Microbats have adult hippocampal neurogenesis, but post-capture stress causes a rapid decline in the number of immature hippocampal neurons expressing doublecortin

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Highlights

- Clear adult neurogenesis was observed in three species of microchiropterans.
- Microchiropteran adult neurogenesis is similar to that of other mammals.
- Capture stress causes a rapid decline in detectable hippocampal neurogenesis.
- Adult hippocampal neurogenesis appears to be a common mammalian neural trait.

Abstract:

A previous study looking for evidence of adult hippocampal neurogenesis in microchiropteran bats failed to reveal the strong presence of this neural trait. As microchiropterans have a high metabolic rate and are small, it is possible that

capture/handling stress could have led to a decrease in the detectable presence of adult hippocampal neurgenesis. In this study we looked for adult hippocampal neurogenesis using immunohistochemical techniques for the endogenous marker doublecortin in 10 species of microchiropterans euthanized and perfusion fixed at specific time points following capture. Our result indicate that when euthanized and perfused within 15 minutes of capture, abundant adult hippocampal neurogenesis could be detected with doublecortin immunhistochemistry. Between 15 and 30 minutes post-capture, the detectable levels of doublecortin dropped dramatically, and after 30 minutes post-capture, immunohistochemistry for doublecortin could not reveal any significant adult hippocampal neurogenesis. Thus, as with all other mammals studied to date, bats, including both microchiropterans and megachiropterans, exhibit substantial levels of adult hippocampal neurogenesis. The present study underscores the concept that, as with laboratory studies, studies of wild-caught animals need to be cognizant of the fact that stress (capture/handling) can induce major changes in the appearance of specific neural traits.

Keywords: adult neurogenesis; doublecortin; Chiroptera; wild-living animals; hippocampus; immunohistochemistry.

Introduction

Studies of adult neurogenesis in wild-living mammals are becoming more common due to the need to understand this process in relation to normal life-history parameters (Bartkowska et al. 2010; Amrein et al., 2011; Kempermann, 2012; Patzke et al. 2013; Chawana et al., 2013; Cavegn et al., 2013). The investigation of wild-living mammals may provide a broader understanding of the dynamics and mechanisms influencing adult neurogenesis for species in their natural habitat and ultimately reveal potential reasons for the presence of adult neurogenesis in the mammalian brain. Wild living mammals are subject to 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 can 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 cortisol levels, physical restraint and translocation leads to significant increases in the stress related release of cortisol (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), although in rats, the reduction in the number of proliferating cells was observed to occur within 2 hours of the acute stress and recover to baseline levels within 24 hours post exposure (Heine et al., 2004).

An earlier study of adult neurogenesis in microchiropterans led to the conclusion that the hippocampus of the species studied had absent to low rates of adult neurogenesis (Amrein et al., 2007). While possible reasons for the absence of adult hippocampal neurogenesis are raised, it appears that no specific conclusion is reached. One issue not raised by Amrein et al. (2007) was whether the stress of capture/handling of these small mammals may have been an important factor in the lack of adult hippocampal neurogenesis. While Amrein et al. (2007) state the the bats were "perfused rapidly after trapping", no estimate of the time that elapsed between trapping and perfusion is 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, although again, no details regarding handling of these bats prior to perfusion is provided. Given that the microchiropterans have high metabolic rates in comparison to other mammals (Austad and Fischer, 1991), 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 of the expression of proteins in the microchiropteran brain, and in the case of Amrein et al. (2007) lead to a false negative report of the absence of adult hippocampal neurogenesis in bat species – 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 the wild-caught microchiropterans using immunohistochemistry for the doublecortin protein (DCX), an endogenous marker of adult hippocampal neurogenesis (Kempermann, 2012).

Experimental procedures

In the current study we examined 38 brains from 10 microchiropteran species including *Miniopterus schreibersii* (n = 2) captured from a wild population in Gauteng, South Africa, *Cardioderma cor* (n = 2), *Chaerephon pumilis* (n = 2), *Coleura afra* (n = 2), *Hipposideros commersoni* (n = 2), and *Triaenops persicus* (n = 2) captured from wild populations in coastal Kenya, *Hipposideros fuliganosas* (n = 2) and *Nycteris macrotis* (n = 2) captured from wild populations in the Yoko Forest near Kisangani, Democratic Republic of the Congo, and *Pipistrellus kuhlii* (n = 2) and *Asellia tridens* (n = 20) captured from wild populations near Unaizah (also spelt Onaizah, Onizah, Unizah or Unayzah), Saudi Arabia. All animals were adults, as judged from epiphyseal closure of the metacarpophalangeal joints (Anthony, 1988). Appropriate permissions to trap and euthanaze the bats were obtained from the Gauteng Department of Nature Conservation, South Africa, the Kenya National Museums, Kenya, the University of Kisangani, DR Congo, and the Saudi Wildlife Authority, Kingdom of Saudi Arabia. All animals were treated and used in accordance with the University of the Witwatersrand Animal Ethics Committee Guidelines (clearance number 2008/36/1) which parallel those of the NIH for the care and use of animals in scientific experiments. All bats were euthanized (overdose of sodium pentobarbital, 100 mg/kg, i.p.) and perfused through the left ventricle with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) at various times following capture. For *H. fuliganosas*, *M. schreibersii*, and *T. persicus*, the animals were perfusion fixed within 15 minutes of capture. For *C. cor* and *C. pumilus*, the specimens were fixed within 30 minutes of capture, and for *C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*, the specimens were fixed within an hour of capture. For *A. tridens*, two animals were sacrificed and perfused at each of the following time points (in minutes) post-capture: 5, 10, 15, 30, 45, 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 sagittal plane into 50 µm thick sections. A one in three series of sections was stained for Nissl substance (cresyl violet) to reveal cytoachitectural features, and immunostained at two different dilutions of the primary antibody to doublecortin (DCX, 1:300 and 1:600) to reveal proliferation of cells and immature neurons.

