, using 1% cresyl violet, or immunohistochemically for DCX. The immunohistological sections were pre-treated for 30 min at room temperature under gentle shaking with an endogenous peroxidase inhibitor (49.2% 0.1M PB, 49.2% methanol, 1.6% of 30% H_2O_2). Following three 10 min rinses in 0.1 M phosphate buffer (PB), the sections were subsequently pre-incubated in a blocking buffer solution (3% normal rabbit serum, 2% bovine serum albumin, 0.25% Triton X-100 in 0.1 M PB) for 2 h under gentle shaking at room temperature to prevent non-specific binding. Sections were then transferred into a primary antibody solution (1:300, goat anti-DCX, in the blocking buffer solution) and were incubated for 48 h at 4°C under gentle shaking. Following incubation, sections were subjected to three 10 min rinses in 0.1M PB before being incubated in secondary antibody solution. The secondary antibody contained a 1:1000 dilution of biotinylated anti-goat IgG (BA-5000, Vector labs, Burlingame, California, U.S.A.) in 3% normal rabbit serum and 2% BSA in 0.1M PB for 2 h at room temperature, under gentle shaking. Following three 10 min rinses in 0.1M PB, the sections were incubated in an Avidin-Biotin solution (1:125 A reactive and 1:125 B reactive, Vector labs, Burlingame, California, U.S.A., in 0.1M PB) for 1 h. The sections were transferred into three 10 min 0.1M PB rinses before being placed in a solution containing 0.05% diaminobenzidine (DAB) in 0.1M PB for 5 min. To each 1 ml of this solution, 3.3 μl of 30% H_2O_2 was added, and chromatic precipitation was visually monitored under a low power stereomicroscope. Development was subsequently arrested by placing the sections in 0.1M PB, followed by a final 10 min rinse in 0.1M PB. Sections were mounted on 0.5% gelatinized slides, left to dry overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure non-specific staining of the immunohistochemical protocol, control sections taken at random were processed in the same manner, but either the primary or secondary antibody was omitted. No labelled cells were observed in either case.

Data Analysis

Sections were analyzed qualitatively with both low and high power microscopy to yield a comparative description of the distribution of DCX positive neurons. Using a stereomicroscope with an attached camera lucida, the architectonic borders were traced according to the Nissl stained sections. The corresponding immunostained sections were then matched to the drawings and the immunopositive DCX neurons were marked. Selected

drawings were then scanned and redrawn using Canvas 8 software. 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.

Abbreviations

VIIv – ventral division of facial nerve nucleus

ac – anterior commissure

Amyg – amygdala complex

AOB – accessory olfactory bulb

AON – anterior olfactory nucleus

C – caudate nucleus

CA – cornu ammonis region of hippocampus

Cb – cerebellum

cc – corpus callosum

Cing – cerebral cingulate cortex

CN – deep cerebellar nuclei

Co – cochlear nucleus

DG – dentate gyrus of hippocampus

DT – dorsal thalamus

f - fornix

GC – central gray matter

GP – globus pallidus

GPe – globus pallidus external division

GPi – globus pallidus internal division

Hbm – medial habenular nucleus

Hyp - hypothalamus

IC – inferior colliculus

icp – inferior cerebellar peduncle

LGd – dorsal lateral geniculate nucleus

LOT – lateral olfactory tract
LV – lateral ventricle
mcp – middle cerebellar peduncle
MG – medial geniculate body
N.Acc – nucleus accumbens
NEO – cerebral neocortex
OB – main olfactory bulb
OC – optic chiasm
OT – optic tract
P – putamen nucleus
PIR – cerebral piriform cortex
Pta – pretectal area
R – thalamic reticular nucleus
RMc – red nucleus, magnocellular division
RMS – rostral migratory stream
SC – superior colliculus
SN – substantia nigra
scn – suprachiasmatic nucleus
scp – superior cerebellar peduncle
TOL – olfactory tubercle
VPO – ventral pontine nucleus
zi – zona incerta

RESULTS:

In the present study we revealed neurons immunoreactive to the endogenous marker doublecortin (DCX) in *A. hottentotus*, *P. capensis*, *E. myurus* and *P. tetradactylus*. Our DCX staining revealed the two commonly reported neurogenic areas, the subventricular zone of the lateral ventricles that gives rise to the rostral migratory stream ending in the olfactory bulb, and the subgranular zone of the hippocampal dentate gyrus. Furthermore, the presence of

DCX positive cells provided evidence of immature or remodelling neurons in cortical brain regions.

Doublecortin immunoreactivity in the hippocampal formation

In all four species examined, a large number of DCX immunopositive neurons were observed at the base of the granular cell layer, in the subgranular zone, which was located between the granular cell layer and the polymorphic layer of the dentate gyrus (Figs. 1-5). These immunopositive neurons were characterized by large, ovoid somata with ramified dendrites extending into the molecular layer (Fig. 1). Occasional DCX immunopositive fibres were observed in the hilus. No apparent differences in DCX immunoreactivity were observed between species in the dentate gyrus. In *P. tetradactylus* densely packed DCX immunopositive processes were observed superior to the stratum pyramidale of the cornu ammonis (CA3), presumably mossy fibres of the newly generated granular cells.

