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Adult Hippocampal Neurogenesis in the Context of Taxonomy, Hippocampal Network, Domestication and Amyloid Precursor Protein

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Abstract

Since its discovery in the 1960s, the field of adult neurogenesis has continued to attract more and more attention. The discovery of adult neurogenesis not only changed the course of our understanding of the central nervous system, but also revealed to us the great potential of adult brains.

New neurons are continuously generated in two brain areas of most adult mammals: the olfactory bulb and the dentate gyrus (DG) of the hippocampus. The hippocampus is the structure associated with learning, memory and emotional function. Therefore, adult hippocampal neurogenesis has been linked with these hippocampal functions. Adult hippocampal neurogenesis (AHN) was extensively investigated particularly in rodent models, and is known to be influenced by many factors, including exercise, stress, environment, GABA signaling, and aging in experimental animals. However, the role of neurogenesis is still debated, and many studies have shown contradictory results regarding the function of AHN, even after decades of extensive study in the field.

One of the potential causes of the contradictory results is the functional heterogeneity of the hippocampus. The septal hippocampus was found to be associated with spatial learning, whereas the temporal hippocampus is more likely to be associated with emotional functions. Another possible cause is the effect of domestication. Previous studies show that voluntary running, which reliably increases adult neurogenesis in laboratory rodents, cannot increase neurogenesis in wild mice. This result indicates that AHN might be modified during the domestication process. This raises the question of whether the findings of AHN studies in laboratory rodents are a true representation of the nature of AHN. A third possible cause is incomparable experiment design, including differing quantification methods, sample size, and in particular, neglecting of confounding variables in analysis such as age or sex. Furthermore, another important point to consider is that the majority of studies have been conducted in rodents, and how these results can be generalized to other species is still an open question.

Until now, most AHN studies have only focused on the dentate gyrus. However, hippocampus circuitry is also composed of other principal cell populations including the subiculum, hilus, cornu ammonis (CA)-3 and CA1. Newly generated granule cells are continuously adding to this circuitry. Regulation of AHN should be investigated as part of this network as a whole.

To address these questions, in project I we looked at the position of AHN as part of the complete hippocampal network. By analyzing and comparing the principal cell populations of different species, we can gain valuable insights into hippocampal connectivity, and into the role of newly born neurons in the network. Moreover, the link of AHN to domestication needs to be addressed alongside septo-

temporal segregation and the connectivity of the hippocampus. In project II of this thesis, these questions have been addressed by examining two groups of silver foxes bred either randomly or selectively for tame/docile behavior. The effects of domestication and aggressive behavior as traits ingrained over many generations are examined, taking into account the functionally segregated divisions of the hippocampus. The third project of this thesis investigates the impact of reported AHN regulators, aging and amyloid precursor protein (APP) expressed by GABAergic interneurons. GABA signaling is known to be a key process regulator for neuronal maturation. Investigating the impact of APP on GABAergic neurons in aged hippocampi may provide some clues concerning the regulation of neuronal maturation process while aging.

The results of this thesis first demonstrate that phylogenetically closely related species are also similar to each other in terms of hippocampal principal cell populations, when we look at the correspondent analysis of these cell populations alone. Unlike the principal cell populations, the levels of hippocampal neurogenesis maintained in the adult brain separate rodent species in a different manner, indicating that the regulation of AHN may follow different rules to both hippocampal principal cell population and phylogeny. Accordingly, study of the silver fox has shown that a higher level of AHN, in particular in the temporal hippocampus, is associated with selection for tameness, a key trait of domestication. Also, by adding the fox data into the previous AHN database across various mammals, a similar level of proliferating cells and a strikingly high level of young neurons were observed in the fox brain. The results indicated prolonged maturation of new-born neurons in foxes compared to primates and rodents. Finally, no difference in AHN between 18-22-month-old mice with APLP2 cKO and APP/APLP2 cdKO on GABAergic interneurons was observed. Our results showed that age was the only factor associated with AHN level. It is difficult to compare with a previous study of APP KO in GABAergic interneurons because age was not taken into consideration in the previous study.

Taken together, the studies in this thesis provide a unique insight into the AHN research field, and also highlight the importance of considering all possible confounding variables, including hippocampal principal cell population, domestication, hippocampal heterogeneity, and age before drawing any conclusions as to how AHN is regulated or functionally linked with behavior.

Zusammenfassung

Seit der Entdeckung der adulten Neurogenese in den 1960er Jahren hat dieses Gebiet immer mehr Aufmerksamkeit auf sich gezogen. Deren Entdeckung hat uns das enorme Potential von adulten Gehirnen zu erkennen gegeben. Das Gebiet der adulten Neurogenese verfügt über breite Anwendungsmöglichkeiten zur Erforschung von Krankheitsursachen, Alterungsprozessen, Lern- und Gedächtnis- Vorgängen, und der Gehirnentwicklung.

Es ist seit langem bekannt, dass bei den meisten adulten Säugetieren Neuronen kontinuierlich neu gebildet werden in den folgenden zwei Hirnregionen: olfaktorischer Bulbus und Hippocampus. Obwohl übereinstimmende Indizien drauf hindeuten, dass im menschlichen olfaktorischen Bulbus keine Neurogenese stattfindet, ist adulte Neurogenese im Hippocampus (ANH) ein häufig beobachtetes Phänomen im menschlichen Gehirn. Hauptsächlich aufgrund von Ergebnissen von Tier-Modellen (hauptsächlich Mäuse) wissen wir, dass ANH von vielen Faktoren beeinflusst wird, wie z.B. körperliche Betätigung, Stress, Umwelt, und Ernährung. Ausserdem wurde ANH mit Verhaltensplastizität in Verbindung gebracht, sowie mit bestimmten Funktionen des Hippocampus wie z.B. Angst und Gedächtnis. Der kausale Effekt der Neurogenese bleibt jedoch auch nach Jahrzehnten von umfangreichen Studien immer noch kontrovers.

Zahlreiche Faktoren wurden entdeckt, welche AHN beeinflussen können, einschliesslich körperliche Aktivität, Umwelteinflüsse, und Alterungsprozesse. Insbesondere körperliche Aktivität ist ein simpler und ausführlich untersuchter Faktor, der die Neurogenese anregt. Unsere früheren Studien haben jedoch gezeigt, dass erhöhte körperliche Aktivität in wilden Mäusen die Neurogenese nicht verstärkt. Dieser negative Befund könnte darauf hindeuten, dass sich die Reglation der AHN bei den Labor-Mausstämmen während des Domestizierungsprozesses verändert hat. Daraus ergibt sich die Frage, ob Labor-Nagetiere überhaupt als translatorisches Tiermodell zur Erforschung von AHN geeignet sind.

Um dieser Frage nachzugehen, und um eine neue Richtung in der AHN Forschung einzuschlagen, konzentrieren wir uns hauptsächlich auf die Untersuchung der natürlichen Eigenschaften von AHN (Basis-Aktivität) in Tierarten, die zuvor nur selten zur Erforschung von AHN verwendet wurden.

Diese Dissertation besteht aus zwei Hauptprojekten und einem Nebenprojekt. Zuerst untersuchten wir den Einfluss von Domestizierung auf AHN an Füchsen mit unterschiedlichem Zahmheitsgrad, und wir haben festgestellt, dass Zahmheit mit erhöhter Basis-AHN einhergeht.

Im zweiten Projekt der Dissertation ermittelten wir die Menge an AHN im Kontext der residenten Zellpopulation im Hippcampus von verschiedenen Tierarten, einschliesslich Labor- und Wild-Tiere.

Unsere Daten zeigen, dass sich die AHN Grundaktivität signifikant zwischen verschiedenen Tierarten unterscheidet.

Im dritten noch nicht publizierten Projekt kehren wir zu einem typischen Labor-Tiermodell zurück, und wenden dabei dieselben Untersuchungsmethoden an, die wir bei den Nicht-Nagetier Studien verwendet haben. Im Fokus dieser Studie steht eine genetisch veränderte Mauslinie bei welcher das Amyloid-Vorläuferprotein (APP) fehlt, weil beta-Amyloid, ein Stoffwechselprodukt von APP, eng mit der Entstehung der Alzheimer-Krankheit verknüpft ist, und AHN eine wichtige Rolle in Lern- und Gedächtnis-Prozessen zu spielen scheint. Wir fanden jedoch keinen Unterschied in der AHN Aktivität (Anzahl neugebildete Zellen) von APP Knockouts (cdKO) und Kontroll-Tieren (APLP2-/-). Obwohl wir keine eindeutige Schlussfolgerung ziehen können mangels einer Wildtyp Kontrolle, so deutet der Vergleich mit früheren Studien mit C57/BL6 Mäusen an, dass der altersbedingte Rückgang von AHN in APLP2-/- Tieren anders verläuft als in C57/BL6 Mäusen.

Unsere Resultate zeigen, dass die AHN Aktivität der Phylogenie folgt, aber sich auch ändern kann, um sich der Umwelt anzupassen. Der Zusammenhang zwischen AHN, Artenvielfalt, und umweltbedingtem Anpassungsdruck deutet an, dass diese Art von Gehirnplastizität eine wichtige Rolle zu spielen scheint bei der Fähigkeit von Tieren sich an verschiedene Umgebungen anzupassen.

Abbreviations

AD: Alzheimer's disease

Aβ: Amyloid beta

AHN: Adult hippocampal neurogenesis

APLP2: Amyloid precursor-like protein 2

APP: Amyloid precursor protein

BDNF: Brain-derived neurotrophic factor

BrdU: Bromodeoxyuridine

CA: Cornu Ammonis

DCX: doublecortin

DG: Dentate gyrus

Dlx: Distal-less homeobox

GABA: Gamma-Aminobutyric acid

GFAP: Glial fibrillary acidic protein

HPA-axis: Hypothalamo-pituitary-adrenal axis

HVC: High vocal center

NPY: Neuropeptide Y

PV: Parvalbumin

SGZ: Subgranular zone

vGAT: Vesicular GABA transporter

1. Introduction

The hippocampus

The hippocampus is located in the medial temporal lobe of the brain. It is present across all mammalian orders and runs along a septal (dorsal)-to-temporal (ventral) axis in rodents and canine, corresponding to a posterior-to-anterior axis in humans (figure 1.1).

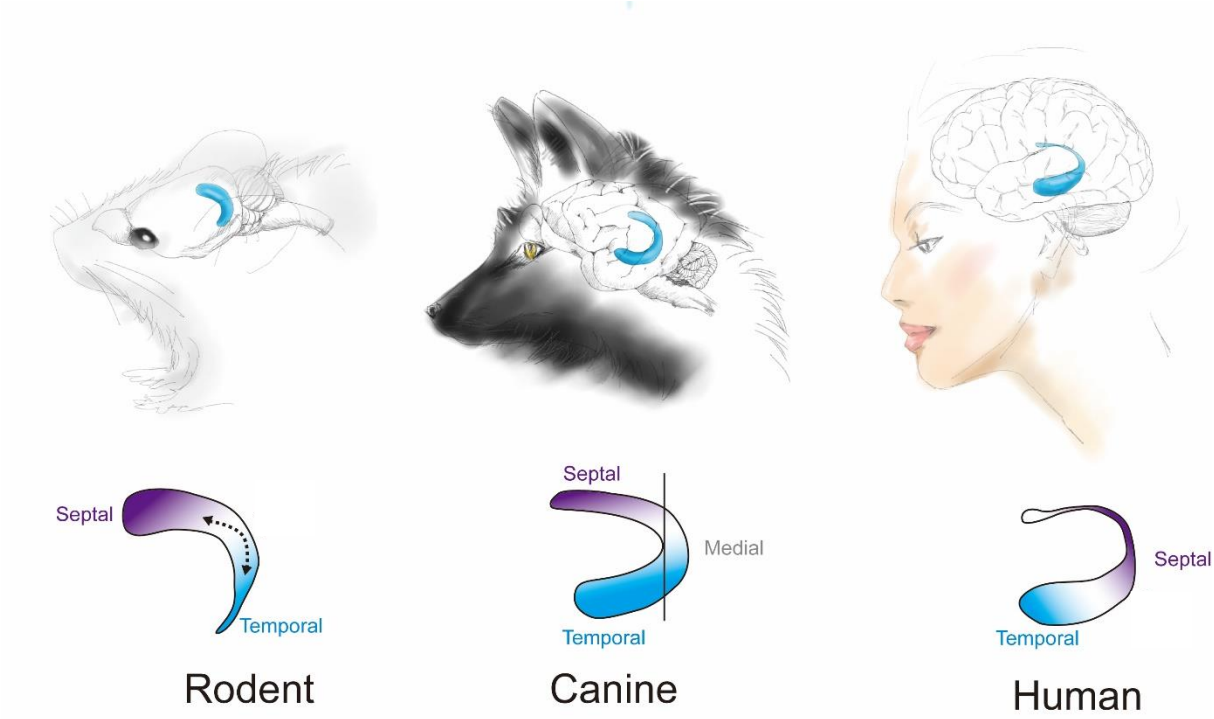

Figure 1.1 Distinct macroscopic anatomical organization between humans, canines and rodents. The most obvious difference in the hippocampi of these three species is the orientation of the hippocampal septo-temporal axis. In non-primate callosal mammals, the major portion of the dorsal hippocampus is tucked under the caudal section of the corpus callosum. However, in prosimian to simian species, this subcallosal flexure diminishes, and is practically absent in humans (Strange, Witter et al. 2014). Canine exhibits an "intermediate" C shaped hippocampus which is in between hippocampus of the rodent and human in terms of shape.

The hippocampus was first linked to memory following a report of the bilateral destruction of the hippocampi of Henry Molaison, known until his death as "Patient H.M."(Scoville and Milner 1957, Squire and Wixted 2011). H.M. underwent surgery in order to relieve epileptic seizures. The seizures were controlled by surgery, but the surgery led to unexpected outcomes. As a result of the surgery, H.M.'s declarative memory, i.e. his memory of facts and events, which can be consciously recalled, was severely impaired. However, his perceptual and cognitive abilities remained intact, as did his

capacities for working memory and perceptual and motor skill learning. Furthermore, H.M. was able to recall information obtained long ago, but had difficulty in recalling events that had occurred recently before the onset of the amnesia. (Scoville and Milner 1957, Squire and Wixted 2011). More studies in humans and monkeys further established that the hippocampus, together with adjacent cortical regions including the entorhinal, perirhinal, and parahippocampal cortices are essential structures in the declarative memory system (Zola-Morgan, Squire et al. 1986, Zola-Morgan, Squire et al. 1994).

In 1971, Marr published a theory about memory and hippocampus which influenced the field for decades onwards. According to Marr's theory, the hippocampus is a device which completes the associative retrieval process to recover the entirety of the original event before the pattern is returned to the neocortex (Marr 1971). This mechanism was afterwards demonstrated in the CA3 system by several experiments (see section **Pattern completion and CA3**). Furthermore, spatial information and spatial navigation are primarily associated with the hippocampus, as proposed by O'Keefe (O'Keefe and Dostrovsky 1971). They proposed that hippocampal activity is correlated to the rat's location within its environment. This idea was further confirmed in rodent studies. Hippocampal lesions resulted in an impairment of spatial navigation tasks depending on distant cues (allocentric navigation) such as navigating a water maze (Morris, Garrud et al. 1982, Morris, Schenk et al. 1990). Today, it is almost universally agreed that the hippocampus is a structure associated with declarative memory, associative memory and spatial coding, although the detailed mechanism still needs to be clarified.

Hippocampal circuitry

The connectivity between the hippocampus, the entorhinal cortex, and the perirhinal and parahippocampal cortices is organized hierarchically (Lavenex and Amaral 2000). The perirhinal and parahippocampal cortices provide major inputs to the entorhinal cortex, which further provides inputs to the hippocampus (Insausti, Amaral et al. 1987). Information processed by the hippocampus then descends back down the hierarchy through reciprocal connections and is distributed to the neocortex through feedback projections (Preston, Shohamy et al. 2005).

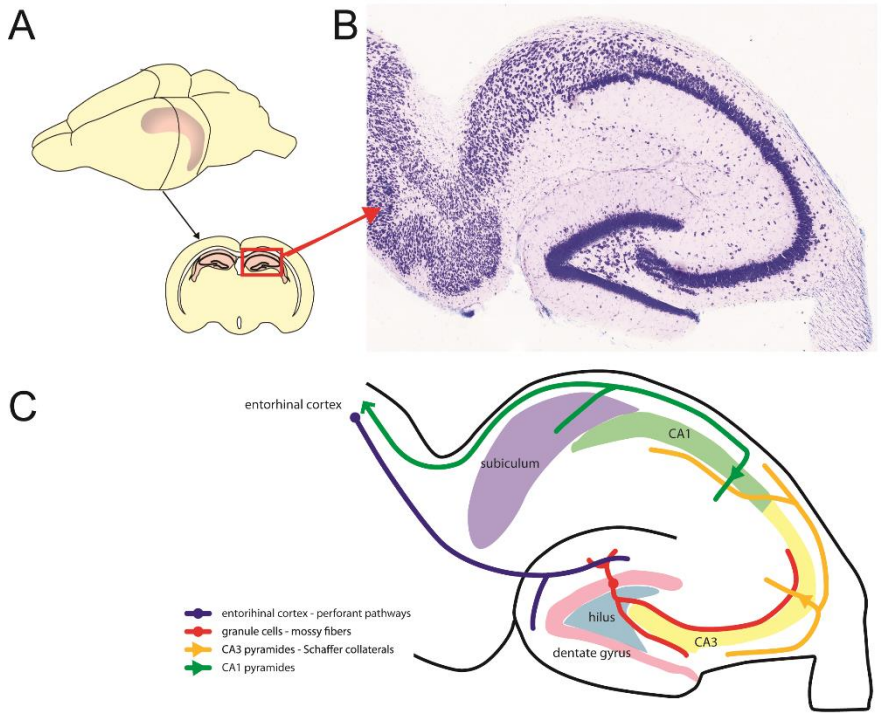

Figure 1.2 The trisynaptic loop in rodent hippocampus (A) Scheme of mouse brain and location of hippocampus. (B) Giemsa staining of hippocampal section. (C) The wiring diagram of the hippocampus is traditionally presented as a trisynaptic loop. The major input from the entorhinal cortex is carried by axons of the perforant path, which convey information from neurons in entorhinal cortex to the dentate gyrus. Perforant path axons then connect to the dendrites of granule cells. Granule cells project through the mossy fibers to the dendrites of CA3 pyramidal cells. CA3 cell project to CA1 pyramidal cells through Schaffer collaterals. CA1 Pyramidal cells send their axons to the subiculum and entorhinal cortex (Amaral and Lavenex 2007, Neves, Cooke et al. 2008).

The output from the entorhinal cortex to the hippocampus is the beginning of a trisynaptic pathway (see details in figure 1.2), and the projections are topographically organized (Preston, Shohamy et al. 2005). The lateral entorhinal cortex projects preferentially to the posterior dentate gyrus, whereas medial entorhinal cortex projections target the anterior dentate gyrus. Different anterior-posterior levels of the entorhinal cortex also project to different proximal and distal regions of CA1 and the subiculum. The parahippocamal cortex, which projects to entorhinal cortex more medially than the perirhinal cortex, may influence more anterior regions of dentate gyrus (Suzuki and Amaral 1994).

Pattern separation and the dentate gyrus

Pattern separation is the ability to discriminate among similar experiences (Yassa and Stark 2011). It is a computational process that has long been associated with the dentate gyrus (DG). Pattern separation occurs when the output firing patterns of a network are less similar to one another than the input firing patterns (Deng, Aimone et al. 2010). The reason is because expansion recoding can

decorrelate input patterns, and this can be performed by a stage of competitive learning with a large number of neurons (Rolls 2007). DG granule cells are numerous (5-10 times more than its input entorhinal cortex, see Amaral, Scharfman et al. 2007), have associatively modifiable synapses (required for a competitive network), and strong inhibition provided by the inhibitory interneurons (Rolls 2007, Deng, Aimone et al. 2010). Furthermore, individual mossy fibers can depolarize downstream CA3 pyramidal neurons (Rolls and Kesner 2006). These results support the theory that the input from DG granule cells on CA3 acts as a competitive network in a way to produce the sparse representation during learning in CA3 neurons, which is required for the autoassociation in CA3 to perform well (Rolls and Kesner 2006, Rolls, Stringer et al. 2006). This mechanism facilitates the hippocampus to store different memories of similar events.

Pattern completion and the CA3

Pattern completion may depend on mechanisms and networking in CA3, CA1, and the subiculum. A leading hypothesis states that the CA3 autoassociative network is the key region responsible for forming connections between event elements, and for subsequently retrieving these representations (pattern completion) (Marr 1971, O'Reilly and Rudy 2001, Preston, Shohamy et al. 2005). CA3 neurons play a crucial role in the rapid hippocampal encoding of novel information for fast learning of one-time experience (Nakazawa, Sun et al. 2003). In CA3-NR1 KO (CA3 NMDA receptor knockout) mice, proper CA1 activity is observed in a full-cue environment, however CA3 neurons are critical for CA1 neurons to display full activity in a partial cue environment (Nakazawa, Quirk et al. 2002). These findings suggest that CA3 plays an important role in pattern completion.

CA1 and memory retrieval

CA1 neurons are critically involved in the recognition, consolidation, and late retrieval of hippocampal-dependent memories (Sladek and Lee 1993, O'Reilly and Rudy 2001, Nakazawa, McHugh et al. 2004, Kesner and Hunsaker 2010). CA3 connects to CA1 by the Schaffer collateral synapse (figure 1.2). Schaffer's collateral connection is highly modifiable which can help the full information present in CA3 to be retrieved in CA1 neurons (Treves 1995, Schultz and Rolls 1999). Experiments have shown that CA1 is required for contextual memory retrieval (Ji and Maren 2008). Studying lesions confined to CA1 in humans also showed that CA1 neurons play a critical role in autobiographical memory retrieval and for re-experiencing detailed episodic memories (Bartsch, Dohring et al. 2011).

A model suggested by Edmund T. Rolls indicates that the modifiable connections from the CA3 neurons to the CA1 neurons allow the whole episode in CA3 to be replayed in CA1. The CA1 neurons would then activate the entorhinal cortex, via their termination in the deep layers of the entorhinal

cortex (Rolls 2013 and see figure 1.3). The entorhinal cortex layer V neurons would then connect to the cerebral cortex (where the retrieval will happen) via their backprojections.

Subiculum and hippocampal connectivity

The subiculum, which lies between CA1 and the presubiculum, is an information center that conveys signals in the hippocampus to down-stream brain regions (Aggleton and Christiansen 2015). The subiculum receives primary input from CA1 and directly projects to the entorhinal cortex and also other brain regions outside the hippocampus such as the anterior thalamic nuclei, the mammillary bodies and the retrosplenial cortex (O'Mara 2005, Aggleton and Christiansen 2015). An earlier experiment, using the Morris Water Maze, also showed that the subiculum plays an important role in encoding spatial memory (Morris, Schenk et al. 1990). A recent study also discovered that subicular involvement in encoding spatial memory was distributed into proximal and distal regions with dissociable molecular, cellular and circuit properties (Cembrowski, Phillips et al. 2018). These results suggest that it is important to consider heterogeneity when studying hippocampal functions and connectivity.

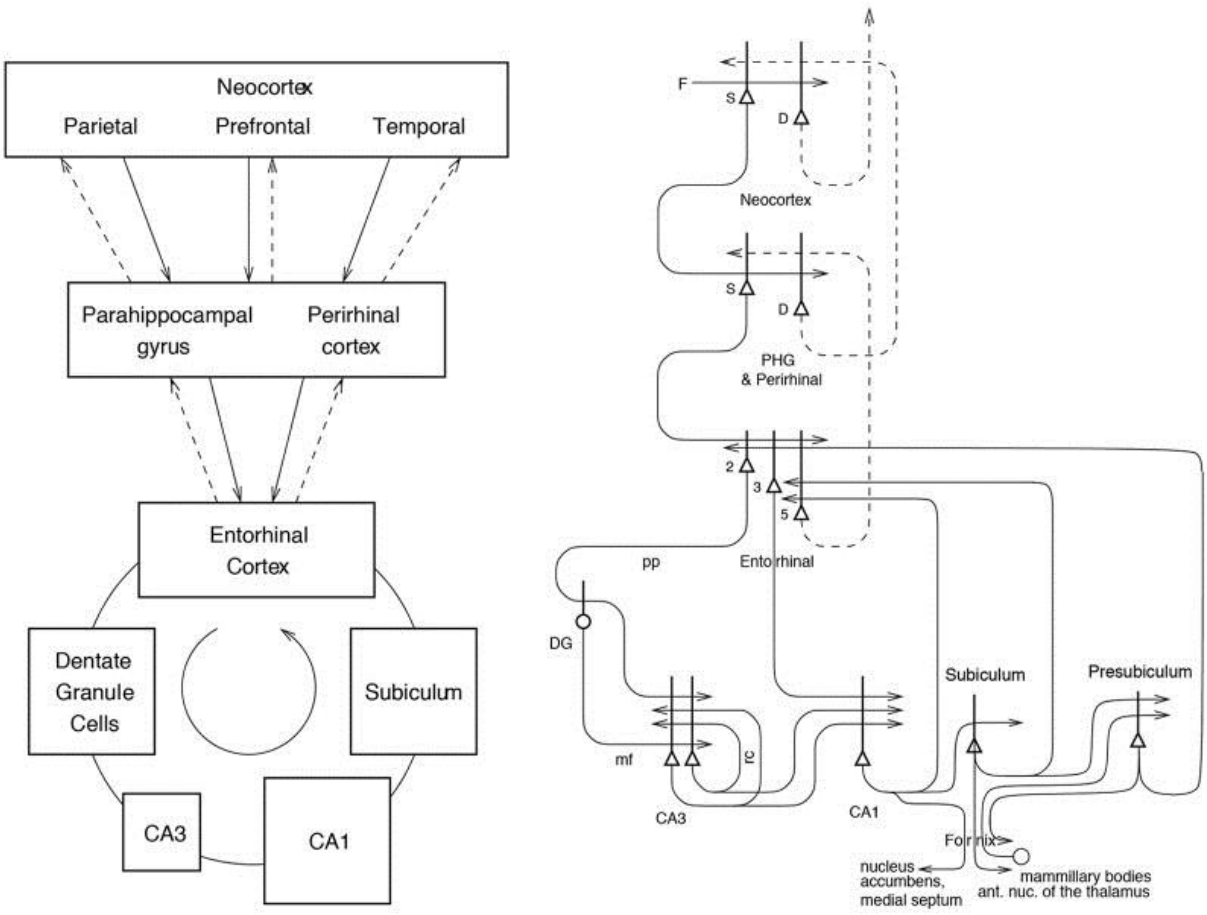

Figure 1.3 A hypothesis of the architecture between cerebral associations and hippocampus. "The feed forward connections from association areas of the cerebral neocortex (solid lines) show major convergence as information

is passed to CA3, with the CA3 autoassociation network having the smallest number of neurons at any stage of the processing. The backprojections (dash lines) allow for divergence back to neocortical areas. During the setting up of a new episodic memory, there would be strong feed forward activity progressing toward the hippocampus. During the episode, the CA3 synapses would be modified, and via the CA1 neurons and the subiculum, a pattern of activity would be produced on the backprojecting synapses to the entorhinal cortex. Here the backprojecting synapses from active backprojection axons onto pyramidal cells being activated by the forward inputs to the entorhinal cortex would be associatively modified. A similar process would be implemented at preceding stages of neocortex, which is in the parahippocampal gyrus/perirhinal cortex stage, and in association cortical areas" (figure 1.3 is from Rolls 2013). "D, deep pyramidal cells; DG, dentate granule cells; F, forward inputs to areas of the association cortex; pp, performant path; rc, recurrent collateral of CA3 hippocampal pyramidal cells; S, superficial pyramidal cells; 2, pyramidal cells in layer 2 of the entorhinal cortex; 3, pyramidal cells in layer 3 of the entorhinal cortex."

Hippocampus and emotional functions

Retention of memory is not passive, but is instead defined by parameters such as context and experience, which both filter the information that is retained, and determine the accuracy of this retention (Richter-Levin and Akirav 2000). The evolution of the mechanisms used by the brain in the storing of information reflects the degree to which information is worth storing, when one considers that, evolutionarily, animals would appear to benefit little from having memories of trivial events that are as strong as those of more important events (Cahill and McGaugh 1996). One important structure which can modulate hippocampal memory is the amygdala. The amygdala can modulate both the encoding and the storage of hippocampal-dependent memories. When emotional stimuli are encountered, the hippocampus can influence the response of the amygdala by interpreting the events and representing the emotional implication (Richter-Levin and Akirav 2000).

Besides the role of the hippocampus in memory, its role in modulating the hypothalamo-pituitary-adrenal axis (HPA axis) has also long been appreciated (Jacobson and Sapolsky 1991). Stress provokes the adrenal cortex to release corticosteroids, which target the hippocampus (De Kloet, Vreugdenhil et al. 1998), and acute stressors impair hippocampal-specific learning (de Quervain, Roozendaal et al. 1998). On the other hand, the hippocampus inhibits the HPA axis, representing a negative feedback regulation loop. Indeed, hippocampal lesions are associated with an increased corticosterone level and an activated HPA axis (Feldman and Conforti 1976). Stimulation of the amygdala and the hippocampus can also increase plasma and urine corticosteroid levels (Rubin, Mandell et al. 1966).

Moreover, the link of the hippocampus and emotional function is mainly associated with the temporal hippocampus. The temporal hippocampus connects directly to the amygdala (Petrovich,

Canteras et al. 2001) and lesion of the temporal hippocampus, but not the septal hippocampus, reduces anxiety in rodents (Kjelstrup, Tuvnes et al. 2002, McHugh, Deacon et al. 2004).

Taken together, the amygdala, the temporal hippocampus and stress hormones play an important interconnected role in learning and the strength of emotional memories. Stress hormones and the amygdala can modify the hippocampal response, and vice versa. Understanding the nature of these dynamic connections and the role of the hippocampus may help us to unveil the mechanism of psychological diseases such as post-traumatic stress disorder and anxiety.

Adult neurogenesis

Neurogenesis occurs continuously in restricted regions of the mammalian brain throughout the postnatal period. The first anatomical evidence of the presence of late postnatal neurogenesis in the rat hippocampus, later termed adult hippocampal neurogenesis (AHN), was shown by Joseph Altman using ^{3}H-thymidine autoradiography (Altman and Das 1965). New cells that they identified as granular neurons continued to be added into the cerebellum, olfactory bulb and dentate gyrus in adult rats (Altman 1970). However, evidence of adult neurogenesis was then largely dismissed until the 1980s. Adult neurogenesis was brought back to the main stage when Nottebohm and his colleagues reported neuronal turnover in adult songbird brains (Goldman and Nottebohm 1983, Alvarez-Buylla and Nottebohm 1988).