In the current study we used immunolabelling of doublecortin (DCX), an endogenous marker of immature neurons, to ascertain the presence or absence of adult neurogenesis. While DCX immunopositive neurons away from the hippocampus may not relate to adult neurogenesis in these regions, such as the piriform cortex (Klempin et al., 2011), it has been established that DCX immunolabelling of granule cells of the dentate gyrus is a good proxy for the presence of adult hippocampal neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005). The presence of DCX also reflects 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.

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 unspecific binding sites the sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal rabbit serum – NRS, 2% bovine serum albumin, BSA, and 0.25% Triton X-100 in 0.1 M PB). Thereafter, the sections were incubated for 48 h at 4°C in the primary antibody solution (1:300 and 1:600, goat anti-doublecortin, DCX, SC-18 Santa Cruz Biotech) under gentle agitation. The primary antibody incubation was followed by three 10 min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated anti-rabbit anti-goat IgG, BA 5000, Vector Labs, in 3% NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin-biotin solution (1:125; Vector Labs), followed by three 10-min rinses in 0.1 M PB. Sections were then placed in 1 ml of a solution containing 0.05% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 µl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low power stereomicroscope. Staining continued until such time as the background stain was at a level that would allow for accurate architectonic matching to the Nissl sections without obscuring the immunopositive structures. Development was arrested by placing sections in 0.1 M PB for 10 min, followed by two more 10 min rinses in this solution. Sections were then mounted on 0.5% gelatine coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody, and sections where we omitted the secondary

antibody. In both cases no staining was observed. It was not possible to undertake Western blot control testing due to the nature of the collection of the tissue from wild populations. Staining patterns of DCX were observed using low power stereomicroscope and digital photomicrographs were captured using Zeiss Axioshop and Axiovision software. No pixilation adjustments, or manipulation of the captured images was undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

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

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

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

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

Results

Doublecortin immunopositive (DCX+) cells in the Microchiropteran hippocampus

Of the 10 microchiropteran species examined in the current study, we found DCX+ cells clearly present in the subgranular zone of the dentate gyrus in 4 species (*A. tridens*, *H. fuliganosas*, *M. schreibersii* and *T. persicus*) which were perfused within 15 minutes of capture, partially present in 2 species (*C. cor*, *C. pumilus*) which were perfused between 15 and 30 minutes of capture, and absent in 4 species (*C. afra*, *H. commersoni*, *N. macrotis* and *P. kuhlii*) all of which were perfused between 30 and 60 minutes post-capture (Figs. 1, 2). In the species where DCX+ cells were observed, the subgranular zone at the base of the granule cell layer was populated by immunopositive cells that had a small soma size with dendritic