DCX immunoreactivity in the subventricular zone (SVZ) of the lateral ventricle, the rostral migratory stream (RMS) and the olfactory bulb

In all four species clusters of DCX positive cells and processes were present in the subventricular zone (SVZ) with the highest density of immunolabelled structures observed towards the rostral end of the lateral ventricle (Figs. 2-5). The labelled cells were characterized by relatively short unipolar and or/bipolar processes. From the SVZ a stream of DCX immunopositive cells could be observed, which we ascribe to the rostral migratory stream (RMS). The RMS originated from the SVZ at the rostral pole of the lateral ventricle, with DCX immunopositive cells found between the dorsorostral aspects of the caudate nucleus and the subcortical white matter. At the rostroventral pole of the caudate nucleus, the “stream” of immunolabelled cells appeared to turn in a rostral direction with the stream ending in the olfactory bulb (Figs. 2-5). The DCX immunopositive cells in the RMS were often obscured by the numerous tangentially oriented fibres of the stream, but when readily viewable were found to be fusiform in shape, small in size and displayed bipolar processes.

In the olfactory bulb (OB) DCX immunoreactivity was evident in all layers in all four species. The majority of DCX-expressing cells were located in the granular cell layer (GCL), exhibiting radially orientated DCX-positive cells and processes (Figs. 2-6). Most of these

cells were bipolar and ovoid in shape. The external plexiform layer of the olfactory bulb (EPL) presented with distinct radial fibres, while the glomerular layer (GL) displayed sparsely distributed DCX immunopositive cells that presumably represent periglomerular cells. There was no evidence of a neurogenic site within the olfactory ventricle, and thus it is assumed that the DCX immunoreactive structures visible in the olfactory bulb are those arising from the rostral migratory stream. In *P. tetradactylus*, DCX positive cells were also visible in the anterior olfactory nucleus (Fig. 5).

DCX immunostaining in the piriform cortex and endopiriform nucleus

In the two elephant shrews, rock hyrax and the golden mole, DCX-immunopositive cells were observed in layer II of the piriform cortex (PIR) (Figs. 2-5). These DCX positive cells in the PIR appear to arise from the SVZ of the ventral portion of the lateral ventricle from where they migrate along the rostral border of the striatum through layer III towards layer II. These DCX immunopositive cells were numerous in the piriform cortex and were densely packed, in clusters, in layer II (Fig. 7). These cells were mostly bipolar or multi-polar in shape, but occasional unipolar cells were present. These DCX immunopositive cells had long processes that were moderately to highly ramified and many of these ramifications extended into layer I. In *P. tetradactylus* loosely packed DCX immunopositive cells with a loosely arranged network of long dendrites were present in the endopiriform nucleus located just dorsal to the piriform cortex. These cells were larger and showed either bipolar or multipolar morphologies.

DCX immunoreactivity in the cerebral neocortex

In the rostral half of the cerebral neocortex, all four species displayed DCX immunopositive cells in layer II (Figs. 2-5, 8), although the extent of these neurons was somewhat less in *A. hottentotus*. In contrast, in *P. capensis* the presence of these DCX immunopositive cortical cells was not restricted just to the rostral half of the neocortex, but could be found throughout the entire neocortical mantle. These cells were readily observed and displayed a diversity of neuronal morphology. The majority appeared to be multipolar with extensive apical dendrites ramifying into layer I, but some horizontal dendritic arbours were also observed (Fig. 8). These DCX immunoreactive cells were predominantly ovoid in shape, but some pyramidal shaped somas were noted.

DISCUSSION:

In the present study we examined DCX immunoreactivity in the adult brains of four different Afrotherian species as a proxy marker for adult neurogenesis or neuronal remodelling. In agreement with previous studies on mammals, DCX immunopositive neurons were found in the two commonly identified regions of adult neurogenesis, the subgranular zone of the dentate gyrus in the hippocampal formation and the subventricular zone of the lateral ventricle that gives rise to the rostral migratory stream which ends in the olfactory bulb. Additionally DCX immunopositive cells were observed in the endopiriform nucleus of one species, and the piriform cortex and neocortex of all species studied. As with our previous observation on other Afrotherian species [Ngwenya et al., 2011; Patzke et al., 2013a,b] no Ki-67 immunoreactivity was observed in the present study, hence we can only make limited suggestions about the proliferation of the newly generated neurons. The non-reactivity of the DAKO Ki-67 antibody (NCL-Ki-67 P) in Afrotherians seems to be related to the phylogenetic specificity of the antibody, and hence might only show reactivity in rodents, megachiropterans and primates [Chawana et al., 2013; Vessal and Darian-Smith, 2010; Wojtowicz and Kee, 2006] without the use of antigen retrieval techniques.

Adult hippocampal neurogenesis and the effect of natural habitats

There is a large body of evidence indicating that the environment of an animal can influence adult hippocampal neurogenesis (AHN). An enriched environment was demonstrated to increase the rate of AHN, whereas an impoverished environment is associated with a decline in ANH. AHN has also been seen to be influenced by stress, exercise, learning and social conditions [Kempermann et al., 1997; van Praag et al., 1999a,b; Olson et al., 2006; Snyder et al., 2009; Gould and Cameron, 1996; Gould et al., 1997, 1998; Pham et al., 2003; Warner-Schmidt and Duman, 2006; Lu et al., 2003]; however, these studies were all conducted on laboratory rodents and thus might have only limited relevance to the natural setting of wild living animals [Konefal et al., 2013]. The question arises whether different environments have a general influence on AHN and hence animals living in a less stimulating/homogenous natural environment/habitat would show a reduced rate of AHN in comparison to animals that live in a highly diverse habitat. Species of the Afrotherian suporder, because they are genetically related, but inhabit different ecological niches and are very diverse in their brain and body size make an interesting set of animals

