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The Distribution of Doublecortin-Immunopositive Cells in the Brains of Four Afrotherian Mammals: the 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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Key Words

Adult neurogenesis · Afrotheria · Doublecortin · Elephant shrew · Golden mole · Habitat diversity · Hippocampus · Hyrax · Mammals · Rostral migratory stream · Sengi

Abstract

Adult neurogenesis in the mammalian brain is now a widely accepted phenomenon, typically occurring in two forebrain structures: the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ). Until 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 SGZ and the SVZ 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 visualise 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 SVZ, from where cells appear to migrate along the rostral migratory stream towards the olfactory bulb (OB). DCX-immunopositive cells were present in the granular cell layer and the glomerular layer of the OB. In the hippocampus, DCX-immunopositive cells were observed in the SGZ 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 cells undergoing cortical remodelling.

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Abbreviations used in this paper

ac	anterior commissure
AHN	adult hippocampal neurogenesis
Amyg	amygdala complex
AOB	accessory olfactory bulb
AON	anterior olfactory nucleus
C	caudate nucleus
CA	cornu ammonis region of the hippocampus
Cb	cerebellum
cc	corpus callosum
Cing	cerebral cingulate cortex
CN	deep cerebellar nuclei
Co	cochlear nucleus
DG	dentate gyrus of the hippocampus
DT	dorsal thalamus
f	fornix
GC	central grey 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	olfactory bulb
OC	optic chiasma
OT	optic tract
P	putamen nucleus
PB	phosphate buffer
PIR	piriform cortex
Pta	pretectal area
R	thalamic reticular nucleus
RMc	red nucleus, magnocellular part
RMS	rostral migratory stream
SC	superior colliculus
scn	suprachiasmatic nucleus
scp	superior cerebellar peduncle
SGZ	subgranular zone
Sn	substantia nigra
SVZ	subventricular zone
TOL	olfactory tubercle
VIIv	facial nerve nucleus, ventral division
VPO	ventral pontine nucleus
zi	zona incerta

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 are, at least in part, linked to learning and memory formation [Gould et al., 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 (OB) [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, hypothalamus and piriform cortex amongst others [Gould, 2007; Shapiro et al., 2007; Migaud et al., 2010; Bonfanti and Peretto, 2011; Patzke et al., 2013a]; thus a comparative analysis of adult neurogenesis might reveal differences in adult neurogenesis correlated with behavioural specialisations or adaptations to specific ecological niches [Bonfanti and Peretto, 2011; Patzke et al., 2013c]. 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 [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 neuro-

genesis 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-eastern regions of southern Africa, where it inhabits rocky outcrops [Stuart and Stuart, 1997; Skinner and Chimimba, 2005]. 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 classic neurogenic sites (SVZ and SGZ) 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 in the SVZ and SGZ, due to specific arrangements of the newly generated cell populations, is a good proxy for the presence of adult neurogenesis [Rao and Shetty, 2004; Couillard-Despres et al., 2005; Bonfanti and Nacher, 2012]. 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. Nevertheless, DCX labelling outside of the classic neurogenic zones may not represent adult neurogenesis, as it may indicate putative morphological plasticity in non-newly generated neurons, since DCX is also expressed in neurons undergoing remodelling [Klempin et al., 2011]. Hence DCX-positive labelling outside of the classic neurogenic zones must be interpreted with caution.

Materials and Methods

Specimen and Tissue Preparation

In the present study, brains from two *A. hottentotus* (brain mass = 1.3 and 1.2 g), two *P. capensis* (brain mass = 20.4 and 20.8 g), two *E. myurus* (brain mass = 1.3 and 1.19 g) and three *P. tetradactylus* (brain mass = 3.05, 2.80 and 2.95 g) were analysed. The *E. myurus* and *P. capensis* were caught in the Limpopo Province, South Africa. The *P. tetradactylus* were caught in the Yoko forest, near Kisangani, Democratic Republic of 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 the 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 4,300 and 4,500 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 and 85 g, for *P. capensis* between 1,500 and 4,300 g, for *E. myurus* between 41 and 98 g, and for *P. tetradactylus* between 160 and 280 g. These body mass ranges are all from the southern African subregion, where all but the *P. tetradactylus* were caught. Based on these 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* from 118.9 to 203.6 g, respectively. These 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 minimise external influences, such as handling stress, on adult neurogenesis, the animals were captured in their natural habitat and euthanised within 30 min 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 0.1 M 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 anti-freeze solution until processing.