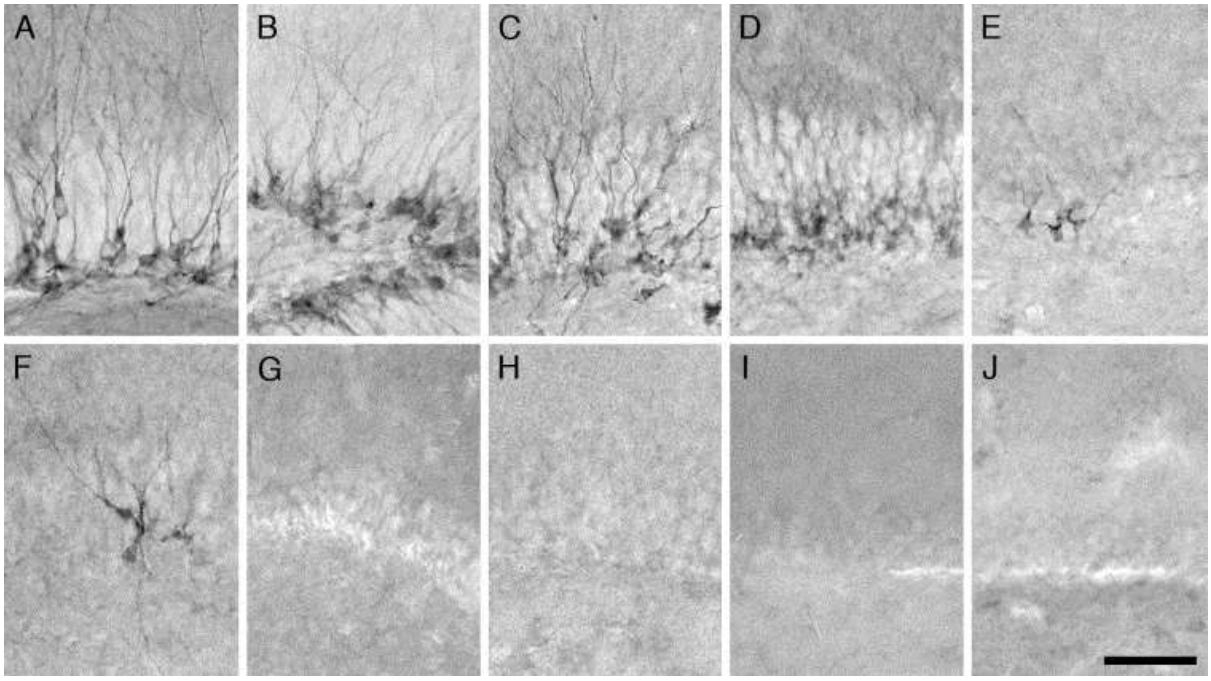

Figure 1: Photomicrographs of doublecortin immunoreacted sections of the dentate gyrus of the hippocampus of the various species of microchiropteran examined in the current study. Doublecortin immunoreactive immature neurons are clearly present in (**A**) *Hipposideros fuliganosas*, (**B**) *Asellia tridens*, (**C**) *Miniopterus schreibersii*, (**D**) *Triaenops persicus*, partially present in (**E**) *Cardioderma cor*, (**F**) *Chaerophon pumilus*, and absent in (**G**) *Coleura afra*, (**H**) *Hipposideros commersoni*, (**I**) *Nycteris macrotis* and (**J**) *Pipistrellus kuhlii*. The scale bar in **J** = 50 µm and applies to all images.

processes that extended through the granule cell layer to ramify into the molecular layer. Additonally, the mossy fibres that emanate from these cells were also observed with DCX immunohistochemistry, indicating that they are in the process of becoming functionally integrated into the hippocampal circuitry (Fig. 2B). The morphology of the DCX+ cells, when present, in the denate gyrus of the microchiropterans were similar to that seen in other mammals studied with the same technique.

In the *A. tridens* time series, (Table 1) DCX+ cells were readily observed at the 10 min post-capture time point (Figs. 2B, 3B and 4A), showing the full range of normal morphology of these immature neurons, including the presence of DCX+ mossy fibres. By 15 min post-capture, the number of DCX+ cells was dramatically reduced (to around 20% of the pre-15 min specimens, Table 1), as were the number of DCX+ dendrites and mossy fibres emanating from these cells (Fig. 3B and 4B). At 20 min post-capture there was a further reduction in the number of DCX+ cells (to around 6% of the pre-15 min specimens, Table 1),