Research into adult neurogenesis (or late postnatal neurogenesis) intensified shortly after two techniques were introduced: the use of bromodeoxyuridine (BrdU) and the introduction of various molecular markers for the identification of the proliferation, differentiation and maturation of newly added neurons (Iball, Morgan et al. 1966, Altman 2011). BrdU labels DNA synthesis, which is similar to the ^{3}H-thymidine method, but which allows for faster investigation without the need to handle radiolabeled materials (Freese, O'Rourke et al. 1994).

Besides the exogenous cell cycle marker BrdU, scientists also discovered markers that expressed endogenously within the cell cycle. Endogenous cell cycle markers include nuclear antigens expressed only in actively dividing cells (namely during the G1, S, and G2 phases of the cell division cycle and during mitosis), which can therefore be used as proliferation markers, for example, Ki67 (Scholzen and Gerdes 2000, Stoeber, Tlsty et al. 2001).

Molecular markers expressed during the adult neurogenesis process have been intensively investigated since the 1990s. Neural stem cell-like primary progenitors (quiescent radial glia), possess astrocytic features and express GFAP (Seri, García-Verdugo et al. 2001). They further develop into slow-dividing type 1 cells or B cells, exhibit radial glia morphology and also express some glial

markers such as GFAP and BLBP (Kempermann, Jessberger et al. 2004, Kriegstein and Alvarez-Buylla 2009). The cells then develop into intermediate progenitors, and start to express some neuronal markers such as Tbr2 and, later, doublecortin (DCX) and PSA-NCAM (Seki, Namba et al. 2007, Hodge, Kowalczyk et al. 2008). Neuronal precursor cells begin to express DCX while still actively dividing. After the terminal division, their neuronal daughter cells continue to express DCX for 2–3 weeks as the cells mature. Downregulation of DCX begins after 2 weeks, and occurs at the same time that these cells begin to express NeuN, a marker for mature neurons (Brown, Couillard-Despres et al. 2003).

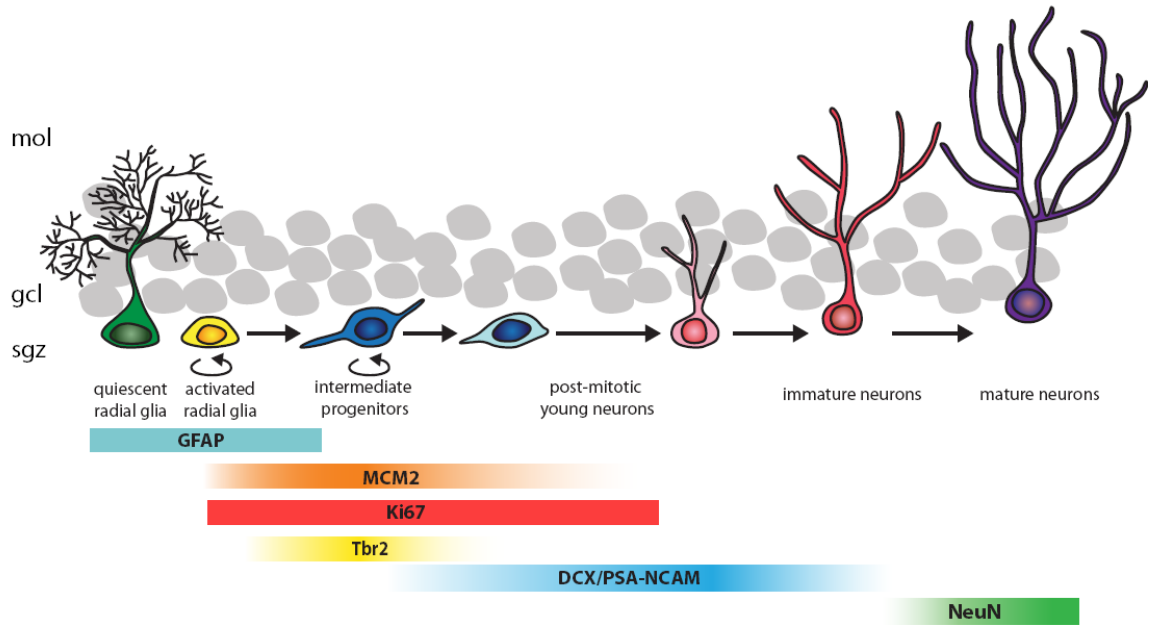

Figure 1.4 Molecular markers expressed by cells in different stages of Adult Neurogenesis (AHN). The figure is modified from (Seki, Namba et al. 2007, Song, Sun et al. 2013). Mol: molecular layer; gcl: granule cell layer; sgz: subgranular zone

Other common markers used for immature neurons include Tuj1 (β-tubulin isoform III) and CRMP (collaspin response-mediated protein 4, also known as TOAD4). Among the markers used for mature neurons are MAP2ab (microtubule-associated protein-2 a and b isoforms) and NeuN (**Neu**ronal **N**uclei). The discovery and development of these cell markers allowed researchers to trace the fate of new born neurons and to follow the AHN process in detail. As a result, the AHN research field became easily assessable. Newborn neurons can be identified by the presence of immature markers and the absence of mature markers of neurons (Kempermann, Jessberger et al. 2004, Ming and Song 2005 ,and figure 1.4).

Regulation of AHN

Is AHN level determined during development or adulthood?

The dentate gyrus develops in a unique manner. In contrast to the ventricular-subventricular zone (V-SVZ), the DG forms through the generation of a dedicated progenitor cell source away from the VZ but in close proximity to the pial surface. (Figure 1.5). This secondary proliferative zone remains active during postnatal stages and eventually becomes the SGZ, which is the site of adult hippocampal neurogenesis (Urban and Guillemot 2014). Due to this reason, it has been theorized that the adult neurogenic niche is actually a subset of cells from the developmental stage. Although embryonic and adult neurogenesis share a lot of common regulators, their responses to regulators are often very different (Urban and Guillemot 2014). Genetic studies in laboratory rodents indicated that AHN is not stable and environmental cues usually have strong impact on stem cell activities (Urban and Guillemot 2014). On the other hand, comparative study across mammals indicated that AHN is rather stable and maintained in wild rodent species (Amrein 2015). These results suggest that domestication may also play a role in sensitivity to AHN regulators in rodents.

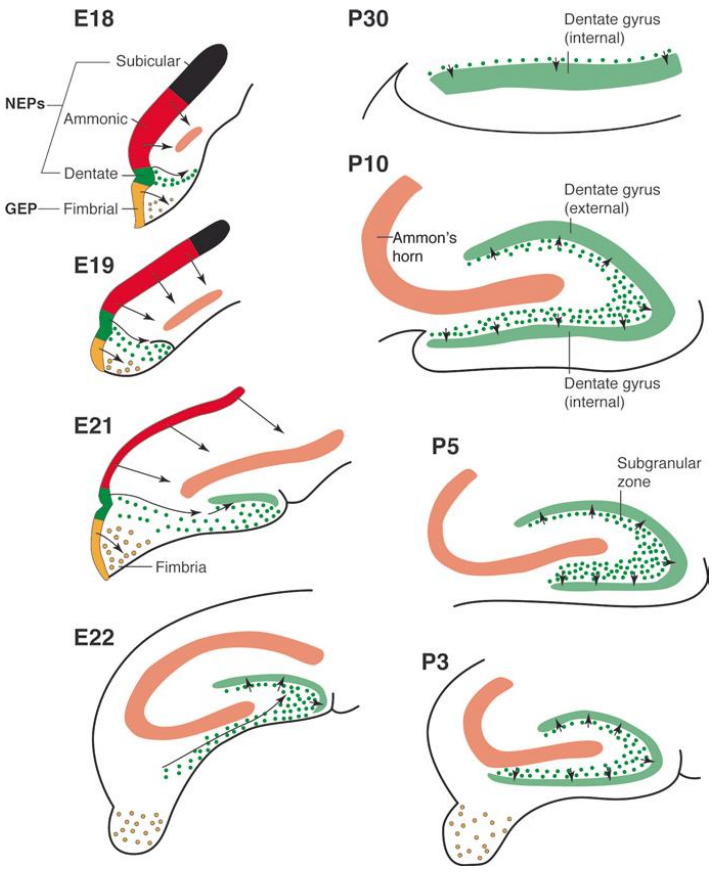

Figure 1.5 The Schematic summary diagram of the primary and secondary germinal matrices of the rodent hippocampus (Figure 1.5 is fromAltman 2011). The primary germinal matrix (neuroepithelium, NEP) includes

the subicular, ammonic, and dentate NEPs and fimbrial glioepithelium (GEP). The dentate NEP then proliferates and these cells migrate to form the subgranular zone (SGZ), which is the main source of AHN.

Regulators of AHN: GABA signaling, APP and aging

AHN is a process which is highly sensitive to the surrounding environment, especially GABA signaling. Young neurons first receive tonic GABA activation, followed by dendritic GABAergic synaptic input, dendritic glutamatergic synaptic inputs and finally perisomatic GABAergic inputs (Ge, Sailor et al. 2008). Granule cells can release both glutamate and GABA (Beltran and Gutierrez 2012), indicating that granule cells may play an important role in regulating the maturation of new born neurons.

An imbalance of GABAergic transmission can alter AHN. For example, in the Alzheimer's Disease (AD) mouse model in which the mice transgenically express human APP (hAPP), researchers observed that altered Aβ causes imbalance between GABAergic and glutamatergic inputs, which may result in impaired morphology and function during later maturation process of adult born granule cells (Sun, Halabisky et al. 2009). APP (Amyloid Precursor Protein) is in a gene family including APLP1 and APLP2 (Aydin, Weyer et al. 2012). Within this gene family, redundancy was observed between APP and APLP2 in terms of their physiological roles (Heber, Herms et al. 2000). A previous study showed that APP expressed in GABAergic neurons regulates different AHN processes. APP knockout (KO) in GABAergic neurons increases cell proliferation but decreases the long-term survival of mature newly born neurons (Wang, Wang et al. 2014). These findings suggest that studying the role of regulators such as APP in GABA singling may be a key to understanding the regulation of AHN.

Aging is one of the best-investigated and convincing negative regulators of AHN (Kuhn, Dickinson-Anson et al. 1996, Ben Abdallah, Slomianka et al. 2010), as this regulatory mechanism appears to be true for all mammals investigated (Amrein, Isler et al. 2011). Indeed, AHN decreases exponentially with age across species including rodents(Ben Abdallah, Slomianka et al. 2010), primates (Ngwenya, Heyworth et al. 2015) and humans (Spalding, Bergmann et al. 2013). The association of AHN to age-dependent decline of cognitive function has drawn a lot of attention in this research field and was encouraged by study in laboratory mice (van Praag, Shubert et al. 2005). However, research in rhesus monkeys showed that no clear relationship between AHN and cognitive impairment was found, and that age was often the best predictors of behavioral performance (Ngwenya, Heyworth et al. 2015). Furthermore, previous study has shown that aging is associated with wide-ranging GABAergic interneuron reduction in DG, CA1 and CA3 (Stanley and Shetty 2004). Another study showed that subunits of the $GABA_A$ receptor are modified with age and AD, which can induce compensatory increases in $GABA_A$ receptor subunits within surrounding cells (Rissman, De Blas et al. 2007).

Running and Domestication effect

Mice housed in an enriched environment, namely a larger cage with a lot of toys, running wheels and sufficient food, showed higher AHN levels than mice housed in a conventional cage (Kempermann, Kuhn et al. 1997). Also, it has been observed that only voluntary running is sufficient to increase cell proliferation in the dentate gyrus (van Praag, Kempermann et al. 1999). As a result of these findings, voluntary running became one of the positive stimulators most often used to study AHN, because it is a very simple and efficient way to increase AHN. However, this well-investigated mechanism cannot be observed in wild rodents (Hauser, Klaus et al. 2009, Klaus, Hauser et al. 2012, Schaefers 2013). This raised a fundamental question about AHN: is the regulation of AHN in laboratory rodents representative to that in other mammals, and ultimately even in humans?

Function of AHN – decades of controversy

Studies in song birds' high vocal center (HVC) showed that new neurons are generated to replace old cells and the replacement is regulated by cell death (Nottebohm 2002). A similar mechanism can be found in the rodent hippocampus. Although new neurons are continuously generated in the dentate gyrus, the total cell number in the dentate remains stable throughout life under normal conditions (Rapp and Gallagher 1996). So, what is the relationship between this neuronal replacement and behavior?

AHN and learning

Because these neurons are generated in the adult dentate gyrus, it is reasonable to link AHN to hippocampal functions such as learning and memory. However, even after the intensive investigation into the possible functional link of AHN over the past decades, the association of AHN with behaviors is still debated (Leuner, Gould et al. 2006). Studies in song birds provided first evidence that adult neurogenesis has a positive relationship with learning. The newly generated neurons in the higher vocal center (HVC) are positively related to sex and seasonal differences in song learning (Goldman and Nottebohm 1983, Alvarez-Buylla, Kirn et al. 1990). Also in the hippocampus of birds, it has been shown that a seasonal fluctuation in the recruitment of new neurons is positively associated with spatial learning behaviors (Barnea and Nottebohm 1994). However, a recent study showed that the number of HVC projecting neurons in adult male zebra finches doubles during the first two years after a song is learned, though the song itself changes relatively little (Walton, Pariser et al. 2012). Similar conflicting results were reported in rodents as well. Mice with the lowest AHN level performed most poorly in a water maze navigation task (Kempermann and Gage 2002). Later studies using radiation or genetic methods to ablate AHN also showed that AHN contributes to spatial memory (Kempermann, Kuhn et al. 1997, Snyder, Hong et al. 2005, Imayoshi, Sakamoto et al. 2008).

But there are also reports to indicate that AHN has no influence in hippocampal dependent spatial learning (Saxe, Battaglia et al. 2006, Shors, Anderson et al. 2012).

AHN and emotional functions

AHN has also been linked to emotional functions in animal models. Besides the cognitive functions, there is accumulating evidence that the hippocampus also plays a direct role in anxiety (Bannerman, Matthews et al. 2004, Engin and Treit 2007). Various reports indicated that either decreasing AHN (in mice) or increasing cell proliferation (in rats) is associated with an increase of anxiety in rodents (Revest, Dupret et al. 2009, Fuss, Ben Abdallah et al. 2010, Snyder, Soumier et al. 2011). However, similar to the above-mentioned studies in spatial learning, several studies showed opposite results that AHN has no impact on emotional behaviors (Holick, Lee et al. 2008, Leasure and Jones 2008).

AHN and neurological diseases

The AHN hypothesis in major depression syndrome gained substantially in popularity in the early 2000s. In patients with a history of several depressive episodes, magnetic resonance imaging (MRI) studies carried out at that time revealed significantly smaller hippocampal volumes in comparison to controls, but with no differences in total cerebral volumes (Sheline 1996, Bremner, Narayan et al. 2000). These evidences raised a popular hypothesis that newly generated neurons may contribute to restore hippocampal cytoarchitecture and volume. Many studies were performed pursuing this idea. It was found that anti-depression treatment can stimulate AHN across various species, including rodents (Malberg, Eisch et al. 2000), primates (Perera, Coplan et al. 2007), and humans (Boldrini, Underwood et al. 2009). Another report even suggested that AHN is necessary for the behavioral effects of antidepressants (Santarelli, Saxe et al. 2003). However, other experiments using different approaches showed that AHN is not causally linked with spatial learning and anxiety-like behavior (Meshi, Drew et al. 2006). Furthermore, studies in post mortem human hippocampal tissue of patients with various types of neuropsychiatric disorders have not been able to find consistent evidence of altered adult neurogenesis in major depression (Lee, Reif et al. 2013). The role of AHN in depression needs to be further clarified.

AHN has also been associated with other psychiatric and neurological disorders, such as drug addiction, epileptogenesis, schizophrenia and Alzheimer's disease (Eisch, Cameron et al. 2008, Martinez-Canabal 2014). On the other hand, more and more studies have shown a lack of causal relationship between AHN and neurological disorders because disruption of AHN cannot lead to any of these disorders (Kempermann, Krebs et al. 2008). Nevertheless, the correlation between AHN and neurological disorders indicates that AHN might still play a role in the pathology of the disease.

Possible reasons for the controversy in AHN's functional role

Species differences in the hippocampus

Studies in basic neurosciences rely heavily upon laboratory rodent models. However, more and more studies suggest that results in rodents are not always applicable to humans especially in neural behavioral studies. There are two main macroscopic differences between the rodent and the primate hippocampus. One is the orientation of the hippocampal long axis in rodents versus humans. The second difference is that the rodent hippocampal cross-sectional area is relatively uniform along the long axis, whereas the anterior (temporal) hippocampus has expanded relative to the posterior (septal) hippocampus in primates, particularly in humans (Strange, Witter et al. 2014).

Besides the anatomical differences, 2D clustering of microarray analysis showed that genes are expressed differentially across hippocampal subdivisions (Hawrylycz, Lein et al. 2012). The expression patterns also differ across species. For example, calcium-binding protein CALB1 is highly expressed in dentate gyrus, CA1 and CA2 in adult mouse and rhesus monkey, but it is selectively expressed in the human dentate gyrus (Hawrylycz, Lein et al. 2012). These results indicate that the molecular organization along the septotemporal axis might also be different in humans, non-human primates and mice.

Most of the knowledge we have about AHN is gained from laboratory rodents, with a little gained from primates (Kempermann, Jessberger et al. 2004, Ming and Song 2005), and only a fraction of mammalian species have been investigated. AHN differs among species. For example, AHN in bats and primates can be completely absent or at a very low level (Amrein, Dechmann et al. 2007, Amrein, Isler et al. 2011). Neuronal turnover has also been observed in the human brain, albeit at a very low level (~700 cells/day) (Spalding, Bergmann et al. 2013). On the other hand, wild red foxes have more young neurons than rodents and primates (Amrein, Isler et al. 2011). Due to lack of knowledge in other mammals, it is very difficult to draw any conclusion with regards to functional link of AHN across mammalian species.

Functional and connectivity difference along the septo-temporal axis in hippocampus

The results of AHN may vary because the hippocampus is not a homogeneous structure, and because most rodent studies have either focused on the septal hippocampus, or have divided the hippocampus in different ways (Tanti and Belzung 2013, Strange, Witter et al. 2014). The cortical and subcortical connections and the functionality of the septal and temporal hippocampus are segregated (Figure 1.6). Previous studies of rats, using anterograde tracing techniques, have shown that rostral cingulate areas primarily project to temporal parts of the entorhinal cortices (Jones and

Witter 2007). Projections from the remaining cingulate areas preferentially target the septal and mid-septotemporal parts of entorhinal cortices as well as the presubiculum and parasubiculum (Jones and Witter 2007). Another study using retrograde tracing has also shown that the rat hippocampus was divided into three domains, which are separated by their descending projections to three different parts of the lateral septum (Risold and Swanson 1996).

Greater cholinergic innervation was found in the septal hippocampus (Amaral and Kurz 1985). The temporal part of the hippocampal formation received a dense dopaminergic innervation whereas no fibers were observed in the septal pole (Verney, Baulac et al. 1985). A greater density of norepinephrine and 5-HT terminals has been reported in the temporal than in the septal hippocampal formation (Gage and Thompson 1980) and a direct connection was found between the amygdala and the temporal hippocampus (Petrovich, Canteras et al. 2001) which indicates that the temporal hippocampus may contribute more in emotional reactions than the septal hippocampus (Tanti, Rainer et al. 2012, Tanti, Westphal et al. 2013).

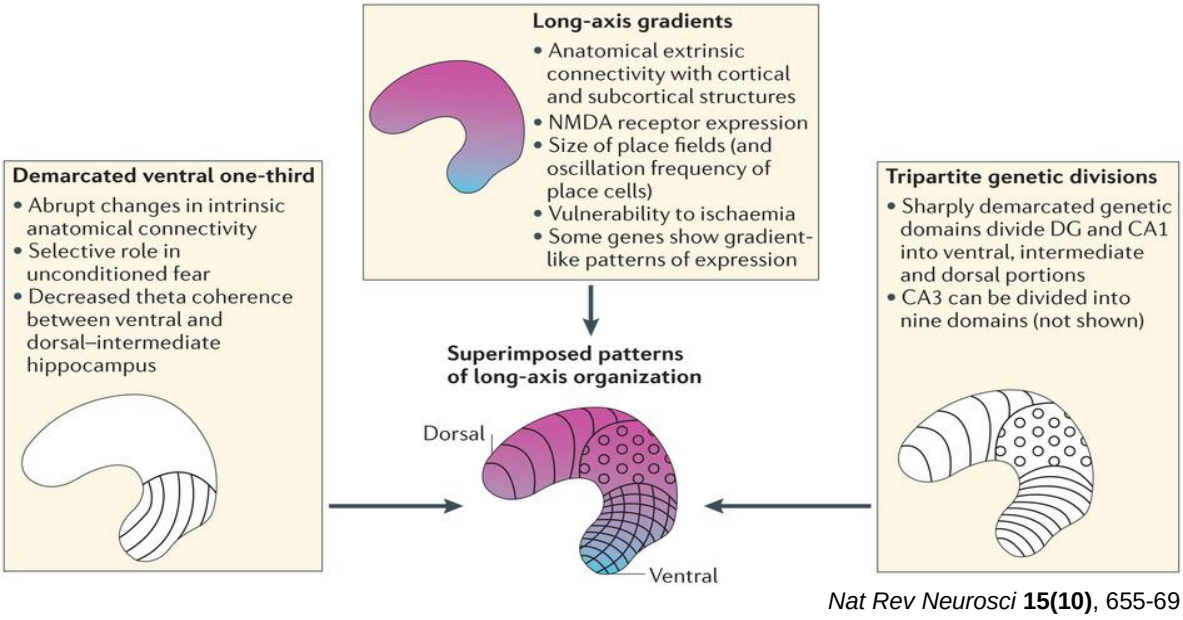

Figure 1.6 The septo-temporal aggregation and gradient of hippocampus. Summary of results from various studies indicated functional divisions or gradients along the hippocampal long axis (Figure 1.6 is from Strange, Witter et al. 2014).

Apart from the anatomical divisions, genetic studies showed that the hippocampus is not only a segregated but a graded structure. Hippocampal gene expression data showed robust septotemporal molecular heterogeneity in CA3 (Thompson, Pathak et al. 2008). In CA1, genetic markers revealed

distinct spatial expression domains and subdomains along the septotemporal axis (Dong, Swanson et al. 2009). The gene expression pattern varies along the septotemporal axis including graded change, segregated change and sharp transitions (Thompson, Pathak et al. 2008). Hippocampal function also shows graded difference along the septotemporal axis. Representation of the spatial information increased almost linearly from small (<1 meter) at the dorsal pole to large (~10 meters) at the ventral pole (Kjelstrup, Solstad et al. 2008). In primates, spacing of grid cells increases with distance from the rhinal sulcus (Killian, Jutras et al. 2012) and is consistent with a septotemporal gradient in rodent and bats (Solstad, Boccara et al. 2008, Yartsev, Witter et al. 2011).

As such, adult hippocampal neurogenesis (AHN) along the septotemporal axis might not only be involved in different functions (Tanti and Belzung 2013) but also in species variations. In rodents, AHN in the septal region is higher or similar to the temporal hippocampus (Snyder, Radik et al. 2009, Jinno 2011, Jinno 2011, Snyder, Ramchand et al. 2011, Klomp, Václavů et al. 2014). In marmosets, a significant gradient of granule cell numbers was observed along the septotemporal axis, but the topography of AHN stay stable along the axis (Amrein, Nosswitz et al. 2015). Analyzing the distribution of AHN along the septotemporal axis may be a key to identify its functional link across species.

Experimental design – Confounding variables

Causes or correlations between AHN manipulations and behavioral outcome are not easy to tease apart. Most methods of manipulating neurogenesis also affect other variables that are known (or could reasonably be expected) to contribute to behavior, which means AHN might just be an epiphenomenon (Lazic 2012), and many studies relating AHN to behavior do not take into account confounding variables such as litter, age or sex (Lazic 2010). The cloud of regulating factors of AHN is large and mostly systematically correlated (Kempermann 2011). Most of the regulating factors can also directly influence other parts of the central nervous system and/or behaviors, i.e. BDNF, GABA, growth hormone, insulin-like growth factor, interleukin 6, etc. It may also result in the difficulty to address any specific "AHN effect" on behavioral tests. Moreover, many experiments used BrdU to label proliferating cells. BrdU is toxic, which may result in morphological and/or behavioral abnormalities. It can trigger cell death, the formation of tetratomas, alters DNA stability, lengthens the cell cycle and has mitogenic, transcriptional and translational effects (Taupin 2007). One cannot exclude the possibility that BrdU might have some impact on AHN due to its nature or due to the stress caused by dosing BrdU. Also, BrdU and thymidine analogs are markers of DNA synthesis and not markers of S-phase of the cell cycle or cell division. Thus, BrdU can be incorporated into DNA during its repair, reentry into the cell cycle and DNA duplication without cell division in the brain (Taupin 2007).

Aim of the thesis

In summary, to address these questions, we need to look at AHN in the context of the hippocampal network. By analyzing the principal cell population between different species, we can gain insights into differences in the relative importance of functional domains and the place of newly born neurons within the network. Based on laboratory rat and mouse data, computational models of hippocampal functions describe the information propagation along converging and diverging cell populations in the hippocampus. In project I, this thesis contrasts the common model with data collected from 20 different mammalian species, evaluates similarities and divergent patterns, and tests the impact of AHN on this network. Also, we investigated cell proliferation by immunostaining of the endogenous nuclear protein Ki67, which is a marker for interphase and cell mitosis (Scholzen and Gerdes 2000), and the neuronal differentiation (immature neurons) by doublecortin (Brown, Couillard-Despres et al. 2003).

Furthermore, the link of AHN to domestication is addressed in the context of septo-temporal segregation of function and the connectivity of hippocampus. Also, investigation of AHN in more non-laboratory animals is also crucial for understanding true AHN function. In the project II, this thesis addresses these questions by examining two groups of silver foxes bred either randomly or selectively for tame/docile behavior. The effects of domestication and aggressive behavior as traits fixed over many generations are examined taking into account the functionally segregated divisions of the hippocampus.

The third project of this thesis investigates the link of AHN to two known regulators – aging and amyloid precursor protein (APP) in relation of GABAergic interneurons. Granule cells are part of hippocampal principal cells and the cells that new born neurons first make contact with. Observing the role of APP and aging on GABAergic neurons may provide insight into the regulation process of AHN.

2. Project I

Taxonomic Separation of Hippocampal Networks: Principal Cell Populations and Adult Neurogenesis

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Abstract

While many differences in hippocampal anatomy have been described between species, it is typically not clear if they are specific to a particular species and related to functional requirements or if they are shared by species of larger taxonomic units. Without such information, it is difficult to infer how anatomical differences may impact on hippocampal function, because multiple taxonomic levels need to be considered to associate behavioral and anatomical changes. To provide information on anatomical changes within and across taxonomic ranks, we present a quantitative assessment of hippocampal principal cell populations in 20 species or strain groups, with emphasis on rodents, the taxonomic group that provides most animals used in laboratory research. Of special interest is the importance of adult hippocampal neurogenesis (AHN) in species-specific adaptations relative to other cell populations. Correspondence analysis of cell numbers shows that across taxonomic units, phylogenetically related species cluster together, sharing similar proportions of principal cell populations. CA3 and hilus are strong separators that place rodent species into a tight cluster based on their relatively large CA3 and small hilus while non-rodent species (including humans and non-human primates) are placed on the opposite side of the spectrum. Hilus and CA3 are also separators within rodents, with a very large CA3 and rather small hilar cell populations separating mole-rats from other rodents that, in turn, are separated from each other by smaller changes in the proportions of CA1 and granule cells. When adult neurogenesis is included, the relatively small populations of young neurons, proliferating cells and hilar neurons become main drivers of taxonomic separation within rodents. The observations provide challenges to the computational modeling of hippocampal function, suggest differences in the organization of hippocampal information streams in rodent and non-rodent species, and support emerging concepts of functional and structural interactions between CA3 and the dentate gyrus.

2.1 Introduction

The highly laminar organization of the mammalian hippocampus has not only facilitated countless physiological and anatomical studies but also made it relatively easy to recognize if observations made in one species diverged from those in another. Individual hippocampal principal cell populations (granule, hilar, CA3, CA1, or subicular cells) have been found to differ in their cytoarchitectural appearance from species to species (Rosene and van Hoesen 1987, Slomianka, Amrein et al. 2011) Similar differences between species have been found in neurochemical signatures (Gall 1990, McNamara, Namgung et al. 1996, Blackshaw, Eliasson et al. 2003, Smits, Terwisscha van Scheltinga et al. 2004, Seress, Ábrahám et al. 2008, Slomianka, Amrein et al. 2011), and the distribution of intrahippocampal efferents (Laurberg and Zimmer 1980, Gaarskjaer, Danscher et al. 1982, van Groen and Wyss 1988). Extrahippocampal afferents of these cell populations also show species differences in their neurochemical (Murakawa and Kosaka 1999) and genomic (Mashiko, Yoshida et al. 2012) signatures and their projection patterns to the hippocampus (Schwerdtfeger 1984, van Groen, Kadish et al. 2002). Because of the mainly qualitative documentation of species differences, it is difficult to incorporate them into computational models of hippocampal function that may reveal their functional impact. Although species differences vividly illustrate the potential for adaptive change, there is, consequently, rarely evidence for their specific adaptive value. This is furthermore hampered by the small number of phylogenetically disjoint species that have been studied, which makes it difficult to judge if the presumed adaptive value should be looked for in the specific species that has been studied or if it may also be found in other taxonomically and/or behaviorally related species.

The volumes of the hippocampus and its subfields are exceptions to the lack of quantitative data in a large species sample. A textbook finding is the apparent expansion of CA1 with primate evolution (Stephan 1983, Seress 1988, West 1990), but even in this case, the structure-function relationship remains elusive. The allometrically progressive development of the hippocampus from insectivores to primates, and larger than expected progression of CA1 in the human brain (Stephan 1983) relates the expected size of CA1 to body weight. If the size of the entire hippocampus is compared to the regions that it is structurally and functionally closely related to, hippocampal size is actually decreasing relative to the neocortex along the primate lineage and smallest in humans (de Winter and Oxnard 2001))—a trend also observed in a taxonomically more diverse species sample (Reep, Finlay et al. 2007). With the further expansion of cortical area, this decline is accentuated in cetaceans (whales and dolphins), in which the hippocampus appears both very small and, at least cytoarchitecturally, not well differentiated (Jacobs, McFarland et al. 1979, Morgane and Jacobs 1986, Patzke, Spocter et al. 2015).

