Trading new neurons for status: adult hippocampal neurogenesis in eusocial Damaraland mole-rats

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Highlights

• The hippocampal architecture of the Damaraland mole-rat corresponds largely to that of other mole-rat species.

• No gender differences are found in hippocampal neurogenesis of Damaraland mole-rats.

• Neurogenesis is unrelated to relative age in Damaraland mole-rats.

• There is an inverse relationship between social status and neurogenesis in Damaraland mole-rats.

Abstract

Diversity in social structures, from solitary to eusocial, is a prominent feature of subterranean African mole-rat species. Damaraland mole-rats are eusocial, they live in colonies that are characterized by a reproductive division of labour and a subdivision into castes based on physiology and behaviour. Damaraland mole-rats are exceptionally long lived and reproductive animals show delayed aging compared to non-reproductive animals. In the present study, we described the hippocampal architecture and the rate of hippocampal neurogenesis of wild derived, adult Damaraland mole-rats in relation to sex, relative age and social status or caste. Overall, Damaraland mole-rats were found to have a small hippocampus and low rates of neurogenesis. We found no correlation between neurogenesis and sex or relative age. Social status or caste were the most prominent modulator of neurogenesis. An inverse relationship between neurogenesis and social status was apparent, with queens displaying the lowest neurogenesis while the worker mole-rats had the most. As there is no natural progression from one caste to another, social status within a colony was relatively stable and is reflected in the level of neurogenesis. Our results correspond to those found in the naked mole-rat, and may reflect an evolutionary and environmentally conserved trait within social mole-rat species.

Keywords: Damaraland mole-rat, castes, hippocampus, neurogenesis, sociality

1. Introduction

Neurogenesis does not only occur in the developing brain but is also conserved in discrete regions in the adult brain (Gage, 2000). In most mammals, proliferation of neuronal progenitor cells takes place throughout life in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Zhao et al., 2008). Newly born neurons originating from these regions are functionally integrated into existing neural circuits in the hippocampus and

the olfactory system (Van Praag et al., 2002, Carleton et al., 2003, Sakamoto et al., 2014). Neurogenesis in the adult brain is a very dynamic process and is characterized by its sensitivity to physiological stimuli at almost all stages of cell lineage progression, from proliferating cells to fully integrated neurons (Ming and Song, 2005, Ming and Song, 2011).

Several extrinsic factors such as physical exercise and environmental enrichment are robust regulators of neurogenesis that increase proliferation of cells and/or neuronal survival in the hippocampus (Kempermann and Kuhn, 1997, Van Praag et al., 1999). In addition, some intrinsic factors such as genetic lineage (Kempermann and Gage, 2002) modulate increases and decreases in hippocampal neurogenesis. Furthermore old age and stress are associated with a decrease in cell proliferation (Kuhn et al., 1996, Gould and Tanapat, 1999). The social environment has been shown to influence adult neurogenesis such that dominant animals normally show more neurogenesis than their subordinate counterparts (Fowler et al., 2002, Kozorovitskiy and Gould, 2004, Wu et al., 2014). Although there is no strong evidence for sex differentiation in adult neurogenesis, some differences have been reported (Chow et al., 2013, Marques et al., 2016). These differences appear to be largely dependent on hormonal variations (Marques et al., 2016). The precise functional significance of adult hippocampal neurogenesis is still a matter of debate, however evidence suggests that newly born neurons may have a more general function by contributing to behavioural flexibility in both wild and laboratory rodents (Garthe et al., 2009, Cavegn et al., 2013) by modulating the animal's reaction to novelty (van Dijk et al., 2015).

African mole-rats from the family Bathyergidae display a wide range of social organizations, with solitary species on one end of the spectrum and eusocial species on the other (Bennett and Faulkes, 2000). African mole-rats are strictly subterranean and inhabit sealed burrow systems (Bennett and Faulkes, 2000). Compared to surface dwelling rodents, a mole-rat tunnel system is a relatively simple but stable habitat devoid of visual landmarks. African mole-rats are therefore valuable models to

investigate the effects of physiological, ecological and behavioural parameters on neurogenesis within the framework of a simplified natural habitat but a complex social environment.

Like naked mole-rats, Damaraland mole-rats (*Fukomys damarensis*) are classified as eusocial and exhibit a caste system similar to that of social insects (Jarvis, 1981, Bennett and Jarvis, 1988, Lacey and Sherman, 1991). In Damaraland mole-rats, a primary reproductive division of labour is evident, and the remainder of the colony can be divided into two distinct morphological and physiological castes, namely disperser and worker castes, and the body mass and social status are reflected in the castes (Bennett and Jarvis, 1988, Scantlebury et al., 2006). It has been suggested that the larger, heavier mole-rats in the colony act as dispersers. After rainfalls when soils are more manageable for digging and dispersal, these animals move away from their natal colony to establish their own colonies. Indeed, disperser Damaraland mole-rats increase their daily energy expenditure significantly after rainfalls whereas smaller, leaner mole-rats maintain a high level of daily energy expenditure throughout the year (Scantlebury et al., 2006). Disperser mole-rats accumulate fat and are significantly heavier than worker mole-rats (Scantlebury et al., 2006) and heavier animals are dominant over lighter animals (Bennett and Jarvis, 1988, Schieffelin and Sherman, 1995).

Compared to most other rodents, mole-rats have unusually long life expectancies, animals may reach ages of up to 20 years in Fukomys species (Dammann and Burda, 2006, Dammann et al., 2011) and more than 30 years in the case of the naked mole-rat (Sherman and Jarvis, 2002). Since neurogenesis declines with age, species with long life spans typically show low rates of neurogenesis as adults (Amrein et al., 2011). However, life spans are not uniform amongst the different castes of a mole-rat colony, breeding animals may live twice as long as their non-breeding counterparts (Dammann et al., 2011). Also, the heaviest animals in a mole-rat colony are not necessarily the oldest. Theoretically, the queen is the oldest female in the colony, and although the worker mole-rats are the lightest animals in the colony, they are not necessarily the youngest. There is not a natural progression from worker to disperser to queens, some animals may remain workers for their

entire lives (Bennett and Jarvis, 1988). The aim of this study was thus to investigate and evaluate the level of neurogenesis in wild-derived Damaraland mole-rats with respect to caste, sex and relative age. Both dominance and sex have been reported to influence neurogenesis, therefore we anticipated differences in neurogenesis in these factors. Age is normally associated with a reduction in neurogenesis, thus we expected lower neurogenesis in older animals. The hippocampal histoarchitecture of the Damaraland mole-rat was also described as it has not been done previously.

2. Experimental procedures

2.1 Trapping and caste determination

Twenty-two adult Damaraland mole-rats (four queens, nine dispersers (5 ♂, 4 ♀), nine workers (4 ♂, 5 ♀)) originating from seven colonies were captured using modified Hickman live traps (Hickman 1979) near Blackrock in the Northern Cape, South Africa (27˚7'S, 22˚52'E). Animals were then allocated into different castes. Breeding females (queens) were easily discernible from the other colony members by the presence of nipples and a perforated vagina. The queens collected for this study were not visibly pregnant and showed no signs of lactation. For the remainder of the colony and separately for each colony, a distinction was made between large, potential disperser animals (longer and with noticeably more body fat) and smaller, leaner and shorter worker animals. A 20g separation was maintained in the body weights between disperser and worker animals within each distinct colony, mole-rats allocated to the disperser caste were always 20g or more, heavier than mole-rats in the worker class. Animals with body weights within the 20g separation zone were released again. We included a smaller male categorised as a disperser in the analysis (green square in Fig. 1a), this colony comprised of only two animals. Since the other animal was a queen, this is likely a newly established colony.