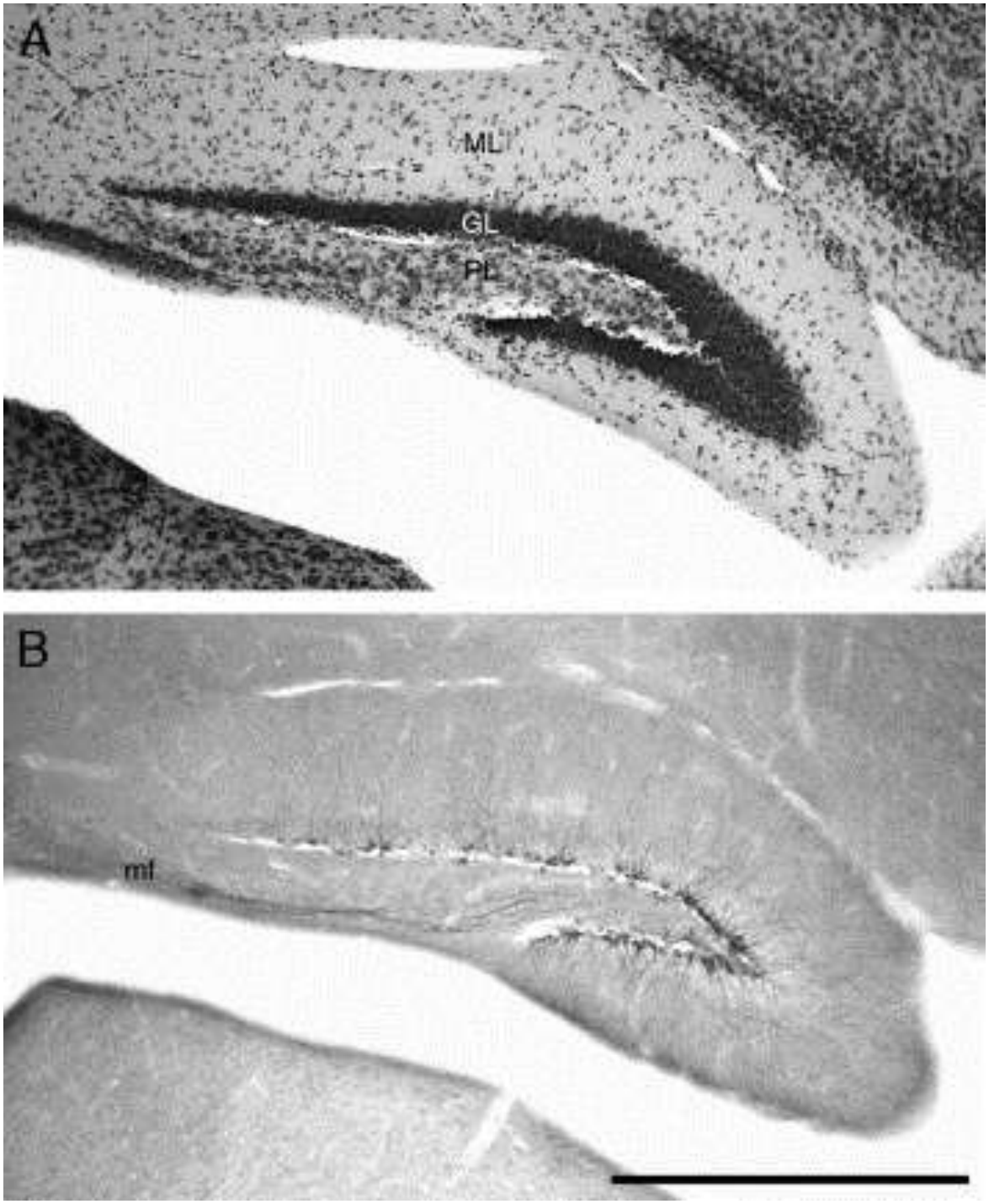

Figure 2: Photomicrographs of adjacent Nissl stained (**A**) and doublecortin immunoreacted (**B**) sections of the dentate gyrus of the hippocampus of *Asellia tridens* at 10 min post-capture. Note the presence of doublecortin immunopositive cells at the base of the granular layer (**GL**), dendrites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