The discrepant views on the size of CA1 reflect two mechanistic models of changes in the size of brain regions—predictable, rule-based changes (linked regularities, Finlay and Darlington 1995) generated by developmental and functional constraints (Finlay and Darlington 1995, Whiting and Barton 2003) and mosaic changes (Harvey and Krebs 1990, Barton and Harvey 2000, de Winter and Oxnard 2001, Rehkämper, Frahm et al. 2008).), which reflect deviations from the size that rules would predict. Linked regularities can explain size relationships across orders of magnitude and quantitatively dominate the variability in the size of brain components across species. Although smaller in size, mosaic changes can reflect both taxonomic relations and life-style groups (Oxnard , de Winter and Oxnard 2001), and anatomically highly localized changes have been associated with speciation events (terminal fields of hippocampal afferents, Slomianka and West 1989)or specific behavioral adaptions (lateral geniculate parvo-/magnocellular cell ratio, Finlay, Charvet et al. 2014).These local differences can reflect the adaptive history of the species (Cahalane, Charvet et al. 2014). In a recent study (Slomianka, Drenth et al. 2013), we observed differences in the relative sizes between hippocampal principal cell populations that in part were shared by a number of taxonomically related species and in part appeared to be species specializations. Three rodent species fell within a tight group that was characterized by a relatively large CA3 cell population. Instead, primates had relatively large hilar and CA1 cell populations, with further emphasis on CA1 but less emphasis on the hilus in humans. One aim of this study was to test if these observations are robust to the inclusion of additional species and to define the cell populations that may quantitatively differentiate species within the rodent group. To this end, we expanded the number of species/strains available for analysis to 20, by generating hippocampal principal cell number estimates for eleven additional rodent species of different taxonomic groups and occupying distinctly different habitats. We also include estimates obtained from one additional primate species.

We previously analysed some of these species for adult hippocampal neurogenesis (AHN; reviewed in Amrein, Isler et al. 2011, Amrein 2015). While differences in AHN could be related to natural habitat differences (e.g. Cavegn, van Dijk et al. 2013) or selective pressures exerted by humans (Huang, Slomianka et al. 2015) , it is not clear how differences in AHN are related to other changes in the network that they are part of. This applies to both the identity and direction of change in other populations as well as to the relative size of changes in the small cell populations that represent AHN as compared to those in other, much larger cell populations. To begin answering these questions, we generated estimates of proliferating cell numbers and young neurons for four species and we extended previous estimates of three additional species. This allowed the joint analysis of AHN-related and principal cell numbers in nine rodent species.

2.2 Materials and Methods

Animals

A total of 18 unique species, with one of the species in three strains, were analyzed in the study. Estimates of hippocampal principal cell numbers of nine species (cotton rat, hamster, sand rat, bank vole, house mouse: wild-type, C57BL/6 and DBA, naked mole-rat, highveld mole-rat, cape mole-rat and marmoset) were performed for this study. Harvesting of brain tissue was performed in agreement with Canton of Zurich veterinary office guidelines. Principal cell number estimates for seven further species were taken from previously published results (sengi, house mouse (C57BL/6), harvest mouse, rhesus monkey, human, brown rats (Wistar and Sprague-Dawley), pigs and dogs). Proliferating (Ki67+) cell and young (DCX+) neuron numbers were estimated in 18 species for this study (hamster, sand rat, cotton rat, muskrat, yellow-necked wood mouse and bank vole). Neurogenesis data for the remaining rodent species were taken from previous publications. Table 2.1 provides a full overview of the species and data sources. The data for the house mouse was analyzed by way of three groups: C57BL/6, DBA and wild-type house mouse, resulting in a total of 20 analyzed groups of species or strains. Sprague-Dawley and Wistar rats were pooled because wild-type estimates are not available. Phylogenetic relations between the species used are illustrated in Figure 2.1.

Table 2.1 Overview of the species

Species	Sex:N	Mean age in month (SD)	Further information
House mouse, wild-type* (*Mus musculus domesticus*)	m:5	3.5	Rodentia, Muridae F1 from wild-caught; (Klaus, Hauser et al. 2012)
House mouse, DBA*	f:6	3	Rodentia, Muridae; (van Dijk, Lazic et al. 2015)
House mouse, C57BL/6	f:11; m:2	3	Rodentia, Muridae; (van Dijk, Lazic et al. 2015) and (Fabricius, Wörtwein et al. 2008)
Rat, Sprague-Dawley (*Rattus norvegicus*)	m:5	~5	Rodentia, Muridae; (Fitting, Booze et al. 2010)
Rat, Wistar	m:5; f:5	1.5(0.5)	Rodentia, Muridae; (West, Slomianka et al. 1991, Hosseini-Sharifabad and Nyengaard 2007)
Yellow-necked wood mouse*[&](*Apodemus flavicolis*)	m:4; f:2	4.3 (0.4)	Rodentia, Muridae; (Amrein, Slomianka et al. 2004)
Harvest mouse (*Micromys minutus*)	n/a:5	n/a, adult	Rodentia, Muridae; (Slomianka, Drenth et al. 2013)
Sand rat*[&] (*Psammomys obesus*)	m:6	2.7 (0.3)	Rodentia, Cricetidae; Harlan Laboratories, Israel
Bank vole*[&] (*Myodes glareolus*)	m:3; f:1	7.4 (6.9)	Rodentia, Cricetidae; (Amrein, Slomianka et al. 2004)

Muskrat*& (*Ondatra zibethicus*)	m:3; f:3	9.5 (4.2)	Rodentia, Cricetidae; wild-caught, Germany
Hamster*& (*Mesocricetus auratus*)	m:6	2.6 (0.2)	Rodentia, Cricetidae; Harlan Laboratories, Netherlands
Cotton rat*& (*Sigmodon hispidus*)	m:6	2 (0.1)	Rodentia, Cricetidae; Harlan Laboratories, Netherlands
Highveld mole-rat* (*Cryptomys hottentotus*)	f:6	20.3 (9.2)	Rodentia, Bathyergidae; (Amrein, Becker et al. 2014)
Cape mole-rat* (*Georychus capensis*)	m:2; f:4	26 (10.7)	Rodentia, Bathyergidae; (Amrein, Becker et al. 2014)
Naked mole-rat* (*Heterocephalus glaber*)	m:4; f:1	39.3 (28)	Rodentia, Bathyergidae; (Amrein, Becker et al. 2014)
Eastern rock sengi (*Elephantulus myurus*)	m:4; f:4	8.8 (1.8)	Macroscelidae, Macroscelididae; (Slomianka, Drenth et al. 2013)
Dog (*Canis lupus familaris*)	n/a:10	109.1 (63.3)	Carnivora, Canidae; (Siwak-Tapp, Head et al.)
Pig, domestic (*Sus scrofa domestica*)	f:5	3.2	Artiodactyla, Suidae; (Holm and West 1994)
Common marmoset* (*Callithrix jacchus*)	m:3; f:2	53.6 (41.6)	Primates, Calitricidae; (Amrein, Nosswitz et al. 2015)
Rhesus monkey (*Macaca mulatta*)	m:8	14 (21.4)	Primates, Cercopithecidae; (Keuker, Luiten et al. 2003)
Human (*Homo sapiens*)	m:56; f:17	777 (260.8)	Primates, Hominidae; (West and Gundersen 1990, West 1993, Harding, Halliday et al. 1998, Korbo, Amrein et al. 2004)

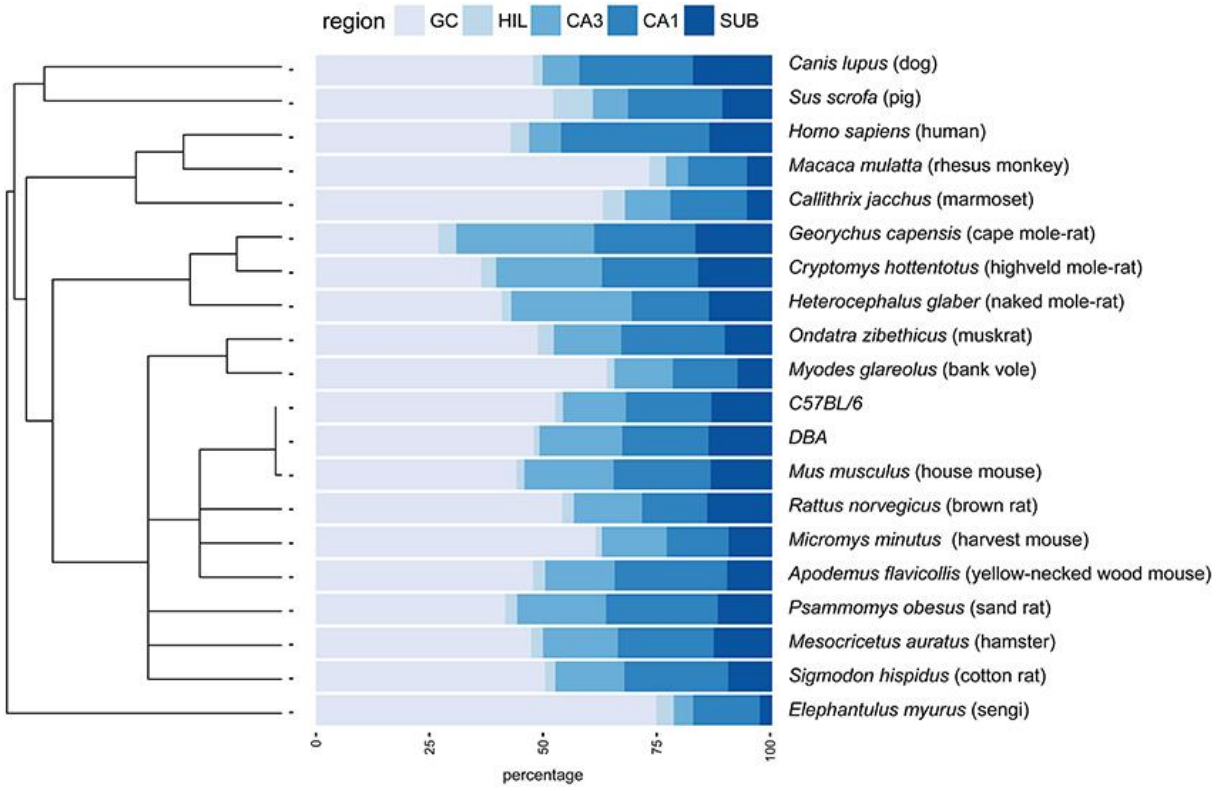

Figure 3.1. Principal hippocampal cell number distribution in the phylogenetic tree. A rooted phylogenetic tree (Fritz, Bininda-Emonds et al. 2009) of the 20 species and strains used in the study is shown along the relative size of the hippocampal cell populations in percentages. Species with extreme relative values for granule cells (GC) disperse over the tree (rhesus monkey, marmoset, bank voles, sengi), while relative high CA3 values are prevalent in rodents. GC, granule cells; HIL, hilus; SUB, subiculum.

Histology and immunohistochemistry

To estimate principal cell population sizes, the left hemispheres of each animal was fixed in 4% paraformaldehyde, dehydrated and embedded in glycol methacrylate (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim/Ts, Germany) according to the manufacturer's instructions but with extended infiltration times depending on brain size (Amrein and Slomianka 2010). Series of every sixth 20 µm sections were mounted, dried and stained in Giemsa solution (1.09204.0500, Merck) diluted 1:10 in 67 mmol KH_2PO_4 buffer (Iñiguez, Gayoso et al. 1985). The stained sections were differentiated 10 sec in 1% acetic acid, dehydrated and coverslipped.

Immunohistochemistry for the neurogenesis markers Ki67 (proliferation) and DCX (neuronal differentiation) was performed as described in detail in previous reports on neurogenesis in these species (table 2.1). Briefly, 40 µm thick sections were cut from the frozen right hemispheres. Complete series of free-floating sections were washed with Tris-Triton (TBS pH 7.4 with 0.05% Triton). Antigen retrieval was performed using 10% citric acid (DAKO) at 90^{o}C for 45 min (Ki67) or by short microwaving (DCX, 2 min). After washes in Tris-Triton, sections were incubated in 0.6% H_2O_2 in TBS with 0.1% Triton to block endogenous peroxidase. After further washes, the sections were incubated in appropriate blocking solution (2% serum, 0.2% Triton in TBS) for 1 hour. Primary antibody concentration was titrated for each species to generate near saturation of the signal at low background levels. Primary antibodies (Ki67: polyclonal rabbit-anti-Ncl Ki-67, Novocastra or Mouse anti-Ki67, BD Pharm and DCX: polyclonal goat-anti-doublecortin, Santa Cruz) were diluted with blocking solution and applied overnight at 4^{o}C. With intermittent washes in TBS, sections were immersed in biotinylated secondary antibodies diluted in TBS with 2% serum, ABC solution (PK-6100, Vector Laboratories) and diaminobenzidine (D4418-50SET, Sigma) following the manufacturers' instructions. All sections were then dehydrated and mounted.

Cell number estimation

Hippocampal principal cell numbers were estimated using the optical Fractionator (West, Slomianka et al. 1991) with StereoInvestigator 10 software (MBF Bioscience, Williston, VT, USA). Every 12th, 18th or 24th section (every 2nd, 3rd or 4th section of the series) was sampled with 10 µm high dissectors and 2 µm top guard zones. Section thickness was estimated at every 10th sampling site. Sampling parameters, cells counts, total number estimates based on number-weighted section thickness

(Dorph-Petersen, Nyengaard et al. 2001) coefficients of error (CEs) of the individual estimates for m = 0 (Gundersen, Jensen et al. 1999, Slomianka and West 2005) and CE^2/CV^2 ratios are listed in table 2.2.

	DBA	C57BL/6	House mouse	Highveld mole-rat	Cape mole-rat	Naked mole-rat	Sand rat	Hamster	Cotton rat	Muskrat	Yellow-necked wood mouse	Bank vole	Marmoset
Granule cells	409,981	438,187	476,984	588,647	48,8676	390,255	611,424	497,717	613,446	1415,956	1437,135	204,2739	249,8487
SD	22,614	137,143	72,686	93,514	77,599	34,806	112,574	88,880	75,937	82,545	213,879	211,439	431,224
Mean CE	0.13	0.13	0.09	0.08	0.11	0.07	0.10	0.09	0.08	0.10	0.07	0.07	0.07
CE^2/CV^2	5.45	1.06	0.34	0.32	0.45	0.70	0.30	0.27	0.42	2.76	0.24	0.40	0.18
Frame/grid size	7/100	7/100	10/100	12/140	18/324	10/120	12/160	12/120	12/120	45/180	10/210	10/210	15/120
Sections	12 (0.8)	11(1.6)	20 (1.1)	15 (0.2)	16 (0)	21 (0.4)	16 (1.2)	15 (0.8)	16 (0.6)	16 (2.8)	31 (1.8)	25 (4.7)	13 (1.5)
Cells counted	145 (60)	144 (16)	204 (99)	216 (30)	164 (62)	353 (23)	210 (40)	221 (27)	240 (34)	213 (52)	263 (33)	380 (88)	697 (78)
Hilar cells	10,487	13,029	18,561	53,219	71,216	19,365	37,742	26,769	27,658	104,394	78,578	53,695	187,457
SD	1212	2267	3099	5697	12,930	2765	5494	5894	3033	15,103	7406	6856	12,391
Mean CE	0.13	0.13	0.11	0.13	0.13	0.15	0.08	0.10	0.08	0.16	0.09	0.13	0.104
CE^2/CV^2	1.37	0.49	0.47	1.26	0.60	1.17	0.33	0.20	0.58	1.32	0.82	1.01	2.52
Frame/grid size	30/70	30/70	30/70	40/120	30/150	40/70	45/140	45/120	45/120	45/210	40/170	40/140	40/200
Sections	12 (1.2)	11 (1.6)	9 (0.9)	9 (0.4)	10.6 (1.2)	9 (0.4)	16 (1.0)	15 (1.7)	15 (0.8)	16 (2.3)	14 (1.7)	8 (1.0)	13 (1.3)
Cells counted	148 (48)	126 (19)	215 (36)	199 (19)	91 (21)	386 (61)	223 (22)	163 (32)	163 (11)	171 (36)	220 (38)	168 (15)	159 (19)
CA3 pyramids	155,129	113,603	21,3077	374,376	55,0413	24,9021	285,537	171,799	184,583	432,250	454,510	409,606	392,291
SD	16,621	29,322	36,953	28,207	71,336	44,854	56,815	27,378	21,957	61,979	47,889	77,249	35,139
Mean CE	0.12	0.11	0.12	0.1	0.1	0.08	0.10	0.10	0.08	0.11	0.09	0.09	0.09
CE^2/CV^2	1.23	3.71	0.51	1.61	0.66	0.18	0.27	0.36	0.47	0.61	0.65	0.26	0.95
Frame/grid size	11/100	11/100	11/100	18/160	15/200	15/130	25/250	25/160	25/160	25/260	15/190	15/140	30/200
Sections	15 (1.2)	13 (1.7)	10 (0.8)	11 (0.4)	12.5 (1.2)	12 (0.4)	18 (1.5)	18 (1.0)	18 (0.8)	20 (2.9)	14 (1.5)	9 (0.5)	13 (1.1)
Cells counted	146 (47)	97 (10)	165 (20)	153 (8)	101 (17)	205 (17)	184 (39)	179 (25)	187 (15)	138 (38)	142 (14)	185 (20)	185 (29)
CA1 pyramids	162,744	155,745	233,670	338,455	415,012	158,693	359,291	221,382	275,219	663,434	740,667	455,249	656,509
SD	21,729	51,368	56,389	29,375	132,131	18,884	70,170	34,246	11,044	81,379	91,568	93,897	41,394
Mean CE	0.13	0.14	0.10	0.12	0.12	0.10	0.11	0.10	0.08	0.10	0.10	0.10	0.12
CE^2/CV^2	1.09	1.51	0.17	2.12	0.15	0.76	0.32	0.44	3.51	0.65	0.60	0.24	4.54
Frame/grid size	11/100	11/100	11/100	18/160	15/180	15/110	25/250	25/160	25/160	25/260	10/160	15/130	30/320
Sections	16 (2.8)	13 (1.7)	12 (0.6)	12 (0.8)	14 (1.4)	13 (0.7)	19 (1.2)	18 (1)	18 (0.8)	20 (2.9)	16 (1.6)	10(0.5)	14 (1)
Cells counted	153 (66)	130 (8)	174 (23)	141 (12)	97 (25)	183 (11)	236 (7)	230 (29)	280 (18)	216 (63)	154 (14)	230 (37)	173 (54)
Subicular cells	119,944	108,479	14,7146	263,138	30,911	13,1505	173,602	134,379	117,120	302,230	295,464	241,765	216,992
SD	12,015	22,932	32,839	33,554	62,378	33,019	28,260	18,230	9493	31,484	38,801	42,119	31,991
Mean CE	0.11	0.10	0.1	0.09	0.10	0.11	0.08	0.07	0.08	0.09	0.09	0.10	0.12
CE^2/CV^2	1.16	0.57	0.21	0.50	0.26	0.13	0.25	0.28	0.95	0.72	0.42	0.30	0.67
Frame/grid size	25/140	18/140	18/140	20/150	20/190	20/130	45/320	45/200	45/190	45/350	20/180	20/140	18/240
Sections	15 (1.4)	12 (1.7)	12 (0.9)	12 (0.5)	13 (1.6)	12 (1.1)	18 (1.2)	18 (0.8)	18 (0.8)	18 (3.4)	15 (1.5)	11 (0.6)	14 (1.7)
Cells counted	156 (59)	128 (13)	145 (24)	154 (20)	112 (22)	192 (35)	259 (100)	286 (32)	282 (20)	179 (23)	176 (23)	183 (20)	132 (60)

Principle cell numbers of animals analyzed for this study. Numbers presented here are means. Frame and grid sizes are in µm. Sections and cells counted are presented as mean (SD).

Table 2.2 Estimates of unilateral hippocampal cell numbers and sampling parameters. Ki67- and DCX-positive cells were quantified either under a x63 oil-immersion lens and either counted manually and exhaustively (Ki67) but avoiding cells in the top focal plane of the section or by using the optical Fractionator (DCX). For details, see table 2.3 and publications in table 2.1.

	DBA	C57BL/6	House mouse	Hamster	Sand rat	Cotton rat	Muskrat	Yellow-necked wood mouse	Bank vole
Proliferating cells	1753	4505	1630	2813	2452	5053	2487	15,030	5373
SD	184	765	268	1240	591	2378	657	3556	2830
Mean CE	0.07	0.06	0.07	0.07	0.10	0.09	0.09	0.06	0.08
CE^2/CV^2	0.42	0.11	0.19	0.03	0.17	0.04	0.11	0.06	0.02
Frame/grid size	Exhaustive counts								
Sections	14 (0.8)	14 (2.1)	13 (1.2)	7 (0.5)	8 (1.3)	8 (1.2)	10 (1.2)	12 (3)	14 (1)
Cells counted	351 (37)	901 (153)	326 (53)	281 (124)	245 (59)	505 (18)	178 (47)	2505 (593)	896 (472)
Young neurons	7372	21,080	10,677	7450	10,850	9103	11,655	57,682	38,833
SD	940	3072	2609	1069	3587	1484	3781	4382	24,082
Mean CE ($m = 0$)	0.11	0.07	0.09	0.07	0.12	0.05	0.08	0.10	0.08
CE^2/CV^2	0.83	0.25	0.14	0.26	0.15	0.10	0.06	1.59	0.02
Frame/grid size	40/100	25/100	30/125	35/60	Exhaustive counts			20/100	30/100
Sections	15 (1.1)	14 (1.0)	13(1.6)	8 (0.8)	9 (1.0)	9 (0.8)	9 (1.2)	7 (0.8)	7 (0)
Cells counted	200 (42)	315 (96)	123 (30)	267 (51)	1085 (359)	902 (155)	817 (255)	210 (47)	376 (93)

Estimated numbers of proliferating cells and young neurons in the hippocampus of rodents. Numbers are given unilateral and represent means; no correction for age was made. Proliferating cell numbers for yellow-necked wood mice and bank voles published earlier (Amrein et al., 2004b) were included for convenience. Frame and grid sizes are in μm. The number of sections used and cells counted to generate estimates of total number are presented as mean (SD).

Table 2.3 Neurogenesis related cell counts.

Age normalization of neurogenesis related cell counts

The decline of neurogenesis with age is independent of life history or the expected life span of the species and can best be described using a negative exponential model (Amrein, Isler et al. 2011). The exponential curve of both Ki67+ and DCX+ cells in C57BL/6 over 9 months was reported by Ben Abdallah, Slomianka et al. (2010). The model was used to extrapolate the neurogenesis-related cell numbers of rodents of different ages to a virtual age of three months:

$$Cell\ Number_{\ 3-month\ age\ estimate} = Cell\ Number_{actual\ estimate} \times e^{[\ln(3)-\ln(actual\ age)]\times Y}$$

For Ki67 and DCX estimates of *Y* were reported to be -1.3933 and -1.2407 respectively. In addition, the decline of both DCX+ and Ki67+ cell numbers was estimated based on all rodents in this study. The ages of wild-caught animals were estimated by the time of capture and breeding time, lens weight (Barker, Wojtowicz et al. 2005) and bone lines (Cavegn, van Dijk et al. 2013). In this data set, *Y* was estimated to be -1.1929 for Ki67 and -1.0798 for DCX.

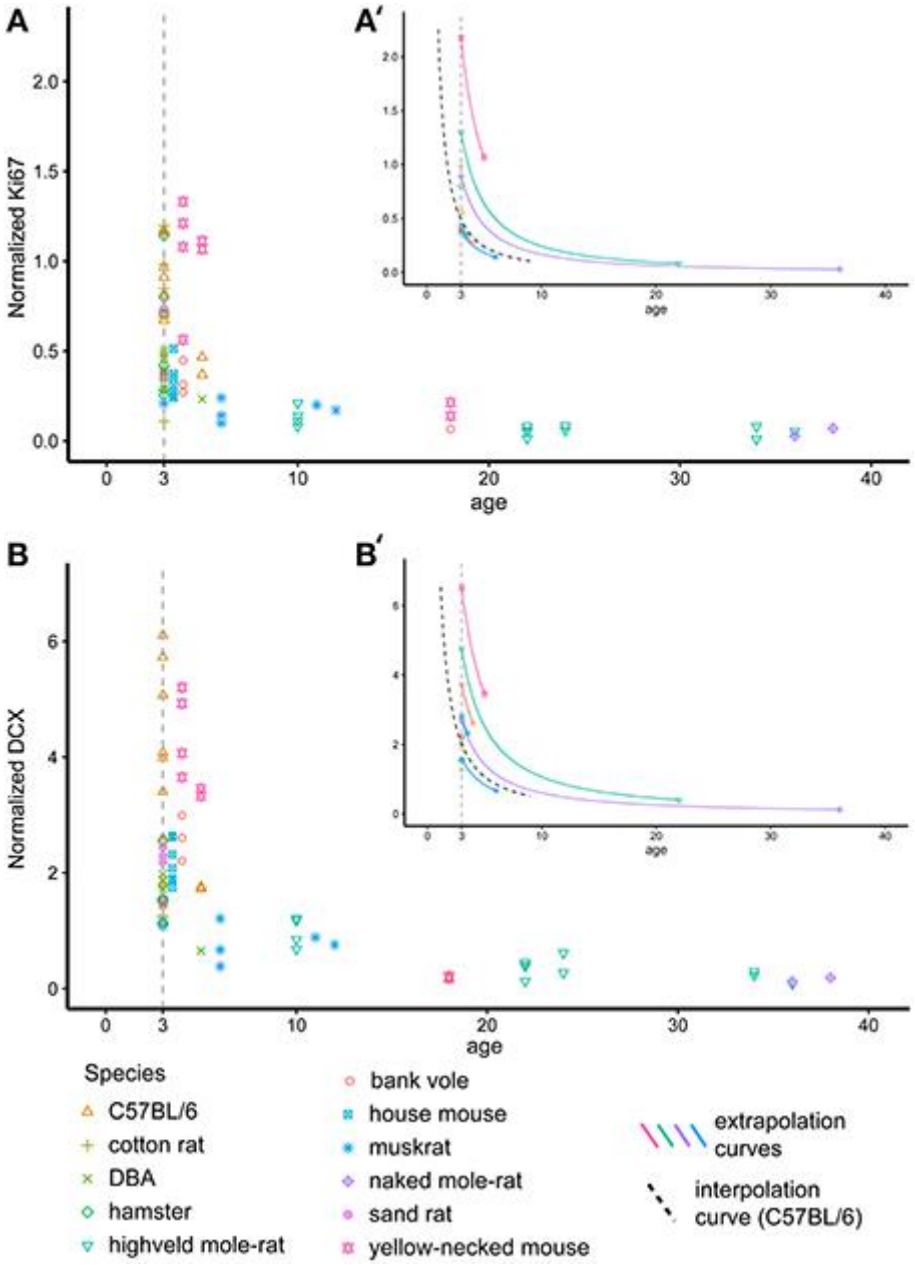

Figure 2.2 Recalculating neurogenesis in rodents to a common age. Hippocampal cell proliferation (Ki67, A) and young neurons (DCX, B) differ extensively due to large age differences between animals. For further analysis, cell numbers were therefore extrapolated for each individual animal to a common age of 3 months based on the negative exponential curve seen in laboratory C57BL/6 (Ben Abdallah, Slomianka et al. 2010, dashed black line in A′,B′). (A′,B′) exemplarily visualizes the procedure on single animals. For graphical presentation, neurogenesis-related cell numbers are given as percentage of total granule cell number (Normalized Ki67 and DCX, respectively).

Statistics

Large differences exist between the principal cell populations within species (e.g., in bank voles granule cells are more than 30 times more numerous than hilar cells) and between species (see Table 2.2). To account for these large differences, values, unless otherwise stated, were log transformed

and scaled by subtracting the mean of all neuron populations of each individual animal from the individual population estimates and dividing the result by the standard deviation of the mean. After this transformation, all animals therefore have cell counts with a mean of zero and a standard deviation of one across cell populations, but the relative size differences between populations within each animal are retained.

A brief numerical example may make the data transformation more accessible. In one specific animal XY, we estimate there to be 90 granule cells, 10 hilar cells, 30 CA3 cells, 40 CA1 cells and 30 subicular cells. The mean of the population estimates in this animal is 40 with a standard deviation (SD) of 30. The mean is subtracted from individual estimates, which results in values of 50 (granule cells), −30 (hilar cells), −10 (CA3 cells), 0 (CA1 cells), and −10 (subicular cells). The new mean of the population is 0. After dividing by the SD, the values are 1.66, −1, −0.33, 0, −0.33. The mean is still 0, but the SD of the data is now 1. Note that absolute differences are preserved after the subtraction of the mean. Also, relative differences still are preserved after the division by the SD. Applying this transformation to all individuals of all species provides directly comparable values that are independent from differences in absolute size and associated differences in variance.

For the analysis of the connectivity of cell populations (convergence or divergence between the functionally connected neurons of granule cells to hilus cells, granule cells to CA3 cells, CA3 cells to CA1 cells, and CA1 cells to subicular cells), ratios were used, requiring no further transformation.

The relationship between species and hippocampal cell population sizes was investigated using a correspondence analysis which is a statistical method for visualizing the associations (degree of correspondence) between the levels of a multi-way contingency table (Greenacre and Hastie 1987). Correspondence analyses are applied using the R package “MADE4” (MADE4R package, Culhane, Thioulouse et al. 2005). It calculates the chi-squared distances between the actual and expected values for both columns (cell numbers in hippocampal regions) and rows (species). Chi-square statistics can be used to examine small tables; correspondence analysis allows for the simplification of large tables with many columns and/or rows (Greenacre and Hastie 1987). This analyses allows complex data to be reduced to a two-dimensional plot while still capturing the majority of the variance in our data (>80%). In all plots presented, the x-axis represents the first dimension of the correspondence analysis while the y-axis represents the second dimension.

2.3 Results

Hippocampal morphology

Because of the variable nomenclature of CA3 pyramidal cells close (proximal) to the dentate gyrus and recent revisions, this region and the borders that it contains is illustrated in Figure 2.2 for the

marmoset monkey. Cytoarchitectural differentiation in marmoset monkeys largely corresponds to that seen in all non-rodent species included in this study. Hippocampal cytoarchitecture and the boundaries between hippocampal fields have not previously been illustrated for the muskrat, cotton rat, sand rat and bank vole and are shown in Figure 2.3. Cytoarchitecture and septotemporal changes in these species follows the general pattern described for the rat and mouse (e.g., Haug 1974, West, Danscher et al. 1978).