Animals were transported to the University of Pretoria and were perfused immediately. Animals were deeply anaesthetised with fluorothane gas (Zeneca, RSA) and perfused transcardially with 0.9%

phosphate buffered saline (pH7.4, Sigma), followed by 4% paraformaldehyde (PFA; Saarchem, RSA) in 0.1M phosphate buffer (pH 7.4; Sigma). The brains were removed from the sculls and post fixed overnight, where after they were stored in 2% PFA. Prior to sectioning, brains were saturated in 30% sucrose for cryoprotection. Right hemispheres were processed for immunohistochemistry whereas the left hemispheres were embedded in hydroxyethylmethacrylate for granule cell counts. Femurs and eye lenses were harvested for relative age determining.

2.2 Histoarchitecture

The left hemispheres of the mole-rat brains were washed in PBS, dehydrated in ascending alcohol solutions over 4 days and embedded in hydroxyethylmethacrylate (HEMA; Technovit 7100, Heraeus Kulzer GmbH, Wehrheim/Ts, Germany). For this, brains were pre-infiltrated overnight with a 1:1 solution of 100% alcohol and HEMA, followed by three changes of HEMA only, for a total period of one month. Embedded brains were cut horizontally at 20 µm. Every 6th section was collected, mounted on slides and dried at 60 °C for 1h. Sections were incubated in a Giemsa staining solution (Giemsa stock solution 1.09204.0500, Merck, Darmstadt, Germany, diluted 1:10 in 67 mmol KH_2PO_4 buffer) at room temperature for 40 min, differentiated in 1% acetic acid for 15s, dehydrated in 3 changes of pure alcohol, cleared in xylol and cover-slipped.

2.3 Immunohistochemistry

Right hemispheres of the mole-rat brains were sectioned in sagittal sections on a sliding microtome (Microm) equipped with a freezing stage at a thickness of 40 µm, sections were collected in series of 12. Two series of sections each were used for immunohistochemical analysis of NCL-Ki67 and PSA-NCAM, while NeuN, Calbindin and Parvalbumin were stained only in selected animals.

Free floating sections were rinsed in Tris buffered saline (TBS) containing 0.05% Triton. For NCL-Ki67 immunohistochemistry, epitope retrieval was required, these sections were incubated in a citrate buffer (DAKO Target Retrieval Solution, pH 6.1) for 40 minutes at 94˚C followed by another rinse in

TBS. For all other antibodies, endogenous peroxidase activity was blocked by incubation of the sections in 0.6% hydrogen peroxidase for 30 minutes and rinsed in TBS. Sections were pre-incubated in TBS containing 0.25% Triton, 2% normal serum of the animal the secondary antibody was raised in and 0.1% bovine serum albumin (BSA) for one hour. Thereafter sections were incubated overnight at 4˚C in primary antibodies in the same diluent (Polyclonal rabbit anti-NCL-Ki-67, Dianova 1:2500; monoclonal mouse anti-PSA-NCAM, Chemicon/Millipore, 1:5000; polyclonal goat anti-NeuN, Santa Cruz Biotechnology 1:1000; polyclonal rabbit anti-Calbindin D28K, Swant 1:1000; monoclonal mouse anti-Parvalbumin, Sigma-Aldrich 1:20'000). After a rinse in TBS, sections were incubated in secondary antibodies (all Vectastain, 1:300) for an hour, followed by avidin-biotin complex (Vectastain Elite ABC kit) and stained with 3,3'diaminobenzidine (DAB) as a chromogen. Sections were mounted, cover slipped and investigated on an Olympus BX 40 microscope.

2.4 Quantification

Granule cell numbers were estimated in the HEMA embedded sections using design based stereology. The optical fractionator method (West et al., 1991) with a 100x oil immersion lens (NA = 1.3) was used in conjunction with StereoInvestigator software (MicroBrightField, Inc., Williston, USA). Granule cells were sampled in every 12th section in 10 μm high and 15 x 15 μm wide dissector and 2 μm top guard zones at 240 μm intervals along the *x*- and *y*-axis. A mean of 17.2 (±0.6) sections per animal were counted (queens 18.7±2.5, dispersers 17.1±0.9, workers 16.7±0.3).

Ki67 positive, proliferating cells and PSA-NCAM positive young neurons located in the granular and subgranular cell layers were counted manually in every 6th section, positively stained cells in the top focal plane were discarded. A mean of 17.2 (±0.4) sections per animal were counted for PSA-NCAM positive neurons (queens 18.25±1.1, dispersers 17.2±0.4, workers 16.7±0.6) and for Ki67 positive cells a mean of 16.8 (±0.4) sections per animal were counted (queens 17.25±1.0, dispersers 16.8±0.7, workers 16.6±0.5).

2.5 Relative age determination

Relative age was determined using the number of adhesion lines in the inner and outer circumferential lamellae of the femur (Barker et al., 2003). Femurs were washed and immersed in a solution for rapid decalcification (Baker, Histograde) for 24h. A 3mm segment was measured and cut from the mid-diaphysis and infiltrated with HEMA. Twenty μm sections were stained with hematoxylin-eosin. Adhesion lines in the inner and outer circumferential lamellae were counted in the bone tissue of cross sectioned femurs using brightfield and fluorescent illumination.

As an additional aging measure, eye lenses were collected. Lenses grow throughout life, and can therefore be used to determine the relative age of an animal (Morris, 1972). The lenses were dried for 5 days at 80°C where after they were weighed. Because of the small size of the lenses, this turned out to be a futile attempt, and the lenses were measured instead using a digital calliper. Lens diameter measurements correlated with the corresponding numbers of adhesion lines in the circumferential lamella and served as verification of relative age determinations.

2.6 Statistical Analysis

The Gundersen-Jensen Coefficient of Error (CE) was determined to assess experimentally introduced variance to granule cell number estimates, using the conservative approach of m=0 (Gundersen et al., 1999). The integrity of the cell counts was assessed by the contribution of the estimation procedure to group variances, conveyed by the ratio CE^2/CV^2 [CV = Coefficient of variation = standard deviation (SD)/mean cell count].

Statistical analyses were performed using SPSS 21.0 (SPSS Inc, Chicago, Illinois). The relationships between bodyweight, age, total granule cell number and positively stained cells for neurogenesis markers were compared using a two-tailed Spearman's r correlation analysis. In addition, body weight in relation to castes was evaluated with a Generalized Linear Model (GLM), as was the analysis of neurogenesis related cells in the dentate gyrus. Here cell numbers were employed as

dependent variables to reveal caste and sex differences in the Damaraland mole-rat hippocampus and relative age was controlled for as a covariate. LSD post-hoc tests were applied and statistical significance was maintained at $P < 0.05$.

3. Results

3.1 Effect of caste on bodyweight, relative age and hippocampal granule cells

Body weights of the three castes differed significantly ($\chi^2 = 23.34$, df = 2, $P < 0.001$). The body weight of the queens and disperser animals did not differ ($P = 0.58$), however worker mole-rats were significantly lighter than both queens and disperser mole-rats (both comparisons $P < 0.001$). Body weight and age were not correlated with one another ($r_{(20)} = 0.062$, $P = 0.783$; Table 1, Figure 1A), and similarly estimated age was not correlated with caste ($r_{(20)} = -0.139$, $P = 0.536$).

Table 1. Estimated cell numbers are provided unilateral and separate for castes and sexes. Granule cell numbers are rounded to the nearest 1000, and young neurons and proliferating cell numbers are rounded to the nearest 10. Values in parenthesis indicate standard errors; CE: Coefficient of error m=0 (Slomianka and West, 2005).

