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.

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.

http://dx.doi.org/10.1016/j.neuroscience.2014.07.063
0306-4522/

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