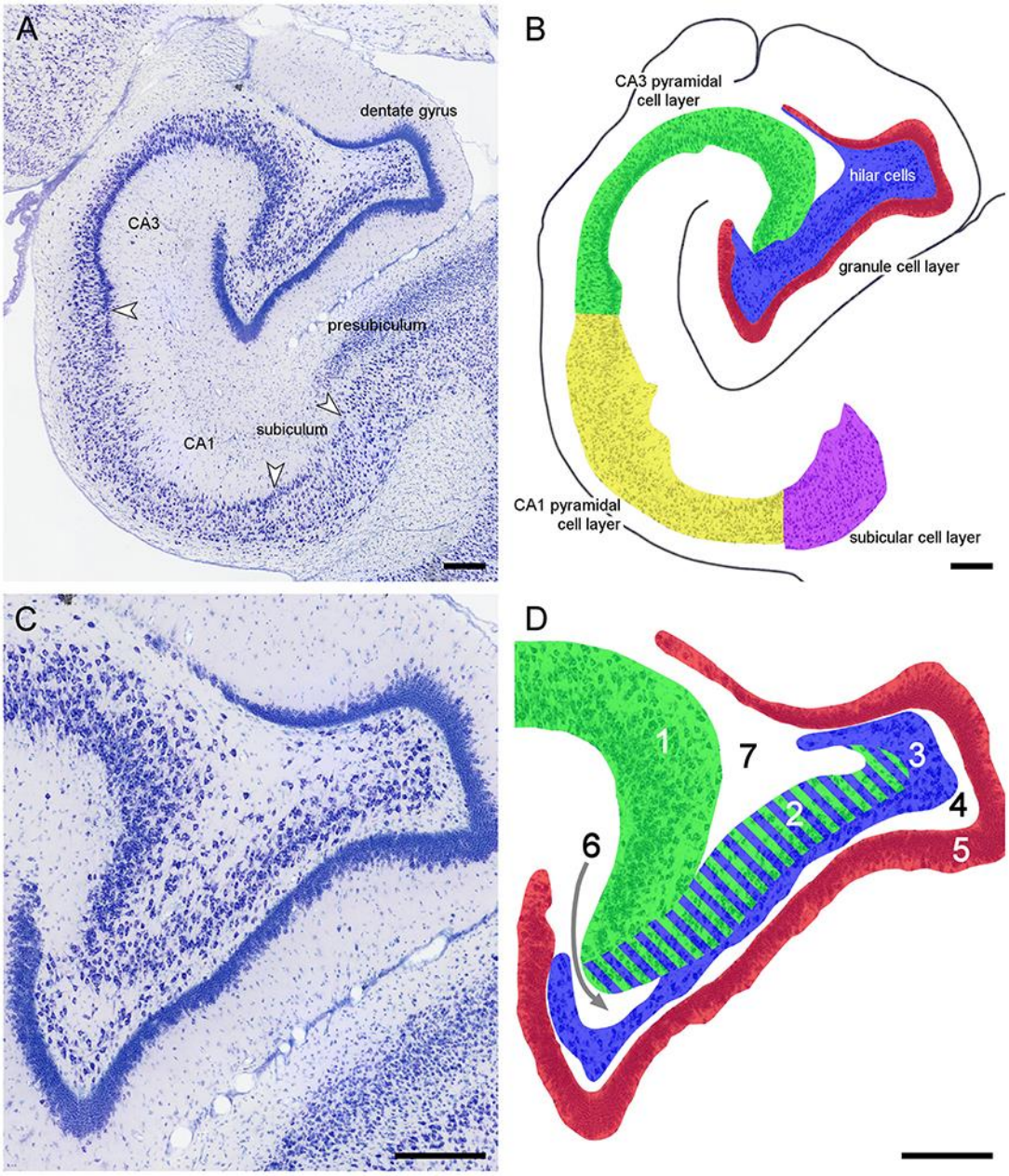

Figure 2.3. Principal hippocampal subdivisions in the marmoset. (A) Major hippocampal subdivisions in Giemsa-stained marmoset mid-septotemporal hippocampus. Arrows mark the boundaries between subdivisions at the level of the cell layer(s). (B) Definitions of the principal cell population in the marmoset mid-septotemporal hippocampus. (C) Complex hilar cytoarchitecture of the marmoset dentate gyrus that is common in non-rodent species. (D) Definitions of the regions that have been used to define CA3 and hilar cell populations within the

dentate gyrus; 1: CA3 or CA3o (outer CA3, Houser, Miyashiro et al. 1990), 2: reflected blade of CA3 (Lorente de Nó 1934) or CA3h (used in this study; Lim, Mufson et al. 1997, Ding and van Hoesen 2015) or CA3i (inner CA3, Houser, Miyashiro et al. 1990)or CA4 (Rosene and van Hoesen 1987), 3: polymorphic cell layer (2+3: CA4 of Lorente de Nó 1934), and 4: plexiform layer (Cajal 1968), 5: dentate granule cells. Stratum radiatum (6) and stratum oriens (7) insert themselves superficial and deep to CA3h. The separation between CA3h and the hilar polymorphic layer is variable in different species and at different septotemporal levels. When the CA3h and the polymorphic cell layer merge, we cannot reliably distinguish CA3h cells from hilar polymorphic cells in Nissl-stained preparations. Scale bars: 250 µm.

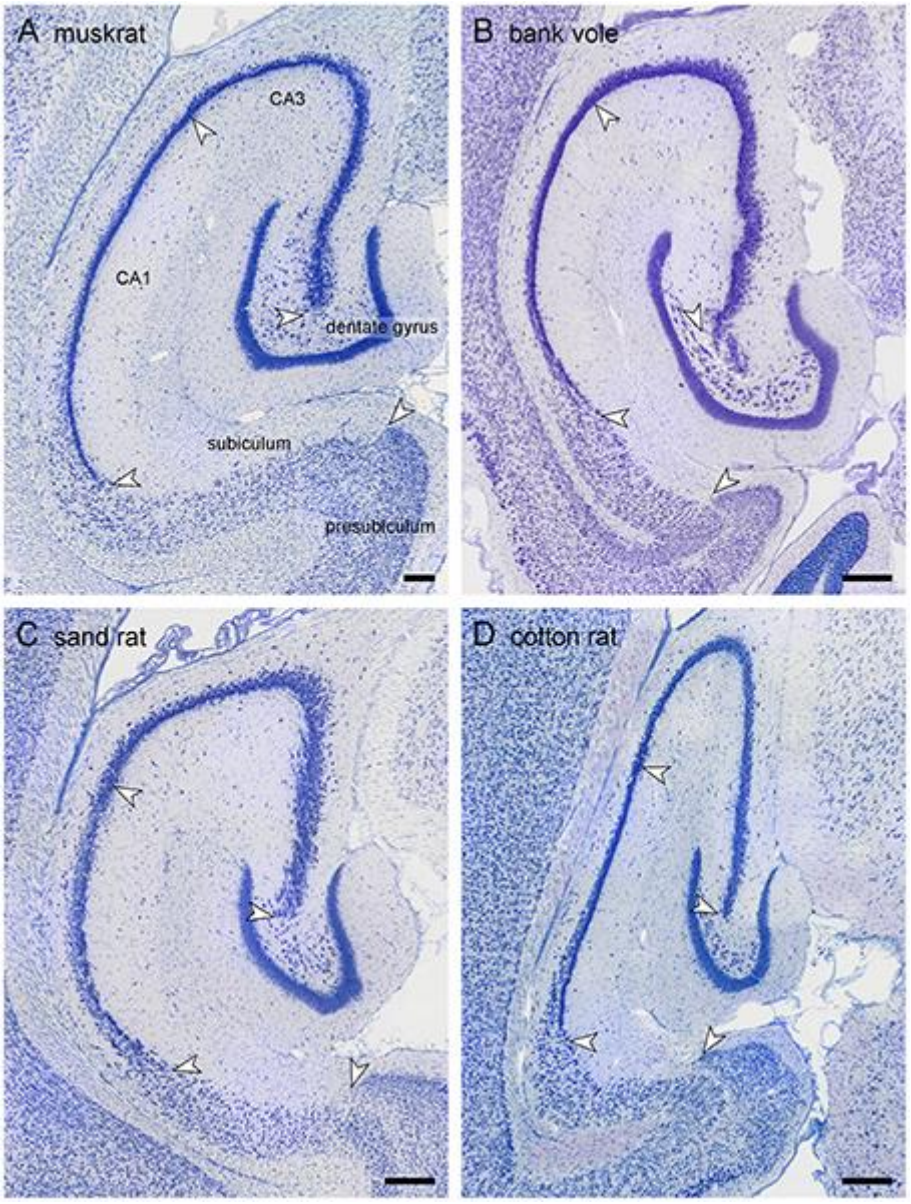

Figure 2.4. Hippocampal morphology of the four rodent species. Mid-septotemporal hippocampus of the four rodent species presented for the first time: (A) muskrat, (B) bank vole, (C) sand rat, and (D) cotton rat. The brains were sectioned horizontally, and images were taken in sections immediately following the disappearance of the

septal pole of the dentate gyrus. Arrows mark the boundaries between subdivisions at the level of the cell layer(s). Scale bars: 250 µm.

Similar to the house mouse, in three of the species (muskrat, sand rat and bank vole) a well-defined cell-poor dentate plexiform layer is located between the granule cell layer and the hilar polymorphic cells. It is not consistently present in cotton rats. Similar to all other rodents included in this study, none of the species show a reflected blade of CA3. In all species, CA1 pyramidal cells are markedly smaller than CA3 pyramids, and the change in cell size was used to define the CA1/CA3 border. A separate CA2 could not be defined reliably in any of the four species based on cytoarchitectural criteria. The CA1 pyramidal cell layer is distinctly divided into deep and superficial sublayers in sand rats and bank voles throughout most of the proximodistal and septotemporal extent of CA1. While the layer appears very compact in bank voles, cells are quite loosely packed in sand rats. More similar to the house mouse and rat, lamination within CA1 develops gradually from septal-distal to proximal-temporal in muskrats and cotton rats. Proximal and distal divisions of the subiculum are better defined in muskrat and cotton rat than in the bank vole or sand rat. Differences in the size of hippocampal fields are readily apparent upon visual examination and reflect the sizes of the principal cell population that are described below.

Principal hippocampal cell numbers

The means of total cell number estimates per hippocampal region are listed in table 2.2. Using a conservative smoothness constant (m) of 0 (Slomianka and West 2005), the overall mean CE of the estimates was ~0.10. CE^2/CV^2 were typically below 0.5, indicating that variability contributed by the estimation procedure was a minor source of the total variability of cell number estimates within each species (table 2.2). Species in which the CE^2/CV^2 were higher than 0.5 were investigated and all cases could be attributed to unusually small sample variation between animals. Several species in the study were composed of animals of both sexes, but low per-sex *n* prevented statistical testing for possible sex effects.

The largest hippocampi with regards to both total cell number and volume are, not surprisingly, found in the largest species in our data set: humans, monkeys, dogs and domesticated pigs. Notable species are the Eastern rock sengi (Slomianka, Drenth et al. 2013), yellow-necked wood mouse and bank vole that are all small animals (< 50 g) with a number of hippocampal cells comparable to much larger species such as dogs, muskrats, and marmosets (Figure 2.5 for rodent comparison). The relative sizes of the cell population are roughly similar between species (see Figure 2.1). Granule cells in the dentate gyrus form the largest population in all species, the second largest cell population is

that of CA1 followed by CA3, the subiculum and last, with the smallest cell population, the hilus. There are however species showing exceptions to this pattern. The three mole-rat species all have CA3 cell numbers that exceed the number of CA1 cells (Figures 2.1, 5). Second exceptions are the four largest species in our data set (human, rhesus monkey, pig and dog, see Figure 2.1), in which subicular cell numbers exceed those in CA3.

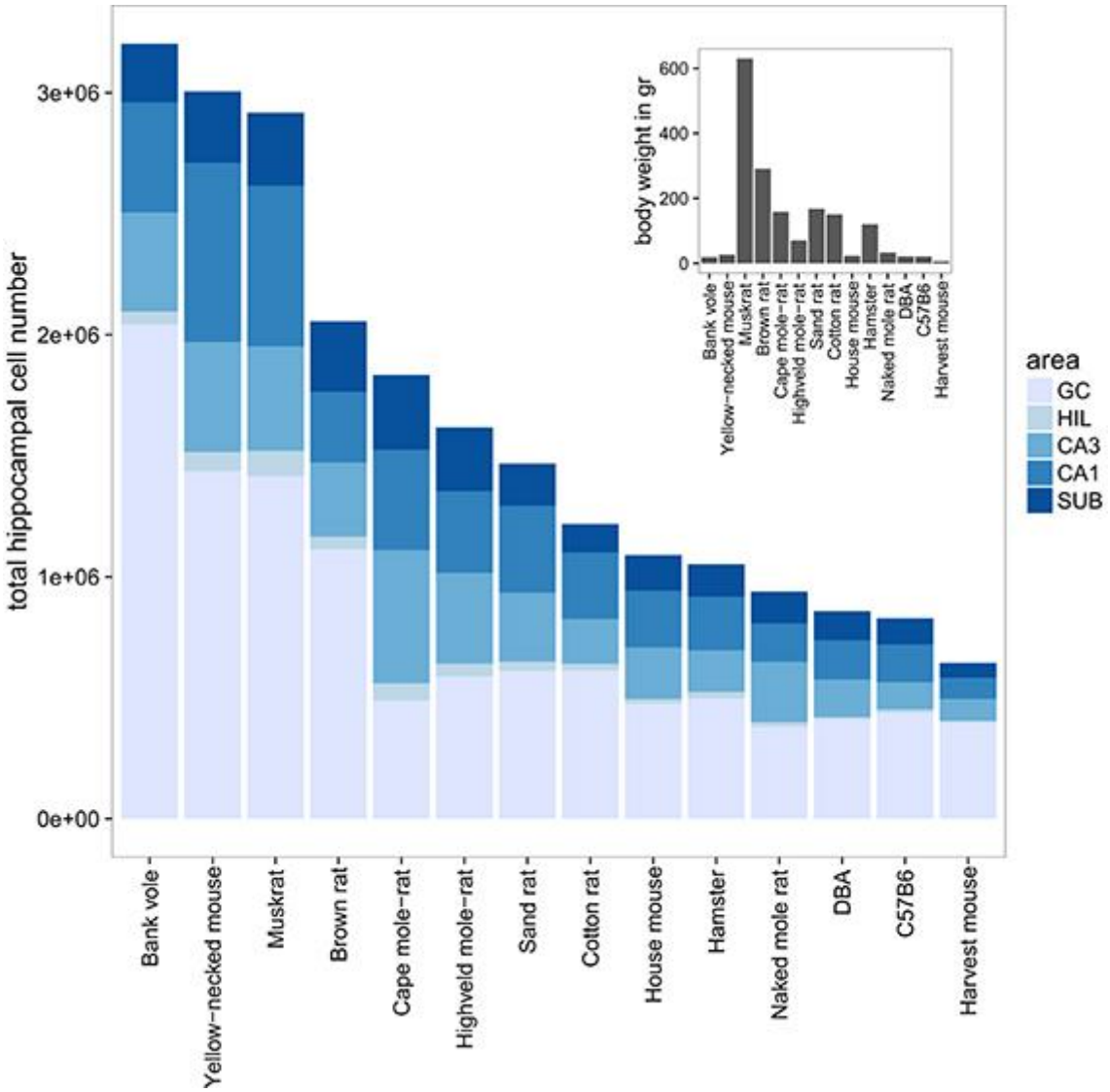

Figure 2.5. Hippocampal cell number and body weight relationships in the rodent group. Total cell numbers were estimated for each of the five main hippocampal region: granule cells (GC), hilus (HIL), CA3, CA1, and the subiculum (SUB). The mean number of estimated cells per region and species are plotted in a bar plot. The 14 rodent species and strains are sorted according to total hippocampal cell number. The insert shows the corresponding mean bodyweight for each of the 14 species and strains.

Correspondence analyses of all species

Total cell numbers for each of the five principal cell populations for all 20 species or strains were compared in a correspondence analysis (Figure 2.6A). Data in this analysis was log transformed and scaled against the five populations per individual animal (mean of 0, standard deviation of 1), resulting in a dimensionless unit representing the relative contribution of each population to total hippocampal cell number. The two axes in Figure 2.6A represent 89% of the variance in the data (axis 1: 64% and axis 2: 25%). Each dot in the plot represents one animal. Hilar and CA3 populations cause the largest separation between species (respectively 38 and 35% of the variation along the first and 21 and 18% along the second axis), and they are able to separate the species into clusters, which largely align with the taxonomic grouping of the species (Figure 2.6A). A proportionally large CA3 in rodents results in a complete separation of all representatives of the order Rodentia from other species along the first axis. The relatively large hilar population found in the Eastern rock sengi, marmosets, rhesus monkey and pigs cause further separation. These four species, together with humans and dogs, also share a reflected blade of the CA3 pyramidal cell layer. In our study, as well as in all sources used in this study, these cells are included in the cell counts of the hilus (see Figure 2.3 and Discussion). Along the second axis, in addition to hilar and CA3 populations, further distinction is made by the subiculum and CA1 (26% and 19%, respectively), which results in a separation of the human hippocampus, marked by both a large CA1 and small hilar cell populations when compared to other primates. Lastly, dogs form a unique group by having both large subicular and CA1 populations. In addition to the correspondence analysis, Figure 2.6B shows the normalized species profiles of population sizes per animal. In these graphs the same patterns can be detected as in the correspondence analyses, where again rodent data show a distinct pattern based on the cellular composition of their hippocampi.

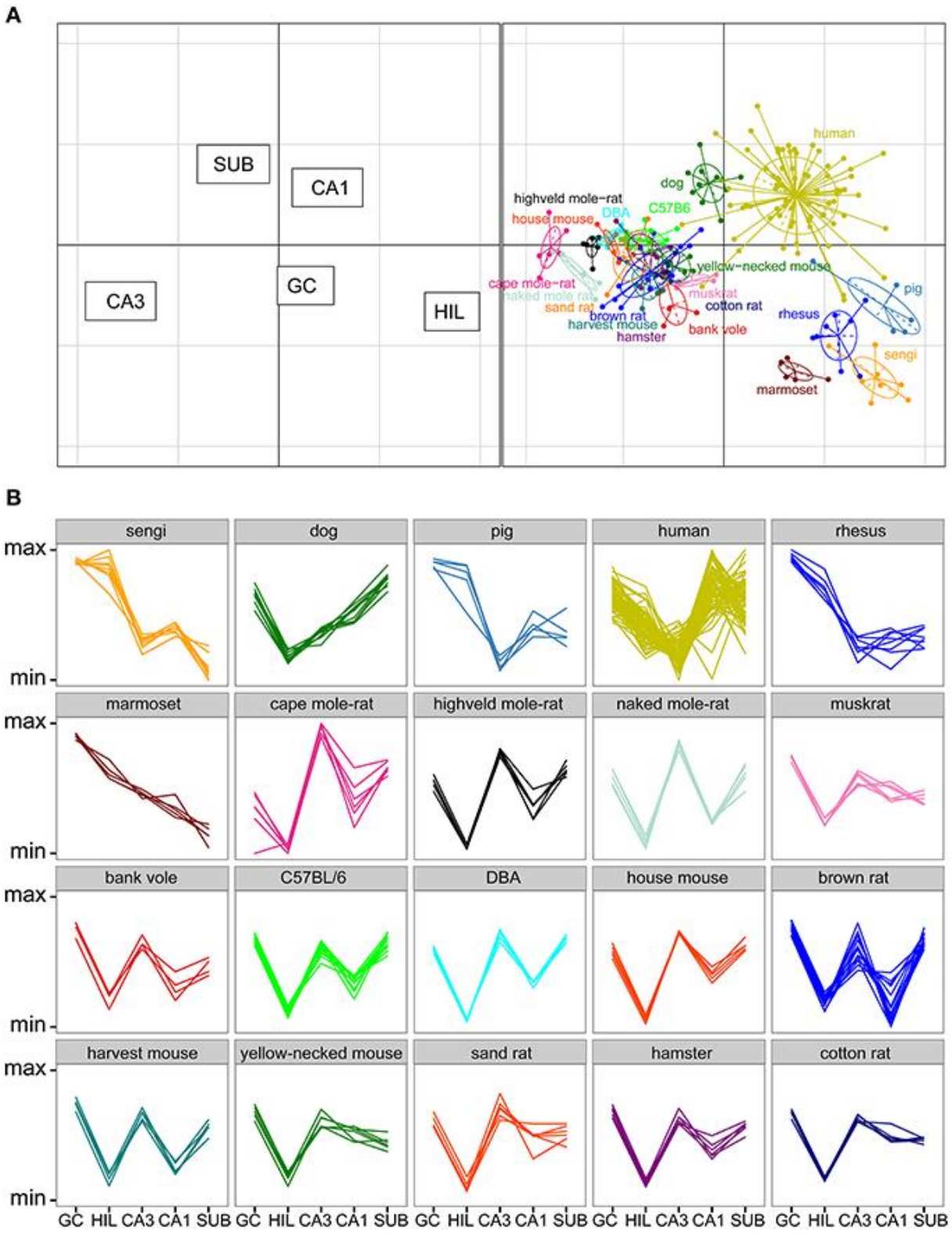

Figure 2.6. Species clusters occupy distinct spaces in the correspondence analysis. (A) Correspondence analysis showing the relationships between species and hippocampal principal neuron numbers. Species form distinct clusters with taxonomically related species such as the rodents clustering close together. The spatial arrangement of hippocampal fields (left graph) can be used to determine which populations are driving the species clustering. Rodents, especially mole-rats, have relatively high numbers of cells in the CA3 and relatively few cells in the CA3h/hilus (right graph). HIL, CA3h/hilar cells; SUB, subicular cells; GC, dentate granule cells. (B) Species

profile plots showing group-specific patterns in the relative composition of hippocampal principal cell populations. The y-axes range from the minimum to the maximum value for each hippocampal field across all species. For example, rodents have relatively larger CA3 than all the other species and humans have relatively larger CA1 than all other species. Each line indicates one individual animal.

Separation within the rodent cluster

The rodents form a tight cluster in comparison to the other species in our data set. To further investigate the rodent group the correspondence analyses was performed using only the rodent data. Figure 2.7A shows this rodent-specific correspondence analysis. In doing so, the contribution of each principal cell populations is reassessed without being skewed by extreme cases seen in the other orders (e.g., the large CA1 and hilus of primates). The two axes in the plot explain 81% of the variation in the data (axis 1: 57% and 2: 24%). Within rodents, hilus and CA3 are still strong separators although their contribution is weaker than in the comparison between taxonomic orders (27% and 30% of variation along the first and 3% and 5% along the second axis). Other populations now account for more of the variation. CA1 is the main differentiator on the second axis (47%) while the granule cells significantly affect separation on both the first (25%) and second axis (11%). The entire rodent group is marked by a large CA3 (Figure 2.6), and CA3 variation is able to further distinguish rodents from one another. Most prominent are the three mole-rat species, which are separated from the other rodents on account of there, even for rodents, large CA3. The center of the correspondence analysis can be seen as an “average” of rodent species, this space is occupied by the two laboratory strains of the house mouse (C57BL/6 and DBA), wild-type house mouse, hamster and harvest mouse. Opposite of the mole-rat species is the brown rat, having both a small CA3 but large hilus and subiculum. Other species marked by a relatively small CA3 but average to large CA1 cell count are the bank vole, muskrat, cotton rat and the yellow-necked wood mouse.

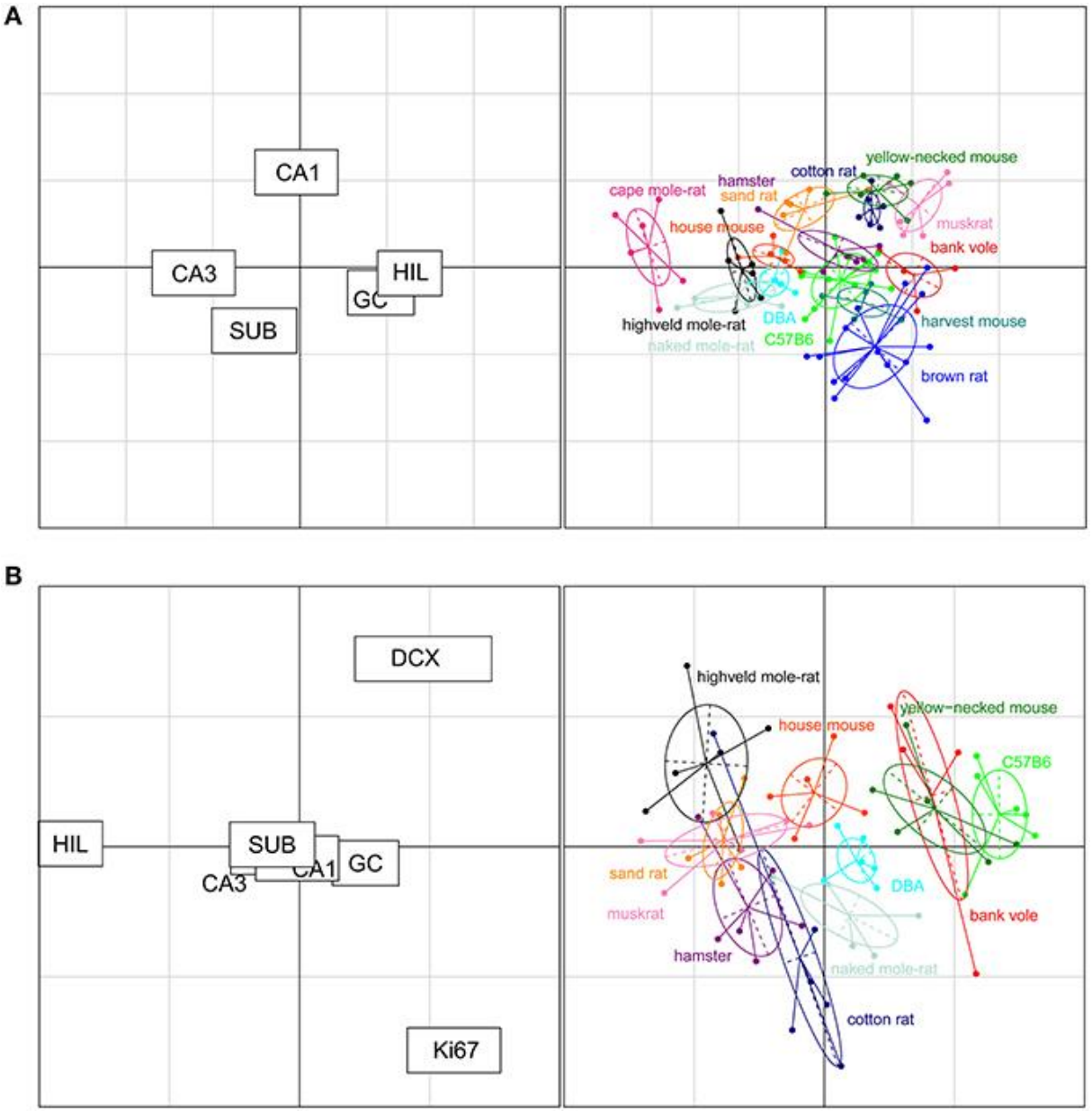

Figure 2.7. Neurogenesis drives the separation within rodent clusters in the correspondence analysis. (A) Separate correspondence analysis of the rodent cluster. Note that the range is now defined by the variability seen in rodents only, providing a higher resolution for the rodent data than in Figure 2.6. All cell populations contribute to the separation within the rodent cluster (left graph). For example, the three mole-rat species, house mice and DBA were pulled toward the negative direction of the x axis due to their relatively large CA3 cell population portion and small granule cell and hilus cell population portion (right graph). (B) When taking the neurogenic cell populations into account, the cell populations that differentiate strongly between rodents are new-born differentiating neurons (DCX+), proliferating cells (Ki67+), and hilar cells (HIL, left graph). The plot places the laboratory mouse strains C57BL/6 and DBA close to yellow-necked wood mice and bank voles. The cotton rats and hamsters (both laboratory bred) show relatively similar patterns to each other. The two mole-rat species, on the other hand, are separated from each other by their distinct levels of neurogenesis and hilar cell populations.

Adult neurogenesis as a separating factor

For 11 out of the 14 rodent groups, adult neurogenesis was assessed using the markers Ki67 for proliferating cells and doublecortin (DCX) for differentiating young neurons. The differences in age between the animals and the consequent differences in neurogenesis, was accounted for by extra-polating cell numbers estimates to an age of 3 months (Figure 2.2). The estimated number of proliferating cells and young neurons were taken together with the five principal hippocampal cell populations, and again analyzed in a correspondence analysis (Figure 2.7B).

The inclusion of the two neurogenesis parameters dramatically changes the results of the analysis due to the larger inter-species variation in neurogenesis compared to the more stable principal cell populations. The first two axes cover 83% of the variation in the data (axis 1: 60% and 2: 23%). DCX+ cells predominantly acts on the first axis (38%, second axis: 21%), and Ki67+ cells act on the second axis (48%, first axis: 2%). Surprisingly, the other hippocampal cell regions lose much of their differentiating power. The only region still having a strong effect is the hilus (first axis: 31% second: 20%). Species can be divided in four groups according to the contribution of the DCX+ cell population; bank voles, C57BL/6 and the yellow-necked wood mouse having the highest relative contribution of DCX+ cells. They are followed by the house mouse, DBA mouse and naked mole-rats having mid-high levels of DCX+ cells, subsequently followed by species with relatively low DCX+ cells: the highveld mole-rat, sand rat, muskrat, hamster and cotton rat. The separating effect of the hilar cells is interestingly enough opposite to that of the DCX+ cell population, species marked by high DCX+ cell numbers have relatively small hilar cell populations and vice versa. The Ki67+ cell population is the main separating factor on the second axis, perpendicular on the DCX+ and hilus driven first axis, where each of the four groups described show a gradient of contribution by the Ki67+ cell population (Figure 2.7B).

Convergence and divergence between connected cell populations

For each set of interconnected cell populations, the degree of convergence/divergence was calculated as ratios (Table 2.4) and analyzed in a correspondence analysis (Figure 2.8). The values represent to what degree information may converge from many-to-few cells or diverge from few-to-many cells in the pathway. The first two axes cover 93% of the variance in the data set (axis 1: 79%, axis 2: 14%). The convergence/divergence between the GC and CA3 populations is the main differentiator with 35% of variation of the first axis, closely followed by the convergence from CA1 to subiculum and from CA3 to CA1 (CA1 to subiculum: 28% on the first and 39% on the second axis; CA3 to CA1: 24% on the first and 47% on the second axis). Rodents again form a tight cluster with the

exception of the mole-rat species, marked by a much larger CA3 cell populations compared to their CA1 populations. Noteworthy is the shift between the primate species. Based on principal cell populations, humans were separated from the other primates on account of their relatively large CA1 population (Figure 2.6). However, the convergence/divergence values of the human data falls between the two other non-human primate species (Figure 2.8).