		Total N	Body weight (g)	Relative age	Granule cells	Young neurons	Proliferating cells
All animals		22	119.36 (5.1)	5.7 (0.4)	489 000 (20 000)	3 500 (430)	620 (90)
					CE 0.11 (0.03)	CE 0.08 (0.01)	CE 0.12 (0.01)
queen	female	4	137.5 (6.74)	6.6 (1.3)	519 000 (99 000)	990 (340)	170 (30)
disperser mole-rat	male	5	137.2 (13.22)	6.3 (0.9)	507 000 (69 000)	3 420 (960)	490 (120)
	female	4	124.25 (5.5)	5.2 (0.6)	500 000 (27 000)	3 600 (1 180)	590 (120)
worker mole-rat	male	4	94.5 (7.75)	5.3 (1)	466 000 (26 000)	4 800 (810)	1 000 (260)
	female	5	103 (3.6)	5.5 (0.6)	511 000 (43 000)	4 120 (610)	820 (190)

Absolute estimations of granule cell numbers in the dentate gyrus of wild Damaraland mole-rats were relatively low, however the coefficients of error (CE = 11 ± 0.03%, Table 1) indicated a good precision (Slomianka and West, 2005). The granule cell numbers in the dentate gyrus were not correlated with body weight ($r_{(20)} = 0.094$, $P = 0.678$) and there were also no correlation with relative age ($r_{(20)} = 0.167$, $P = 0.458$; Figure 1B).

Figure 1. Correlations of relative age X body weight, total granule cells X relative age and proliferation X young neurons in Damaraland mole-rats.

(A) Body weight of Damaraland mole-rats is not correlated with the relative age. Instead, body weight separates the castes independently of the relative age. The distribution nicely illustrates the lack of a natural progression in social status with ageing. The different symbols indicate the respective colonies, and the castes are shown in different colours (See legend on Fig 1c). (B) Total granule cells numbers are not correlated with relative age, and (C) a scatter plot of proliferating cells (Ki67) and young neurons (PSA-NCAM) of the different castes in the Damaraland mole-rat.
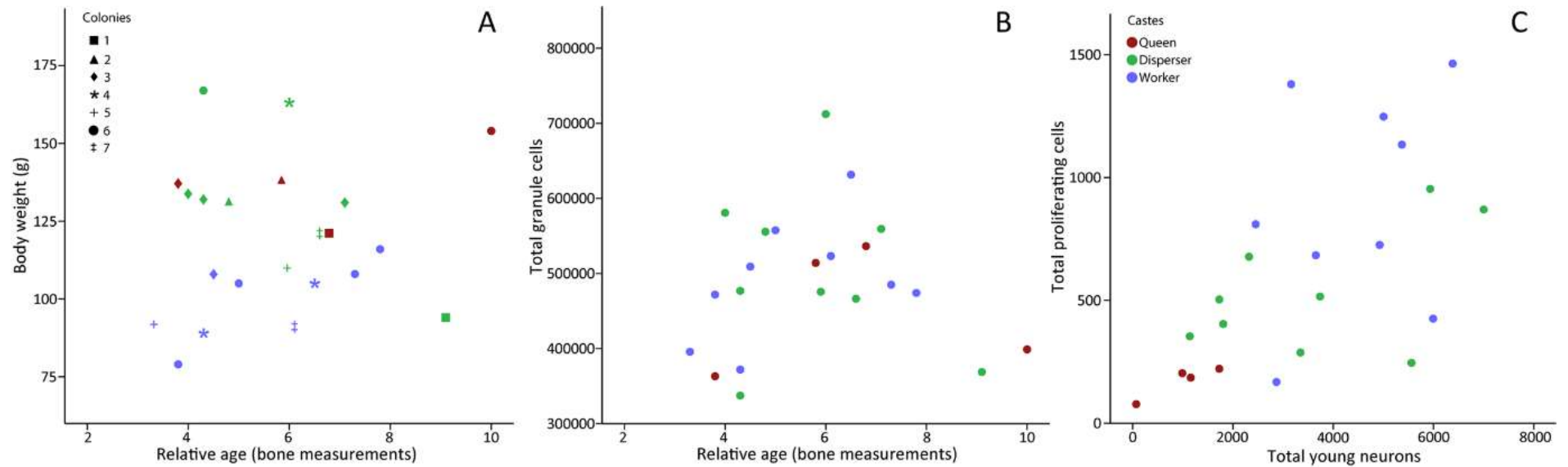
3.2 Caste affiliation had the strongest effect on hippocampal neurogenesis

ANOVA analysis of neurogenesis related cells in the dentate gyrus was assessed with castes and sex as fixed factors and relative age as a covariate. Castes played a significant role in the numbers of Ki67 positive proliferating cells (χ^2=19.57, df=2, P < 0.001, Figure 1C, 2A), and post-hoc analysis revealed that worker mole-rats possess higher numbers of proliferating, Ki67 positive cells than both disperser mole-rats (P = 0.003) and queens (P < 0.001). The number of proliferating cells did not differ between queens and disperser mole-rats (P = 0.079; Figure 3B). The number of proliferating cells was not different between males and females when queens were included (χ^2 = 3.02, df=1, P = 0.129) or excluded from the analysis (χ^2 = 0.05, df=1, P = 0.817). The interaction between sex and caste showed an effect on proliferating cells (χ^2 = 20.06, df=4, P < 0.001). Post hoc tests showed that queens have significantly less proliferating cells than male (p<0.001) and female (p = 0.001) worker mole-rats and male dispersers have less proliferating cells than female workers (p=0.03).

Castes were also identified as a contributing factor in the numbers of PSA-NCAM positive young neurons (χ^2=14.59, df=2, P = 0.001, Figure 1C, 2B). Both worker mole-rats (p<0.001) and disperser mole-rats (p=0.01) displayed a higher number of PSA-NCAM positive young neurons compared to queens, but no difference was observed between worker and disperser mole-rats (P= 0.134; Figure 3C). Males had significantly more young neurons compared to females (χ^2 = 3.91, df=1, P = 0.048), however this difference disappeared when queens were not included in the analysis (χ^2 = 0.113, df=1, P = 0.737). The sex and caste interaction indicated an influence on the young neurons (χ^2 = 15.02, df=4, P = 0.005). Queens have less young neurons than male (p=0.021) and female (p=0.028) dispersers and male (p<0.001) and female (p=0.003) workers.

Relative age was not correlated with the number of proliferating cells ($r_{(20)}$=-0.278, p=0.211) or young neurons ($r_{(20)}$=-0.025, p=0.910). The total number of granule cells are similar in all groups and are not affected by any of the factors investigated (Table 1, Figure 3A).