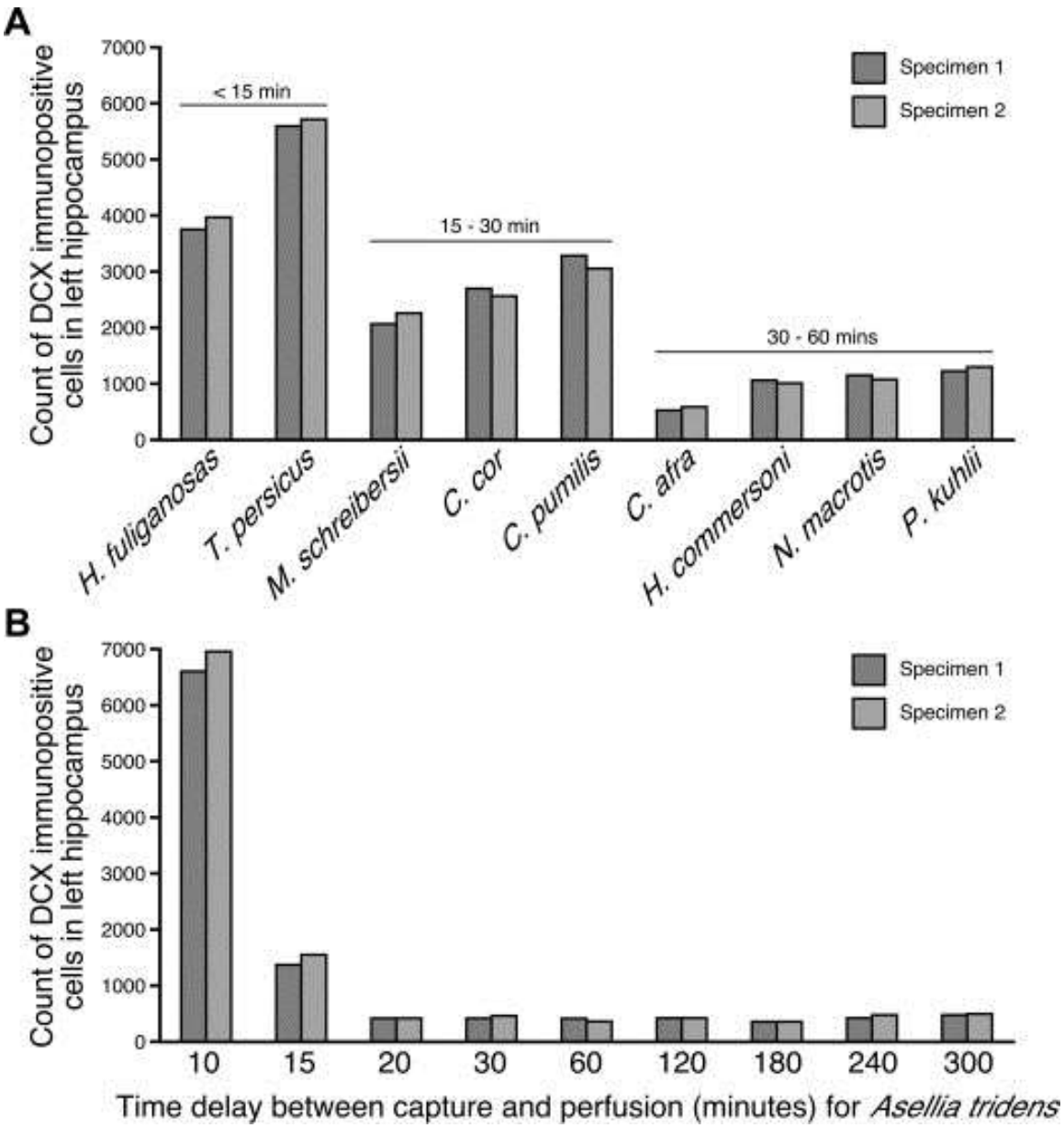

Figure 3: Bar graphs showing the results of our quantitative analysis of the number of doublecortin immunopositive neurons in the left hippocampus of a range of microchiropteran species. **(A)** This bar graph shows significant levels of DCX immunoreactive cells in the hippocampus of two species (*H.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

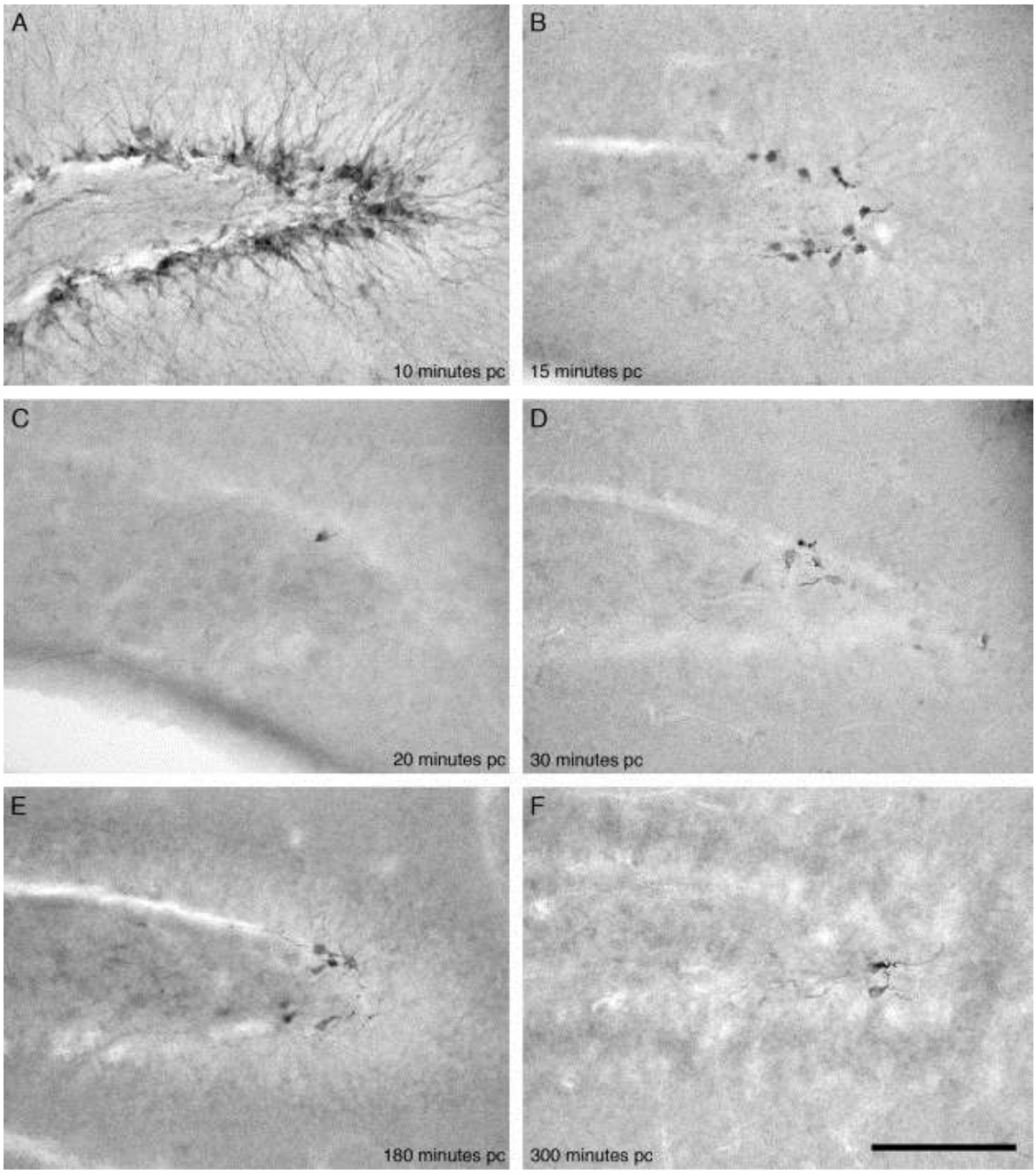

Figure 4: Photomicrographs of doublecortin immunoreacted sections of the dentate gyrus of the hippocampus of *Asellia tridens* at different time points post-capture (pc). At 10 min post-capture (A), numerous cells immunopositive for doublecortin are found throughout the entire dentate gyrus. These cells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