Tissue Staining and Immunohistochemistry

The brains were examined immunohistochemically using antibodies directed against the intrinsic marker DCX. DCX is a microtubule-associated phosphoprotein that is expressed from 2 weeks up to 6 months in actively dividing neuronal precursor cells and their neuronal daughter cells [Brown et al., 2003; Rao and Shetty, 2004; Kohler et al., 2011]. 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 [Bartkowska et al., 2010]. Goat anti-DCX (C-18; Santa Cruz Biotechnology, Dallas, Tex., USA) 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 0.1 M 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 pretreated for 30 min at room temperature under gentle shaking with an endogenous peroxidase inhibitor (49.2% 0.1 M PB, 49.2% methanol, 1.6% of 30% H_2O_2). Following three 10-min rinses in 0.1 M 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 0.1 M PB before being incubated in secondary antibody solution. The secondary antibody contained a 1:1,000 dilution of biotinylated anti-goat IgG (BA-5000; Vector Laboratories, Burlingame, Calif., USA) in 3% normal rabbit serum and 2% bovine serum albumin in 0.1 M PB for 2 h at room temperature under gentle shaking. Following three 10-min rinses in 0.1 M PB, the sections were incubated in an avidin-biotin solution [1:125 A reactive and 1:125 B reactive (Vector Laboratories) in 0.1 M PB] for 1 h. The sections were transferred into three 10-min 0.1 M PB rinses before being placed in a solution containing 0.05% diaminobenzidine in 0.1 M PB for 5 min. To each 1 ml of this solution, 3.3 µl of 30% H_2O_2 were added, and chromatic precipitation was visually monitored under a low-power stereomicroscope. Development was subsequently arrested by placing the sections in 0.1 M PB, followed by a final 10-min rinse in 0.1 M PB. Sections were mounted on 0.5% gelatinised slides, left to dry overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure that non-specific staining was not affecting the results, control sections taken at random were processed in the same manner, but either the primary or the secondary antibody was omitted. No labelled cells were observed in either case.

Data Analysis

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

Results

In the present study, we revealed neurons immmunoreactive to the endogenous marker DCX in *A. hottentotus, P. capensis, E. myurus* and *P. tetradactylus*. Our DCX staining revealed the two commonly reported neurogenic areas, the SVZ of the lateral ventricles that gives rise to the rostral migratory stream (RMS) ending in the OB and the SGZ of the hippocampal dentate gyrus. Furthermore, the presence of DCX-positive cells provided evidence of immature or remodelling neurons in cortical brain regions.

DCX 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 SGZ, which was located between the granular cell layer and the polymorphic layer of the dentate gyrus (fig. 1–5). These immunopositive neurons were characterised by large, ovoid somata with ramified dendrites extending into the molecular layer (fig. 1). Occasional DXC-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 SVZ of the Lateral Ventricle, the RMS and the OB

In all four species, clusters of DCX-positive cells and processes were present in the SVZ, with the highest density of immunolabelled structures observed towards the rostral end of the lateral ventricle (fig. 2–5). The labelled cells were characterised 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 RMS. The RMS originated from the SVZ at the rostral pole of the lateral ventricle, with DCX-immunopositive cells found between the rostrodorsal 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 OB (fig. 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 and small in size, and displayed bipolar processes.

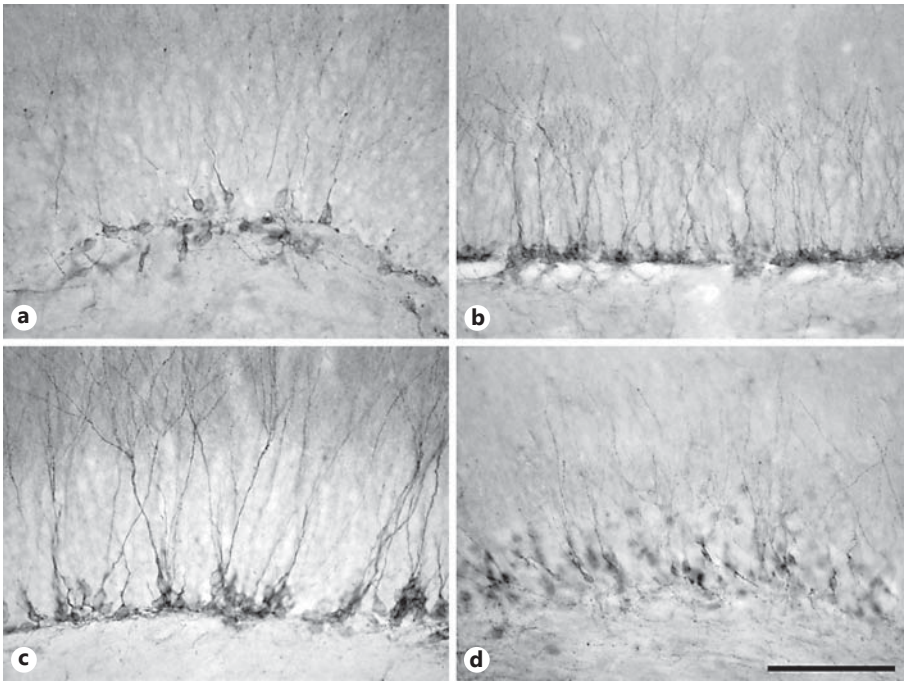


Fig. 1. High-power photomicrographs of DCX-positive cells located in the SGZ and the granular layer of the dentate gyrus of four afrotherian species. **a** Hottentot golden mole *(A. hottentotus)*. **b** Eastern rock sengi *(E. myurus)*. **c** Four-toed sengi *(P. tetradactylus)*. **d** Rock hyrax *(P. capensis)*. Scale bar in **d** is 100 μm and applies to all.