Figure 2. Neurogenesis-related markers in the Damaraland mole-rat dentate gyrus

Representative examples of immunopositive cells in the subgranular layer (SGL) of the Damaraland mole-rat dentate gyrus for Ki67 positive proliferating cells (A), and PSA-NCAM positive young neurons (B). Counterstain with hematoxylin in B allows the identification of PSA-NCAM positive cell bodies versus dendritic staining. The extensive ramification of the dendritic tree of young neurons into the molecular layer (ML) can be observed, the staining also visualises the axons of young neurons extending into the polymorphic hilar region (H). Scale bars A: 10 µm, B: 50 µm. GC: Granule cell layer; SGL: Subgranular layer; H: Hilus; ML: Molecular layer.
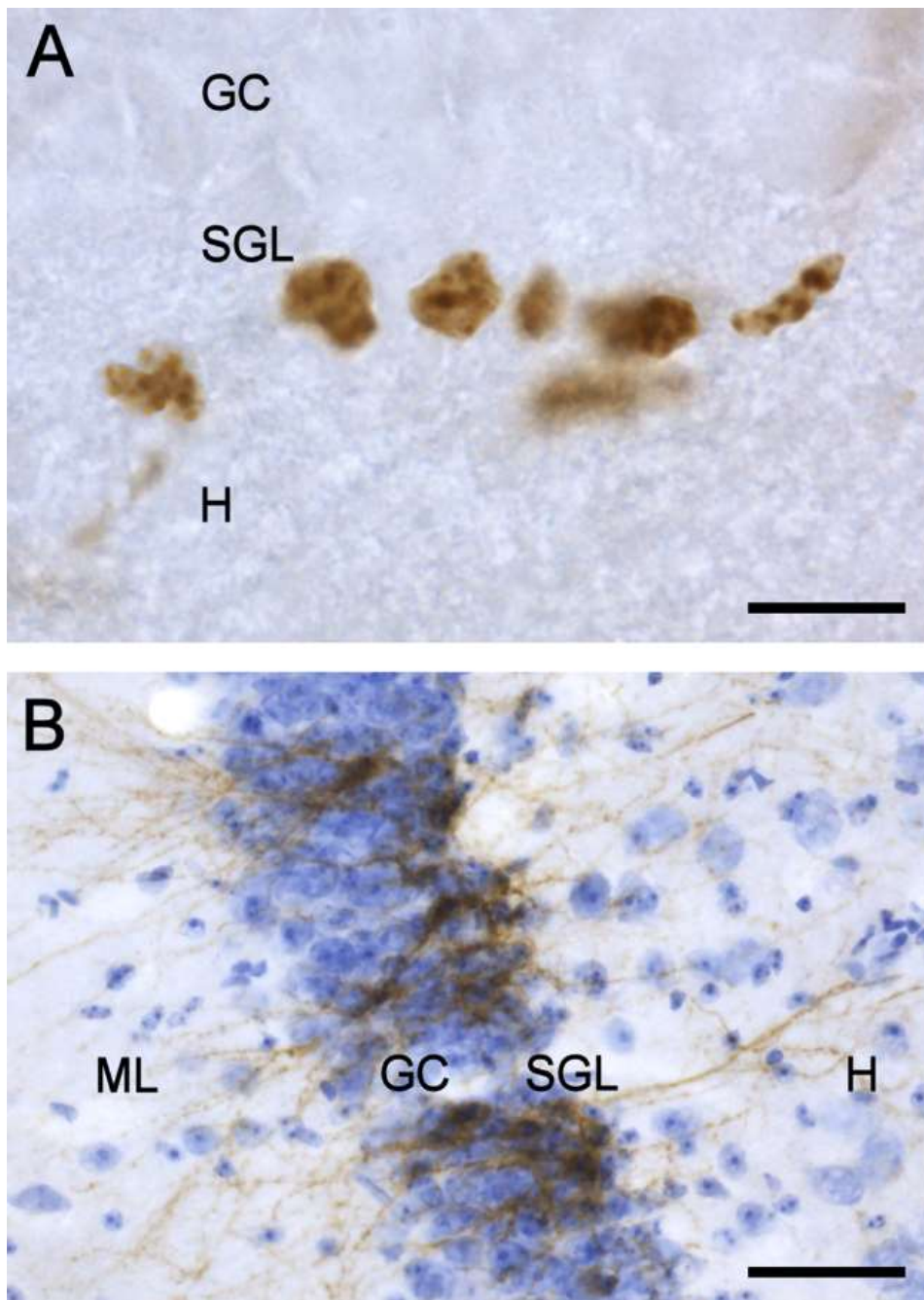
Figure 3. Granule cell numbers and neurogenesis-related cell numbers in the Damaraland mole-rat

(A) The total number of granule cells is similar in the three castes of Damaraland mole-rats, while (B) worker mole-rats have more proliferating cells than both the disperser mole-rats and the queens. Numbers of proliferating cells do not differ between dispersers and queens. (C) Worker and disperser mole-rats have more young neurons than queens, but the number of young neurons in disperser mole-rats do not differ from the worker mole-rats. For exact p-values see result section.
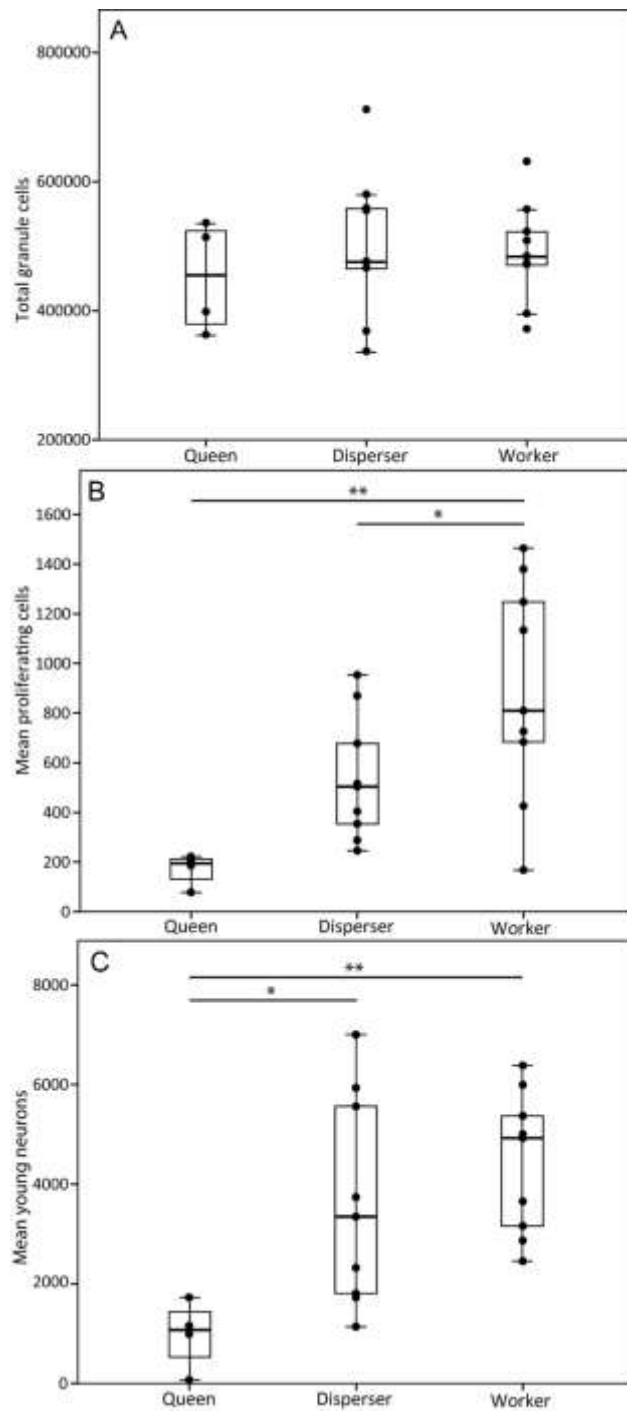
Figure 4. Cell distribution in the Damaraland mole-rat hippocampal formation