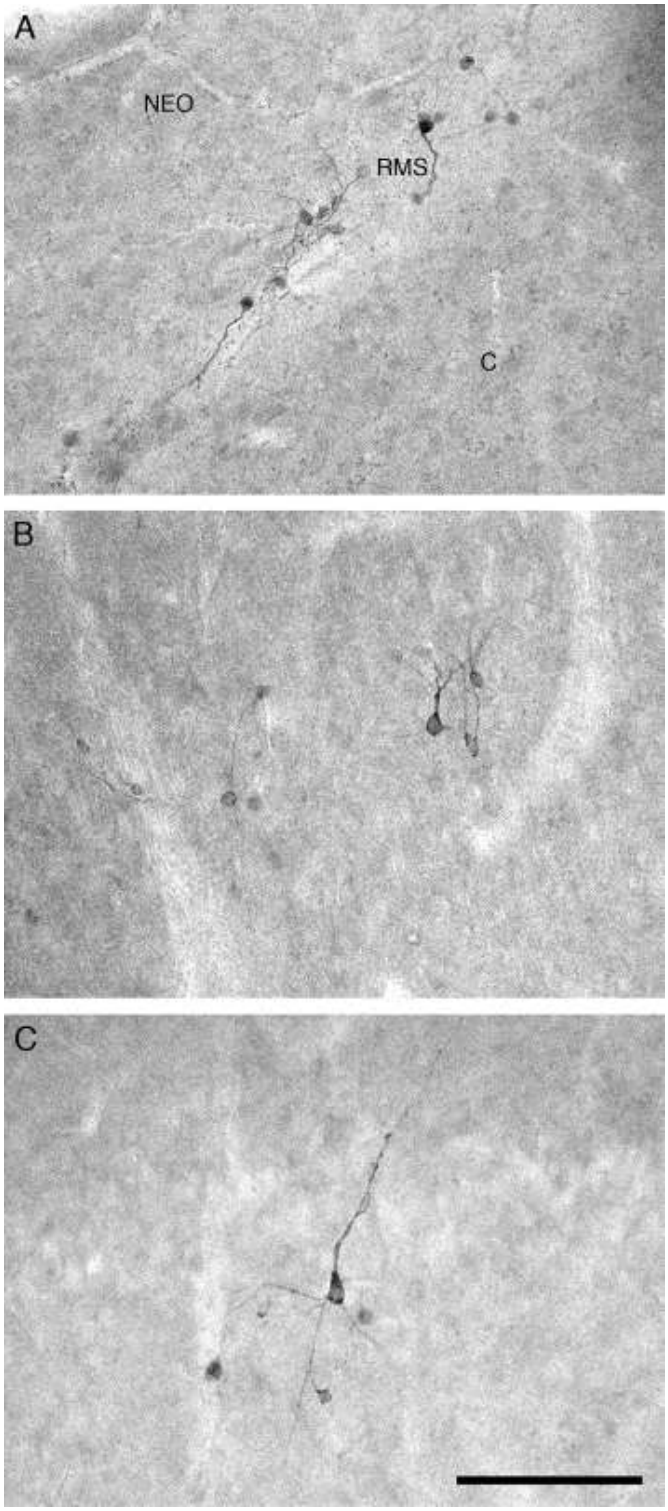

Figure 5: Photomicrographs of doublecortin immunoreacted sections in different regions of the brain of *Asellia tridens* at 10 min post-capture**. (A)** Doublecortin immunopositive cells and fibers in the rostral migratory stream (RMS) located between the caudate nucleus **(C)** and the cerebral neocortex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

dendrites and mossy fibres (Fig. 3B and 4C). By 30 min post-capture (Fig. 3B and 4D; Table 1), only a few DCX+ cells remained, and the DCX+ dendrites and mossy fibres were almost absent. The remaining time points examined, up to 300 minutes post-capture, evinced DCX immunostaining similar to that seen in the 30 min post-capture time point, with only a few persistent DCX+ cells, dendrites and mossy fibres (Fig. 3B and 4; Table 1). Similar to the trend observed with all species provided above, our comparison of DCX-labeled cells in *A. tridens* only showed that those animals perfused more than 15 min after capture (n = 14) was associated with a 10 times decline in cell number (median = 401 cells and range = 345–513 cells) when compared to those perfused within 15 min of capture (n = 4; median = 4078 cells and range = 1380–6945 cells) (Mann–Whitney test z = 2.974 and p = 0.0029).).