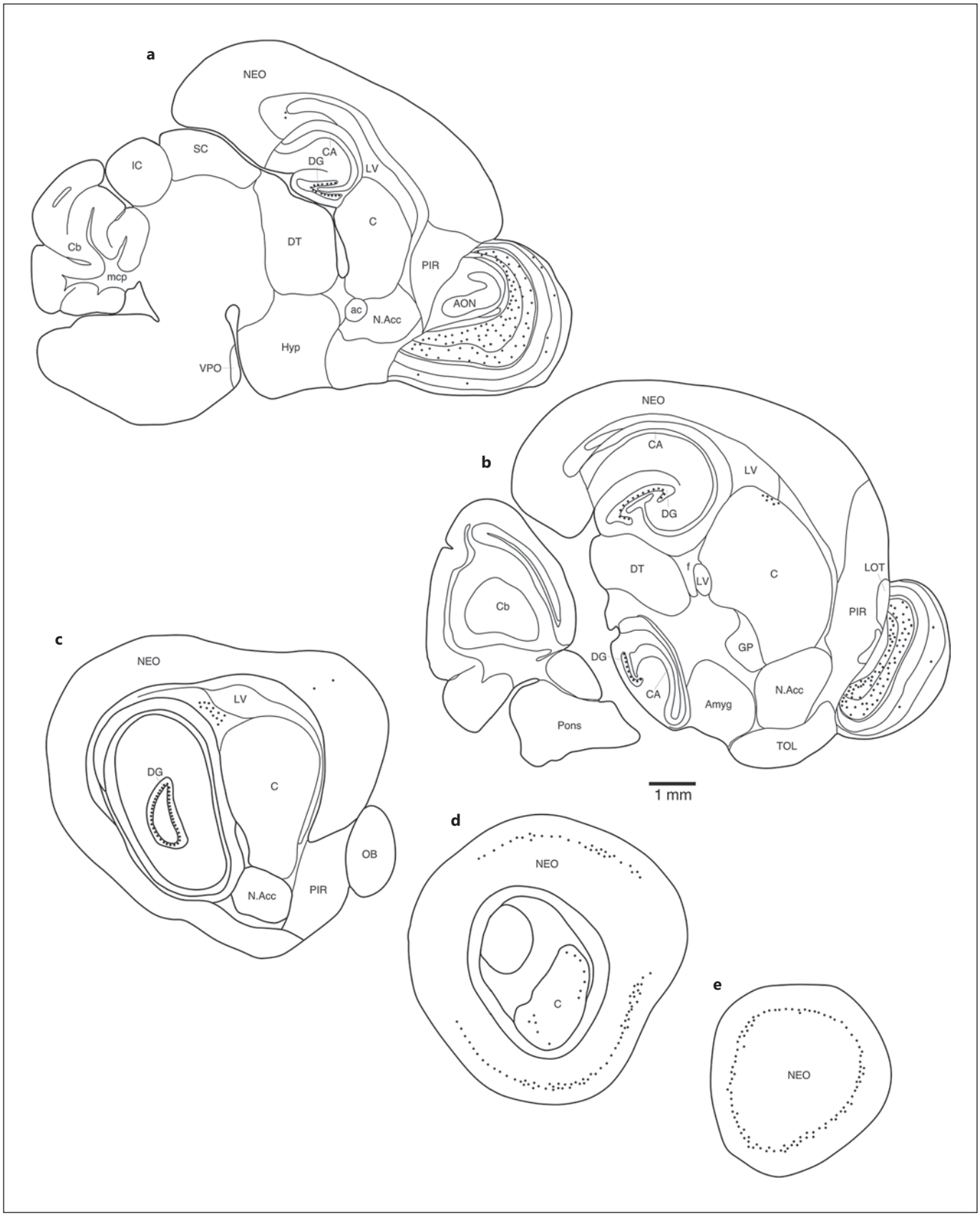
In the main OB, DCX immunoreactivity was evident in all layers in all four species. The majority of DCX-expressing cells were located in the granular cell layer, exhibiting radially orientated DCX-positive cells and processes (fig. 2–6). Most of these cells were bipolar and ovoid in shape. The external plexiform layer of the OB presented with distinct radial fibres, while the glomerular layer 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 OB are those arising from the RMS. 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 sengis, rock hyrax and the golden mole, DCX-immunopositive cells were observed in layer II of the piriform cortex (fig. 2–5) and along a line running from the SVZ of the ventral portion of the lateral ventricle, along the rostral border of the striatum, through layer III towards layer II of the piriform cortex, suggesting that the DCX-positive cells in the piriform cortex arise from the SVZ. 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 multipolar in shape, but occasionally 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 bi- 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 (fig. 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.