with which to address this question. The two elephant shrews, rock hyrax and the golden mole analysed in this study, as well as the giant other shrew, a semi aquatic Afrotherian previously examined [Patzke et al., 2013a], show, qualitatively, similar amounts of DCX immunoreactivity in the hippocampus. In the African elephant [Patzke et al., 2013b], DCX immunoreactive cells were present at lower density in comparison to the other Afrotherian species examined, but this could be related to either the age (25 years), since age was demonstrated to be one factor to influence AHN [Seki and Arai, 1995; Kuhn et al. 1996], or to the overall size of the hippocampus, which is several times larger in the elephant than the other Afrotherian species studied [Patzke et al., 2013b]. Despite these potential differences, our results suggest that the different natural environments inhabited by the different species might have little effect on the number of newly generated neurons in the dentate gyrus observed during migration and maturation, the stages of adult neurogenesis that are visualised by DCX expression. Even though it is evident that short-term changes in the environment have an effect in the laboratory setting, the different ecological niches with their diversity in environmental stimuli do not appear to have an influence on the overall rate of AHN. Hence, it would appear that it is not environmental complexity itself that directly effects neurogenesis, but rather the individual interactions between each species and its' respective environment. As discussed by Kempermann [2012], new neurons may provide the cognitive adaptability required to be able to successfully survive in different ecological niches. Thus, it would appear that basal levels of adult hippocampal neurogenesis in different mammalian species are dependent upon two factors (besides intrinsic factors like age and genotype): (1) the size and or the neuronal numbers found within the hippocampus; and (2) the phylogenetic history of the animal under study. In addition, short-term up or down regulations in the rate of AHN in response to the novel interactions of a mammal with its environment is likely to affect rates of adult neurogenesis within the hippocampus (extrinsic factors).

Neurogenesis in olfactory areas

Throughout adulthood the olfactory bulb incorporates new neurons that arise in the SVZ of the lateral ventricle and migrate along the RMS to the olfactory bulb. From the periventricular layer of the olfactory bulb, these new neurons migrate radially into the granular and glomerular layers where they become functionally integrated into the olfactory bulb circuitry [Peretto et al., 1997; Bedard and Parent, 2004; Lledo et al., 2006]. This continuous supply of new neurons to the olfactory bulb has been reported in all mammalian species studied to date including the four Afrotherian species studied herein [e.g. Pencea et

al., 2001; Bedard et al., 2002; Bedard and Parent, 2004; Alpár et al., 2010; Bartowska et al., 2010; Ngwenya et al., 2011; Patzke et al., 2013a,b]; however, this rostral migration seems to be absent in humans [Eriksson et al., 1998; Bergmann et al., 2012], with some studies suggesting that newly generated neurons in the human olfactory bulb are generated locally [Bedard and Parent, 2004].

In addition to the immature olfactory bulb neurons, immature or remodelling neurons, as revealed with DCX immunohistochemistry, were observed in the secondary olfactory structures of the species examined: endopiriform nucleus (*P. tetradactylus*) and layer II of the piriform cortex (*P. capensis*, *P. tetradactylus*, *E. myurus* and *A. hottentotus*). These findings agree with previous reports in mice and rats [Shapiro et al., 2007], primates [Gould et al., 1999], moles and hedgehogs [Bartowska et al., 2010], the hedgehog tenrec [Alpár et al., 2010] and the giant otter shrew [Patzke et al., 2013a]. In the four Afrotherian species analyzed in this study, DCX immunopositive neurons appear to emanate from the SVZ at the caudoventral portion of the lateral ventricle and migrate towards the piriform cortex. A migration from the SVZ towards the piriform cortex was previously observed in rodents [Shapiro et al., 2007], non-human primates [Bernier et al., 2002], megachiropteran bats [Chawana et al., 2013], the giant otter shrew [Patzke et al., 2013a] as well as moles and hedgehogs [Bartowska et al., 2010]. In rodents [Shapiro et al., 2007] and in the moles and hedgehogs [Bartowska et al., 2010] these newly generated cells appear to emanate from the RMS and migrate along a ventrolateral migratory stream towards the piriform cortex. In contrast, in primates [Bernier et al., 2002] and megachiropterans [Chawana et al., 2013] the cells seem to emanate from the temporal horn of the lateral ventricle and migrate along the temporal stream to the piriform cortex. In the species studied herein, and the giant other shrew [Patzke et al., 2013a], the newly generated neurons seem to migrate from the SVZ of the caudal portion of the lateral ventricle towards the PIR, as seen in rodents. Our findings, together with the previous studies, indicate that the DCX neurons in the PIR are not locally generated or remodelling neurons, but rather arise from the SVZ of the lateral ventricles from where they migrate to the PIR; however, local proliferation and/or remodelling cannot be ruled out at this stage. As in the giant other shrew [Patzke et al., 2013a], in *P. tetradactylus* DCX immunoreactive neurons, were also observed in the endopiriform nucleus, seemingly supplied by the migratory stream from the SVZ of the lateral ventricle; however, no DCX immunopositive cells were observed in endopiriform nucleus in the the other three Afrotherian species analyzed.

Doublecortin immunoreactive neurons in the cerebral neocortex – new or remodelling neurons?