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

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

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

Discussion

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

The present study indicates that the potential problem encountered by Amrein et al (2007), leading to a false-negative report regarding adult hippocampal neurogenesis in the microchiropterans, was the length of post-capture stress and handling experienced by the animals prior to euthanasia and fixation of the neural tissue. The four species for which we did not observe any adult hippocampal neurogenesis were those that were perfused at a time point greater than 30 minutes post-capture. The two species in which partial adult hippocampal neurogenesis was observed were perfused between 15 and 30 minutes post-capture, but the four species for which we saw significant adult hippocampal neurogenesis were all perfused within 15 minutes of capture. The idea that post-capture stress and handling leads to a rapid decline of detectable adult hippocampal neurogenesis in the microchiropterans is fully supported by the time series study of *A. tridens* undertaken herein, where at 10 min post-capture DCX immunostaining revealed extensive adult hippocampal neurogenesis, but that by 15 min post-capture, the extent of staining had decreased dramatically and was near absent in subsequent time points. Similar effects in the decrease in the detectable presence of doublcortin in immature neurons have been observed in the retrosplenial cortex of the rat after 15 min exposure to actue stress (Kutsuna et al., 2012). The observation that post-capture stress rapidly diminishes the detectable presence of adult hippocampal neurogenesis in the microchiropterans may also explain other unusual results in

wild-caught species, such as the low proliferation rate, but high differentiation rate seen in wild caught South African rodents (Cavegn et al., 2013), where capture stress may have reduced the detectable presence of newly born neurons using Ki-67 immunohistochemistry, but had no specific effect on the differentiating neurons, as the doublecortin immunohistocehmistry used to detect differentiating neurons can be present in these neurons over a much longer period.

That the detectable presence of adult hippocampal neurogenesis in the microchirotperans disappeared so rapidly is of interest. As mentioned, the microchiropterans have a very high metabolic rate in comparison to other mammals (Austad and Fischer, 1991), and it is possible that the stress associated with capture or handling of wild animals, or animals not accustomed to being handled, when combined with a high metabolic rate, may lead to the rapid cessation of cell proliferation and the rapid breakdown of proteins associated with cell differentiation/maturation such as doublecortin, but perhaps not cell death (Kutsuna et al., 2012). Studies on standard laboratory animals often seek to eliminate any potential stressors from the protocol as it is well known that introduced stress can influence the experimental outcome (Balcombe et al., 2004). Similar care should clearly be taken when examining wild-caught species, as capture, handling and removal from a familiar environment can lead to high rates of stress (Morton et al., 1995). In the case of the microchiropterans, it appear that this has led to a false-negative report regarding the presence of adult hippocampal neurogenesis (Amrein et al., 2007). If care is not taken when examining wild-caught species, theories built on the observations made are likely to be incorrect, or misleading at best.

Reports detailing the presence of adult hippocampal neurogenesis across mammalian species are becoming more numerous, and in each case, it would appear that adult hippocampal neurogenesis is present (reviewed in Kempermann, 2012; see also Patzke et al., 2013; Chawana et al., 2013). Thus, at this stage, with the presence of adult hippocampal neurogenesis in the microchiropterans, it would appear that this neural trait is a common feature of mammalian brains; however, as mentioned by Kempermann (2012), certain species, such as cetaceans that live in homogeneous environments, do need to be examined to determine whether there is phylogenetic variability in this trait. If further comparative studies do reveal variation in this trait, these variations may help to understand whether adult hippocampal neurogenesis relates to either specific aspects of the environment of the species examined (extreme heterogeneity or extreme homogeneity), or whether other explanations

may account for this potential variation. Thus, at present, adult hippocampal neurogenesis may be thought of as a standard feature of mammalian brains and hippocampal function, but if variations are indeed present when further species are examined, these possible variations may shed more light regarding functional aspects of this interesting neural phenomenon in the adult mammal brain.

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References

Amrein, I., Dechmann, D.K., Winter, Y. & Lipp, H.P. 2007 Absent of low rate of adult neurogenesis in the hippocampus of bats (Chiroptera). *PLoS One* **2**, e455 (DOI: 10.1371/journal.pone.0000455)

Amrein, I., Isler, K. & Lipp, H. P. 2011 Comparing adult hippocampal neurogenesis in mammalian species and orders: influence of chronological age and life history stage. *Eur. J. Neurosci.* **34**, 978-987 (DOI: 10.1111/j.1460-9568.2011.07804.x.)

Anthony, E. L. P. 1988 Age determination in bats. In *Ecological and Behavioral Methods for the Study of Bats* (ed. T. H. Kunz), pp. 47-58. Washington DC: Smithsonian Institution Press,

Austad, S. N. & Fischer, K. E. 1991 Mammalian aging, metabolism, and ecology – evidence from the bats and marsupials. *J. Gerontol.* **46**, B47-B53.

Balcombe, J. P., Barnard, N. D. & Sandusky, C. 2004 Laboratory routines cause animal stress. *Contemp. Top. Lab. Anim. Sci.* **43**, 42-51.