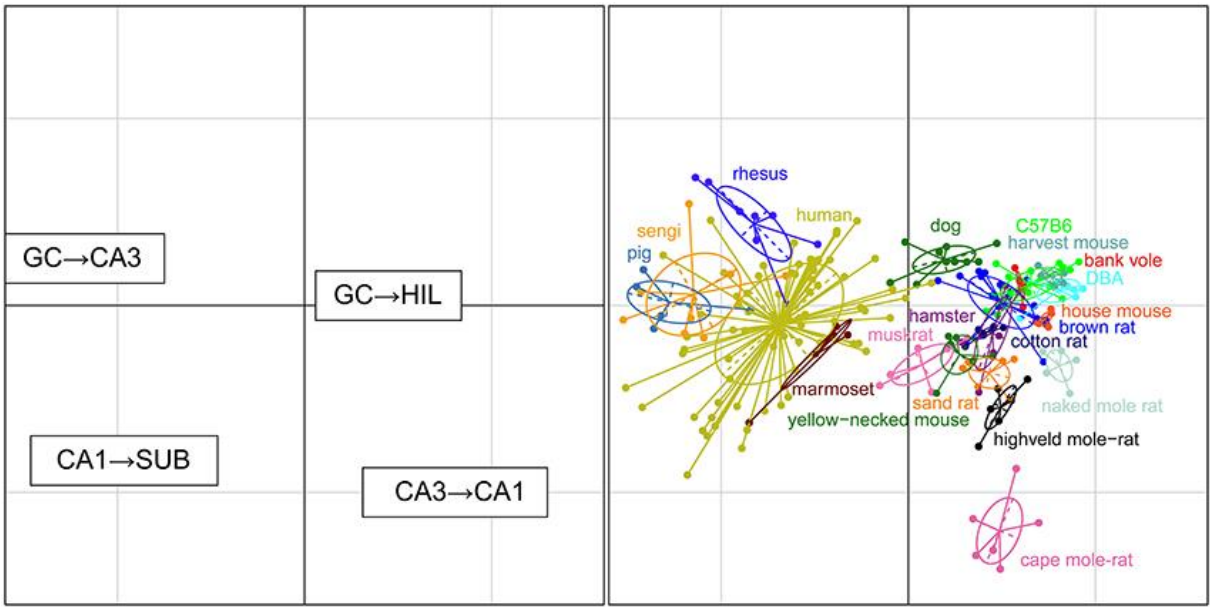

Figure 2.8. Convergence and divergence of hippocampal cell populations across species. While all rodents form a tight cluster similar to the figures plotting principal cell populations, all primates including humans now cluster together due to similar degrees of convergence. This indicates that individual cell populations can differ to a large degree between species, while stable convergence/divergence relationships are retained in phylogenetically related species.

Species	GC→HIL	GC→CA3	CA3→CA1	CA1→SUB
Dog	0.041	0.165	3.044	0.694
Pig	0.166	0.144	2.686	0.513
Human	0.087	0.152	4.194	0.379
Rhesus monkey	0.048	0.061	2.599	0.421
Marmoset	0.074	0.158	1.667	0.323
Cape mole-rat	0.143	1.122	0.717	0.756
Highveld mole-rat	0.09	0.639	0.898	0.766
Naked mole-rat	0.05	0.635	0.64	0.803
Muskrat	0.072	0.302	1.526	0.452
Bank vole	0.026	0.194	1.089	0.527
C57BL/6	0.029	0.263	1.31	0.719
DBA	0.028	0.361	1.113	0.722
House mouse	0.039	0.443	1.081	0.629
Brown rat	0.047	0.231	1.013	0.993
Harvest mouse	0.022	0.228	0.942	0.697
Yellow-necked mouse	0.054	0.313	1.608	0.396
Sand rat	0.061	0.46	1.221	0.481
Hamster	0.05	0.331	1.274	0.606
Cotton rat	0.045	0.301	1.49	0.423
Sengi	0.051	0.055	3.443	0.171

Functional connectivity of the principal cell numbers in the hippocampus can be expressed as a ratio of one cell population to the next one. Convergent ratios (many cells to few cells, white in the table) apply to the granule cells to hilus cells (GC→HIL), granule cells to CA3 pyramidal cells (GC→CA3), and CA1 pyramidal cells to subicular cells (CA1→SUB). For example, C57BL/6 has a GC→CA3 convergence ratio of 0.26 that means roughly four granule cells for each CA3 cell. Divergent ratios (few cells to many cells, gray background in the table) are found for CA3 pyramidal cells projecting to CA1 (CA3→CA1). For example, C57BL/6 has a CA3→CA1 divergence ratio of 1.3, which means 1.3 CA1 neurons for each CA3 neuron. Note that in particular the mole-rat species do not follow the overall convergence—divergence pattern, see Discussion. For data sources see ***Table 1****.*

Additional validation

Data for humans and C57BL/6 mice originated from different studies (see Table 2.1 for references). To test if the data sets are comparable between studies, we reanalyzed the data treating each study as a separate group. In all instances, data from different studies for a single species overlap and share the same characteristics (not illustrated). Furthermore, in addition to the primates (humans, rhesus monkeys and marmosets) in our data set, available mean values of hippocampal cell counts from an

additional species (Macaca nemestrina; (Leverenz, Wilkinson et al. 1999)) and one additional group of rhesus monkeys (Jabès, Banta Lavenex et al. 2011) were included in the analysis. The data points grouped with the marmosets and rhesus monkeys respectively, supporting the outcomes of our analysis.

In view of the reported age-related changes in neuron numbers of the human hippocampus (West 1993, Simic, Kostovic et al. 1997), we analyzed the combined human data set for age related differences in principal cell population numbers. Using study as a covariate, the hilar [$F(1, 67) = 6.71$, $p = 0.01$], CA1 [$F(1, 67) = 22.56$, $p < 0.001$] and subicular [$F(1, 67) = 15.70$, $p < 0.001$] cell numbers show a significant decrease with age. We also analyzed the effect of this age related decline in cell numbers on the positioning of humans in the correspondence analyses by comparing both age-quartiles and low-age (< 30) vs. high-age (>80) groups. Both quartiles and low-age vs. high-age did show largely overlapping distributions (not illustrated).

Neurogenesis-related cell numbers in rodents of different ages where extrapolated to the age of 3 months based on two curve estimation. We compared the neurogenesis calculations (see Figure 2.2) based on the curve estimation of published data (Ben Abdallah, Slomianka et al. 2010) and in addition the curve estimation based on the data from this study (for details see in the Materials and Method Section). Using the two slopes, we found no difference in the positioning of the species relative to each other (not illustrated). While extrapolation to a common age usually did not require changing the age by more than a few months (see Figures 2.2A ,B′ for examples), the age of the mole-rats had to be changed by years. To investigate the impact of possible exponentially accumulating errors in extrapolated cell number estimates, the rodent sample was also analyzed excluding the mole-rats. Proliferating cells, young neurons and hilar cells remained the most powerful separating factors also when mole-rats were excluded from the species sample (not illustrated).

2.4 Disscussion

Correspondence analyses of total principal cell numbers show that the major hippocampal separating factors in either the survey of all mammalian brains included in this study or within the rodent species alone are the cell populations in the hilus and CA3. For the rodent species, neurogenesis was a key separating factor. The inclusion of new-born neurons in the analysis dramatically increased separating power of the hilar cells. Species profile plots cluster phylogenetically close species, both when comparing all species and within the rodent cluster.

Mosaic changes in hippocampal cell composition

In rodents and primates hippocampal principal cell numbers do not decline with age (Rapp and Gallagher 1996, Rasmussen, Schliemann et al. 1996, Keuker, Luiten et al. 2003, Keuker, de Biurrun et al. 2004). Humans seem to be an exception in that the hilar, CA1 and subicular cell numbers decrease across the age range of 13 to 99 years. Both the large and selective effects reported previously by (West 1993) or (Simic, Kostovic et al. 1997)could be confirmed. Due to the concerted age-related changes, which largely preserved relative population sizes, there was no age-related impact on the position of humans relative to other species.

The visualization of complex data free from effects of absolute size by correspondence analyses allows comparisons of the relative compositions of the hippocampal cell populations. The analyses provided a clear separation of a tight cluster formed by rodents from other taxonomic groups and evidence for mosaic changes within the hippocampus, which agree with comparative studies at a more gross anatomical level (Barton and Harvey 2000, de Winter and Oxnard 2001, Reep, Finlay et al. 2007). Yet, when measured against absolute cell numbers these relational differences are overshadowed by the differences that exist in total hippocampal size. Limits to the growth of individual cell populations independent of the growth of functionally related brain areas fit with the concerted view of brain development where developmental constrains cause coordinated changes in size (Finlay and Darlington 1995, Finlay, Darlington et al. 2001). A similar dynamic between evolutionary constraints and a complete structure can be seen in other structures of the brain (Gómez-Robles, Hopkins et al. 2015) or neurocranium (Mitteroecker and Bookstein 2008). Considering the tight functional linkage between hippocampal principal cells, which has been equated with tight constraints on independent phylogenetic development (Whiting and Barton 2003, Gómez-Robles, Hopkins et al. 2015), it is notable that we are still able to see strong separation of species based on convergence and divergence. Finding these relationships also illustrates the suitability of this data to study possible relationship between the different hippocampal regions and ecological and life-history variables.

Within the rodent group, the only exceptional species are the mole-rats that have unusually large CA3 cell numbers (discussed below). Interesting and perhaps reassuring is that quantitative relations in the common laboratory rodents: C57BL/6 mice, DBA mice, Wistar rats, and Sprague Dawley rats (brown rat) are at the center of the rodent cluster and, as far as quantitative relations are concerned, can be considered representative of rodents.

A known outlier species is the Sengi, which in size and habitat is similar to a rodent but shares quantitative relations closer to those of primates and the domesticated pig (Slomianka, Drenth et al. 2013). Of the three primate species included in this study, the two monkey species share similar quantitative relations and, together with two additional data points from the literature (Leverenz, Wilkinson et al. 1999, Jabès, Banta Lavenex et al. 2011), provide further evidence for a quantitatively distinct monkey hippocampus. Humans are separated from all other species on account of a relatively large CA1, fitting with previous assessments of the human hippocampus (Stephan 1983, Seress 1988). However, CA1 has a rather low impact on overall species separation. What separates humans from other primates is less an exceptionally large CA1 but an exceptionally small hilus.

We feel that the number of species included does warrant the identification of a rodent cluster separate from other mammals in this study. The number of species in other taxonomic groups is however not sufficient to determine if quantitative relations in the hippocampus will be able to differentiate between these groups.

Convergence and divergence

Primates, including humans, cluster tighter when we look at the relationship between connected areas by mapping the convergence and divergence in cell numbers. They share similar degrees of convergence of CA3 to CA1 cells, demonstrating that individual cell populations can differ to a large degree between species, while stable convergence/divergence relationships are retained in phylogenetically related species.

An observation relevant to current ideas about intrahippocampal information processing is the degree of convergence and divergence between the number of neurons in CA3 and CA1 in the three mole-rat species. (Treves and Rolls 1994)have put forward the idea that a robust and noise-free down-stream transmission of information can be achieved if each CA1 neuron has to code less information compared to the CA3 neurons. Computational analysis of the information carrying capacity of the Schaffer collaterals show that an expansion rate of two, i.e., two CA1 neurons for every CA3 neuron, allows information to be passed without a significant loss. Increasing the expansion rate above two only leads to limited gains, while an expansion rate below one results in a rapid deterioration of information transfer between CA3 and CA1 (Treves 1995, Schultz and Rolls 1999). We observed four species with an ‘expansion’ rate below one, of which the three most extremes cases are mole-rats. Mole-rats are known for having a relatively small overall brain size, which has been linked to reduced sensory input due to their strictly subterranean habitat (Harvey, Clutton-Brock et al. 1980). With little information available on the hippocampal circuitry of mole-rats,

one can only speculate if the limited sensory input puts a greater demand on the auto associative capabilities of the CA3 region or if it results in simpler CA1 ensemble codes requiring fewer cells. Also, it is not clear if and how this would be compatible with a loss of information in the transmission from the CA3 to the CA1.

As mentioned above, information transfer capability does not improve much at expansion rates above two and rapidly deteriorates at values below one. Yet, all non-rodent species that we analyzed exceed the upper value, with humans reaching just above four. As pointed out by Schultz and Rolls (1999), these relations would allow CA3 cells to serve not only one but multiple segregated information streams. Quantitative observations relate well to the recent definition of subsets of CA1 pyramidal cells based on overlapping cytoarchitectural, neurochemical, and connectional criteria (Deguchi, Donato et al. 2011, Slomianka, Amrein et al. 2011, Lee, Marchionni et al. 2014), physiological characteristics (Mizuseki, Diba et al. 2011, Hongo, Ogawa et al. 2015, Valero, Cid et al. 2015) and gene-expression data (Thompson, Pathak et al. 2008, Dong, Swanson et al. 2009, Zeisel, Muñoz-Manchado et al. 2015). In the non-rodent CA1, quantitative relations would allow streams to be represented by non-overlapping cell populations that each can be served by the entire CA3 population. In rodents, in which most of the data pertaining to information streams were generated, the developmental matching of interconnected CA3 and CA1 cells (Deguchi, Donato et al. 2011) may prevent the deterioration predicted by theoretical models. Clear answers on how excessive redundancies or information deterioration are avoided will require refinements of the anatomical data and/or theoretical models.

Do hilar cells punch above their weight?

The results of the present study suggest that changes in hilar cell numbers are associated with many taxonomic shifts. While the definitions of the principal cell populations appear rather straight forward in the rodents, this is not the case for non-rodents species with a reflected blade of CA3 (Figure 2.3D, number 2). The reflected blade of CA3 has also been named CA3h (Lim, Mufson et al. 1997, Ding and van Hoesen 2015), avoiding the ambiguity associated with the heterogeneously defined alternative term CA4 (e.g., Lorente de Nó 1934, Rosene and van Hoesen 1987). In all the sources that we have used and in the non-rodent species that we assessed ourselves, CA3h cells have been included in the estimates of hilar cell numbers (Figure 2.3D, number 2 and 3). In our case and most likely in the sources as well, this decision was made because a reliable border between CA3h and the remainder of CA3 is far easier to define than the border between CA3h and the hilar polymorphic cell layer that contains the bulk of the “proper” hilar cells. This must raise the question how an apparently arbitrary, technical border does impact on the interpretation of the outcomes. Some observations in our dataset suggest that the impact may be limited. First, hilar and CA3 cells

differentiate between rodents in the same manner (similar strength and opposite directions) as they do between rodents and the remaining species. Second, hilar cells also differentiate species within the group that is characterized by inclusion of CA3h in hilar cell number estimates. Similar separations despite different definitions suggest that there is more to including CA3h cells in hilar estimates than technical reasons.

It would be difficult to argue a CA3 pyramid into being hilar polymorphic cell, because of their distinct morphologies (Amaral 1978, Buckmaster and Amaral 2001), connectivities (Blackstad 1956), electrophysiological properties (Scharfman 1993, Buckmaster and Amaral 2001) and development (Li, Berger et al. 2008). On the other hand, some of the ideas about these subfields have changed. The unidirectionality of information flow from the dentate gyrus to CA3 and the assignment of distinct functions to either the dentate gyrus (pattern separation) or CA3 (pattern completion) have softened enough to allow for bidirectional functional interactions between the dentate gyrus and proximal CA3 cells. Backprojections from CA3 to the dentate gyrus (reviewed in Scharfman 2007) in the form of axon collaterals of proximal CA3 pyramidal cells to both the hilus (Ishizuka, Weber et al. 1990, Li, Somogyi et al. 1994) and deep dentate molecular layer (Li, Somogyi et al. 1994, Buckmaster and Amaral 2001) provide feedback to hilar mossy cells, interneurons and granule cells (Scharfman 1994, Kneisler and Dingledine 1995, Scharfman 1996). Notably, CA3 pyramidal cells extending dendrites into the dentate molecular layer have been found in CA3h of primates (Lim, Mufson et al. 1997, Buckmaster and Amaral 2001, Buckmaster 2005). The suggestion that backprojections may provide a mechanism for CA3 to influence pattern separation (Myers and Scharfman 2009) has been tested and used in computational models of CA3-dentate interactions (Myers and Scharfman 2011, Petrantonakis and Poirazi 2015), in which backprojections improve pattern separation in CA3. Consistent with these models, studies on the functional differentiation along the proximal to distal axis of CA3 found an emphasis on pattern completion in distal CA3 and on pattern separation in proximal CA3 (Lee, Wang et al. 2015, Lu, Igarashi et al. 2015), and both studies argue for a tight functional integration of proximal CA3 and the dentate gyrus in pattern separation. Functionally, this integration is also reflected in similar behavioral deficits following proximal CA3 and dentate lesions (Hunsaker, Rosenberg et al. 2008) and *arc* expression in proximal as compared to distal CA3 pyramids in relation to pattern separation demands of changing environments (Marrone, Satvat et al. 2014).

Therefore, it appears that, instead of hilar cells punching above their weight, it is more likely the interaction between proximal CA3 and the dentate and, consequently, the number of CA3 cells involved in this interaction that is a strong taxonomic separator between species. This idea would predict a much sharper functional differentiation between proximal and distal pyramidal cells in species in which proximal CA3 pyramids form a distinct CA3h. This idea could be tested in guinea pigs

or rabbits, which both possess a well-defined CA3h (Geneser-Jensen 1973, Geneser 1987, Buckmaster, Kunkel et al. 1994). Lastly, it should be considered if a CA3h is indeed absent in rodents. We have previously discussed the possibility that cytoarchitectural changes in the temporal rodent hippocampus, in which backprojections are also stronger than septally (Li, Somogyi et al. 1994), suggest the presence of a CA3h (Slomianka, Drenth et al. 2013). Lorente de Nó (1934) did identify a CA3h (the first reflected blade of his CA4; his Figure 2.2) in the mouse using the hippocampus temporal to the appearance of the lateral entorhinal cortex in his illustration.

Neurogenesis as a separating factor

The stability of hippocampal principal cell numbers contrasts with an exponential age-related decline in adult hippocampal neurogenesis (AHN) in all mammals that have been investigated (Ben Abdallah, Slomianka et al. 2010, Amrein, Isler et al. 2011). To allow for comparison of cell numbers, neurogenesis related cell counts were extrapolated to a common age of three month, representing the approximate mean of the rodents in our data set. To test the overall robustness of the estimates, additional analyses (testing different slopes for the decline of AHN and excluding mole-rats in which the error may be largest) were performed. Invariably and despite the small sizes of the cell populations representing AHN, neurogenesis is a major contributor to the separation of species in the rodent cluster. In mice, cell proliferation (marked by Ki67) and neuronal differentiation (marked by DCX) represent distinct stages of AHN, that can be differentially regulated to adjust the number of newly formed young neurons (van Praag, Kempermann et al. 1999, Kronenberg, Reuter et al. 2003) and their maturation (Plümpe, Ehninger et al. 2006). The selective regulation is also reflected in our analysis in that DCX and Ki67 are independent separating factors, indicating that species use different strategies in the regulation of proliferation versus survival and differentiation of new-born neurons. Differences in the regulation of AHN have been observed between and within taxonomic units before. Red foxes (Amrein and Slomianka 2010) and non-human primates (Ngwenya, Peters et al. 2006, Ngwenya, Rosene et al. 2008, Kohler, Williams et al. 2011, Amrein, Nosswitz et al. 2015, Ngwenya, Heyworth et al. 2015) show a prolonged maturation phase of new-born neurons compared to rodents. Within rodents, habitat variability can be associated with different numbers of young neurons despite similar numbers of proliferating cells (Cavegn, van Dijk et al. 2013). Unexpectedly, when neurogenesis is added to the comparison, of all principal cell populations only hilar cells retain their power to separate between rodent species. Adult neurogenesis may provide an ontogenetic mechanism to adapt faster to changes in the ecological niche compared to the phylogenetic time scales that mediate changes in the principal hippocampal cell populations.

Without age-series for all the species that are included in this study, one remaining caveat will always be the possibility that it is not the number of new-born neurons at a particular age that is different

between species, but rather the rate at which neurogenesis declines in a particular species. However, this does not change the separating power of AHN in the correspondence analyses and the subsequent interpretation of the data. In this scenario, it would be the differences in the rates of decline that serve as the plastic mechanism to adapt to specific ecological niches.

Perspectives

Changes in the composition of the hippocampus in terms of the size of its' principal cell populations, the degrees of convergence and divergence of interconnected cell populations and adult neurogenic cell populations separate species groups at multiple taxonomic ranks. The value of this information lies in the provision of data points that may be useful in the computational modeling of hippocampal function and the issues raised in the contexts of the emerging concepts of hippocampal information streams and the functional differentiation of the CA3/dentate network. We have mostly abstained from speculations about the functional significance of these differences at the species level, which, more often than not, are difficult to substantiate and remain anecdotal. Provided that the species sample is large enough, statistical methods have become available that allow the detection of phylogenetic signals in the character distribution across a species sample (Pagel 1999, Blomberg and Garland 2002). Rank-ordering traits according to phylogenetic stability would, e.g., be one rational way to also rank them as targets of translational efforts. Also, large databases have been generated that define the life histories of species and the biotic and abiotic factors that characterize the niches that they occupy (Jones, Bielby et al. 2009, Botero, Dor et al. 2014). Incorporating phylogenetic information (Freckleton, Harvey et al. 2002), it has become possible to statistically associate ecological parameters with brain traits (Hutcheon, Kirsch et al. 2002, Finlay, Charvet et al. 2014, Weisbecker, Blomberg et al. 2015), and we are currently extending our species sample to allow these techniques to be applied to the hippocampal cell populations.

3. Project II

Selection by tameness increases AHN in mid-septotemporal and temporal hippocampus in silver fox brain

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Abstract

Work on laboratory and wild rodents suggests that domestication may impact on the extent of adult hippocampal neurogenesis and its responsiveness to regulatory factors. There is, however, no model of laboratory rodents and their nondomesticated conspecifics that would allow a controlled comparison of the effect of domestication. Here, we present a controlled within-species comparison of adult hippocampal neurogenesis in farm-bred foxes (*Vulpes vulpes*) that differ in their genetically determined degree of tameness. Quantitative comparisons of cell proliferation (Ki67) and differentiating cells of neuronal lineage (doublecortin, DCX) in the hippocampus of foxes were performed as a proxy for neurogenesis. Higher neurogenesis was observed in tameness-selected foxes, notably in an extended subgranular zone of the middle and temporal compartments of the hippocampus. Increased neurogenesis is negatively associated with aggressive behavior. Across all animals, strong septotemporal gradients were found, with higher numbers of proliferating cells and young neurons relative to resident granule cells in the temporal than in the septal hippocampus. The opposite gradient was found for the ratio of DCX/Ki67- positive cells. When tameness-selected and unselected foxes are compared with rodents and primates, proliferation is similar, while the number of young neurons is higher. The difference may be mediated by an extended period of differentiation or higher rate of survival. On the background of this species-specific neurogenic pattern, selection of foxes for a single behavioral trait key to domestication, i.e. genetic tameness, is accompanied by global and region-specific increases in neurogenesis.

3.1 Introduction

Domestication is a relationship between humans and animals that leads to morphological and behavioral changes in an animal population (Bökönyi 1969, Ingold 1980, Clutton-Brock 1992). Taming, which includes less fear and aggression in captivity, is often a necessary prerequisite for domestication, and selection of tameness is an important step during the domestication of laboratory mice (Ingold 1980, Russell 2002, Goto, Tanave et al. 2013). The farm-fox experiment, started by Belyaev in the 1950s, used tameness in farm-bred silver foxes (a color variant of the red fox *Vulpes vulpes)* as selection criterion and forty years after the initiation of the farm-fox experiment, 70 to 80 percent of the cubs are genetically tame (Belyaev 1979, Trut 1999, Trut, Oskina et al. 2009). The main correlated response to selection for tameness was a change in the functional state of the key stress hormone system - the HPA axis. It has changed at all levels – from the central regulation of the pituitary to peripheral blood levels of glucocorticoids. A delay of the early postnatal maturation of the glucocorticoid status is positively correlated with later ontogenetic manifestation of fear reactions to humans (Trut, Oskina et al. 2009). In laboratory mice, adult-born neurons in the hippocampus are necessary for the normal hippocampal inhibitory regulation of the HPA axis (Snyder, Soumier et al. 2011). Consequently, a lower reactivity of the HPA axis in domesticated animals may involve an increase in adult hippocampal neurogenesis (AHN).

Several findings suggest that the regulation of AHN is modified during domestication. Physical activity reliably up-regulates hippocampal neurogenesis in laboratory mice (van Praag, Christie et al. 1999, van Praag, Kempermann et al. 1999), whereas this phenomenon could not be observed in wild rodents (Hauser, Klaus et al. 2009, Klaus, Hauser et al. 2012, Schaefers 2013). However, when comparing the AHN levels between wild and laboratory/domesticated conspecifics, the results are ambiguous. Wild Norway rats (*Rattus norvegicus*) show a similar level of adult hippocampal neurogenesis as laboratory Norway rats (Epp, Barker et al. 2009). Most laboratory mice strains were established from European house mice, *Mus musculus domesticus* (Ferris, Sage et al. 1983, Frazer, Eskin et al. 2007). The rate of AHN in wild house mice can be below or above that in laboratory mouse strains (Kempermann, Kuhn et al. 1997, Kempermann and Gage 2002, Klaus, Hauser et al. 2012, Schaefers 2013). Results might vary because AHN is influenced by genetic background (Kempermann, Kuhn et al. 1997) or because AHN in laboratory rodents is ontogenetically influenced by diet and environmental conditions (Kempermann, Kuhn et al. 1997, Kempermann, Kuhn et al. 1998, Yon, Mauger et al. 2013). In order to clarify how the degree of domestication in terms of behavioral traits impacts AHN, a well-controlled experiment (i.e. a selected group and its unselected control) is necessary.

AHN along the hippocampal septo-temporal axis may play different roles. The cortical and subcortical connections and the functionality of the dorsal and ventral hippocampus are segregated (Fanselow and Dong 2010, Poppenk, Evensmoen et al. 2013). In laboratory rodents, the septal hippocampus is preferentially associated with spatial learning and contextual memory, and the temporal part with emotional functions such as regulation of the stress response or fear (Bannerman, Yee et al. 1999, Kjelstrup, Tuvnes et al. 2002, Kjelstrup, Solstad et al. 2008, Tanti and Belzung 2013). Graded functional dissociations in terms of behavioral outcomes are likely to be mediated by superimposed differences in genetic, connectional and physiological patterns that can be observed along the septo-temporal axis (Strange, Witter et al. 2014). The role of AHN along the septo-temporal axis is still debated because the anatomical subdivision of the hippocampus is not consistent across studies (Fanselow and Dong 2010, Tanti and Belzung 2013). A well-defined regional comparison study is necessary to address this question.

In this report, we studied AHN of tameness-selected and unselected adult farm-bred foxes by quantification of cells expressing the proliferation marker Ki67, and the young neuron marker doublecortin (DCX). The dentate gyrus of the silver fox was divided into three regions along its longitudinal (septo-temporal) axis and two regions along the radial axis. The six regions allowed an allocation of hippocampal neurogenesis to defined divisions within canine hippocampal anatomy (Figure3.1A). By also assessing the granule cell numbers in the different regions, region-specific neurogenesis can be expressed as a percentage of local granule cells, assigning a functional weight to the population of newly generated cells.

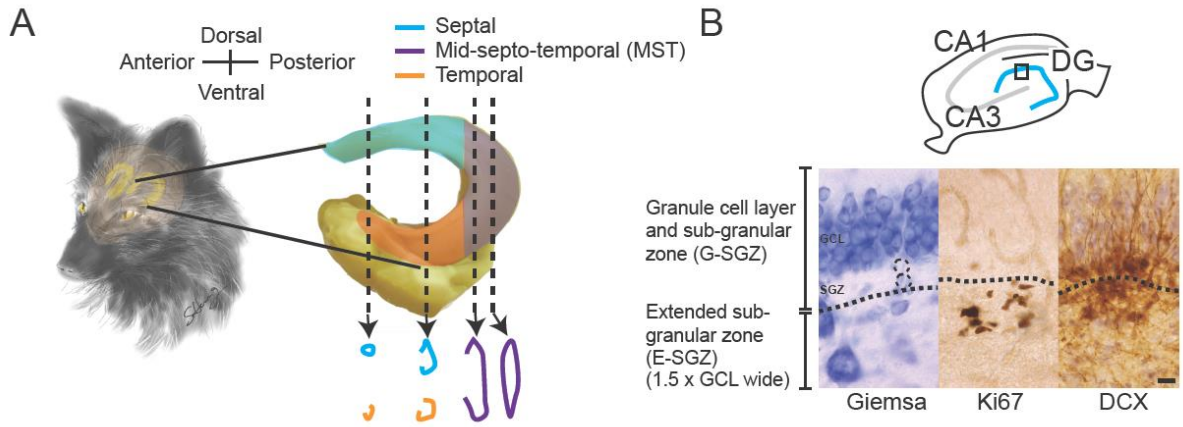


Figure 3.1 Scheme of hippocampal septo-temporal and radial axis (A) The septo-temporal axis. Granule cells, DCX and Ki67 positive cells in the dentate gyrus were quantified in three divided areas along the hippocampus: septal, mid-septo-temporal (MST) and temporal. The hippocampus was sliced coronally and tissues in the blue, purple and orange area were considered as septal, MST and temporal, respectively. (B) The radial axis. For the DCX and Ki67 positive cell quantification, the dentate gyrus was separated into two regions: (1) granule cell layer

and sub-granular zone (G-SGZ), and (2) the extended SGZ (E-SGZ). DG: dentate gyrus; CA: Cornu Ammonis; GCL: granule cell layer; SGZ: sub-granular zone. Scale bar: 10µm.

3.2 Materials and Methods

Animals

17 male farm-bred foxes (*Vulpus vulpes*, 8 tame, 9 unselected for behavior) from the fox colony maintained by the Institute of Cytology and Genetics (ICG), Siberian Branch of the Russian Academy of Science in Novosibirsk, Russia, are included in the project. Females were not included in the experiment, due to their use as breeders. Animal experiments were conducted following the international guiding principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences (CIOMS) and were also in compliance with to the laws, regulations, and policies of the "Animal welfare assurance for humane care and use of laboratory animals", permit number A5761-01. Tame animals are the result of long-term selection (>50 years) for the one main trait – tolerant and then affiliative behavior toward humans. The distinction of behavior between tame and unselected foxes, as well as principles of behavioral scoring has been described earlier in detail (Trut 1980, Trut 1999, Trut, Oskina et al. 2009). Tests for behavior have the aim to discriminate the degree of tameness or fear of and aggression towards humans. All foxes are tested at the age of 6-7 months in the home cage. At this age the behavioral reaction toward humans is formed and stays stable in most animals during their later life. An experimenter stays near the cage, opens the door and tries to touch the animal. The reaction is rated from +0.5 for less tame animals to +4.0 for animals who demonstrate dog-like behavior (wagging tails, licking, sniffing and whining to attract attention). Under the ICG scoring system, non-selected foxes were rated for their aggressive response (the scale is from 0, indicating no aggression, to - 4.0, indicating most aggression). The fear reaction was also rated according to the extent to which contact with humans was avoided. All tame foxes in this study scored +4.0 for tameness and 0 for fear reaction. Two unselected animals showed no fear (0), whereas 6 unselected foxes showed a weak fear reaction (0.5). Unselected foxes scored between -1.0 and -2.0 for aggressive behavior.