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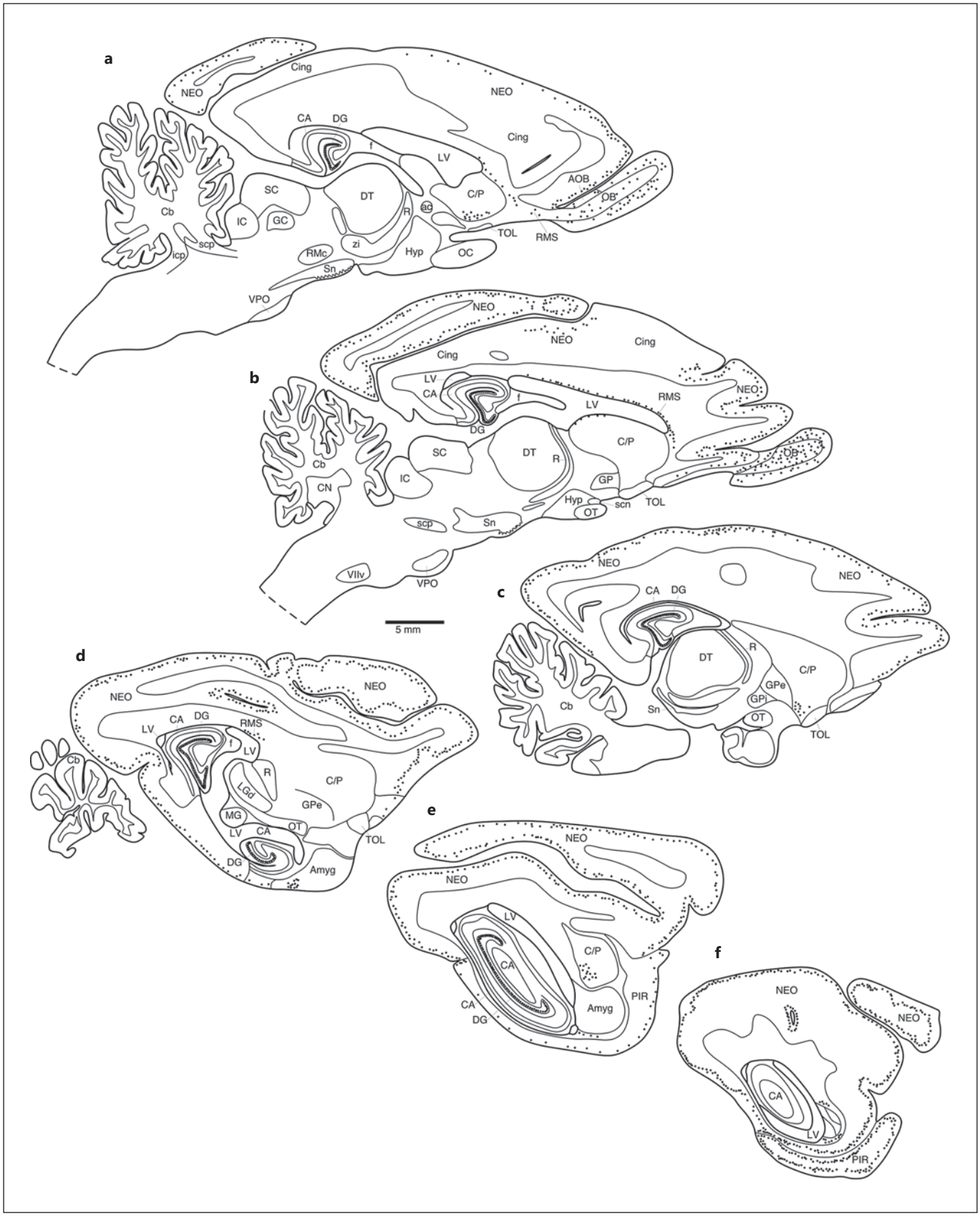
Discussion

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 SGZ of the dentate gyrus in the hippocampal formation and the SVZ of the lateral ventricles that gives rise to the RMS which ends in the OB. 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–c], 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. Ki-67 is an endogenous protein expressed in dividing cells during late G1, S, G2 and M phases of the cell cycles in all mammalian species and is used to identify proliferating cells [Scholzen and Gerdes, 2000]; however, the absence of Ki-67 labelling does not mean that no proliferation occurs in the adult brain of Afrotheria, but is rather related to the species-specific affinity of the antibody used. The non-reactivity of the DAKO Ki-67 antibody (NCL-Ki-67 P) in Afrotheria seems to be related to the phylogenetic specificity of the antibody, and hence might only show reactivity in rodents, megachiropterans and primates [Wojtowicz and Kee, 2006; Vessal and Darian-Smith, 2010; Chawana et al., 2013] 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 AHN [van Praag et al., 2000]. AHN has also been seen to be influenced by stress, exercise, learning and social conditions [Gould and Cameron, 1996; Gould et al., 1997; Kempermann et al., 1997; Gould et al., 1998; van Praag et al., 1999; Lu et al., 2003; Pham et al., 2003; Olson et al., 2006; Warner-Schmidt and Duman, 2006; Snyder et al., 2009]; 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; Patzke et al., 2013c]. 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 [Patzke et al., 2013c]. Species of the afrotherian superorder, 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 sengis, rock hyrax and the golden mole analysed in this study, as well as the giant otter shrew, a semi-aquatic afrotherian mammal previously examined [Patzke at 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 in the other afrotherian species studied [Patzke et al., 2013b, c]. 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 basal 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 AHN levels in different mammalian species are dependent upon two factors (besides intrinsic fac-

Fig. 2. A series of sagittal drawings from the brain of the Hottentot golden mole *(A. hottentotus)* showing the location of DCX-immunopositive cells (dots, where one dot represents one cell): medial (**a**) to lateral (**e**), each figurine being approximately 1 mm apart. Note the presence of DCX-immunopositive neurons in the hippocampus (dentate gyrus), RMS and OB, piriform cortex and neocortex. See list for abbreviations.