The presence of DCX immunoreactive neurons in layer II of the cerebral neocortex in Afrotherians is in accord with reports in rodents [Kutsuna et al., 2013], primates [Bloch et al., 2011; Zhang et al., 2009], cats [Cai et al., 2009], megachiropteran bats [Chawana et al., 2013], guinea pigs [Xiong et al., 2008] and the giant otter shrew [Patzke et al., 2013a]. Recent studies demonstrated that DCX positive cell in the layer II of the neocortex, using double labelling with neuronal markers, are of a neuronal identity, rather than glial, since no double labelling was observed with glial markers [Xiong et al., 2008]; however, it is still under debate if these DCX positive cells are newly generated, are generated during development and remain in an immature state, or are mature neurons undergoing neuronal remodelling. Previous studies using BrdU and neuronal markers suggest that the immature neurons in the neocortex arise from the SVZ of the lateral ventricle and migrate through the subcortical white matter towards layer II of the neocortex [Kakita and Goldman, 1999; Gould et al., 1999; Gould et al., 2001]. In contrast Kornack and Rakic [2001] proposed that the newly generated cells in the cortex are rather endothelial cells lining longitudinally cut capillaries, since they failed to verify the neuronal character of the newly generated cells. In the current study in all 4 Afrotherian species as well as in the giant other shrew [Patzke et al., 2013a], using DCX immunoreactivity, no stream of presumably newly generated neurons from the SVZ towards the neocortex could be observed; however, this does not exclude that these cells might be generated in the SVZ. In addition, in the rock hyrax DCX positive cells were not restricted to the rostral portion of the neocortex but were present throughout the entire neocortex. Future studies are needed to clarify if these DCX immunopositive cells in the layer II of the neocortex are newly generated or remodelling, using improved birth-dating methodology.

Acknowledgements:

This work was mainly supported by funding from the South African National Research Foundation (P.R.M., N.C.B.), the Swiss-South African Joint Research Program (P.R.M.), the Belgian co-operation service (D.G.D.) at the Royal Museum for Central Africa (E.G.), and by a fellowship within the Postdoctoral-Program of the German Academic Exchange Service, DAAD (N.P.).

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Figure Legends:

Figure 1: High-power photomicrographs of DCX-positive cells located in the subgranular zone and the granular layer of the dentate gyrus of four Afrotherian species. **(A)** hottentot golden mole (*Amblysomus hottentotus*), **(B)** eastern rock sengi (*Elephantulus myurus*), **(C)** four-toed sengi (*Petrodromus tetradactylus*), and **(D)** rock hyrax (*Procavia capensis*). Scale bar in **D** = 100 μ m and applies to all.

Figure 2: A series of sagittal drawings from the brain of the hottentot golden mole (*Amblysomus hottentotus*) showing the location of doublecortin immunopositive cells (dots, where one dot represents one cell). **A** is medial, **E** is lateral, each figurine being approximately 1 mm apart. Note the presence of DCX immunopositive neurons in the hippocampus, rostral migratory stream and olfactory bulb, piriform cortex and neocortex. See list for abbreviations.

Figure 3: A series of sagittal drawings from the brain of the rock hyrax (*Procavia capensis*) showing the location of doublecortin immunopositive cells (dots, where one dot represents one cell). **A** is medial, **F** is lateral, each figurine being approximately 3 mm apart. Note the presence of DCX immunopositive neurons in the hippocampus, rostral migratory stream and olfactory bulb, piriform cortex and throughout the neocortex. See list for abbreviations.