At the age of 7 months, the animals were euthanized with 5% sodium-thiopental, decapitated and perfused bilaterally via the carotid arteries with heparinized phosphate-buffered saline followed by 4% paraformaldehyde in PBS. Brains were dissected and post-fixed in changes of fixative over 4 days. Dissected hippocampal formations of randomly selected hemispheres were cryoprotected and processed for immunohistochemistry; hippocampal formations of the other hemispheres were dehydrated and embedded into methacrylate for dentate granule cell number estimations.

Immunohistochemistry

The hippocampus and entorhinal cortex of each animal were separated into two blocks (anterior and posterior) and cryoprotected in 30% sucrose solution, frozen and preserved at -80^{o}C until processing. 16 series (the anterior blocks) or 24 series (the posterior blocks) of 40µm coronal sections were cut and preserved in cryoprotectant (25% glycerol, 25% ethylene glycol and 50% 0.1M phosphate buffer) at -20^{o}C until staining.

Complete series of free-floating sections from each animal were washed with Tris-Triton (TBS pH 7.4 with 0.05% Triton). Antigen retrieval was performed using 10% citric acid (DAKO) at 90^{o}C for 45 min (for Ki67) or by short microwaving (for DCX, 1 min). After washing in Tris-triton, the sections were incubated in 0.6% H_2O_2 in TBS with 0.1% Triton to block endogenous peroxidase. After further washes, the sections were incubated in appropriate blocking solution (2% serum, 0.2% Triton in TBS) for 1 hour. Primary antibodies for Ki67 (rabbit anti-Ki67, 1:1000; NCl-Ki67p #6013874, Novocastra) and DCX (goat anti-DCX, 1:250; SC-8066 #J0112, Santa Cruz) were diluted with blocking solution and applied overnight at 4^{o}C. After washing in TBS, the sections were immersed in biotinylated secondary antibodies (goat anti-rabbit, 1:300; rabbit anti-goat IgG, 1:300, Vector Laboratories) diluted in TBS with 2% serum. After washing in TBS, ABC solution (PK-6100, Vector Laboratories) and diaminobenzidine staining (D4418-50SET, Sigma) was applied following the manufacturer's instructions. The sections immunostained with DCX were counter-stained with hematoxylin (Fluka 51275). All sections were then dehydrated and mounted.

Granule cell number in dentate gyrus

Fixed hippocampal formations were washed in phosphate buffer saline and dehydrated in ascending concentration of ethanol for 5 days. The tissues were then infiltrated in 1:1 solution of hydroxyethylmethacrylate (HEMA, Technovit 7100, Heraeus Kulzer GmbH) and 100% ethanol for 24 hours, transferred into HEMA solutions and changed after 3 days and 1 week. 2 weeks after the last change, tissue embedding was performed following the manufacturer's instruction. The blocks were cut coronally at 20 µm and every 10th section was collected. Sections were mounted, dried and

stained in Giemsa solution (1.09204.0500, Merck) which was diluted 1:6 in 67 mmol KH_2PO_4 buffer. The stained sections were differentiated 10 sec in 1% acetic acid, dehydrated and mounted.

Quantitative analysis

Hippocampi were cut coronally from anterior to posterior along the septo-temporal axis. For quantitative assessment, the dentate gyrus was subdivided along the septo-temporal and the radial axis (from pia/obliterated hippocampal fissure to hilus)., The following criteria were used: in sections in which the dentate gyrus granule cell layer appeared twice, they were, according to their position, considered as septal (blue in Figure 3.1A) or temporal (orange in Figure 3.1A) hippocampus. The mid-septo-temporal hippocampus is represented by the posterior sections, in which the granule cell layer formed one continuous band (purple in Figure 3.1A). Along the radial axis, the region G-SGZ in this study is composed of the granular cell layer and the subgranular zone which is as two granule-cell-wide region below the granular cell layer. The region E-SGZ (extended SGZ) was defined as 1.5 times GCL wide zone below G-SGZ, extending into the hilus (Figure 3.1B).

Ki67 positive cells in G-SGZ (Figure 3.3 and 2.4B) and E-SGZ (Figure 3.3 and 3.4C) were counted exhaustively in every 16th (septal/temporal) or 24th (mid-septotemporal) section using a x40 oil-immersion lens (ZEISS EC Plan-NEOFLUAR 40x/1.3 oil, ∞/0.17). Cells observed in the top focal plane of the section were omitted from the counts. Counts were multiplied by 16 or 24 to estimate the total number of labelled cells.

DCX positive cells (Figure 3.5 and 3.6) were counted in the same regions as Ki67 using the optical fractionator (West, Slomianka et al. 1991). Every 16th or 24th section was counted using a counting frame of 30 x 30 µm, a dissector height of 5 µm. Disector probes were placed at 280 µm intervals along the x- and y-axis.

Total granule cell numbers were estimated in HEMA embedded sections using the optical fractionator. Every 10th section was analyzed using a counting frame of 15 x 15 µm, a dissector height of 10 µm and x- and y- steps of 280 µm.

The data visualization was performed using R (version 3.1.0) package ggplot2 (Wickham 2009).

Statistics

Statistical analyses were done using R (version 3.1.0). We performed analyses of variance (ANOVA) with tameness selection as factor on Ki67 positive cells, DCX positive cells and total granule cells. For comparison of the different anatomical regions of the hippocampus, two-way or three-way ANOVA (tame and unselected foxes as between group factor, and septo-temporal division/radial axis division

as the within-subject factors) were performed using the ez package in R (Lawrence 2013). Post hoc analysis using Tukey's HSD was also performed in R. Mauchly's test was used to test for equal variances of group differences. We did not find any violations of sphericity (Mauchly 1940). Simple linear regression was performed to analyze the relationship between total or regional DCX and Ki67 positive cells and aggression scores. For analysis of the fear reaction in relation to neurogenesis, logistic regression was performed due to the binary behavioral scorings. Coefficients of error (CE) that provide a measure of the precision of the cell number estimates were calculated as described previously (Gundersen, Jensen et al. 1999, Slomianka and West 2005).

3.3 Results

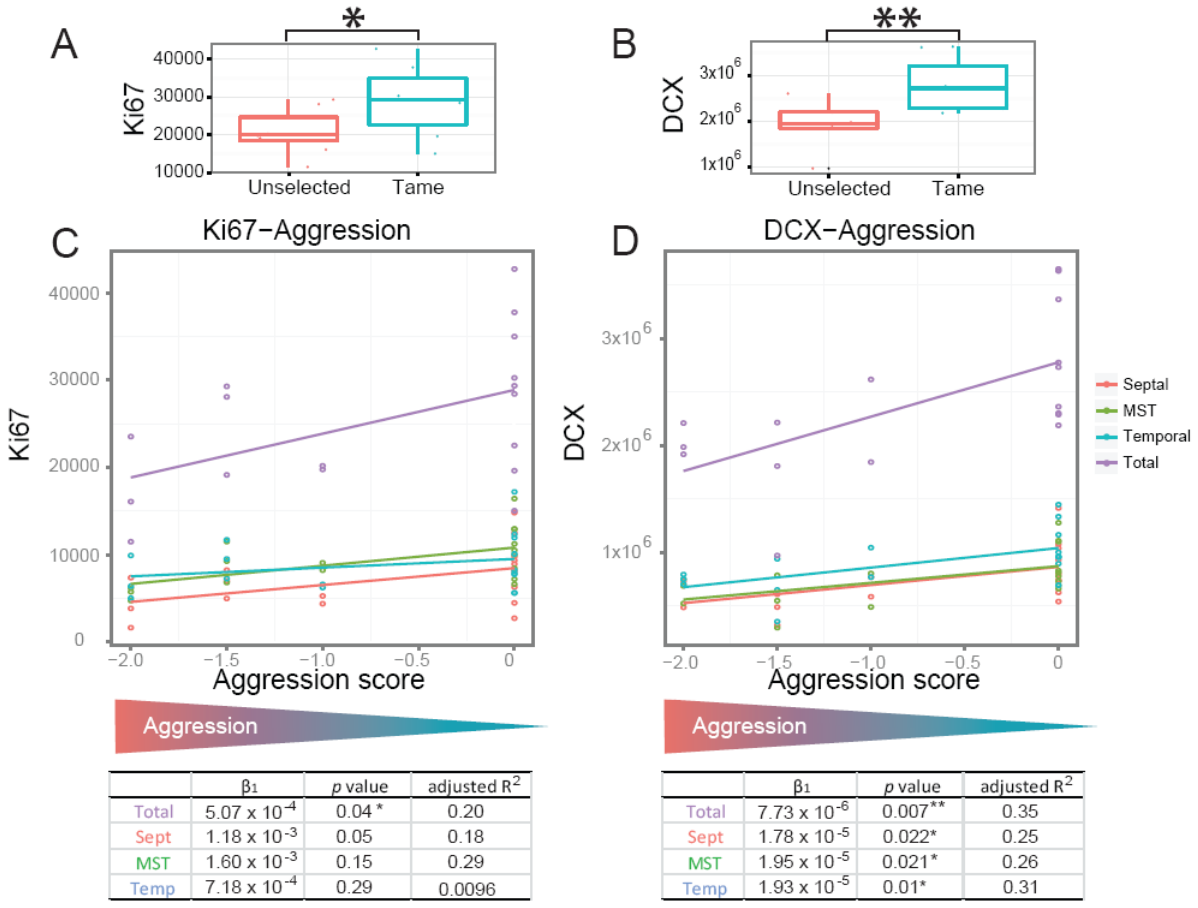
Table 3.1 Animal profile

	Body Weight (kg)	Brain weight (g)	Age (Days)	Total GCs	Total Ki67	Total DCX	Aggression score
Tame (n=9)	Mean: 4.51 SD: 0.30	Mean: 49.6 SD: 1.99	Mean: 224.89 SD: 6.37	Mean: $7.06x10^6$ SD: $1.40x10^6$ CE: 0.06±0.01	Mean: $2.89x10^4$ SD: $8.85x10^3$ CE: 0.07±0.02	Mean: $2.79x10^6$ SD: $5.78x10^5$ CE: 0.11±0.05	0
Un-selected (n=8)	Mean: 5.36 SD: 0.50	Mean: 48.52 SD: 4.29	Mean: 219.50 SD: 16.31	Mean: $6.56x10^6$ SD: $1.42x10^6$ CE: 0.07±0.01	Mean: $2.10x10^4$ SD: $5.91x10^3$ CE: 0.07±0.03	Mean: $1.95x10^6$ SD: $4.73x10^5$ CE: 0.13±0.06	1.5±0.5

AHN is increased in tame foxes and correlates negatively with aggression

Total numbers of the proliferating, Ki67 positive cells and DCX positive young neurons in the tame fox dentate gyrus were significantly higher than in the unselected fox dentate gyrus (Figure 3.3, 2.4A, B and Table 3.1, total Ki67: p=0.047, total DCX: p=0.0049). Linear regression revealed that the number of DCX positive cells had a significant negative relationship with aggressive behavior (for p values see Figure 3.2D), both overall and in all septo-temporal divisions. A negative relation between aggressive behavior and cell proliferation was observed overall, but not within divisions (Figure 3.2C). The regression of total DCX positive cells fit the linear model better than regional DCX positive cells and

Ki67 positive cells. In contrast to the aggressive behavior, the logistic regression for fear behavior did not show a relation to subdivisions or total DCX or Ki67 positive cell numbers (odds ratio for fear response with both global and regional Ki67 positive cells and DCX positive cells were all equal to 1.0.)



	β1	*p* value	adjusted R^2
Total	5.07×10^{-4}	0.04 *	0.20
Sept	1.18×10^{-3}	0.05	0.18
MST	1.60×10^{-3}	0.15	0.29
Temp	7.18×10^{-4}	0.29	0.0096

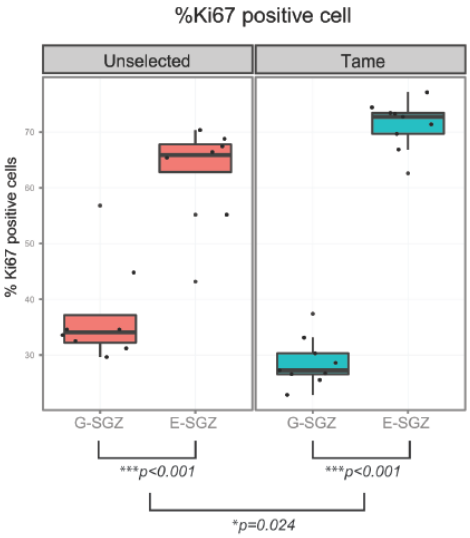
	β1	*p* value	adjusted R^2
Total	7.73×10^{-6}	0.007**	0.35
Sept	1.78×10^{-5}	0.022*	0.25
MST	1.95×10^{-5}	0.021*	0.26
Temp	1.93×10^{-5}	0.01*	0.31

Figure 3.2 Neurogenesis is up-regulated in the tame fox hippocampus and negatively correlated with aggressive behavior. Boxplot figures for total Ki67(A) and DCX (B) positive cells in tame and unselected foxes. Upper and lower hinges correspond to the first and the third quartiles and the upper/lower whisker extends from the hinge to the highest/lowest value that is within 1.5 inter-quartile ranges. Data beyond the ends of the whiskers are outliers (as specified by Tukey.) Significantly higher neurogenesis (both proliferation and new neuron numbers) could be observed in tame fox hippocampus. Ki67: *p=0.047, DCX: **p=0.0049. Area-specific Ki67 (C) and DCX (D) positive cell counts of the hippocampus in relation to aggression scores of farm-bred foxes. Red, green and blue circle and line represent septal, mid-septo-temporal (MST), and temporal hippocampus, respectively. The table below shows the slope coefficient estimates β1, p values and adjusted R squared of simple linear regression.

The increase in AHN in tame foxes changes along the septo-temporal and radial axis

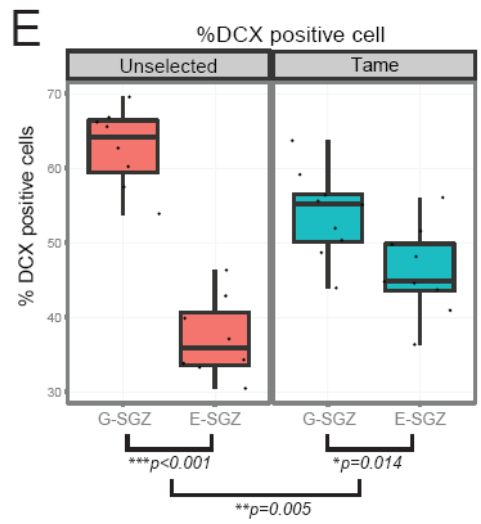
For Ki67 positive cells, results of a three-way ANOVA showed significant main effects for tameness selection ($F(1, 80)=4.67$, $p=0.047$), along the septo-temporal axis ($F(2, 80)=6.30$, $p = 0.005$) and also

radial axis (F(1, 80)=56.00, p<0.001). Interactions between tameness selection and radial axis (F(1, 80)=9.22, p = 0.008), septo-temporal axis and radial axis (F(2, 80)=10.56, p < 0.001) and all the three (F(2, 80)=3.45, p=0.045) were also found.



Unpublished Figure A Percentage of Ki67 positive cells along the radial axis. Common to both tame and unselected foxes was a large percentage of Ki67 positive cells in E-SGZ (tame: 71.28%, unselected: 62.77%, Figure 2.5). The percentage of Ki67 positive cells was significantly higher in the E-SGZ of tame foxes compared to unselected controls (p=0.024).

Most of the cells form clusters near blood vessels (Figure 3.3 A, arrowhead) which might correspond to the vascular niche for adult hippocampal neurogenesis in rodents (Palmer, Willhoite et al. 2000). Along the septo-temporal axis, the number of Ki67 positive cells distributes similarly in tame and unselected foxes in the G-SGZ (Unpublished Figure A and 3.3B). Along the radial axis however, post hoc comparison showed significantly higher numbers of proliferating cells in the E-SGZ of the mid-septo-temporal (MST, figure 3.3C) subdivision in tame foxes (p=0.01).



Unpublished Figure B Percentage of DCX positive cells along the radial axis. Within selection groups, the regional percentage of DCX positive cells indicated that more DCX positive cells could be observed in the E-SGZ in tame foxes' dentate gyrus compared to unselected controls (tame: 46.17%, unselected: 37.25%, p=0.0053).

For DCX positive cells, results of a three-way ANOVA showed significant main effects for tameness selection (F(1, 80)=10.83, p=0.005), septo-temporal axis (F(2, 80)=7.11, p = 0.003) and radial axis (F(1, 80)=27.89, p<0.001). A significant interaction between group and radial axis division was also found

(F(1, 80) = 4.87, p = 0.04). Compared to the proliferating cells, the distribution of young neurons (Figure 3.3D) along the radial axis was inverted: overall, more DCX-positive cells were observed in G-SGZ than E-SGZ (Unpublished Figure B).

Along the septo-temporal axis, post hoc comparison showed significantly higher numbers of DCX positive cells in the E-SGZ of tame animals in MST and temporal divisions of hippocampus (Figure 3.3F).

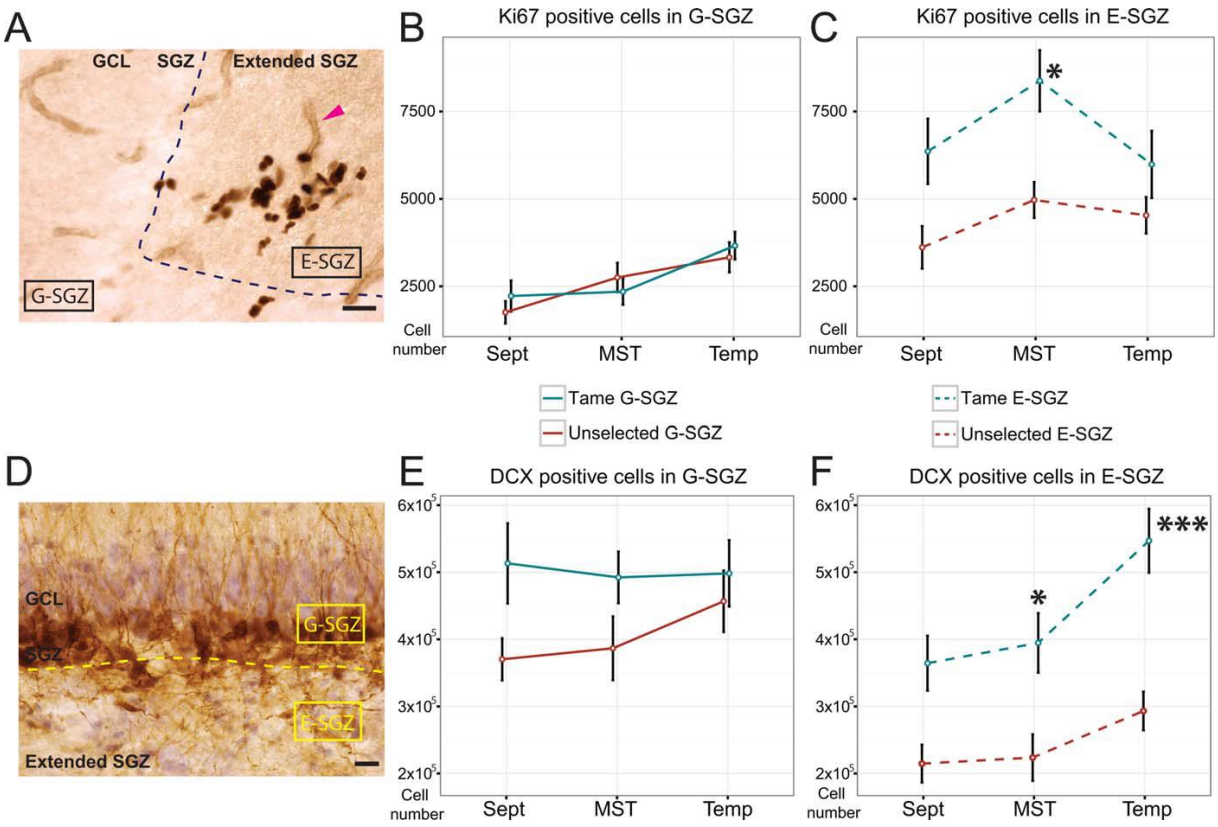


Figure 3.3 Robust increase of proliferating cells and young neurons in the tame fox extended subgranular zone.. (A) The immunohistochemistry staining for the proliferation marker Ki67. Cells were quantified in two regions: the granule cell layer plus sub-granular zone (G-SGZ) and extended sub-granular zone (E-SGZ). Arrow head: blood vessel. Distribution of the proliferating cells in G-SGZ (B) and E-SGZ (C). Significantly higher numbers of proliferation cells were observed in E-SGZ (C) of the septal mid-septo-temporal hippocampus of the tame fox. (D)The immunohistochemistry staining for the young neuron marker doublecortin (DCX, brown). Cells were quantified in two regions: G-SGZ and E-SGZ. Distribution of the young neurons in G-SGZ(E) and E-SGZ (F). Significantly higher numbers of young neurons were observed in E-SGZ (C) of the mid-septo-temporal (MST *p=0.04) and temporal (***p < 0.001) hippocampus of the tame fox. Bars: standard error of mean. Scale bars: 10µm.

Higher ratio of DCX /Ki67 positive cells in the septal hippocampus of all foxes

The ratio was calculated by dividing DCX positive cells by Ki67 positive cells. Results of the three-way ANOVA showed no significant difference between tame and unselected animals. Significant main effects were found along the septo-temporal axis ($F(2, 80)=6.60$, $p = 0.004$), the radial axis ($F(1, 80)=46.06$, $p<0.001$) and the interaction between the two axis ($F(2, 80)=10.10$, $p = <0.001$). The DCX/Ki67 ratio dropped along the septo-temporal axis in the G-SGZ in both groups (Figure 3.4A), post-hoc analysis indicated a higher DCX/Ki67 ratio in septal than temporal G-SGZ ($p<0.001$) in all foxes. The ratio was relatively stable in the E-SGZ with no differences along the axis (Figure 3.4B).

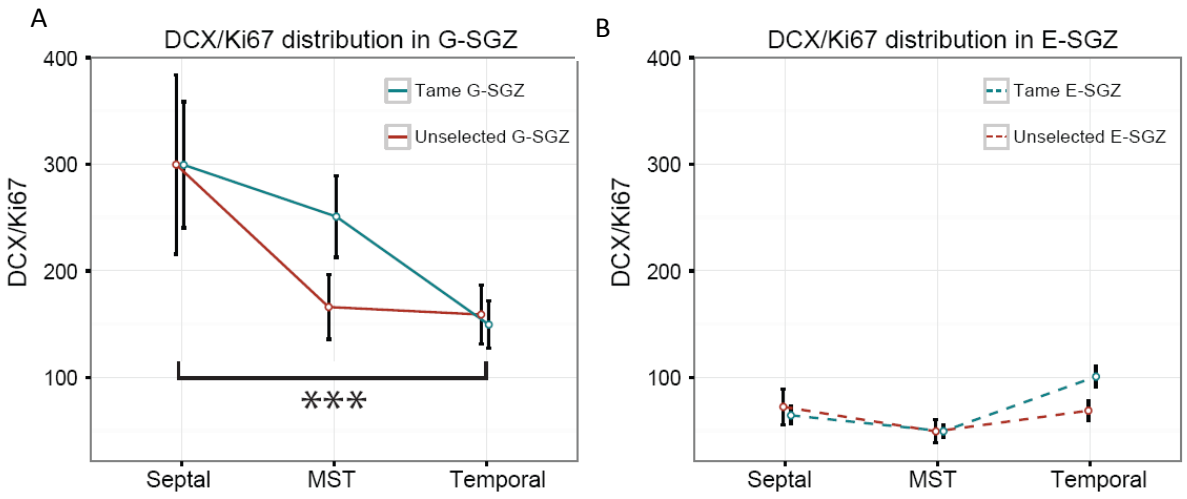


Figure 3.4 The regional relationship between DCX and Ki67 positive cells along the septo-temporal axis. The ratio of DCX/Ki67 in tame and unselected fox brains along the septo-temporal axis in G-SGZ (A) and E-SGZ (B). There is no significant difference between tame and unselected foxes. Higher DCX/Ki67 ratio in septal G-SGZ than temporal G-SGZ was observed (***$p < 0.001$). Bars: standard error of mean.

Morphology of the farm-bred silver fox dentate gyrus

Similar to an earlier red fox study (Amrein and Slomianka 2010), two distinct granule cells (large and small cells) could be observed in the silver fox dentate gyrus (Figure 3.5D) and were therefore quantified separately. Although fewer sections contained septal than temporal dentate gyrus (average number of analyzed sections, Sept: 13; Temp: 15), more granule cells could be observed in the septal hippocampus of silver foxes (both tame and unselected groups, Table 3.2). There was no significant difference in cell numbers (both large and small cells) between tame and unselected foxes in any of the three regions of the fox dentate gyrus (Figure 3.5C). Unlike the results in the red fox study (Amrein and Slomianka 2010), the majority of the granule cells were large cells. Total numbers of granule cells in farm-bred foxes were however considerably lower than in wild red foxes ($p<0.001$),

even though brain weight did not differ between tame, unselected (Table 3.1) and wild foxes (Tukey's pair wise comparison, wild-unselected: p = 0.29; tame-unselected: p = 0.74; tame-wild: p = 0.08).

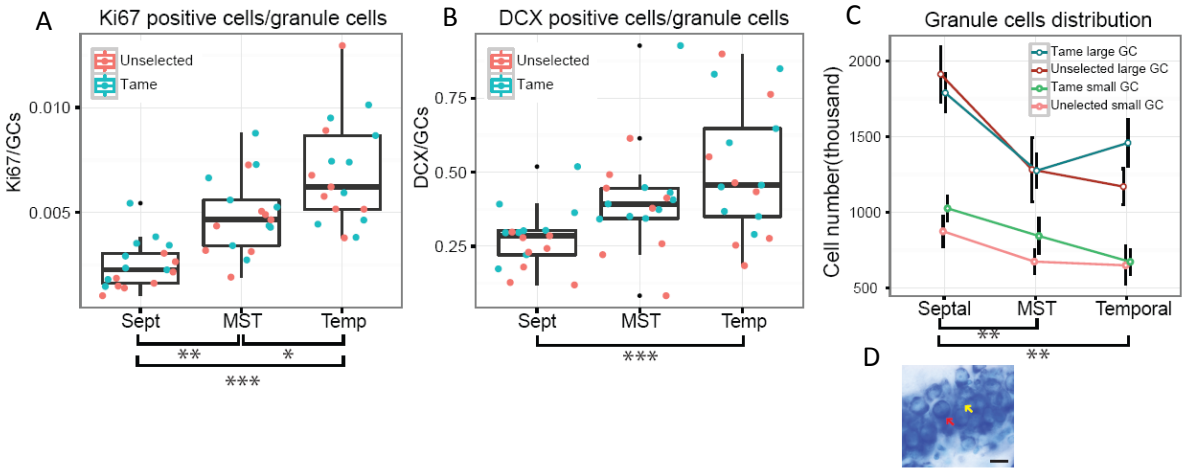


Figure 3.5 Strong longitudinal gradients in neurogenesis and granule cells. In relation to resident granular cells, the septal hippocampus has less neurogenesis, whereas there is a higher percentage of neurogenesis at the temporal hippocampus in both groups. (A) Boxplot figure for the Ki67 positive/granule cells ratio along the septo-temporal axis. Sept-MST: p = 0.002, MST-Temp: p = 0.01, Temp-Sept: p < 0.001.(B) Boxplot figure for the DCX positive/granule cells ratio along the septo-temporal axis. Temp-Sept: p < 0.001. (C) The quantification results indicate that there is no significant difference in small granule cells (small GC), large GC or total GC between the tame and the unselected foxes, but that there is a septo-temporal gradient in the number of total granule cells (Sept-MST: p = 0.003, Temp-Sept: p = 0.001). (D) In the fox dentate gyrus, two different granule cell types can be observed: small GC (yellow arrow) and large GC (red arrow). Bars: standard error of mean. Scale bar: 10µm.

General comparisons

Results were further compared with other mammalian species. The plot of normalized proliferating cells in relation to age showed that the percentage of proliferating cells declines similarly in foxes as in other species (Figure 3.6A, foxes: orange; green: sengi (Macroscelidea); primate: blue and rodent: purple). However, percentages of young neurons were far above all the other species in both wild and domesticated fox brains (Figure 3.6B, orange).

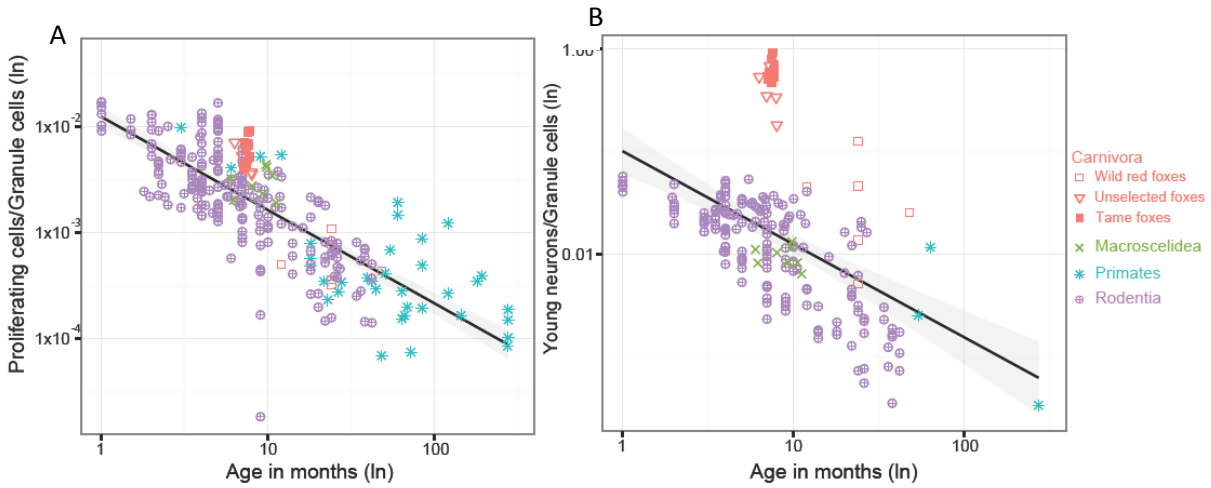


Figure 3.6 More young neurons in fox brains, but not proliferating cells. (A) Proliferating cells/granule cells ratio in relation to absolute age in the rodent, sengi, primate and fox hippocampus. Slope coefficient β1 = -0.77, multiple R squared = 0.62, $p < 0.001$, n=265. (B) The young neurons/granule cells ratio against age of fox is higher than all the other species. Slope coefficient β1 = -0.91, multiple R squared = 0.25, $p < 0.001$, n=189. Data of other species were from previous studies (Amrein and Slomianka 2010, Amrein, Isler et al. 2011, Slomianka, Drenth et al. 2013, Amrein, Becker et al. 2014).