Overview of the hippocampal formation in Damaraland mole-rats in Giemsa stained sections (A), and corresponding sections immunohistochemically stained for NeuN (B), calbindin (C) and parvalbumin (D). Arrows in A mark the proximal and distal boundaries of the mole-rat typical large CA3 region. In the NeuN stained section (B), the segregation of the CA1 pyramidal cell layer in a deep and superficial tier is particularly clear, arrows mark the proximal and distal boundaries of the CA1 region. Calbindin (C) and parvalbumin (D) staining in Damaraland mole-rats is similar to Cape mole-rats (see Amrein et al 2014), with the exception of an even neuropil staining of the molecular layer without a darker staining of the medial performant path (arrow) seen in Cape mole-rats. Scale bars all 250 μm.
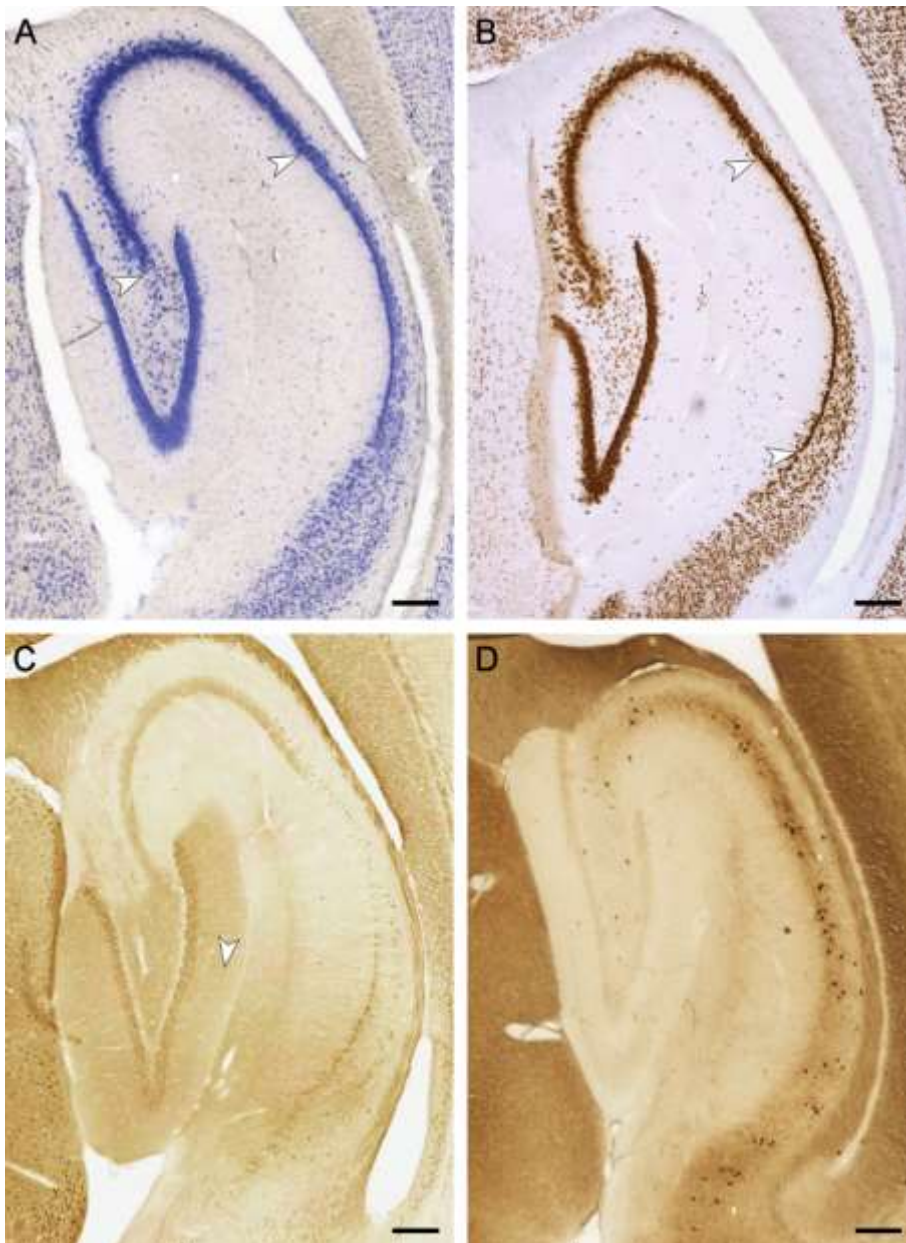
3.3 Histoarchitecture of the Damaraland mole-rat hippocampus

The dentate gyrus of the Damaraland mole-rat appeared to be relatively small while the CA3 is extensive (Figure 4). This was also characteristic of the other three mole-rat species previously investigated by us (Amrein et al., 2014). Within the dentate gyrus, Damaraland mole-rat hilar polymorphic cells extended from the tip of CA3 as a band beneath the dentate granule cells. This band widened to almost fill the centre of the hilus, similar to what was observed in the Cape mole-rat (Figure 5A, B). The pronounced division of the CA1 pyramidal cell layer into separate superficial and deep tiers seen in Cape- and Highveld mole-rats was far less obvious in Damaraland mole-rats, where the histoarchitecture of CA1 very closely resembled that seen in naked mole-rats. We assessed the distribution of calbindin (Figure 5C) and parvalbumin (Figure 5D) as markers of different populations of interneurons and principal cells. In the Damaraland mole-rat, their distribution patterns closely resembled the pattern that we described for the Cape mole-rat. The only clear distinction between the two species was the absence, in the Damaraland mole rat, of the dark calbindin-positive band at the border between the outer and middle dentate molecular layers seen in the Cape mole rat.

4. Discussion

African mole-rats possess a number of unusual features that set them apart from other rodent species. Perhaps the most prominent and relevant characteristic to this study is the extreme form of social organization exhibited by eusocial mole-rat species. Damaraland mole-rats display a morphological and physiological caste system which is largely independent of age (Bennett and Faulkes, 2000). In addition, mole-rats are extraordinary long-lived with negligible senescence. Neuronal numbers of the granule cell layer as well as cells related to neurogenesis in the dentate gyrus of the eusocial Damaraland mole-rat will be discussed in context of the above.

4.1 Neurogenesis in a natural rodent population

The importance of studying free living populations to properly understand the functional and adaptive significance of neurogenesis has been emphasized repeatedly (Boonstra et al., 2001, Nottebohm, 2002, Barnea, 2010, Amrein, 2015). Simultaneously, it has also been recognised that when conducting research under natural conditions, the environment is not controlled and can introduce a large range of unknown factors that may potentially influence results (Gould et al., 2000, Barnea, 2010, Amrein, 2015). These environmental effects become evident when investigating single species that occur in a wide variety of ecological niches. For example in chickadees, the results indicate that populations that live in harsher environmental conditions show increased neurogenesis (Chancellor et al., 2011). In rodents, a different approach has been followed where various species from diverse habitats were compared, and once again rodents species resident in more challenging environments show higher neurogenesis (Cavegn et al., 2013). Compared to these surface dwelling rodents, mole-rats have the lowest level of neurogenesis (Amrein et al., 2014, Penz et al., 2015). The Damaraland mole-rats presented here fit well into the pattern of mole-rat species. Our finding is therefore in agreement with the habitat complexity hypothesis since mole-rats occupy a very stable and uniform habitat, and even in the more complex tunnel systems of eusocial mole-rat species, the animals are subjected to vastly less environmental stimuli compared to those of the habitats of surface dwelling rodents. The fact that within the eusocial Damaraland mole-rats, caste members again show different patterns in neurogenesis as discussed below is particularly interesting as this natural within-species variation could be used for testing the functional significance of adult hippocampal neurogenesis in behaviour under natural conditions.