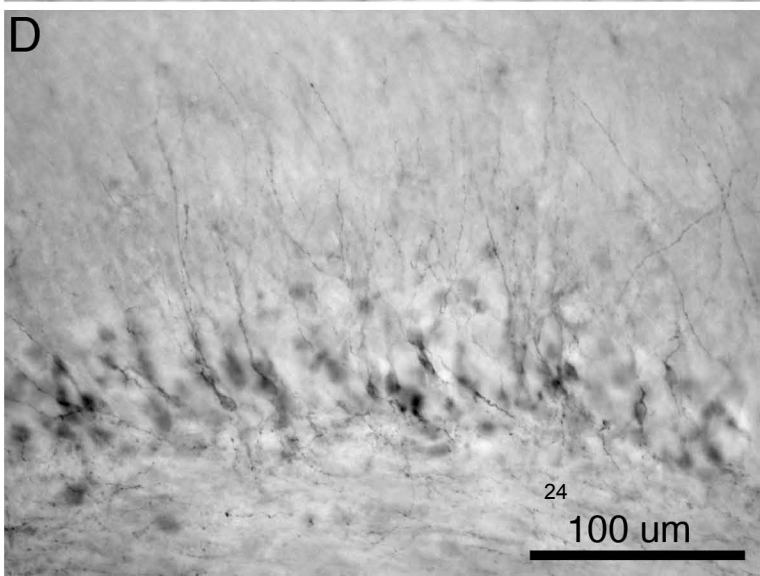
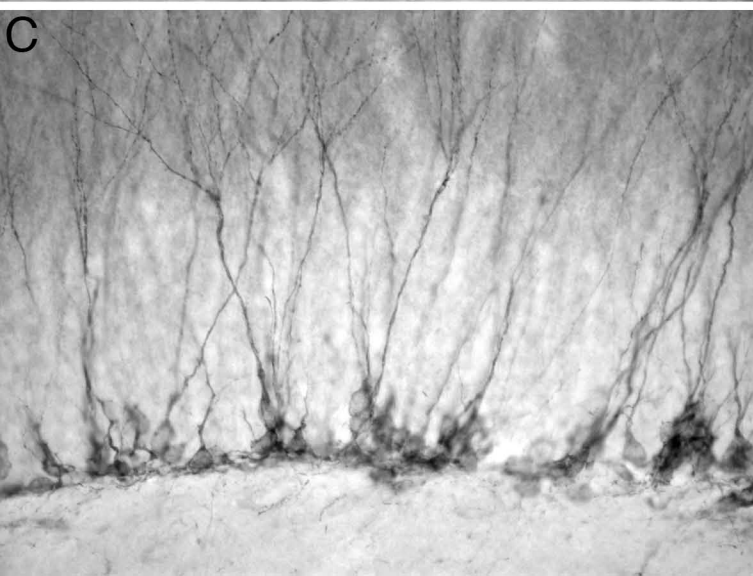
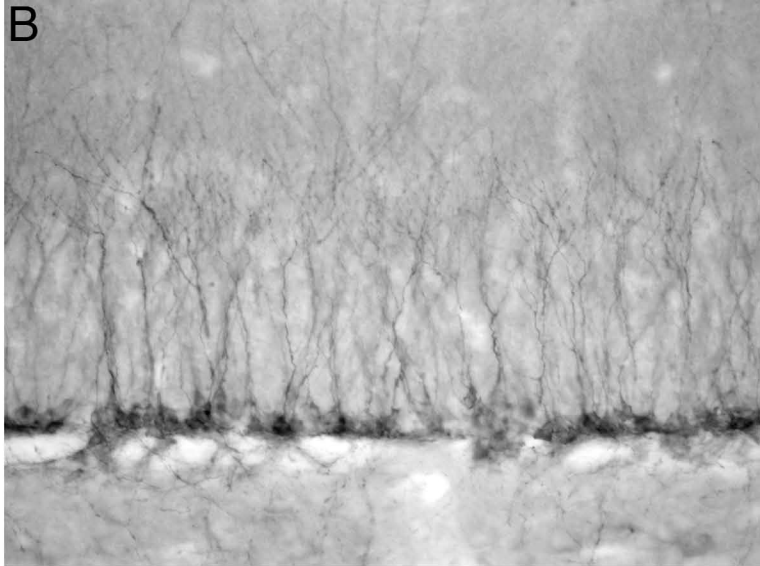
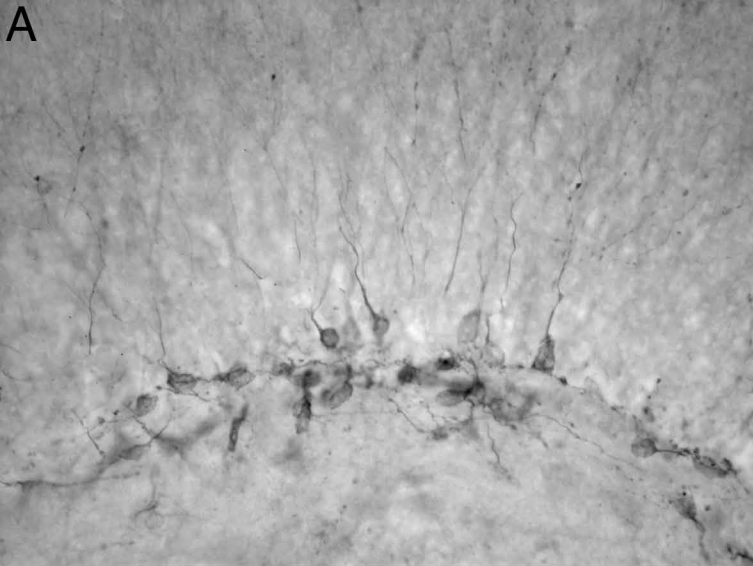
Figure 4: A series of sagittal drawings from the brain of the eastern rock sengi (*Elephantulus myurus*) showing the location of doublecortin immunopositive cells (dots, where one dot represents one cell). **A** is medial, **F** is lateral, each figurine being approximately 1.5 mm apart. Note the presence of DCX immunopositive neurons in the hippocampus, rostral migratory stream and olfactory bulb, piriform cortex and the rostral half of the neocortex. See list for abbreviations.