Table 3.2 The quantification results

	Area	GCs (big)	GCs (Small)	Region	Ki67	DCX
Tame (n=9)	Sept	Mean:1.79×10^6 SD: 3.77×10^5 CE: 0.09± 0.01	Mean:1.03×10^6 SD: 2.40×10^5 CE: 0.12±0.02	I	Mean:2.22×10^3 SD: 1.33×10^3 CE: 0.15±0.07	Mean:5.13×10^5 SD: 1.79×10^5 CE: 0.12±0.03
				II	Mean:6.36×10^3 SD: 2.80×10^3 CE: 0.15±0.04	Mean:3.64×10^5 SD: 1.23×10^5 CE: 0.18±0.06
	MST	Mean:1.27×10^6 SD: 3.25×10^5 CE: 0.25±0.11	Mean:8.43×10^5 SD: 3.43×10^5 CE: 0.32±0.15	I	Mean:2.35×10^3 SD: 1.13×10^3 CE: 0.17±0.06	Mean:4.92×10^5 SD: 1.15×10^5 CE: 0.32±0.20
				II	Mean:8.38×10^3 SD: 2.62×10^3 CE: 0.14±0.05	Mean:3.95×10^5 SD: 1.33×10^5 CE: 0.37±0.21
	Temp	Mean:1.46×10^6	Mean:6.72×10^5	I	Mean:3.66×10^3	Mean:4.98×10^5

		SD: $4.65x10^5$ CE: 0.13±0.03	SD: $2.45x10^5$ CE: 0.19±0.03		SD: $1.18x10^3$ CE: 0.07±0.01	SD: $1.48x10^5$ CE: 0.17±0.06
				II	Mean:$5.98x10^3$ SD: $2.87x10^3$ CE: 0.07±0.02	Mean:$5.47x10^5$ SD: $1.43x10^5$ CE: 0.24±0.10
Un-Selected (n=8)	Sept	Mean:$1.91x10^6$ SD: $5.17x10^5$ CE: 0.10±0.01	Mean:$8.74x10^5$ SD: $2.78x10^5$ CE: 0.13±0.01	I	Mean:$1.76x10^3$ SD: $0.90x10^3$ CE: 0.19±0.10	Mean:$3.70x10^5$ SD: $8.88x10^4$ CE: 0.25±0.20
				II	Mean:$3.61x10^3$ SD: $1.71x10^3$ CE: 0.13±0.05	Mean:$2.15x10^5$ SD: $7.95x10^4$ CE: 0.33±0.25
	MST	Mean:$1.28x10^6$ SD: $5.60x10^5$ CE: 0.28±0.14	Mean:$6.73x10^5$ SD: $2.29x10^5$ CE: 0.37±0.19	I	Mean:$2.75x10^3$ SD: $1.18x10^3$ CE: 0.23±0.25	Mean:$3.87x10^5$ SD: $1.35x10^5$ CE: 0.27±0.15
				II	Mean:$4.97x10^3$ SD: $1.45x10^3$ CE: 0.14±0.03	Mean:$2.24x10^5$ SD: $9.81x10^4$ CE: 0.39±0.20
	Temp	Mean:$1.17x10^6$ SD: $3.33x10^5$ CE: 0.15± 0.02	Mean:$6.49x10^5$ SD: $3.57x10^5$ CE: 0.22±0.08	I	Mean:$3.33x10^3$ SD: $1.21x10^3$ CE: 0.07±0.01	Mean:$4.57x10^5$ SD: $1.29x10^5$ CE: 0.19±0.06
				II	Mean:$4.53x10^3$ SE:$5.19x10^2$ CE: 0.07±0.01	Mean:$2.93x10^5$ SD: $8.13x10^4$ CE: 0.30±0.15

3.4 Discussion

Genetic studies suggest that genes affecting brain and neuronal development have been particularly targeted during the domestication process (Carneiro, Rubin et al. 2014), The volume of the hippocampus is consistently reduced, often even more so than other brain regions, in highly domesticated mammals such as pigs and sheep (Kruska and Rohrs 1974, Kruska 1988, Price 2002) indicating that the hippocampus might play an important role in the convergent development of the tame behavior in domesticated animals. In this report, we demonstrated that domestication by selection for tameness in foxes is associated with an up-regulation of neurogenesis. The findings will be discussed below in relation to physiological and behavioral changes during domestication.

Domesticated foxes have lower glucocorticoid level and higher AHN

Domestication is considered to evolve through a sequence of processes that includes hunting, captivity-rearing of wild-derived animals, taming (or training/selecting for other targeted behaviors) and finally leading to domesticated animals (Clutton-Brock 1992, Clutton-Brock 1994, Price 2002). Three different degrees of taming were defined by Hemmer (Hemmer 1988): tameness by acquiring confidence in the presence of people (i.e. by hand-rearing young animals), tameness by reduction of distrust (i.e. taming adult animals) and genetically determined tameness. Hemmer suggests that only genetically acquired tameness indicates “real” domestication. Belyaev believed that the key trait that humans used for selection was not size or reproduction but behavior, i.e. tameness (Belyaev 1979, Belyaev, Plyusnina et al. 1985, Trut 1999). While AHN is negatively correlated with aggressive behavior, there is no relationship between AHN and defensive fear response to humans. Our findings indicate that offensive aggression and defensive fear response are differently regulated, and only the offensive aggression behavior is negatively correlated with AHN in foxes. Concomitant with changes in behavior, neurotransmitter systems were also altered in tame foxes. Higher 5-hydroxyindoleacetic acid (5-HIAA, a main metabolite of serotonin) levels were found in the hippocampus (Popova, Voitenko et al. 1991), and lower plasma cortisol and adrenocorticotropic hormone (ACTH) levels were observed as well (Gulevich, Oskina et al. 2004). Increased endogenous plasma glucocorticoids have been linked to the down-regulation of adult neurogenesis (David, Samuels et al. 2009, Anacker, Cattaneo et al. 2013). Wild house mice that have lower plasma corticosterone and dentate gyrus glucocorticoid receptor levels also have a higher baseline cell proliferation in the dentate gyrus (Veenema, De Kloet et al. 2007). Laboratory C57BL/6J mice which have a higher rate of AHN than DBA/2J (Kempermann and Gage 2002) also show a lower baseline level of plasma corticosterone than DBA/2J mice (Matthews, Morrow et al. 2008). In wild living animals, the baseline of glucocorticoids changes seasonally and has been linked with different life events such as feeding, migration, metabolism or reproduction (Landys, Ramenofsky et al. 2006). The plasma cortisol and

corticosterone increase during reproduction and lactation in free living chipmunks (Kenagy and Place 2000) and maternal experience have also been associated with down-regulation of AHN (Leuner, Mirescu et al. 2007, Cavegn, van Dijk et al. 2013). Even though glucocorticoids are known as a classic endocrine responders to stress (Munck, Guyre et al. 1984, Sapolsky, Romero et al. 2000), stress does not always decrease AHN (Lagace, Donovan et al. 2010, Eisch and Petrik 2012). Taken together, these findings suggest that the high level of AHN in the tame fox corresponds with low baseline levels of plasma cortisol and ACTH - whether as a consequence or a concomitantly regulated process has still to be addressed.

Septo-temporal gradients in fox AHN

Functional and connectional segregations or gradients along the longitudinal axis of the hippocampus are well documented in rodents and humans (Moser and Moser 1998, Fanselow and Dong 2010, Poppenk, Evensmoen et al. 2013, Strange, Witter et al. 2014) and may be similar in carnivores (Siegel, Edinger et al. 1974). E.g., the scale of spatial representation increased almost linearly from small (<1 meter) at the dorsal pole to large (~10 meters) at the ventral pole (Kjelstrup, Solstad et al. 2008). We observe a graded effect of tameness selection in that young neuron numbers increase along the septo-temporal axis. The effect is restricted to the E-SGZ, and might be due to a higher local survival rate, slower outbound migration of young neurons, or slower differentiation of the DCX positive cells in the temporal E-SGZ. It is tempting to think that in the tame foxes we observe a prolongation of the phase in which young neurons possess their unique properties (Ming and Song 2011) as has been shown in rats, in which the maturation of adult-born neurons is prolonged in the temporal compared to the septal dentate gyrus (Snyder, Ferrante et al. 2012). In the tame foxes, this might be regulated by genes that are targeted during domestication (Carneiro, Rubin et al. 2014) and that are expressed heterogeneously along the septo-temporal axis. For example, one of the domestication-targeted genes, Ephrin A5 (Carneiro et al., 2014), is expressed mainly in the ventral CA3 (Thompson, Pathak et al. 2008), and Ephrin A5-EphA5 receptor signaling pathway was found to regulate synaptogenesis in the hippocampus during development (Akaneya, Sohya et al. 2010).

In all foxes, neurogenesis shows a continuous increase along the septo-temporal axis, supporting an additional gradient in AHN (Strange, Witter et al. 2014). Both absolute and relative AHN is markedly higher in the temporal hippocampus of all foxes. This is in contrast to what has been found in adult rodents, were AHN in the septal (or dorsal) subdivision is higher or similar to the temporal hippocampus (Snyder, Radik et al. 2009, Jinno 2011, Jinno 2011, Snyder, Ramchand et al. 2011, Klomp, Václavů et al. 2014). The temporal hippocampus has been associated with odor memory, anxiety-related and social behavior (Sams-Dodd, Lipska et al. 1997, Kjelstrup, Tuvnes et al. 2002, Kesner, Hunsaker et al. 2011). Previous reports have indicated that neurogenesis in the temporal

hippocampus may contribute to the regulation of these emotional functions (Tanti, Rainer et al. 2012, Tanti and Belzung 2013).

Neurogenesis along the radial axis and behavioral neoteny

During the early postnatal stage of dentate gyrus formation, neurogenesis occurs in the early hilar neurogenic niche and along the dentate migration stream (Altman and Bayer 1990). In the adult rodent brain, the neurogenic niche is situated in close proximity to the granule cells and is composed of vasculature, astrocytes and microglia (Ming and Song 2011). In our study, most of the Ki67 positive cells and many DCX positive cells were found in the E-SGZ. This morphology is similar to that found in neonatal mice brains (Overstreet-Wadiche, Bensen et al. 2006), and is, to some extent, reflected in young (3 month-old) dogs (see figure 1F in (De Nevi, Marco-Salazar et al. 2013)). Even older foxes show a wider neurogenic area in the SGZ than that usually observed in rodents (see Figure 2e,f in (Amrein and Slomianka 2010)).

Accelerated physical maturation (especially sexual maturation) and the retention of juvenile behavioral characteristics (neoteny) are usually observed in domesticated animals (Price 2002). Similar observations were also made in farm-bred foxes. Tameness-selected farm-bred foxes exhibited developmental changes in physiological functions including earlier eye-opening, auditory response and delayed onset of the fear response, and exhibited cub-like behavior such as tail wagging, and seeking physical contact with humans (Trut 1999, Trut, Oskina et al. 2009). These juvenile behaviors might link with higher neurogenic activity in the E-SGZ. On the other hand, naked mole rats which reflect a higher degree of physiological neoteny such as closed eyes of infancy throughout life and hairless skins (Michel and Moore 1995) still have levels of AHN as low as other mole rat species (Amrein, Becker et al. 2014). Behavioral neoteny is regulated differently from physiological neoteny and might be associated with higher AHN in the fox hippocampus.

Heterogeneity in the dentate granule cells

A previous study revealed a heterogeneity of granule cells defined by different protein expressions in their axon terminals (Rekart and Routtenberg 2010). Also, distinct morphologies of granule cells in mice were reported, although the functional difference of the two populations remains unclear (Becker, Willems et al. 2012). Interestingly, even newly formed dentate granule cells can be divided into two functionally different groups (Brunner, Neubrandt et al. 2014). Different sizes of granule cells were also observed in the rodent (Perez-Delgado, Serrano-Aguilar et al. 1992), primate (Jabes, Lavenex et al. 2010) and wild red fox dentate gyrus (Amrein and Slomianka 2010). The percentage of large granule cells in farm-bred foxes exceeds that in wild red foxes. This difference may be due to

the extended period required by early postnatally formed granule cells to reach their adult size after an initial overshoot (Perez-Delgado, Serrano-Aguilar et al. 1992, Jabes, Lavenex et al. 2010).

In the fox brains, we observe a strong septo-temporal gradient in granule cells which is in accordance with the findings in rats, where the number of granule cells is relatively high septally and declines at the temporal dentate gyrus (Gaarskjaer 1978).

Species comparison

The silver fox is a color variant form (about 10 percent of the species) of *Vulpes vulpes*, which we see more regularly in its red form — red foxes. Wild red foxes reach adulthood at the age of six to seven months (Nowak 2005), and domesticated foxes reach sexual maturity about a month earlier than non-domesticated foxes (Trut 1999). Their potential natural longevity is around 12 years (Mulder 2004), though few individuals live more than 3-4 years in locations where the species is heavily hunted. Adult neurogenesis decreases with absolute age in both short- and long lived species (Amrein, Isler et al. 2011) and foxes do not differ from other mammals in terms of proliferation activity. However, young neurons of foxes (both wild red foxes and farm-bred silver foxes) are far above the average compared with other species. The farm-bred foxes therefore retain their species-specific neurogenesis pattern when compared to other mammals.

The red fox has the widest geographical distribution of any member of the order Carnivora covering nearly 70 million km², and has been recorded in habitats as diverse as tundra, desert and forest, as well as in city centers (Macdonald and Reynolds 2008). According to a study suggesting that AHN promotes the degradation of hippocampus-dependent memories in mammalian brains (Akers, Martinez-Canabal et al. 2014), high level of AHN in the fox brain might be associated with its ability to modify old memories, which is in turn linked to its behavioral plasticity to adapt to different environments. Taming foxes apparently increases the flexibility further, extending its ecological flexibility to man-made environmental conditions.

4. Project III

The amyloid precursor protein (APP) and adult neurogenesis

Abstract

It is well established that abnormal processing of the beta-amyloid precursor protein (APP), which generates beta-amyloid, and aging, plays an important role in the pathogenesis of Alzheimer's disease (AD). By contrast, loss of APP functions contributes to reductions in brain and body weight, grip strength deficits, alterations in circadian locomotor activity, exploratory activity, and the impairment of spatial learning and long-term potentiation. AHN was thought to contribute to learning, memory, and behavioral plasticity, and was known to be regulated by aging. Recently, increasing evidence suggests that molecular players in AD, including APP and its metabolites, play a role in adult neurogenesis. GABAergic signaling plays an important role during the neuronal maturation process of AHN. A previous study also showed that APP knockout (KO) in GABAergic neurons in 4- to 6-month-old mice can increase cell proliferation and impair neuron survival. Therefore, we hypothesize that the influence of APP deficiency in GABAergic neurons on AHN might be enhanced with aging, as aging is one of the main risk factors for AD.

APP and Amyloid precursor-like protein 1 and 2 (APLA1, APLP2) share many common features and overlapping functions. Especially, APP and APLP2 show a large expression overlap during development and in adulthood in many tissues with a particularly high expression in neurons. Thus, in this project, we studied APP cKO in GABAergic neurons on a APLP2 cKO background to eliminate the possible compensation from APLP2.

However, we were unable to find any difference in the number of new neurons between animals which lack APP and APLP2 in GABAergic inhibitory neurons, and their APLP2-/- background control. Although we cannot make a clear conclusion due to the lack of a wild type control, comparisons with previous C57/BL6 results have shown that the rate of AHN decline might be different in APLP2 -/- animals. Moreover, our results show that age was the only factor associated with ANH level even in 18- to 22-month-old mice, suggesting the importance of considering all the confounding variables in order to generate comparable and meaningful results across different studies.

4.1 Introduction

Amyloid precursor protein (APP), and amyloid precursor-like proteins 1 (APLP1) and 2 (APLP2) are type I transmembrane protein belonging to a small gene family. The three family members share several common features such as similar structural organization and overlapping functions (Müller, Deller et al. 2017). Among the three, APP and APLP2 show a large expression overlap during development and in adulthood in many tissues with a particularly high expression in neurons (Muller and Zheng 2012). In contrast to these, APLP1 is specifically expressed in neurons (Lorent, Overbergh et al. 1995).

The cleavage of APP within the extracellular domain by either α- or β- secretase leads to the release of soluble fragment of APPsα or APPsβ (collectively called APPs or sAPP fragments) (Müller, Deller et al. 2017) . Along the non-amyloidogenic pathway, α-secretase processed APP in the amyloid-β (Aβ) region, release APPsα, generate p3 and APP intracellular domain (AICD) (Müller, Deller et al. 2017). Along the amyloidogenic pathway, APP is processed through both β- secretase and γ-secretase cleavage and releases neurotoxic Aβ peptide and AICD (Müller, Deller et al. 2017).

Various studies have shown that APP and other proteins related to APP-processing pathway influence adult neurogenesis. APP and APPsα under non-pathological conditions are potent factors in increasing neuronal precursor cell (NPC) proliferation embryonically, and, in adults, shows neuroprotective functions and possibly plays an important role in cell lineage commitment (Reviewed by Lazarov and Demars 2012). Decreased proliferation and cell survival was found in the dentate gyrus of adult mice co-expressing ACID fragment and its binding partner Fe65. (Ghosal, Stathopoulos et al. 2010). Overexpressing APP and/or presenilin-1(PS1, part of γ-secretase complex) can reduce cell proliferation and differentiation in the hippocampus of 2-month old mice (Verret, Jankowsky et al. 2007)

APP is highly expressed in the majority of hippocampal gamma-aminobutyric acid (GABA)-ergic interneurons (Wang, Wang et al. 2014). It has been well established that $GABA_A$ receptor-mediated signaling plays an important role in mediating the regulation of adult neurogenesis (Dieni, Chancey et al. 2013). The function for GABA signaling during neurogenesis is initially an early trophic factor. Following the progressive stages of granule cell maturation, its function shifts from excitation to inhibition (Ming and Song 2011, Dieni, Chancey et al. 2013). In the dentate gyrus, a subpopulation of GABAergic interneurons express parvalbumin (PV) (Milenkovic, Vasiljevic et al. 2013). Although the percentage of GAD67 labeled GABAergic interneurons that express PV in the dentate gyrus is relatively low compared with those in CA1 and CA3 in mice (Jinno and Kosaka 2002), among the PV+

GABAergic interneurons have been shown to regulate stem cell quiescence via $GABA_A$ receptor-mediated tonic GABA signaling (Song, Zhong et al. 2012). Optogenetic activation of PV+ expressing cells, but not somatostatin or vasoactive intestinal peptide expressing cells, can dictate the radial glia choice between quiescence or activation (Song, Zhong et al. 2012). Other than the PV+ interneurons, some GABAergic interneurons also express neuropeptide Y (NPY) which is found primarily in GABAergic neurons in the rat hippocampus (Freund and Buzsáki 1996). It has been reported that NPY increases cell proliferation in post-natal mouse hippocampus (Howell, Scharfman et al. 2003). The interneuron population containing SOM/NPY was preferentially affected in APP/ Presenilin-1 (PS1) transgenic mice model (Ramos, Baglietto-Vargas et al. 2006). Loss of GABA is a well-demonstrated change in AD and may be related to behavioral symptoms or dementia (Lanctot, Herrmann et al. 2007), and selective deletion of GABAergic APP disrupts adult hippocampal neurogenesis in 16-20 week-old mice (Wang, Wang et al. 2014).

The major risk factor for AD is age, with a sharp increase in incidence in people over 60 years old (Kawas, Gray et al. 2000). Earlier study also indicated functional defects in neurotransmission and in long-lasting synaptic plasticity in aged, APP depleted mice but not in young ones (Ring, Weyer et al. 2007). Thus, we expect the consequences of a lack of APP/APLP2 to be intensified in aged animals.

In this study, we analyzed both adult hippocampal neurogenesis and GABAergic interneurons in 18-22 month-old mice with conditional APP KO on an APLP2 cKO background to circumvent the early postnatal lethality of combined constitutive mutants (Heber, Herms et al. 2000). Neurogenesis level was accessed by cell proliferation marker Ki67 and young neuron marker doublecortin (DCX). For GABAergic interneurons, we focus on PV and NPY expressing cells in the dentate molecular layer, granule cell layer and hilus. All the cell quantification was performed using stereological methods (West, Slomianka et al. 1991) .

4.2 Materials and Methods

Animals

The mice used in this study were 18-22 months old double KO (APP/APLP2 cdKO) Dlx5/6/Cre;ßAPPlx/lx;APLP2-/-, that is a Dlx-Cre-driven conditional APP KO specific for GABAergic neurons on a APLP2 cKO background. APLP2 cKO animals thus constitutively lack APLP2 (ßAPPlx/lx;APLP2-/-). Genetic background strains are C57BL/6 for the Dlx5/6/Cre, and C57BL/6J x 129P2/Ola for ßAPPlx/lx;APLP2-/-. 14 males (8 APP/APLP2 cdKO; 6 APLP2 cdKO) and 22 females (10 APP/APLP2 cdKO; 12 APLP2 cKO) were analyzed.

Animals were deeply anesthetized with pentobarbital (50 mg/kg), weighed and perfused transcardially using phosphate buffer saline (PBS, pH 7.4) followed by cold 4% paraformaldehyde solution in PBS with 15% picric acid (PFA-PA). The brains were removed, weighed, separated into hemispheres and post-fixed overnight.

Histology and Immunohistochemistry

The right hemispheres were transferred to 20% Glycerol 0.1M PBS pH7.2 for cryoprotection and subsequently embedded in a gelatin matrix (Smiley and Bleiwas 2012). The embedded tissues were placed into cryoprotectant (20% glycerol in PBS) over night at 4^{o}C. After cryoprotection, the embedded hemispheres were frozen using dry ice and cut coronally into 8 series of 40 µm thick sections. Sections were collected in cryoprotectant (25% glycerol, 25% ethylene glycol and 50% 0.1M phosphate buffer) and stored at -20°C until further processing.

Sections were first heated to 80°C in citrate buffer (Target Retrieval Solution, DAKO; 1:10, pH 6.0) for 40 min, washed with Tris-Triton (TBS pH 7.4 with 0.05% Triton) and incubated in blocking buffer (1% normal serum with 0.25% Triton in TBS) for 1 hour at room temperature. The sections were then incubated overnight at 4°C with primary antibodies against Ki67 (polyclonal Mouse anti Ki67, BD Pharmingen, IG754441E, 1:300), DCX (polyclonal Goat anti Doublecortin, Santa Cruz H1804, 1:250), neuropeptide Y (rabbit anti-Neuropeptide Y, Sigma Aldrich N9528, 1:8000) or parvalbumin (Mouse anti parvalbumin, Sigma Aldrich 100M4797, 1:10000). Incubation in secondary antibody (1:300) and ABC solution (Vectastain Elite Kits, Vector Laboratories, Burlingame, CA, USA) followed the manufacturer's instructions. Finally, sections were diaminobenzidine-stained, dehydrated and mounted.

Quantitative Procedures

Immunopositive cells were counted exhaustively in every 4th (Ki67) or 8th (DCX, PV and NYP) section using a x40 oil-immersion lens (ZEISS EC Plan-NEOFLUAR 40x/1.3 oil) along the entire septotemporal axis of the hippocampal formation. On average, in each animal 17 sections were analyzed in the Ki67-stained series, and 9 sections in the DCX-, PV- or NPY-stained series. Cells observed in the top focal plane of the section were omitted from the counts. Counts were multiplied by 4 or 8 to estimate the total number of labelled cells. Parvalbumin and NPY positive cell were quantified in three separate regions: hilus, subgranular zone/granular zone, and molecular layer. Ki67 and DCX positive cells were assessed in the subgranular layer of the dentate gyrus.

Statistics

Statistical analyses were done using R (version 3.2.1). We performed multiple linear regressions between Ki67-, DCX-, NPY- and PV-positive cells and explanatory variables including age, sex, and genotypes. Data visualization were performed using R (version 3.2.1) package ggplot2 (Wickham 2009).

4.3 Results

No differences in neurogenesis between APP/APLP2 cdKO and APLP2 cKO mice

We compared total numbers of the proliferating cells (figure 4.1A, Ki67 positive cells) and young neurons (figure 4.1B, DCX positive cells) and we found no differences between double knockout mice APP/APLP2 cdKO and APLP2 cKO mice (figure 4.1C and D, DCX: p = 0.34; Ki67: p= 0.41).

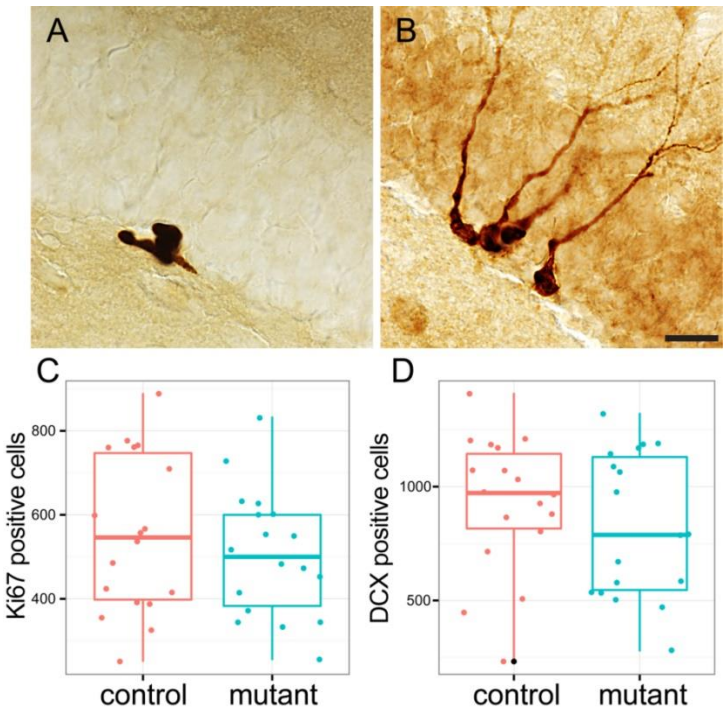


Figure 4.1 GABAergic APP KO does not change cell proliferation and young neuron number in mice. Immunohistochemistry and stereological quantification results of the proliferating cell marker Ki67 (A) and young neuron marker DCX (B) showed there is no significant difference between the mutant (APP/APLP2 cdKO) and control (APLP2 cKO) mice (C and D). Scale bar: 10 µm

Young neuron numbers and survival rates still decline with age in old mice

Results of the multiple linear regression indicated that young neuron numbers are negatively associated with age (figure 4.2A, *P < 0.0001*, $R^2 = 0.5141$), corresponding to a decline of 17% within 4 months in 18-22 month-old mice. There was no difference between male and female mice. Furthermore, the DCX/Ki67 ratio was also negatively associated with age (figure 4.2B, p = 0.001, $R^2 = 0.3531$). On the other hand, the association between age and Ki67 positive cell numbers was not significant (*p* = 0.4; $R^2 = 0.05$).

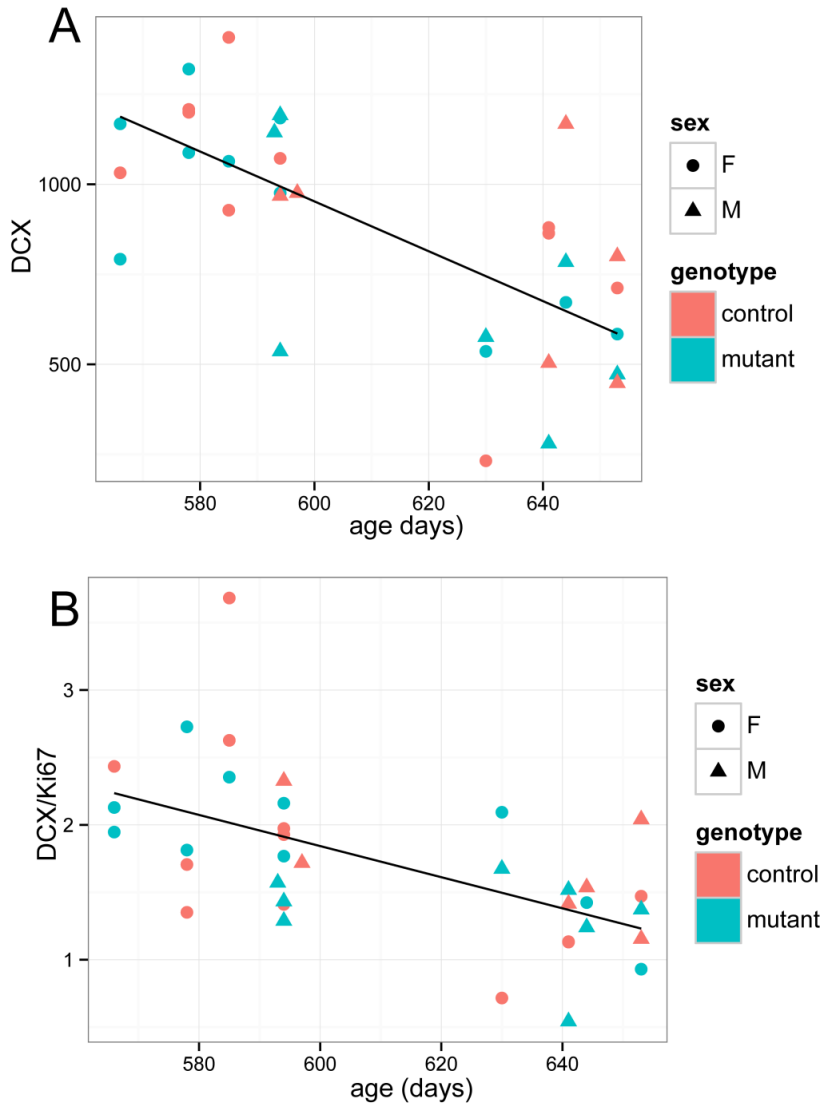


Figure 4.2 DCX+ young neuron numbers and DCX/Ki67 ratio decline with age in old mice. Across all animals, we found both DCX + cells (A, p =2.34x 10^{-5}) and DCX/Ki67 (B, p = 0.001) still decline with age. There is no difference between mutants (APP/APLP2 cdKO) and controls (APLP2 cKO), males and females.

If the results were compared with previous data of avarage DCX+ and Ki67+ cells in C57BL/6 (blue line), both APLP2 cKO and APP/APLP2 cdKO show higher proliferating cell numbers (Figure 4.3).