Mole-rats have remarkably long lifespans for their body size (Buffenstein and Jarvis, 2002, Sherman and Jarvis, 2002, Dammann and Burda, 2006). They reach adult weight relatively later in their lives compared to other similar sized rodents (Bennett and Jarvis, 1988) and also show prolonged brain maturation (Penz et al., 2015). Generally, ageing has a very strong effect on hippocampal

neurogenesis, with aged animals showing a drastically lower level of neurogenesis than young individuals (Kuhn et al., 1996, Ben Abdallah et al., 2010). In rats and mice, neurogenesis declines exponentially to reach a low but relatively constant level in animals older than 7 to 12 months (Rao et al., 2006, Ben Abdallah et al., 2010). The mole-rats in the present study are all adults, and our data suggest that these long-lived species also reach a low but stable rate of neurogenesis.

4.2 Effect of social status on neurogenesis

Social status is known to influence the rate of adult neurogenesis in the dentate gyrus. The majority of studies indicate that dominant animals show more neurogenesis than subordinate ones (Gould et al., 1997, Gould et al., 1998, Kozorovitskiy and Gould, 2004, Thomas et al., 2007, Wu et al., 2014). In contrast, social isolation of group-living species has also been shown to reduce neurogenesis (Stranahan et al., 2006, Spritzer et al., 2011, Lieberwirth et al., 2012, Lieberwirth and Wang, 2012, Cinini et al., 2014). In stark contrast to what has been found in other rodent species, cooperatively breeding mole-rats exhibit an inversed relationship between neurogenesis and dominance hierarchy. Significant differences in proliferating cells and young neurons are observed between the different castes in Damaraland mole-rats. Queens have the lowest levels of proliferating cells and young neurons and the subordinate worker mole-rats have the highest number of proliferating and young neurons. Thus the rate of neurogenesis appears to progressively decrease as the level of dominance increases. Our findings are in agreement with findings in laboratory-bred naked mole-rats, the breeding animals, in particular the queens, were found to have a reduced number of young neurons compared to non-breeding subordinate colony members (Peragine et al., 2014).

Neurogenesis declines with age (Kuhn et al., 1996, Zitnik and Martin, 2002, Galvan and Jin, 2007), and in social mole-rat species the breeding animals are typically the oldest animals in the colony. By studying wild populations, the exact age of the animals is not known and is typically restricted to age classes (Amrein, 2015). This effect is on the one hand exaggerated by the long life expectancies of social mole-rats and on the other hand countered by the delayed ageing of reproductive mole-rats

(Dammann and Burda, 2006, Dammann et al., 2011). Contrary to what was expected, in our current sample we did not find a correlation between age and any of the cell numbers counted, suggesting that our results are independent of age. Nevertheless, our sample was relatively small thus an age effect cannot be completely disregarded, and would require further investigation.

The low neurogenesis in the dominant queen in the Damaraland mole-rat colony may be related to the fact that this animal is the only female that is engaged in mating, pregnancy, and lactation, activities that has been linked to reduced neurogenesis in a wide range of species (Lieberwirth and Wang, 2012, Cavegn et al., 2013). Gestation in Damaraland mole-rats is up to 92 days, which is unusually long for rodents (Bennett and Jarvis, 1988). Even though pups are born precocial, they are only weaned at 21-28 days after birth (Bennett and Jarvis, 1988). Considering that Damaraland mole-rats are aseasonal breeders that can produce up to four litters per year (Bennett and Faulkes, 2000), the queen can literally be either pregnant or lactating at any time of the year. Thus, neurogenesis in queens might be limited by the physiological and energetic burden of reproduction despite their dominant status within the colony.

Oestrogen plays a significant role in the regulation of hippocampal neurogenesis, affecting both cell proliferation and cell survival (Galea, 2008). However, the effects of oestrogen depend on a variety of factors such as the sex of the animal, the time of exposure and the amount of oestrogen (Galea et al., 2006). Typically, oestrogen has an enhancing effect on neurogenesis. For example, in meadow voles, higher oestrogen levels in the breeding season promoted cell survival in both males and females (Ormerod and Galea, 2001, Ormerod and Galea, 2003) and also increases the cell survival in males (Ormerod et al., 2004). Acute doses of oestrogen initially enhances neurogenesis and then suppresses it (Ormerod et al., 2003). The inversed relationship between social status and hippocampal neurogenesis observed in our study contrasts previous findings. In social mole-rats, the reproductive female or queen is the only animal in the colony that ever have significant levels of oestrogen present. Non-reproductive Damaraland mole-rat females have low oestrogen levels

throughout the year whereas reproductive females show elevated oestrogen levels during pregnancy (Bennett, 1994). Previous studies investigating the influence of testosterone on neurogenesis are inconsistent, with some showing no effect and others increases and decreases (Ormerod and Galea, 2003, Spritzer and Galea, 2007, Benice and Raber, 2010, Buwalda et al., 2010, Spritzer et al., 2011, Hamson et al., 2013). Nonetheless, Bennett showed that testosterone levels do not differ between males in Damaraland mole-rat colonies (Bennett, 1994). Peragine and colleagues found no relationship between gonadal hormones and neurogenesis in the naked mole-rat (Peragine et al., 2014). The combined findings of our study and that of the naked mole-rats suggest that the reduced neurogenesis in eusocial mole-rat species are unrelated to gonadal hormones.

Regrettably stress hormones were not measured in this study and would have provided some insight regarding the stress levels of the respective castes. Hippocampal cells are sensitive to stress, and although some positive effects have been reported, numerous studies in many different species provide evidence for a stress-induced reduction in cell proliferation in the dentate gyrus (Gould et al., 1997, Gould et al., 1998, Tanapat et al., 2001, Malberg and Duman, 2003, Brain et al., 2004, Mitra et al., 2006). Social stress also has been shown to cause a reduction in locomotor activity, social motivation and interactions (Blanchard et al., 2001, Denmark et al., 2010, Sandi and Haller, 2015). In a previous study, Damaraland queens were found to have the lowest or almost the lowest levels of cortisol among the females in the colony, although this difference is not significant in most colonies (Clarke et al., 2001). However, worker mole-rats with the lowest status in the colony shows the largest number of proliferating cells and young neurons and are the most active. Thus it is unlikely that stress plays a role in the caste dependent differentiation in neurogenesis.

The rate of neurogenesis present in the different castes of social mole-rats may potentially also be related to physical activity and the utilization of space of individual animals in a colony. Although tunnel systems can be very complex and extensive in social mole-rat species (Bennett and Faulkes, 2000), individuals from a colony show distinct differences in the extent to which they use the tunnel

system. Work by our Czech colleagues suggests that in social species, breeding animals and large non-breeders spend more time in the nest and their activity were mostly concentrated closer to the nest, while smaller non-breeders travel further from the nest and make use of a larger part of the burrow system (Šklíba et al., 2015). The lean worker Damaraland mole-rats are also responsible for the majority of the extensions of the tunnel systems. Higher neurogenesis rates in this non-breeding mole-rat caste may provide some flexibility to explore novel areas and to quickly accommodate for the extended tunnels they have to navigate (Amrein, 2015). Retaining worker status for a lifetime may actually be advantageous for animals, since high physical activity while ageing has repeatedly been shown to mediate beneficial effects not only for increased neurogenesis, but a wide array of neurobiological processes (Voss et al., 2013).

4.3 Hippocampal cytoarchitecture of mole-rats

The hippocampal cytoarchitecture of Damaraland mole-rats resemble that of other mole-rats (both solitary and social) to a large extent (Amrein et al., 2014). Mole-rats species possess a relatively small dentate gyrus with low numbers of resident granule cells. In fact, Damaraland mole-rats harbours similar numbers of granule cells than laboratory mice (Calhoun et al., 1998, Abusaad et al., 1999, Fabricius et al., 2008) despite a body weight up to six times higher than that of laboratory mice. Like other mole-rats, Damaraland mole-rats show a prominent CA3 layer and lamination of the CA1 layer (Slomianka et al., 2011). Calbindin and parvalbumin staining of the Damaraland mole-rat is similar to that of the solitary Cape mole-rat, the deep calbindin+ pyramidal cells present in the CA3 of naked mole-rats are however absent in the Damaraland mole-rat (Amrein et al., 2014). Based on the species available, we did not observe cytoarchitectonic patterns that could be related to social structure in mole-rats, however all mole-rats share characteristics that appear distinct from other rodents.