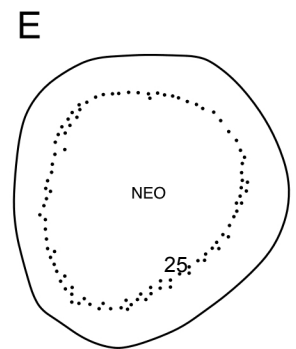
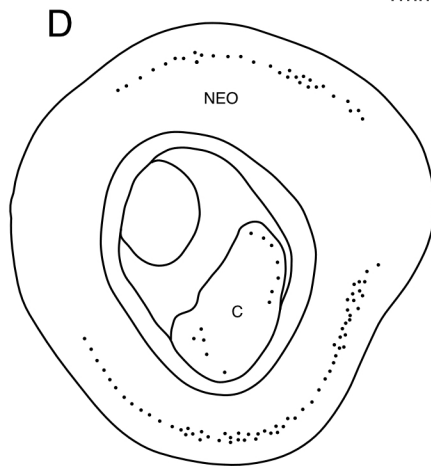
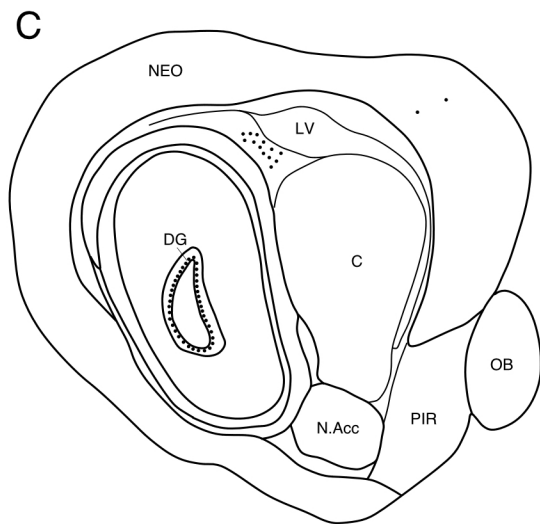
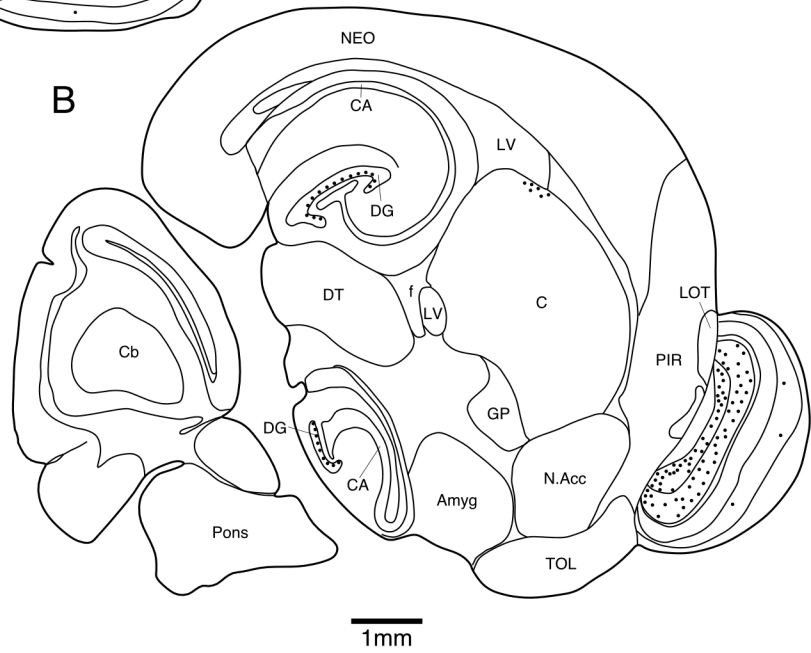
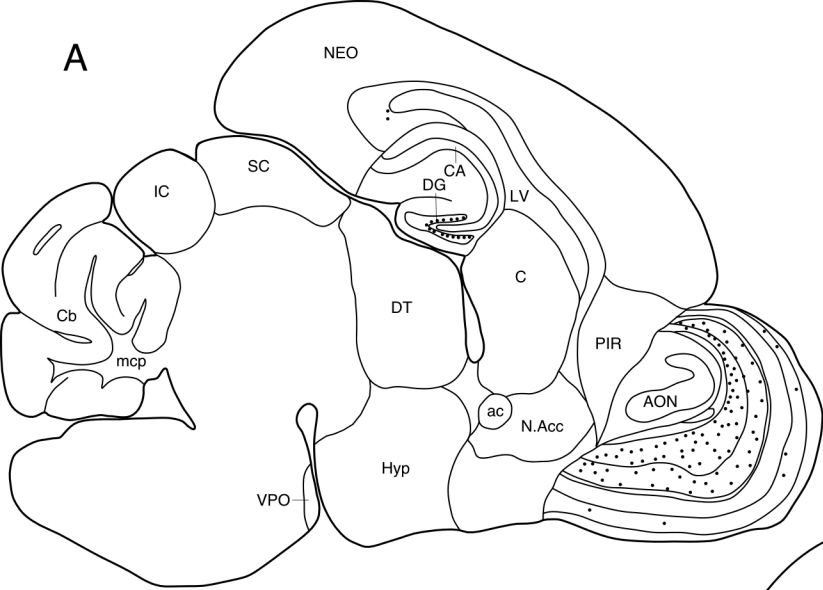
Figure 5: A series of sagittal drawings from the brain of the four-toed sengi (*Petrodromus tetradactylus*) showing the location of doublecortin immunopositive cells (dots, where one dot represents one cell). **A** is medial, **F** is lateral, each figurine being approximately 2 mm apart. Note the presence of DCX immunopositive neurons in the hippocampus, rostral migratory stream and olfactory bulb, piriform cortex and the rostral half of the neocortex. See list for abbreviations.