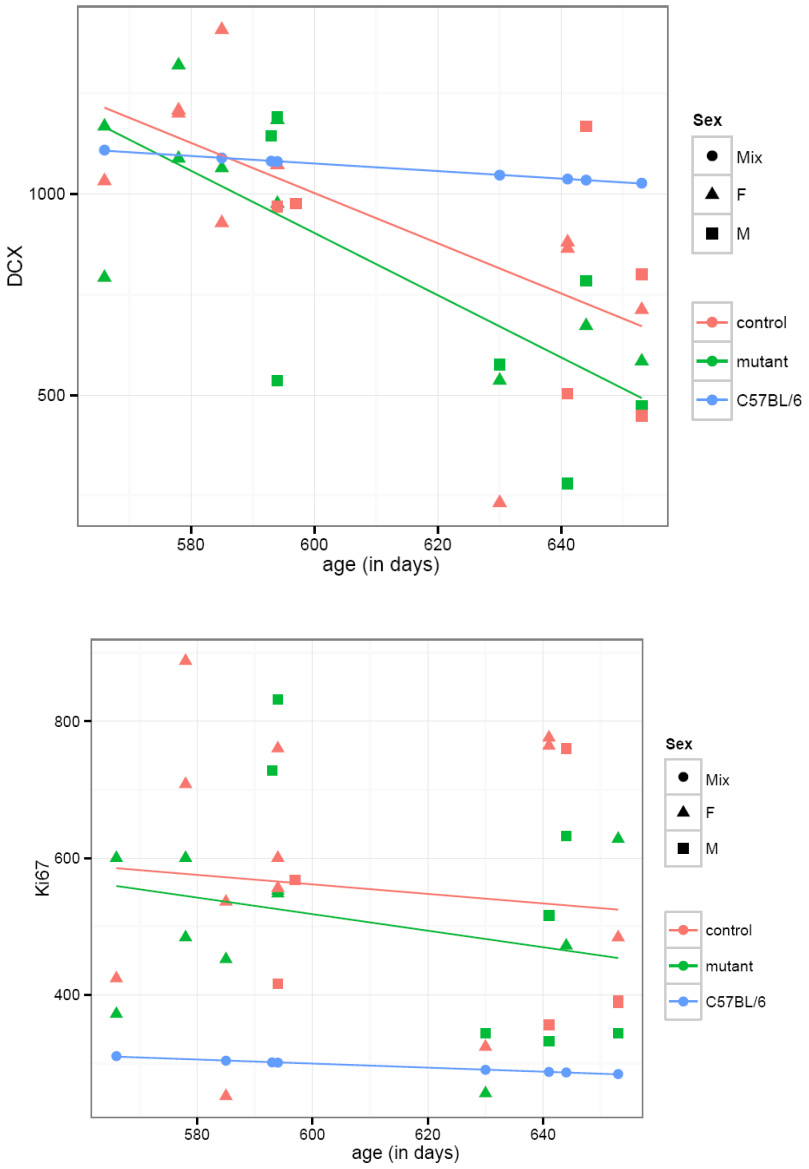


Figure 4.3 Cell proliferation level may be higher in APLP2 cKO (control) mice and APP/APLP2 cdKO (mutant) mice. AHN is known decline exponentially with age (Ben Abdallah, Slomianka et al. 2010, Amrein, Isler et al. 2011). Therefore we use data of C57BL/6 and using linear regression to predict AHN level into the same age range as the animals in this experiment. Mix: mix data from male and female. M: Male; F: Female.

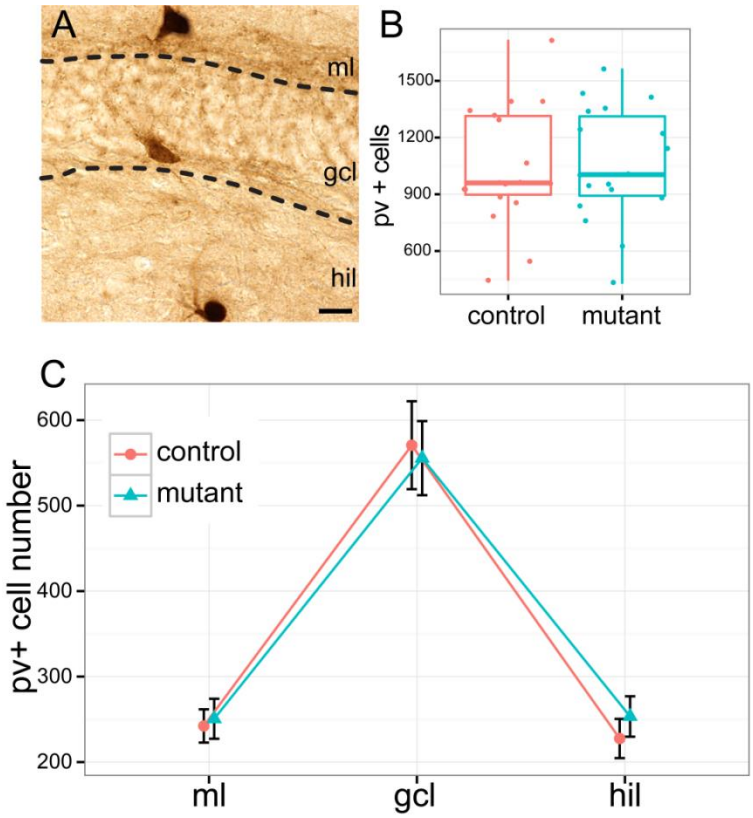


Figure 4.4 No difference of PV positive cells between APP/APLP2 cdKO and control APLP2 cKO mice (A)We quantified PV+ in hilus (hil), granule cell layer (gcl) and molecular layer (ml) in dentate gyrus and we found there is no difference of PV+ in terms of total cell number (B) and also regional cell numbers (C) between the mutant (**APP/APLP2 cdKO**) and control (APLP2 cKO) mice. Scale bar: 10 µm

No differences in parvalbumin and neuropeptide Y positive cell numbers between APP/APLP2 cdKO and APLP2 cKO mice

Cell bodies of interneurons in the different layers of the dentate gyrus are not evenly distributed. E.g., PV+ interneurons are located primarily near the base of the granule cell layer or located near the junction of the granule cell to molecular layers, and granule cell layer to hilus (Houser 2007). As a consequence, we assessed the PV+ and NPY+ interneurons separately in the three regions: molecular layer, granule cell layer and hilus.

Quantitative results of both PV+ and NPY+ cells showed that there was no significant difference between APP/APLP2 cdKO and APLP2 cKO mice (figure 4.4B, 4.5A). With respect to the three regions (figure 4.4A), we found most of the PV+ cells in the granule cell layer (figure 4.4C), whereas most of the NPY+ cells are in the hilus (figure 4.5B). Again, there was no significant difference between APLP2 cKO and APP/APLP2 cdKO mice in any region (figure 4.4C, 4.5B). There were also no differences in both PV+ and NPY+ cells with respect to age and sex.

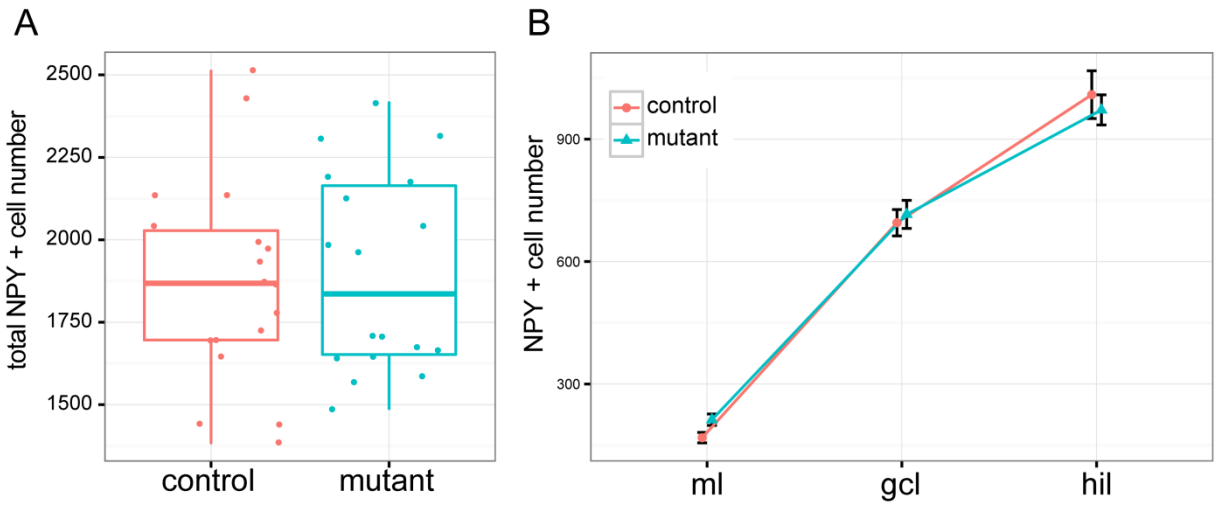


Figure 4.5 No difference of NPY positive cells between mutant (APP/APLP2 cdKO) and control (APLP2 cKO) mice. NPY+ in hilus (hil), granule cell layer (gcl) and molecular layer (ml) in dentate gyrus were quantified and we found again there is no difference of NPY+ cells in terms of total cell number (A). Most NPY+ cells were found in hilus (hil:52.5%; gcl:37.5%; mol: 10%)

Young neuron numbers are negatively associated with parvalbumin positive cell numbers in molecular layer

We added the PV+ cell numbers in each area into the multiple linear regression model and we found that DCX positive young neurons were negatively correlated with PV+ cells in the molecular layer (figure 4.6, $p = 0.048$; $R^2 = 0.57$), but not with the PV+ cells in the dentate gyrus or hilus. No such correlation could be found for NPY+ cells, e.g. no global or regional significant correlation with DCX+ or Ki67+ cell or DCX/Ki67 ratio was observed.

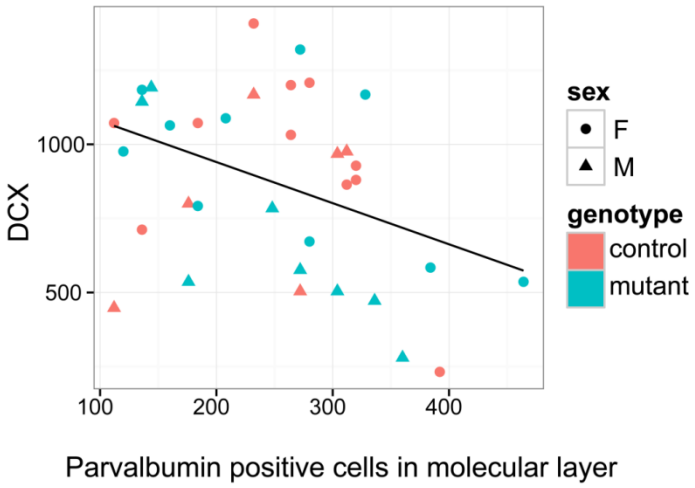


Figure 4.6 DCX+ young neuron numbers are negatively associated with PV+ cells in dentate molecular layer. When we included PV+ cell number into analysis, in addition to age effect ($p = 0.00015$), we also observed a negative association between DCX+ cell numbers and PV+ cell numbers in molecular layer ($p = 0.048$). M: Male; F: Female.

4.4 Discussion

Summary

Our results demonstrated that the depletion of APP specifically in GABAergic neurons on a constitutive APLP2 cKO background did not influence the number of proliferating cells or young neurons in aged (18-22 month-old) mice further. It also did not influence the number or spatial distribution of PV and NPY positive interneurons in the dentate gyrus. A correlation between PV positive cells in the molecular layer and young neuron numbers was observed. In addition, our results indicated that young neuron numbers continuously decreased while ageing even in 18-22 month-old mice.

Effect of genotype on neurogenesis

Neurogenesis

Our results showed there is no difference in cell proliferation and young neuron numbers between APLP2 cKO and the APP/APLP2 cdKO.

The NPY positive cells

In the rat dentate gyrus, hilar NPY positive cells account for the majority (60-70%) of NPY-positive cells, with stratum moleculare containing the smallest (7%) proportion (Deller and Leranth 1990, Freund and Buzsáki 1996). The distribution is very similar to the relations reported in mice, where NPY+ cells in the hilus (68%) exceed by far those in the granule cell layer (22%) or molecular layer

(10%) (Jinno and Kosaka 2006). Unfortunately, numbers of NPY+ cells in the latter study and presented here can not be compared directly due to methodological differences. The relative distribution of NPY+ cells indicate that in the cDKO and cAPLPL2 dentate gyrus NPY+ cells in the hilus are reduced (52%), whereas cells in the molecular layer are maintained (10%). It has been shown that interneurons co-expressing somatostatin and NPY are the most damaged subpopulation in the hippocampus of APP/PS1 tg mice (Ramos, Baglietto-Vargas et al. 2006). We did not see differences in NPY + cells between APP/APLP2 cdKO and APLP2 cKO mice here, which suggests that APP KO on a APLP2 background does not impair the population of NPY+ cells in the dentate gyrus further. Whether the APLP2 cKO itself is responsible for the relative reduction in NPY+ cells in the hilus still needs to be clarified.

Vulnerability of Parvalbumine positive cells in AD patients might not be associated with APP expressed in GABAergic neurons

Reduced numbers of PV+ interneurons in the dentate gyrus were linked to various diseases. In human epileptic dentate gyrus, the number of PV+ cells is reduced compared with healthy controls (Wittner, Maglóczky et al. 2001) and many studies have shown that patients with AD are at increased risk for developing epilepsy (Pandis and Scarmeas 2012). In AD patients' brains, a 60% decrease in the number of PV+ interneurons in the dentate gyrus/CA4 and CA1-CA2 subfields has been reported (Brady and Mufson 1997). In the AD related mouse model investigated here, we did not see quantitative or region specific differences in PV+ interneurons between the APP/APLP2 cdKO and APLP2 cKO. Our results are similar to a recent study in TgCRND8 mice (Swedish KM670/671NL and Indiana V717F mutations in the Aβ precursor encoding gene) reporting PV expressing neurons are the least vulnerable to Aβ accumulation compared with other GABAergic neurons (Albuquerque, Mahar et al. 2015). In addition, another study performed in 10-month-old APP/PS1 mice (mice that carry M233T/L235P knocked-in mutations in presenilin-1(PS1) and overexpress a mutated human beta-amyloid precursor protein (APP)), showed also no change of PV+ neurons in dentate gyrus (Takahashi, Brasnjevic et al. 2010). These results suggest that PV+ interneurons are not vulnerable to either overexpression of APP/PS1 or lack of APP on a APLP2 KO background in mice. An interesting observation however arises if the proportion of PV+ cells within the dentate layers are compared between our mutant mice and C57BL/6. Although the vast majority of PV+ cells can still be found in the granular cell layer as shown before (Jinno and Kosaka 2006), the proportions are similarly reduced as with NPY. A considerable proportion of PV+ cells could be seen in the dentate gyrus molecular layer in our mice (24%), which is strikingly more than what has been reported in rats (Kosaka, Katsumaru et al. 1987). Whether the reduction of the PV+ and NPY+ cells in the most

prominent strata of our mutants is due to ageing as shown for PV+ interneurons in rats (Shetty and Turner 1998) or to genotype has to be investigated further.

Parvalbumine positive cells and adult hippocampal neurogenesis

The PV+ interneurons in the hippocampus have been shown to be critical for spatial representations and working memory (Korotkova, Fuchs et al. 2010) and maintaining a normal level of adult hippocampal neurogenesis (Song, Sun et al. 2013). Quantitative data presented here do not indicate that neurogenesis and overall PV+ cells are correlated. Surprisingly, however, we found a negative correlation between DCX+ cells and PV+ cells exclusively in the dentate molecular layer. Not only are the PV+ cells in the molecular layer more numerous than expected, many GABAergic interneurons in the outer molecular layer project from the dentate gyrus to the subiculum across the hippocampal fissure and may participate in an oscillating cross-regional interneuron network (Ceranik, Bender et al. 1997). Ivn/neurogliaform cells, a special population of interneurons in the dentate molecular layer which can not be classified using a single marker mediate an activity–dependent regulation of neurogenesis and coordinate the activation of newborn and granule cells (Armstrong, Szabadics et al. 2011, Markwardt, Dieni et al. 2011). It has also been proposed that the precise properties of GABAergic interneuron signaling to immature granule cells have important consequences for determining their role in network activity (Marin-Burgin, Mongiat et al. 2012, Dieni, Chancey et al. 2013). GABA input from PV+ neurons can thus regulate cell survival during neurogenesis (Song, Sun et al. 2013). Reduced inhibition of GABA input on dentate granule cells can cause temporal lobe epilepsy (Kobayashi and Buckmaster 2003) and it is known that epileptic seizures increase AHN (Parent, Valentin et al. 2002, Jessberger and Parent 2011).

On the other hand, we cannot eliminate the possibility that the regulation or the functional link of AHN is activity-dependent rather than cell number-dependent. Electrophysiology experiments have shown that AHN is modulated by PV positive GABAergic neurons (Song, Sun et al. 2013). However, our results do not show correlation between cell numbers of AHN markers and PV+ cells in the subgranular zone, where it is known that new neurons are continuously generated. The only correlation in cell numbers was found between numbers of young neuron and PV+ cells in dentate molecular layer. If this regulation by PV+ cells is activity dependent, the output of newly generated granule cells within the wiring circuit in hippocampus could also be independent from cell numbers. Also, interneurons expressed certain molecular markers, such as PV, that do not always exhibit similar morphological and connectivity patterns in mice neocortex (Jiang, Shen et al. 2015). Mixed cell populations within the PV+ interneurons may also be a reason that the results showed no correlation between SGZ / total PV positive cells and AHN.

In conclusion, our results provided no evidence that elimination of APP in GABAergic interneurons on a constitutive APLP2-null background alters AHN, or the number and distribution of PV+ and NPY+ interneurons. This might be further associated with unchanged AHN because PV+ and NPY+ cells were shown as an important regulator of AHN (Howell, Scharfman et al. 2003, Howell, Doyle et al. 2005, Song, Sun et al. 2013). The comparison between the results in both APP/APLP2 cdKO and APLP2 cKO to predicted Ki67 or DCX positive cell number in C57BL/6 indicates that APLP2 cKO alone may impact the AHN level and APP was not able to conpensate the impact of APLP2 in aged mice. However a wild type control is needed in order to draw a conclusion. Finally, our results show that age was the only factor associated with the number of new born neurons, which suggests the importance of taking age into account as a confounding factor when studying AHN in mice not born on the same day.

5. Discussion

AHN in the hippocampal circuitry (Project I)

For a long time, many people have often referred to modification of synaptic strength when discussing neuronal plasticity. In the hippocampus, it has been well established that synapse modification plays an important role during the formation of new memories (Bailey and Kandel 1993). However, if the modification of synaptic strength is referred to as functional plasticity in the mammalian brain, why do our brains need to replace a whole cell with a new one?

A recent hypothesis has pointed out that mathematically speaking, learning and memory cannot be only associated with synapse plasticity (Trettenbrein 2016). Based on previous observations, the signals computed by neurons are rather stereotyped and reflect only the most elementary properties of the stimulus. Therefore, it is difficult to assume that this signal alone can carry the rich variety of information needed for complex behavior (Kandel, Barres et al. 2013). A mouse model lacking NMDA receptor in DG and dorsal CA1 has shown intact performance in a water maze task (Bannerman, Bus et al. 2012). This result also challenged the long-believed theory that NMDAR-dependent synaptic plasticity, particularly LTP, is the unit of coding associative memory. It seems feasible to assume much more information is processed inside the neurons, and that the relationship between numbers of neurons may represent neuronal plasticity and neuronal circuitry better than LTP. This may also explain why our brains need to replace a whole cell in the hippocampus with a new one. New cells may be a unit to represent a certain specific function. Moreover, this idea could also explain the observation in convergence/divergence of three mole rat species in Project I. The “in theory” rapid deterioration of information transfer between CA3 and CA1 might be compensated by intracellular mechanisms.

If a cell, not a synapse, is a unit of forming new memory, or is linked to a certain hippocampal function, the numerical relationship between newly generated cells and principal cell numbers in the hippocampus might be a good indicator to represent the functional dynamics of the hippocampus. Indeed, the results of correspondence analysis indicate that the numeric relationship between the principal cell population follows the phylogeny. When AHN is factored in, the picture that emerges of hippocampal principal cells (i.e. DG, CA1, CA3, hilus and subiculum) is completely different than the picture that emerges when we consider hippocampal principal cells alone. Despite the relatively small population, proliferating cells and new-born neurons together with the hilus became the best separating factors between rodents. The proliferating cells and new-born neurons separate the species independently, indicating that the AHN regulating strategies in cell proliferation and differentiation of new-born neurons have great species variation. Similar results were also found in

project II. AHN in both the red fox and silver fox seem to have prolonged cell differentiation stages compared to primates and rodents. Compared to embryonic neurogenesis or neurogenesis in the olfactory bulb, neuronal maturation in AHN is a very slow process (Zhao, Deng et al. 2008, Ming and Song 2011). Although the physiological significance of the prolonged differentiation/maturation process is still unclear, the pace of cell maturation process seems important. An increase in the pace of cell maturation sometimes leads to aberrant integration of newborn neurons in the adult hippocampus (Ming and Song 2011). Taken together, the findings in this thesis suggest that regulation of AHN is a key trait to differentiate species and shows great potential of incorporating AHN regulation, ecology and behavior into future cross-species comparison study.

Link of AHN to tame behavior and domestication – in non-rodent species

Previous studies suggest that voluntary running, which is one of the most broadly used positive regulators of AHN, can increase AHN in laboratory mice (van Praag, Christie et al. 1999, van Praag, Kempermann et al. 1999) but not wild mice (Hauser, Klaus et al. 2009, Klaus, Hauser et al. 2012, Schaefers 2013). These results indicate that laboratory mice and wild mice may use a different strategy in regulating AHN when coping with the same situation. In project II, we found that higher levels of AHN are associated with selection for tameness, which is believed to be one of the key traits of domestication. We still do not know whether these foxes were born with a higher level of AHN that is maintained throughout life, or if AHN was up-regulated during the postnatal period. In cats, the hippocampus is known to be involved in mediating defensive rage behavior and predatory attack behavior by its direct or indirect projection to the medial hypothalamus and midbrain periaqueductal gray, and perifornical lateral hypothalamus (Gregg and Siegel 2001). If AHN is genetically high in tame foxes, it is possible that a high level of AHN is involved in natural tame behavior towards humans in domesticated animals. On the other hand, if AHN is up-regulated postnatally, AHN is more likely associated with coping with captivity or artificial environments. It is known that an increase of plasma stress hormone level is linked to down-regulation of AHN (David, Samuels et al. 2009, Anacker, Cattaneo et al. 2013). Domesticated animals usually exhibit a smaller response of the hypothalamo-pituitary-adrenocortical (HPA) system than their wild counterpart (Gulevich, Oskina et al. 2004, Kaiser, Hennessy et al. 2015). This trait in domesticated animals may be associated with their less changeable environment because the robustness of the stress response system and stress hormones can provide animals more energy and accelerate their reaction to changing habitats in the wild. Also, if a higher level of AHN is associated with the robustness of animals' stress response system, it is highly possible that AHN in domesticated and wild animals will react differently in behavioral experiments. Future experiments to observe the change of AHN in fox brains through time and in combination with stress hormone measurement may clarify these questions.

In addition, we also found a strikingly higher level of young neurons in the fox brain. Red foxes are known for their highly adaptive ability. They are distributed throughout the entire northern hemisphere, from arctic nature to urban areas (St-Georges, Nadeau et al. 1995, Walton, Samelius et al. 2017). This feature could also be with a consequence of their highly flexible behavior. However, we cannot exclude that the high level of AHN observed in the fox is a carnivore-associated feature. This question needs to be further clarified based on experiments on more carnivore species, in combination with comparisons between wide-spread species such as *Vulpes vulpes* (red fox) (Macdonald and Reynolds 2008) and those with restricted habitats.

Domestication effect in temporal hippocampus

Our study of the silver fox showed a graded increase of new-born neurons along the septo-temporal axis. This result indicates that the domestication effect might mainly impact the temporal hippocampus. The temporal hippocampus has been associated with odor memory, anxiety-related and social behavior (Sams-Dodd, Lipska et al. 1997, Kjelstrup, Tuvnes et al. 2002, Kesner, Hunsaker et al. 2011). These are key traits linked to domestication (Kaiser, Hennessy et al. 2015). Domestication may not only impact AHN but also hippocampal principal cell population. In Project I of this thesis, we included data from sengi, dogs, pigs, humans, rhesus monkeys, marmosets, mole rats and another 11 different rodent species to the species profile plot analysis. And we can see that one of the most successful domesticated animals – the dog – distances itself from all the other species, due to the relatively large number of neurons in its subiculum. In rats, bilateral temporal subicular lesions are associated with reduced AHN (Kapgal, Prem et al. 2016). This result suggests that the temporal subiculum may be linked with the regulation of AHN.

The subiculum links the hippocampus with the entorhinal cortex and many other cortical and subcortical areas (Quintero, Diaz et al. 2011). Specifically, the temporal subiculum contains glutamatergic projections to the medial nuclei of the bed nucleus of the stria terminalis (BNST), which sends GABAergic projections to the medial parvicellular part of the paraventricular nucleus (mpPVN). The connection is involved in negative feedback to the HPA axis (Choi, Furay et al. 2007, Lebow, Neufeld-Cohen et al. 2012). On the other hand, the septal subiculum receives input from septal CA1 and may be involved mainly in integration of hippocampal spatial information and whole-body-movement related information (O'Mara 2005). These results suggest that the large portion of subiculum in domesticated dogs might be associated more with the temporal subiculum, if there is a link between the subiculum and domestication. The data of hippocampal principal cell population from tame and unselected foxes are not yet investigated nor included in comparison study in project I. A future comparison study for the temporal subiculum between the two groups of silver foxes could be a simple way to test this hypothesis.

AHN and hippocampal heterogeneity along the septo-temporal axis

The results of project II also showed the importance of considering heterogeneity along the hippocampal septo-temporal axis when studying hippocampal function. A recent finding also suggests that the septo-temporal distribution of AHN is different even between closely-related rodent species (Wiget, van Dijk et al. 2017). Due to differences in connectivity along the septo-temporal axis, alteration of AHN along the axis could modify signals to very different down-stream brain regions. AHN along the septo-temporal axis can also be regulated by different regulator because heterogeneity along the axis is not only at circuit level, but also cellular and molecular level (Tanti and Belzung 2013). Therefore, if we do not consider these differences along the axis, the local effect might be neutralized and result in no significant global observation when the alteration of AHN is not so dramatic.

AHN, regulators and confounding variables

Another way to gain a broader picture of the AHN-regulator relationship is to observe the long-term impact when applying any regulators. In project III, we have chosen a reported regulator – APP expressed by GABAergic neurons – to observe the impact on aged mice. This project extends previous studies in several respects. First, APLP2, that is suggested to be redundant with APP (Heber, Herms et al. 2000) was knocked out completely. A compensation of APLP2 for the conditionally knocked out APP can therefore be excluded. Second, APP was reported to have an impact on LTP in aged animals but not young animals (Ludewig and Korte 2016) suggesting that effects of the manipulation of APP do increase or first appear with age. Surprisingly, no significant difference in terms of cell proliferation, young neurons, NPY+ or PV+ cell number can be found between APLP2 cKO and APP/APLP2 cdKO mice. There are several possibilities that could cause the negative results. First, the impact of APP on AHN is an age-dependent mechanism. Mice in project III are considerably older (18-22 months old in project III) than those used in a previous study (4-6 month old in Wang, Wang et al. 2014). Also, GABA production is reduced in the aged brain (Stanley and Shetty 2004). If APP impacts AHN via GABA signaling, reduced GABA levels in the aged brain may result in insignificant changes of AHN level. Different results could also be due to different promotors in the experiments. In previous study(Wang, Wang et al. 2014), APPf/f;vGAT-Cre driven APP KO was used and we used Dlx 5/6-Cre-driven conditional APP KO model. vGAT is a GABA transporter localizing to synaptic vesicles in glycinergic and GABAergic neurons; and Dlx 5/6 (Distal-less homeobox 5/6) is expressed by inhibitory interneurons. Although both are commonly used to target GABAergic interneurons, we cannot exclude that there might be a difference between the two promotors. Experiments have shown that during granule cell development, mRNAs encoding glutamate, glutamate's vesicular transporter (vGlut-1), GABA, its synthetic enzyme (GAD67) and the vesicular

transporter (vGAT) coexist (Gutierrez 2016). Using vGAT as promotor might knockout the APP in young neurons, that directly influence neuronal maturation process. On the other hand, Dlx 5/6 targets only matured inhibitory interneurons. If it is true, it may suggest that the impact of APP on AHN is cell-autonomous.

Third, the negative observation could be due to experimental design. In a previous study performed by (Wang, Wang et al. 2014), age and sex was not reported together with AHN data even though age and sex was known to modulate AHN (Ben Abdallah, Slomianka et al. 2010, Mahmoud, Wainwright et al. 2016). The results in project III showed that age was the only factor correlated with AHN. If a decline of AHN can still be observed at 18 to 22-month old mice, the manner of AHN decline should be even sharper at 4- to 6-month-old because AHN declines exponentially (Ben Abdallah, Slomianka et al. 2010). The other possible cause is that the impact on AHN was already made by APLP2, and APP was not able to compensate this effect. However, a comparison between APLP2 cKO, APP/APLP2 cdKO, and WT control is required to support this assumption. Finally, we cannot exclude that there might still be a difference if we can compare the results to wild type control or analyze the AHN data in detail along the septo-temporal axis. At least we could conclude there was no significant global differences regarding AHN observed between APLP2 cKO and APP/APLP2 cdKO mice.

Conclusion

In conclusion, this thesis has provided insights into AHN that complement and extend those provided by traditional rodent study. We have observed that a natural level of AHN is associated with species variety, domestication, emotional functions, and age across a variety of mammals. We have also noted the importance of looking at AHN in the "whole" hippocampal network. Our results provide a firm foundation for future studies to focus on the functional link of different strategies between different species with regards to the regulation of AHN. Furthermore, our results showed that increase of AHN, especially at the temporal hippocampus, might be a key feature of domestication. This thesis provides evidence of hippocampal heterogeneity along the long axis. It suggests that AHN along the septo-temporal axis needs to be taken into consideration when investigating any functional link of AHN. Finally, this thesis also points out the importance of experimental design in order to obtain meaningful and comparable results across various studies.

AHN can also be found in the human brain, although it was still debated among recent studies (Spalding, Bergmann et al. 2013, Boldrini, Fulmore et al. 2018, Sorrells, Paredes et al. 2018). The main cause of the debate is again experimental design that are difficult to compare (e.g. quantification methods or sample preparation methods) (Boldrini, Fulmore et al. 2018, Sorrells, Paredes et al. 2018). It also supports the argument we discussed above.

Furthermore, although AHN may be found in the human brain, it is very difficult to address the functional link of AHN in humans using current experimental methods and the technology that is commonly used in animal experiments. Most AHN studies in humans have been performed postmortem, which makes it more difficult to link AHN with any specific factor.

To address these questions, a comparative approach, coupled with a study of the nature of AHN, the functional complexity of the hippocampus, might be the key, or might at least help us to find an answer as to what the functional link of adult hippocampal neurogenesis is in the human brain.

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