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tors 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-regulation 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 OB incorporates new neurons that arise in the SVZ of the lateral ventricle and migrate along the RMS to the OB. From the periventricular layer of the OB, these new neurons migrate radially into the granular and glomerular layers where they become functionally integrated into the OB circuitry [Peretto et al., 1997; Bedard and Parent, 2004; Lledo et al., 2006]. This continuous supply of new neurons to the OB has been reported in all mammalian species studied to date, including the four afrotherian species studied herein [Pencea et al., 2001; Bedard et al., 2002; Bedard and Parent, 2004; Alpár et al., 2010; Bartkowska 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 OB are generated locally [Bedard and Parent, 2004].

In addition to the immature OB 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 [Bartkowska et al., 2010], the hedgehog tenrec [Alpár et al., 2010] and the giant otter shrew [Patzke et al., 2013a]. In the four afrotherian species analysed 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, as indicated by the presence of DXC-positive cells along this migratory pathway. 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 in moles and hedgehogs [Bartkowska et al., 2010]. In rodents [Shapiro et al., 2007], moles and hedgehogs [Bartkowska 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 in the giant otter 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 piriform cortex, as seen in rodents, indicating that the DCX neurons in the piriform cortex are not locally generated or remodelling neurons, but rather arise from the SVZ of the lateral ventricles; however, local proliferation and/or remodelling cannot be ruled out at this stage. As in the giant otter 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 the endopiriform nucleus of the other three afrotherian species analysed.

DCX-Immunoreactive Neurons in the Cerebral Neocortex – New or Remodelling Neurons?

The presence of DCX-immunoreactive neurons in layer II of the cerebral neocortex in Afrotheria is in accord with reports in rodents [Kutsuna et al., 2013], primates [Zhang et al., 2009; Bloch et al., 2011], 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 cells in 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 or generated during development and remain in an immature state, or are mature neurons undergoing neuronal remodelling. Previous studies using 5-bromo-2′-deoxyuridine and neuronal markers suggest

Fig. 3. A series of sagittal drawings from the brain of the rock hyrax *(P. capensis)* showing the location of DCX-immunopositive cells (dots, where one dot represents one cell): medial (**a**) to lateral (**f**), each figurine being approximately 3 mm apart. Note the presence of DCX-immunopositive neurons in the hippocampus (dentate gyrus), RMS and OB, piriform cortex and throughout the neocortex. See list for abbreviations.

