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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.

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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