The dentate gyrus of the hippocampus: roles of transforming growth factor beta1 (TGFβ1) and adult neurogenesis in the expression of spatial memory.

by
Alonso Martinez Canabal
A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy (PhD)

Institute of Medical Science University of Toronto

I. Abstract

The dentate gyrus of the hippocampus: roles of transforming growth factor beta1 (TGFβ1) and adult neurogenesis in the expression of spatial memory.

Alonso Martinez-Canabal,

Doctor of Philosophy,

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University of Toronto, 2013.

The dentate gyrus is a region that hosts most of the hippocampal cells in mammals. Nevertheless, its role in spatial memory remains poorly understood, especially in light of the recently-studied phenomenon of adult hippocampal neurogenesis and its possible role in aging and chronic brain disease. We found that chronic over-expression of transforming growth factor β1 (TGFβ1), a cytokine involved in neurodegenerative disease, results in several modifications of brain structure, including volumetric changes and persistent astrogliosis. Furthermore, TGFβ1 over-expression affects the generation of new neurons, leading to an increased number of neurons in the dentate gyrus and deficits in spatial memory acquisition and storage in aged mice. Nonetheless, reducing neurogenesis via pharmacological treatment impairs spatial memory in juvenile mice but not in adult or aged mice. This suggests that the addition of new cells to hippocampal circuitry, and not the increased plasticity of these cells, is the most relevant role of neurogenesis in spatial memory. We tested this idea by modifying proliferation in the dentate gyrus at several ages using multiple techniques and evaluating the incorporation of newborn neurons into hippocampal circuitry. We found that all granule neurons, recently generated or not, have the same probability of being incorporated. Therefore, the number of new neurons participating in memory circuits is proportional to their availability. Our conclusion is that adult-generated cells have the same functional relevance as those generated during development. Together, our data show that the dentate gyrus is important for memory processing and that adult neurogenesis may be relevant to its functionality by optimizing the number of neurons for memory processing. The equilibrium between neurogenesis and optimal dentate gyrus size is disrupted when TGFβ1 is chronically increased, which occurs in neurodegenerative pathologies, leading to cognitive impairment in aged animals.

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V. Acknowledgments

IV.I Supervision

Dr. Paul Frankland PhD (Primary Supervisor and Thesis Advisory Committee member)

Dr. JoAnne McLaurin PhD (Thesis Advisory Committee member)

Dr. Howard Mount PhD (Thesis Advisory Committee member)

Dr. Wei-Yang Lu MD, PhD (Thesis Advisory Committee member)

Dr. John Yeomans PhD (Thesis Advisory Committee member, special invitation for last committee meeting)

V.II Contributions

Alonso Martinez-Canabal solely prepared this thesis. All aspects of this body of body of work presented, including planning, execution, analysis, writing and figures preparation was performed in whole or in part by the author. The following contributions by other individuals are formally and inclusively acknowledged:

Dr. Paul Frankland PhD – Mentorship, laboratory resources; guidance and assistance in planning, execution, analysis of experiments and manuscripts preparation.

Dr. Katherine Akers PhD – assistance in planning, execution, analysis and manuscript preparation for experiments in section 4 and Appendixes I and II.

Dr. Anne Wheeler PhD – assistance in analysis presented in section 5.4.2

Dr. Jason Lerch PhD – assistance in planning, execution and analysis in section 5.4.2.

Dr. Nohjin Kee PhD – assistance in planning, execution and analysis of experiments for section 4.

Dani Sarkis – Assistance in analysis presented in section 5.4.3

Jinah Choi – Assistance in execution and analysis in section 3.

Dr. Tony Wyss-Coaray PhD and Dr. Marion Buckwalter M.D PhD provided the TGFβ1 overexpression mice used in section 5.

Dr. Sheena Josselyn PhD- Assistance in planning and analysis of experiments, also laboratory equipment, guidance and manuscripts revision.

V. III Manuscript preparation

I appreciate the valuable suggestions and corrections made to this manuscript by Dr. Katherine Akers, Dr. Melanie Sekeres, Dr. Loren de Vito, Dr. Leigh Botley and Mattew Florczynski.

V. IV Technical and administrative assistance

I appreciate the technical support of Antonietta deCristoffaro, Russell Braybon and Melissa Thibault. Also the administrative assistance of Delizia Ferri, Ann Victory, Lisa Alano and Institute of Medical Science staff and the laboratory animal services of The Hospital for Sick Children.

V.VI Permissions

John Wiley and Sons publishing group generously allowed us to reproduce the published work in this thesis (2976930537257).

IV.V Funding

This studies have been possible thanks to the generous funding support of CONACyT (Consejo Nacional de Ciencia y Tecnología, Mexican government foreing graduate scholarship to Alonso Martinez-Canabal), Institute of medical science open awards and International fee waivers to Alonso Martinez-Canabal. EJLB foundation and CIHR (Canadian Institutes of Health Research) research grants to Paul Frankland.