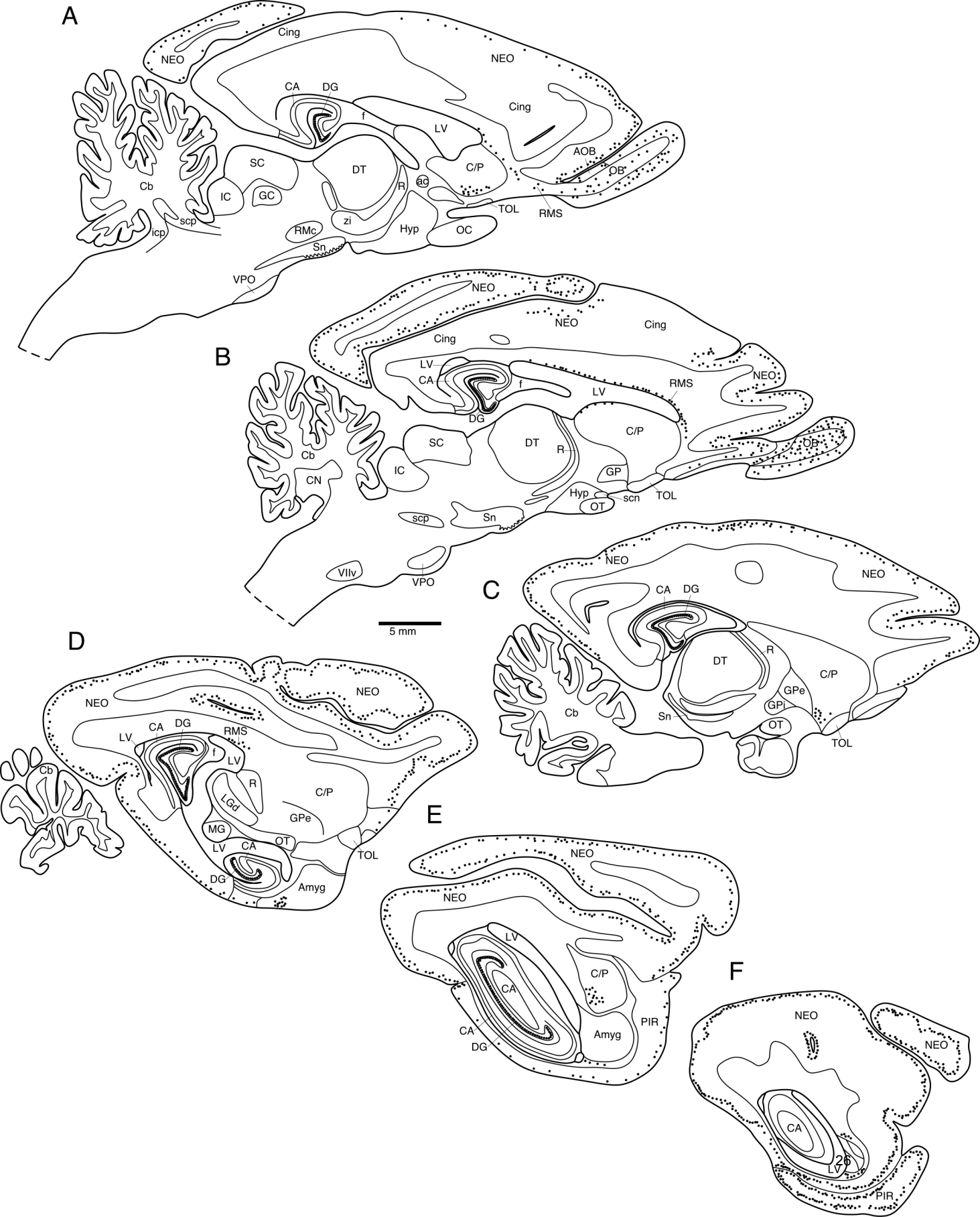
Figure 6: Photomicrographs of DCX immunostained sagittal sections of the olfactory bulb of four Afrotherian species. DCX-positive cells were mostly observed in the granule cell layer and the glomerular layer. (A) hottentot golden mole (*Amblysomus hottentotus*), (B) eastern rock sengi (*Elephantulus myurus*), (C) four-toed sengi (*Petrodromus tetradactylus*), and (D) rock hyrax (*Procavia capensis*). Scale bar in **D** = 500 μm and applies to all.

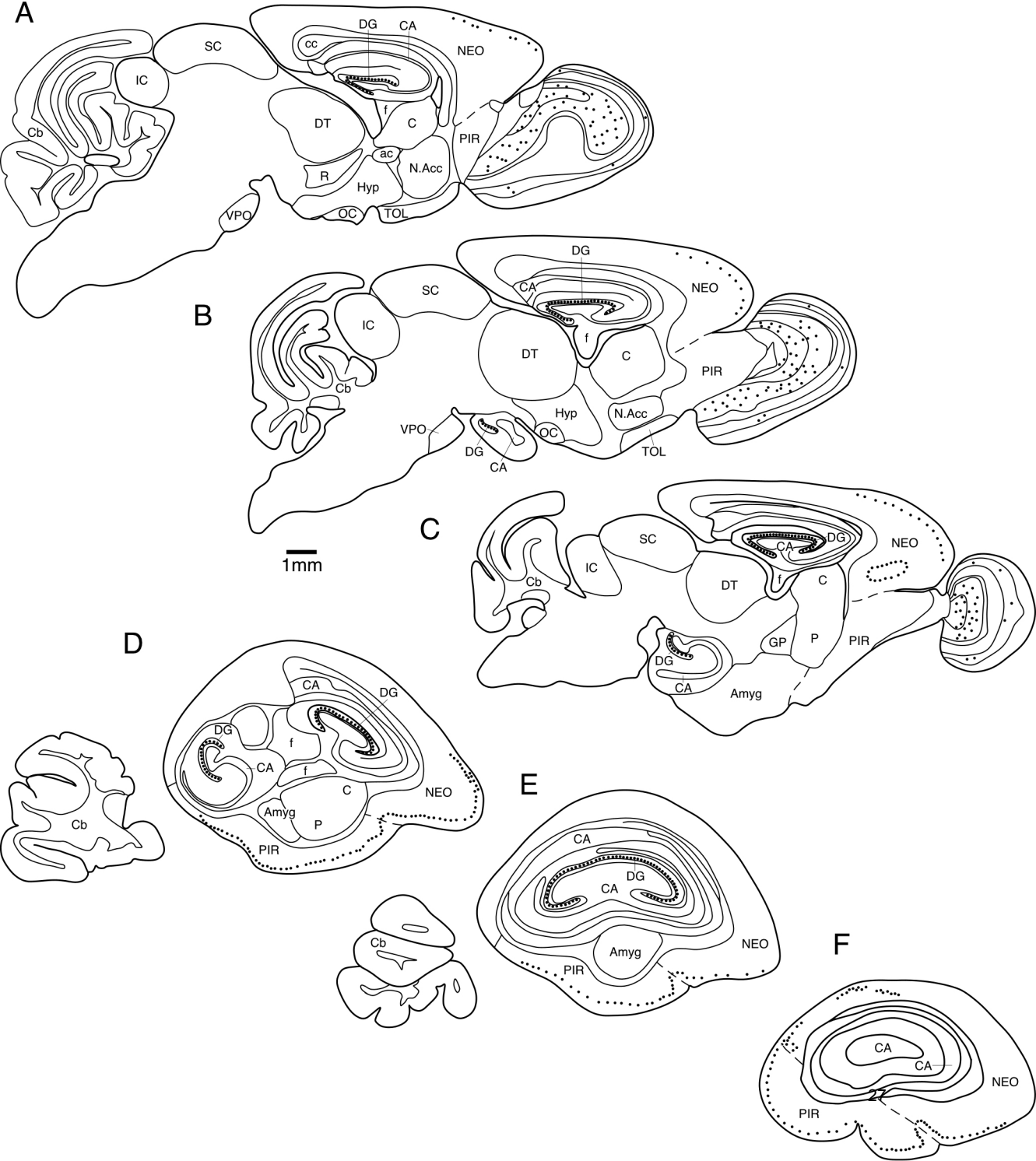
Figure 7: High-power photomicrographs of DCX-positive cells located in the piriform cortex of four Afrotherian species. (A) hottentot golden mole (*Amblysomus hottentotus*), (B) eastern rock sengi (*Elephantulus myurus*), (C) four-toed sengi (*Petrodromus tetradactylus*), and (D) rock hyrax (*Procavia capensis*). Scale bar in **D** = 100 μm and applies to all.