4.4 Concluding remarks

We found that adult Damaraland mole-rats have overall low numbers of hippocampal granule cells, proliferating cells and young neurons. Castes manifested as a prominent modulator of neurogenesis in this social mole-rat species. Neurogenesis in Damaraland mole-rats is inversely correlated to social status in the colony, with subordinate worker mole-rats exhibiting more proliferating cells and young neurons than disperser mole-rats and queens. Neurogenesis appears to be unrelated to age and gonadal hormones, two factors which usually have a significant modulating effect on neurogenesis. The higher rate of neurogenesis in worker mole-rats may be related to the larger areas in the tunnel systems that they utilize compared to the other two castes, whereas low neurogenesis in queens may be related to their permanent engagement in reproduction. The similarity of our results to that of the naked mole-rats may indicate an evolutionary and environmentally conserved trait within social mole-rat species.

5. Acknowledgments

6. Reference list

Abusaad I, MacKay D, Zhao J, Stanford P, Collier DA, Everall IP (1999) Stereological estimation of the total number of neurons in the murine hippocampus using the optical disector. Journal of comparative neurology 408:560-566.

Amrein I (2015) Adult hippocamal neurogenesis in natural populations of mammals. Cold Spring Harb Perspect Biol 7:a021295.

Amrein I, Becker AS, Engler S, Huang SH, Muller J, Slomianka L, Oosthuizen MK (2014) Adult neurogenesis and its anatomical context in the hippocampus of three mole-rat species. Front Neuroanat 8:39.

Amrein I, Isler K, Lipp HP (2011) Comparing adult hippocampal neurogenesis in mammalian species and orders: influence of chronological age and life history stage. Eur J Neurosci 34:978-987.

Barker JM, Boonstra R, Schulte-Hostedde AI (2003) Age determination in yellow-pine chipmunks (Tamias amoenus): a comparison of eye lens masses and bone sections. Canadian Journal of Zoology 81:1774-1779.

Barnea A (2010) Wild neurogenesis. Brain Behav Evol 75:86-87.

Ben Abdallah NM, Slomianka L, Vyssotski AL, Lipp HP (2010) Early age-related changes in adult hippocampal neurogenesis in C57 mice. Neurobiol Aging 31:151-161.

Benice TS, Raber J (2010) Castration and training in a spatial task alter the number of immature neurons in the hippocampus of male mice. Brain Res 1329:21-29.

Bennett NC (1994) Reproductive suppression in social Cryptomys damarensis colonies - a lifetime of socially-induced sterility in males and females (Rodentia: Bathyergidae). Journal of Zoology, London 234:25-39.

Bennett NC, Faulkes CG (2000) African mole-rats: ecology and eusociality. Cambridge, UK: Cambridge University Press.

Bennett NC, Jarvis JU (1988) The social structure and reproductive biology of colonies of the mole-rat, Cryptomys damarensis (Rodentia, Bathyergidae). Journal of Mammalogy 69:293-302.

Blanchard RJ, McKittrick CR, Blanchard DC (2001) Animal models of social stress: Effects of behavior and brain neurochemical systems. Physiology and behavior 73:261-271.

Boonstra R, Galea L, Matthews S, Wojtowicz JM (2001) Adult neurogenesis in natural populations. Can J Physiol and Pharm 79:297-302.

Brain MJ, Dwyer SM, Rusak B (2004) Restraint stress affects hippocampal cell proliferation differently in rats and mice. Neuroscience Letters 368:7-10.

Buffenstein R, Jarvis JU (2002) The naked mole-rat - a new record for the oldest living rodent. Sci Aging Knowledge Environ 21:7.

Buwalda B, van der Borght K, Koolhaas JM, McEwen BS (2010) Testosterone decrease does not play a major role in the suppression of hippocampal cell proliferation following social defeat stress in rats. Physiol Behav 101:719-725.

Calhoun ME, Kurth D, Phinney AL, Long JM, Hengemihle J, Mouton PR, Ingram DK, Jucker M (1998) Hippocampal neuron and synaptophysin-positive bouton number in aging C57BL/6 mice. Neurobiol Aging 19:599-606.

Carleton A, Petreanu LT, Lansford R, Alvarez-Buylla A, Lledo P-M (2003) Becoming a new neuron in the adult olfactory bulb. Nature Neurosci 6:507-518.

Cavegn N, van Dijk RM, Menges D, Brettschneider H, Phalanndwa M, Chimimba CT, Isler K, Lipp HP, Slomianka L, Amrein I (2013) Habitat-specific shaping of proliferation and neuronal differentiation in adult hippocampal neurogenesis of wild rodents. Front Neurosci 7:59.

Chancellor LV, Roth TC, LaDage LD, Pravosudov VV (2011) The effect of environmental harshness on neurogenesis: A large-scale comparison. Dev Neurobiol 71:246-252.

Chow C, Epp JR, Lieblich SE, Barha CK, Galea L (2013) Sex differences in neurogenesis and activation of new neurons in response to spatial learning and memory. Psychoneuroendocrinology 38:1236-1250.

Cinini SM, Barnabe GF, Galvao-Coelho N, de Medeiros MA, Perez-Mendes P, Sousa MB, Covolan L, Mello LE (2014) Social isolation disrupts hippocampal neurogenesis in young non-human primates. Front Neurosci 8:45.

Clarke FM, Miethe GH, Bennett NC (2001) Reproductive supression in female Damaraland mole-rats Cryptomys damarensis: dominant control or self-restraint? Proceedings of the Royal Society London 268:899-909.

Dammann P, Burda H (2006) Sexual activity and reproduction delay ageing in a mammal. Curr Biol 16:R117-118.

Dammann P, Sumbera R, Masmann C, Scherag A, Burda H (2011) Extended longevity of reproductives appears to be common in Fukomys mole-rats (Rodentia, Bathyergidae). Plos ONE 6:e18757.

Denmark A, Tien D, Wong K, Chung A, Cachat J, Goodspeed J, Grimes C, Elegante M, Suciu C, Elkhayat S, Bartels B, Jackson A, Rosenberg M, Chung KM, Badani H, Kadri F, Roy S, Tan J, Gaikwad S, Stewart A, Zapolsky I, Gilder T, Kalueff AV (2010) The effects of chronic social defeat stress on mouse self-grooming behavior and its patterning. Behav Brain Res 208:553-559.

Fabricius K, Wörtwein G, Pakkenberg B (2008) The impact of maternal separation on adult mouse behaviour and on the total neuron number in the mouse hippocampus. Brain Struct Funct 212:403-416.

Fowler CD, Liu Y, Ouimet C, Wang Z (2002) The effects of social environment on adult neurogenesis in the female prairie vole.

Gage FH (2000) Mammalian Neural Stem Cells. Science 287:1433.

Galea LA (2008) Gonadal hormone modulation of neurogenesis in the dentate gyrus of adult male and female rodents. Brain Res Rev 57:332-341.

Galea LA, Spritzer MD, Barker JM, Pawluski JL (2006) Gonadal hormone modulation of hippocampal neurogenesis in the adult. Hippocampus 16:225-232.