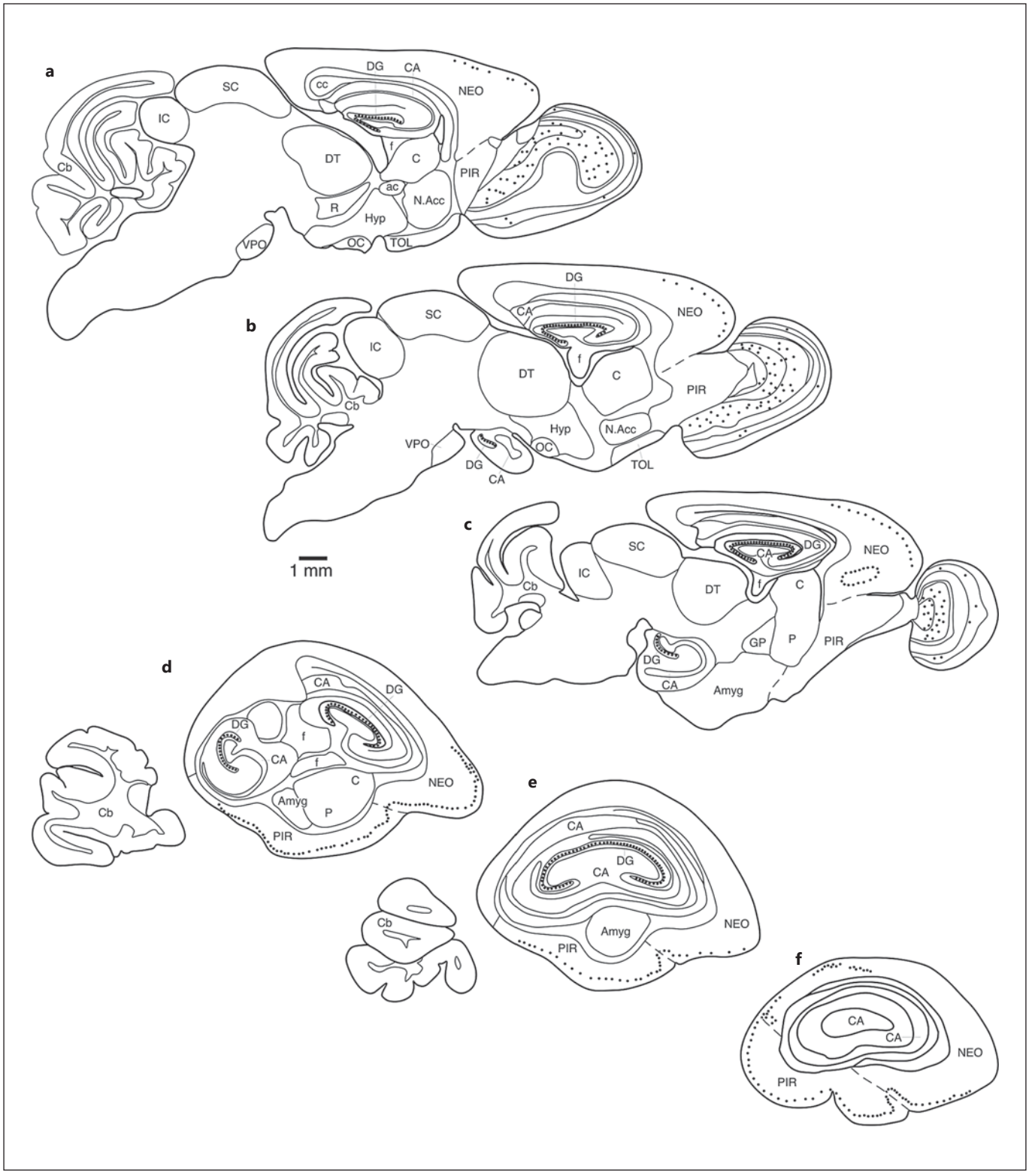

Fig. 4. A series of sagittal drawings from the brain of the eastern rock sengi *(E. myurus)* showing the location of DCX-immunopositive cells (dots, where one dot represents one cell): medial (**a**) to lateral (**f**), each figurine being approximately 1.5 mm apart. Note the presence of DCX-immunopositive neurons in the hippocampus (dentate gyrus), RMS and OB, piriform cortex and the rostral half of the neocortex. See list for abbreviations.