Bartkowska, K., Turlejski, K., Grabiec, M., Ghazaryan, A., Yavruoyan, E. & Djavadian, R. L. 2010 Adult neurogenesis in the hedgehog (*Erinaceus concolor*) and mole (*Talpa europaea*). *Brain Behav. Evol.* **76**, 128-143 (DOI: 10.1159/000320944)

Bonfanti, L. & Peretto, P. 2011 Adult neurogenesis in mammals – a theme with many variations. *Eur. J. Neurosci.* **34**, 930-950 (DOI: 10.1111/j.1460-9568.2011.07832.x)

Cavegn, N., van Dijk, R. M., Menges, D., Brettschneider, H., Phalanndwa, M., Chimimba, C. T., Isler, K., Lipp, H. P., Slomianka, L. & Amrein, L. 2013 Habitat-specific shaping of proliferation and neuronal differentiation in adult hippocampal neurogenesis of wild rodents. *Front. Neurosci.* **7**, 59 (DOI: 10.3389/fnins.2013.00059)

Chawana, R., Patzke, N., Kaswera, C., Gilissen, E., Ihunwo, A. O. & Manger, P. R. 2013 Adult neurogenesis in eight Megachiropteran species. *Neuroscience* **244**, 159-172 (DOI: 10.1016/j.neuroscience.2013.04.020)

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H. G. & Aigner, L. 2005 Doublecortin expression levels in adult brain reflect neurogenesis. *Eur. J. Neurosci.* **21**, 1-14 (DOI: 10.1111/j.1460-9568.2004.03813.x)

Falconer, E. M. & Galea, L. A. 2003 Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. *Brain Res.* **975**, 22-36 (DOI: 10.1016/S0006-8993(03)02542-3)

Gatome, C. W., Mwangi, D. K., Lipp, H. P. & Amrein, I. 2010 Hippocampal neurogenesis and cortical cellular plasticity in Wahlberg's epauletted fruit bat: a qualitative and quantitative study. *Brain Behav. Evol.* **76**, 116-127 (DOI: 10.1007/s00429-010-0239-z)

Gould, E., Tanapat, P., McEwen, B. S., Flugge, G. & Fuch, E. 1998 Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc. Natl. Acad. Sci. U. S. A.* **9**, 3168–3171.

Heine, V. M., Maslam, S., Joels, M. & Lucassen, P. J. 2004 Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation. *Neurobiol. Aging* **25**, 261-375 (DOI: 10.1016/S0197-4580(03)00090-3)

Kempermann, G. 2012 New neurons for ‘survival of the fittest’. *Nat. Rev. Neurosci.* **13,** 727-736 (DOI: 10.1038/nrn3319)

Kim, J. B., Ju, J. Y., Kim, J. H., Kim, T. Y., Yang, B. H., Lee, Y. S. & Son, H. 2004 Dexamethasone inhibits proliferation of adult hippocampal neurogenesis in vivo and in vitro. *Brain Res.* **1027**, 1-10 (DOI: 10.1016/j.brainres.2004.07.093)

Klempin, F., Kronenberg, G., Cheung, G., Kettenmann, H. & Kempermann, G. 2011 Properties of doublecortin-(DCX)-expressing cells in the piriform cortex compared to the neurogenic dentate gyrus of adult mice. *PLoS ONE* **6**, e25760 (DOI: 10.1371/journal.pone.0025760)

Kohler, S. J., Williams, N. I., Stanton, G. B., Cameron, J. L. & Greenough, W. T. 2011 Maturation time of new granule cells in the dentate gyrus of adult macaque monkeys exceeds six months. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 10326-10331 (DOI: 10.1073/pnas.1017099108)

Kutsuna, N., Suma, T., Takada, Y., Yamashita, A., Oshima, H., Sakatani, K., Yamamoto, T. & Katayama, Y. 2012 Decrease in doublecortin expression without neuronal cell death in rat retrosplenial cortex after stress exposure. *NeuroReport* **23**, 211-215 (DOI: 10.1097/WNR.0b013e32834fca3a)

Morton, D. J., Anderson, E., Foggin, C. M., Kock, M. D. & Tiran, E.P. 1995 Plasma-cortisol as an indicator of stress due to capture and translocation in wildlife species. *Vet. Rec.* **136**, 60-63.

Patzke, N., Kaswera, C., Gilissen, E., Ihunwo, A. O. & Manger, P. R. 2013 Adult neurogenesis in a giant otter shrew (*Potamogale velox*). *Neuroscience* **244**, 159-172 (DOI: 10.1016/j.neuroscience.2013.04.020)

Powers, A. S. 2013 Adult neurogenesis in mammals and nonmammals. *Brain Behav. Evol.* **in press** (DOI: 10.1159/000350932)

Rao, M. S. & Shetty, A. K. 2004 Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *Eur. J. Neurosci.* **19**, 234-246 (DOI: 10.1111/j.0953-816X.2003.03123.x)

Tanapat, P., Hastings, N. B., Rydel, T. A., Galea, L. A. & Gould, E. 2001 Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats viaan adrenal hormone-dependent mechanism. *J. Comp. Neurol.* **437**, 496-504.

Widmaier, E. P. & Kunz, T. H. 1993 Basal, diurnal, and stress-induced levels of glucose and glucocorticoids in captive bats. *J. Exp. Zool.* **265**, 533-540.