Galvan V, Jin K (2007) Neurogenesis in the aging brain. Clinical interventions in aging 2:605-610.

Garthe A, Behr J, Kempermann G (2009) Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PLoS One 4:e5464.

Gould E, McEwen BS, Tanapat P, Galea LAM, Fuchs E (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J Neurosci Methods 17:2492-2498.

Gould E, Tanapat P (1999) Stress and hippocampal neurogenesis. Biol Pshychiatry 46:1472-1479.

Gould E, Tanapat P, McEwen B, Flügge G, Fuchs E (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci USA 95:3168-3171.

Gould E, Tanapat P, Rydel T, Hastings N (2000) Regulation of hippocampal neurogenesis in adulthood. Biol Psychiatry 48:715-720.

Gundersen H, Jensen E, Kieu K, J N (1999) The efficiency of systematic sampling in stereology-reconsidered. J Microsc 193:199-211.

Hamson DK, Wainwright SR, Taylor JR, Jones BA, Watson NV, Galea LA (2013) Androgens increase survival of adult-born neurons in the dentate gyrus by an androgen receptor-dependent mechanism in male rats. Endocrinol 154:3294-3304.

Jarvis JUM (1981) Eusociality in a mammal: cooperative breeding in naked mole-rat colonies. Science 212:571-573.

Kempermann G, Gage FH (2002) Genetic influence on phenotypic differentiation in adult hippocampal neurogensis. Dev Brain Res 134:1-12.

Kempermann G, Kuhn HG (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493.

Kozorovitskiy Y, Gould E (2004) Dominance hierarchy influences adult neurogenesis in the dentate gyrus. J Neurosci 24:6755-6759.

Kuhn HG, Dickenson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027-2033.

Lacey EA, Sherman PW (1991) Social organization of naked mole-rat colonies: Evidence for a division of labor. In: The Biology of the Naked Mole-rat (P.W. Sherman, J. U. M. J., R.D. Alexander, ed), pp 275-336 Princeton, New Jersey: Princeton University Press.

Lieberwirth C, Liu Y, Jia X, Wang Z (2012) Social isolation impairs adult neurogenesis in the limbic system and alters behaviors in female prairie voles. Horm Behav 62:357-366.

Lieberwirth C, Wang Z (2012) The social environment and neurogenesis in the adult mammalian brain. Front Hum Neurosci 6:118.

Malberg JE, Duman RS (2003) Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. Neuropsychopharmacology 28:1562-1571.

Marques AA, Bevilaqua MCdN, da Fonseca AMP, Nardi AE, Thuret S, Dias GP (2016) Gender Differences in the Neurobiology of Anxiety: Focus on Adult Hippocampal Neurogenesis. Neural Plasticity 2016:1-14.

Ming G, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223-250.

Ming GL, Song H (2011) Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70:687-702.

Mitra R, Sundlass K, Parker KJ, Schatzberg AF, Lyons DM (2006) Social stress-related behavior affects hippocampal cell proliferation in mice. Physiol Behav 89:123-127.

Morris P (1972) A review of mammalian age determination methods. Mamm Rev 2:69-104.

Nottebohm F (2002) Why are some neurons replaced in the adult brain? J Neurosci 22:624-628.

Ormerod BK, Galea L (2001) Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. Neurosci 102:369-379.

Ormerod BK, Galea LAM (2003) Reproductive status influences the survival of new cells in the dentate gyrus of adult male meadow voles. Neurosci Lett 346:25-28.

Ormerod BK, Lee TT, Galea LA (2003) Estradiol initially enhances but subsequently suppresses (via adrenal steroids) granule cell proliferation in the dentate gyrus of adult female rats. J Neurobiol 55:247-260.

Ormerod BK, Lee TT, Galea LA (2004) Estradiol enhances neurogenesis in the dentate gyri of adult male meadow voles by increasing the survival of young granule neurons. Neurosci 128:645-654.

Penz OK, Fuzik J, Kurek AB, Romanov R, Larson J, Park TJ, Harkany T, Keimpema E (2015) Protracted brain development in a rodent model of extreme longevity. Sci Rep 5:11592.

Peragine DE, Simpson JA, Mooney SJ, Lovern MB, Holmes MM (2014) Social regulation of adult neurogenesis in a eusocial mammal. Neurosci 268:10-20.

Rao MS, Hattiangady B, Shetty AK (2006) The window and mechanisms of major age-related decline in the production of new neurons within the dentate gyrus of the hippocamus. Aging Cell 5:545-558.

Sakamoto M, Kageyama R, Imayoshi I (2014) The functional significance of newly born neurons integrated into olfactory bulb circuits. Front Neurosci 8:121.

Sandi C, Haller J (2015) Stress and the social brain: behavioural effects and neurobiological mechanisms. Nat Rev Neurosci 16:290-304.

Scantlebury M, Speakman JR, Oosthuizen MK, Roper TJ, Bennett NC (2006) Energetics reveals physiologically distinct castes in a eusocial mammal. Nature 440:795-797.

Schieffelin JS, Sherman PW (1995) Tugging contests reveal feeding hierarchies in naked mole-rat colonies. Anim Behav 49:537-541.

Sherman PW, Jarvis JU (2002) Extraordinary life spans of naked mole-rats (Heterocephalus glaber). J Zool 258:307-311.

Šklíba J, Lövy M, Burda H, Šumbera R (2015) Use of underground space by a eusocial mole-rat. In: The 12th African Small Mammal symposium Mantasoa, Madagascar.

Slomianka L, Amrein I, Kneusel I, Sorensen JC, Wolfer DP (2011) Hippocampal pyramidal cells: the reemergence of cortical lamination. Brain Struct Funct 216:301-317.

Slomianka L, West MJ (2005) Estimators of the precision of stereological estimates: an example based on the CA1 pyramidal cell layer of rats. Neurosci 136:757-767.

Spritzer MD, Galea LA (2007) Testosterone and dihydrotestosterone, but not estradiol, enhance survival of new hippocampal neurons in adult male rats. Dev Neurobiol 67:1321-1333.

Spritzer MD, Ibler E, Inglis W, Curtis MG (2011) Testosterone and social isolation influence adult neurogenesis in the dentate gyrus of male rats. Neurosci 195:180-190.

Stranahan AM, Khalil D, Gould E (2006) Social isolation delays the positive effects of running on adult neurogenesis. Nat Neurosci 9:526-533.

Tanapat P, Hastings NB, Rydel TA, Galea LA, Gould E (2001) Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. J Comp Neurol 437:496-504.

Thomas RM, Hotsenpiller G, Peterson DA (2007) Acute Psychosocial Stress Reduces Cell Survival in Adult Hippocampal Neurogenesis without Altering Proliferation. Journal of Neuroscience 27:2734-2743.

van Dijk RM, Lazic SE, Slomianka L, Wolfer DP, Amrein I (2015) Large scale phenotyping links adult neurogenesis to the reaction to novelty Hippocampus in press.

Van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nature Neurosci 2:266-270.

Van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-1034.

Voss MW, Vivar C, Kramer AF, Van Praag H (2013) Bridging animal and human models of exercise-induced brain plasticity. Trends Cogn Sci 170:525-544.

West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the opical fractionator. Anat Rec 231:482-497.

Wu MV, Shamy JL, Bedi G, Choi C-W, Wall MM, Arango V, Boldrini M, Foltin RW, Hen R (2014) Impact of social status and antidepressant treatment on neurogenesis in the baboon hippocampus. Neuropsychopharmacology 39:1861-1871.

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645-660.

Zitnik G, Martin GM (2002) Age-related decline in neurogenesis: old cells or old environment? J Neurosci Res 70:258-263.