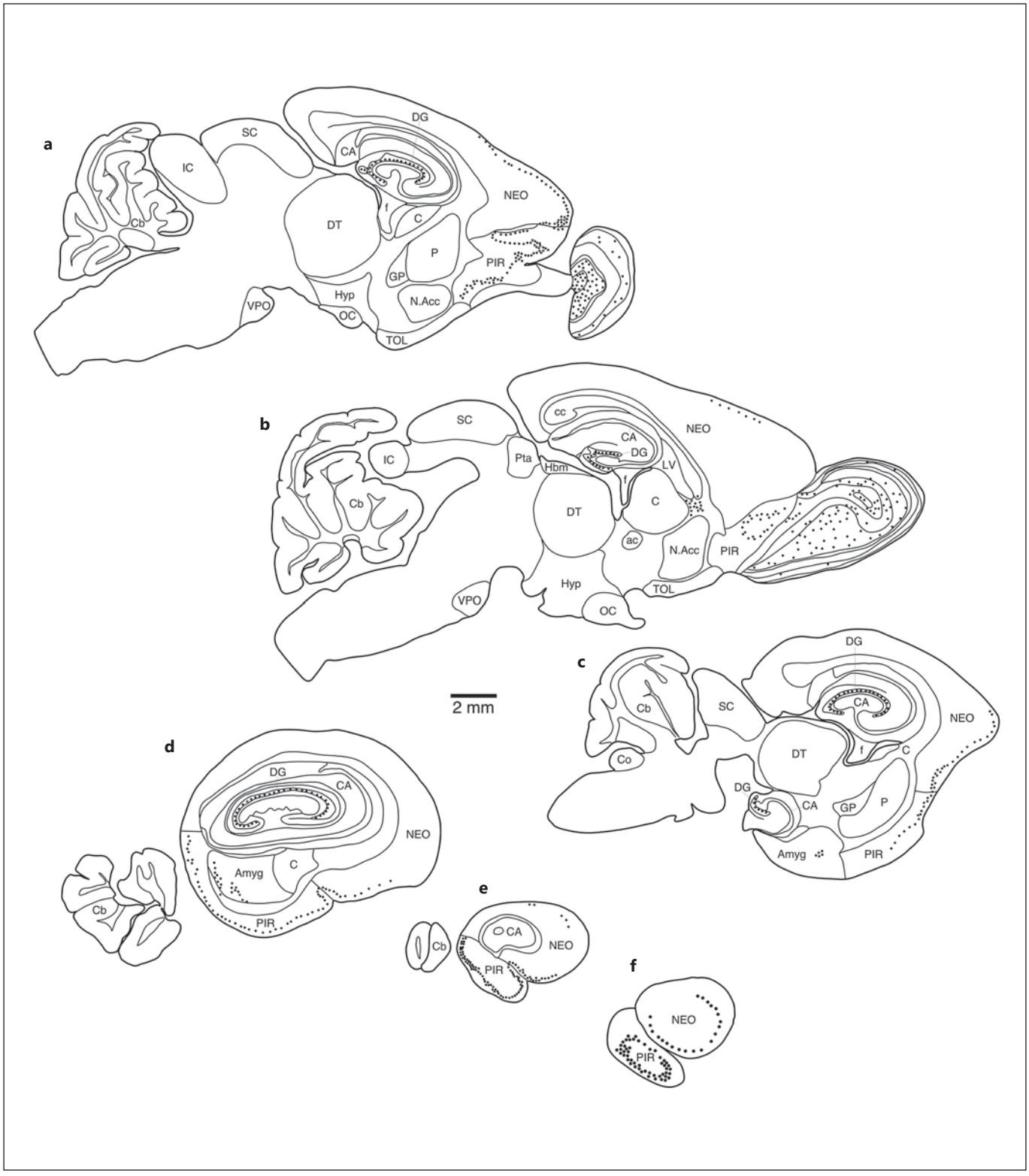

Fig. 5. A series of sagittal drawings from the brain of the four-toed sengi *(P. tetradactylus)* showing the location of DCX-immunopositive cells (dots, where one dot represents one cell): medial (**a**) to lateral (**f**), each figurine being approximately 2 mm apart. Note the presence of DCX-immunopositive neurons in the hippocampus (dentate gyrus), RMS and OB, piriform cortex and the rostral half of the neocortex. See list for abbreviations.

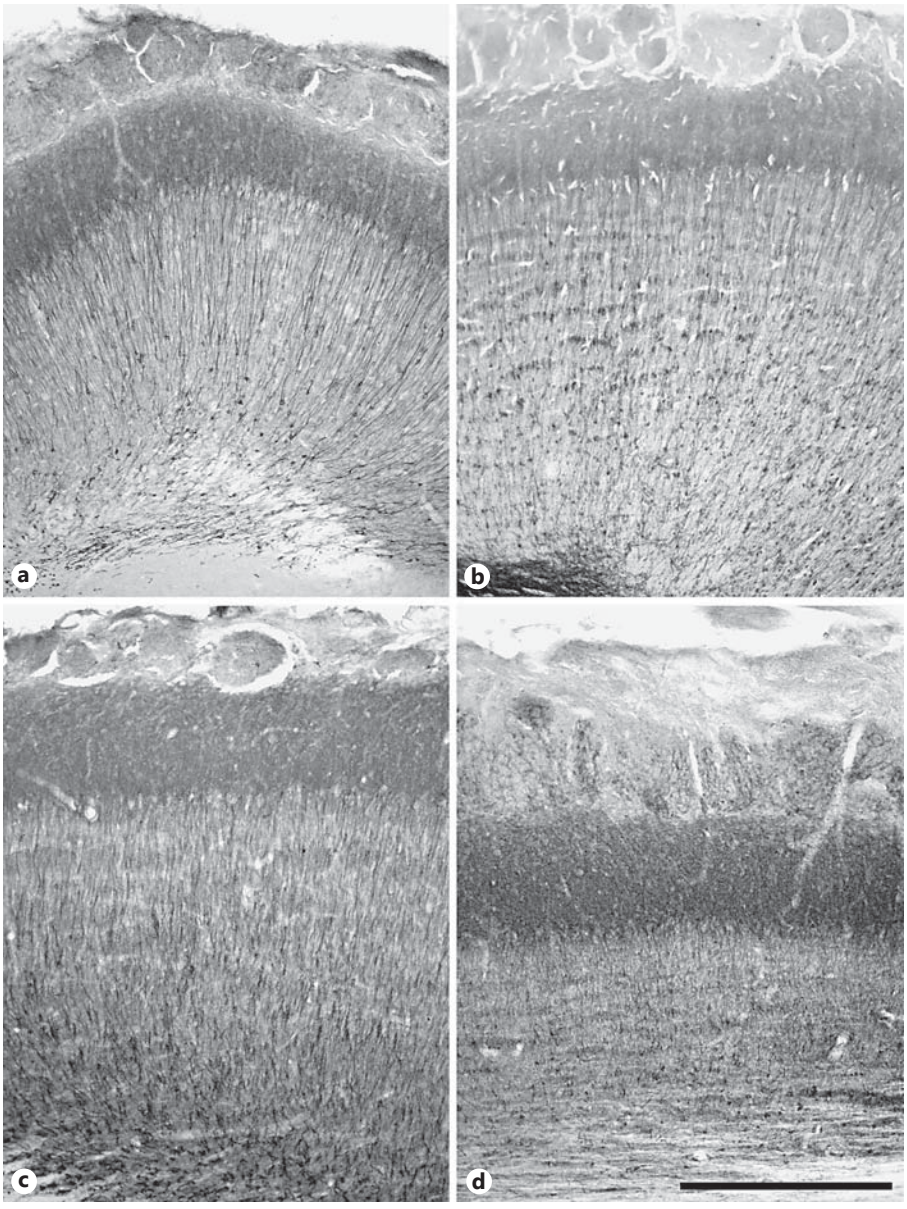

Fig. 6. Photomicrographs of DCX-immunostained sagittal sections of the OB of four afrotherian species. DCX-positive cells were mostly observed in the granule cell layer and the glomerular layer. **a** Hottentot golden mole *(A. hottentotus)*. **b** Eastern rock sengi *(E. myurus)*. **c** Four-toed sengi *(P. tetradactylus)*. **d** Rock hyrax *(P. capensis)*. Scale bar in **d** is 500 μm and applies to all.