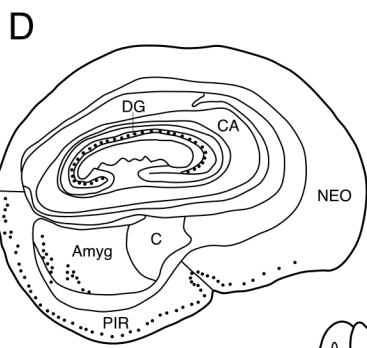
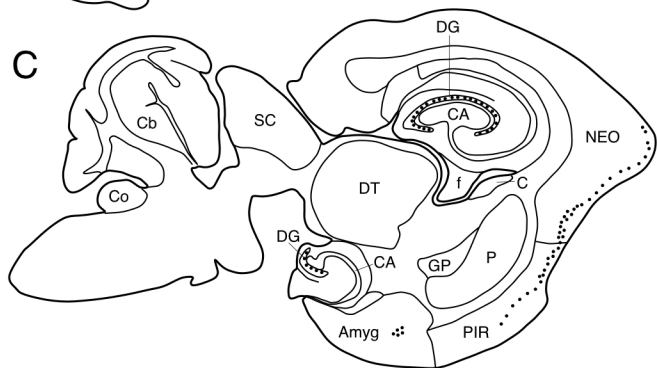
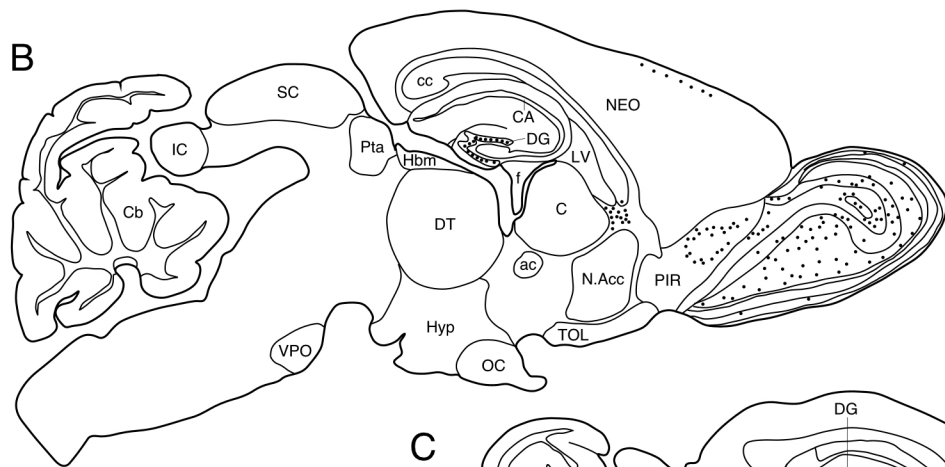
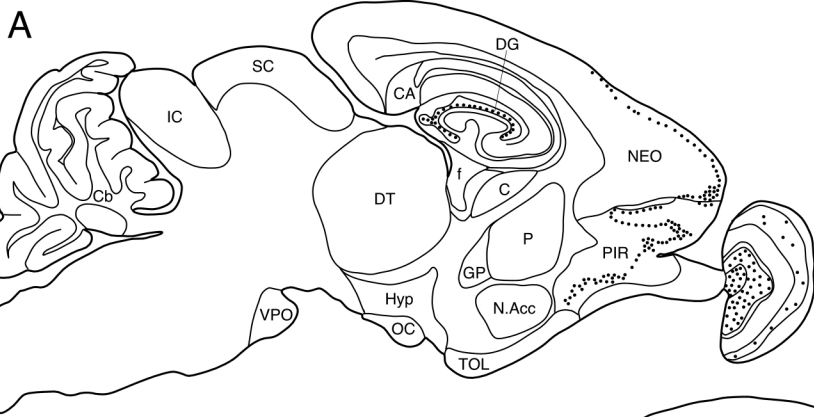
Figure 8: High-power photomicrographs of DCX-positive cells located in the layer II of the neocortex of four Afrotherian species. (A) hottentot golden mole (*Amblysomus hottentotus*), (B) eastern rock sengi (*Elephantulus myurus*), (C) four-toed sengi (*Petrodromus tetradactylus*), and (D) rock hyrax (*Procavia capensis*). Scale bar in **D** = 100 μm and applies to all.











2 mm