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 [Gould et al., 1999; Kakita and Goldman, 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 four afrotherian species as well as in the giant otter shrew [Patzke et al., 2013a], no stream of presumably newly generated neurons from the SVZ towards the neocortex could be observed using DCX immunoreactivity; 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 whether these DCX-immunopositive cells in layer II of the neocortex are newly generated or remodelling, using improved birth-dating methodology.

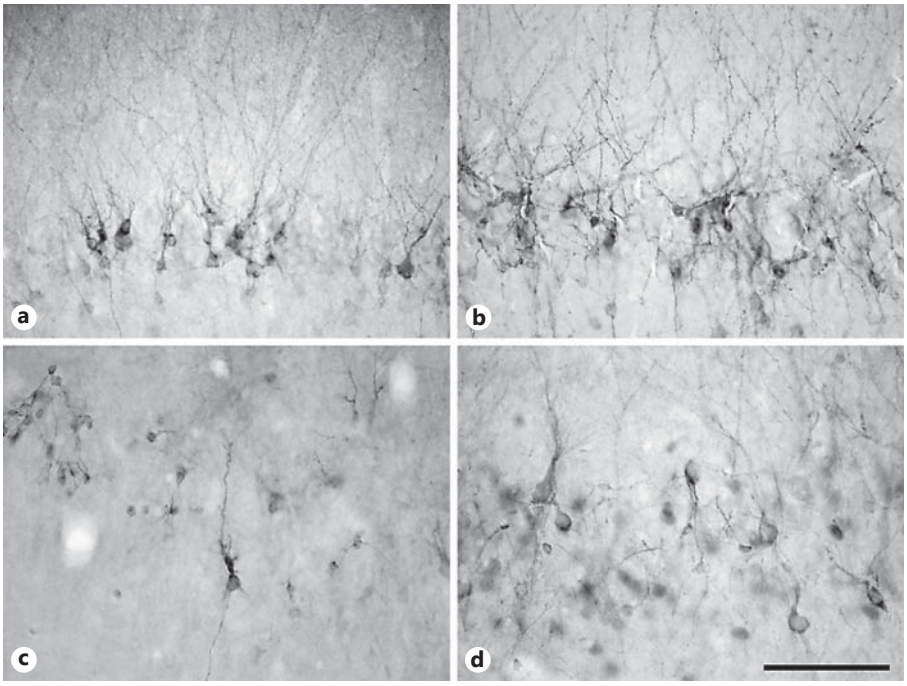

Fig. 7. High-power photomicrographs of DCX-positive cells located in the piriform cortex of four afrotherian species. **a** Hottentot golden mole *(A. hottentotus)*. **b** Eastern rock sengi *(E. myurus)*. **c** Four-toed sengi *(P. tetradactylus)*. **d** Rock hyrax *(P. capensis)*. Scale bar in **d** is 100 μm and applies to all.

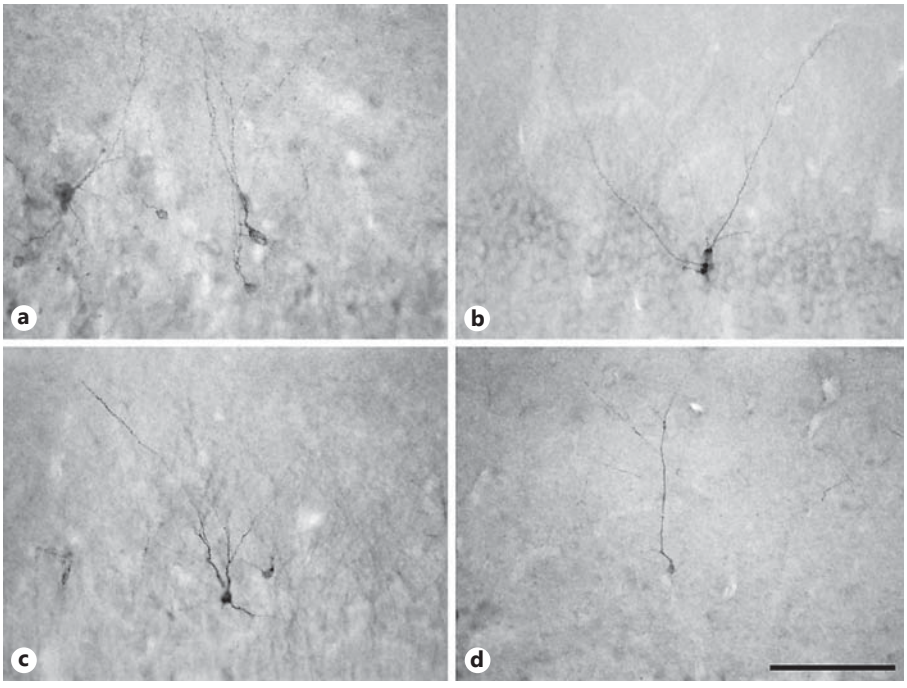

Fig. 8. High-power photomicrographs of DCX-positive cells located in layer II of the neocortex of four afrotherian species. **a** Hottentot golden mole *(A. hottentotus)*. **b** Eastern rock sengi *(E. myurus)*. **c** Four-toed sengi *(P. tetradactylus)*. **d** Rock hyrax *(P. capensis)*. Scale bar in **d** is 100 μm and applies to all.

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