VI. List of abbreviations

AD Alzeimer's disease

ALK1 Activin-like type 1 receptor

ALK2 Activin-like type 2 receptor

ALS Amyotrophic lateral sclerosis

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ARC ***A****ctivity-****R****egulated* ***C****ytoskeleton-associated protein*

BCL-2 B-cell lymphoma 2

BMPR1 Bone morphogenetic protein receptor 1

BMPs Bone morphogenetic proteins

catFISH Compartmentalized fluorescent *in situ* hybridization

CA Cornu ammonis

CAA Cerebral amyloid angyopathy

CCL3 C-C motif chemokine 3, formerly known as : macrophage inflammatory protein 1α (MIP-1α)

CDK Cyclin dependent kinase

CGL Granule cell layer

CIITA Class II transactivator

CNS Central nervous system

COX-2 Cyclooxygenase-2

CREB c-AMP response element binding protein

co-SMAD Common partner SMAD

CR Conditioned reaction

CRMP4	Collapsing response-mediated protein 4
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
DG	Dentate gyrus (Fascia dentata)
DAB	Diaminobenzidine
DGCs	Dentate granule cells
DCX	Doublecortin
DS	Down syndrome
DVR	Decapentaplegic-Vg1 Related Family
DVXX	Death associated protein-6
ECM	Extracellular matrix
EAAT1/2	Excitatory amino acid transporters 1 and 2
EAE	Experimental allergic encephalitis.
EGFP	Enhanced green fluorescent protein
EGF	Endothelial growth factor
EPSPs	Excitatory postsynaptic potentials
ER	Estrogen receptor
FSH	Follicle stimulating hormone
FDA	Federal drug administration
FGF	Fibroblast growth factor
fMRI	Functional Magnetic resonance imaging
GABA	γ-Aminobutyric acid
GAD67	Glutamic acid- decarboxylase 67

GDFs Growth and differentiation factors

GDNF Glial cell-line-derived neurotrophic factor

GFAP Glial fibrilary acidic protein

GFP Green fluorescent protein

GM-CSF Macrophage/granulocyte colony stimulation factor

hAPP Human amyloid precursor protein

HD Huntington's disease (chorea)

HTT *huntingtin* gene

IEGs Immediate early genes

IFN-□□□□□□□□□□ Interferon gamma

Il-1β Interleukin-1β

Il-2 Interleukin-2

I-SMAD Inhibitory SMAD

LacZ gene of β-galactosidase

LIF Leukemia inhibitor factor

LTD Long-term depression

LTP Long-term potentiation

MAM Methylazoxymethanol

MHC I Major histocompatibility complex I

MIP-1□ Macrophage inflammatory protein 1□

MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRI Magnetic resonance imaging

MS Multiple sclerosis

MTL	Medial temporal lobe
NCAM	Neural cell adhesion protein
NK	Natural killer cells
NMDA	*N*-Methyl-D-aspartic acid
NMTS	Non matching to sample
NR1	NMDA receptor subunit 1
NR2	NMDA receptor subunit 2
NSAID	Non steroideal antiinflammatory drug
NSCs	Neural stem cells
PAI-1	Plasminogen activator inhibitor 1
PD	Parkinson's disease
PET	Positron emission tomography
RIAP-1	Rat inhibitor-of-apoptosis protein 1
RIAP-3	Rat inhibitor-of-apoptosis protein 3
RMS	Rostral migratory stream
SGZ	Sub granular zone
SMADs	Small mothers against decapentoplegia
SON	Sensory olfactory neurons
SOD-1	Superoxide dismutase-1
SOX-2	Sex determining region Y-box 2
SVZ	Subventricular zone
TGFα	Transforming-growth factor alpha
TGFβ1	Transforming-growth factor beta 1

TNFα	Tumoral necrosis factor alpha
t-PA	Tissue plasminogen activator
TMZ	Temozolomide
TMX	Tamoxifen
TSE	Transmissible spongiform encephalopathy (Prion disease).
TUNEL	**T**erminal deoxynucleotidyl **U**TP **n**ick **e**nd **l**abelling
TβR1	Transforming-growth factor β receptor 1
TβR2	Transforming-growth factor β receptor 2
US	Unconditional stimulus
UCR	Unconditional response
Wnt	Wingless-integration, developmental gene pathway
WT	Wild type
ZIF268	Zinc finger 225

VII. Preface

In this thesis, the relationship between hippocampal neurogenesis and memory was approached experimentally by manipulating aging, running, and presence of transforming growth factor β1 (TGFβ1), a cytokine that is expressed in the brain in response to acute or chronic injury. TGFβ1 was chosen for study because it is up-regulated in humans during Alzheimer 's disease and reported to be a strong inhibitor of cell proliferation in the adult hippocampus. No report to date has linked this cytokine with behavioural processes in non-human mammals.

Two parts of the literature review are dedicated to explaining the biology and medical implications of TGFβ1 (sections 1.4 and 1.5) and of adult neurogenesis in the hippocampus (sections 1.6 and 1.7). Three independent, but interrelated, studies are presented. The first explores the incorporation of newly-born neurons in the adult hippocampus, when rates of neurogenesis are down- or up-regulated (section 3). The second explores the anatomical and behavioural consequences of TGFβ1 over-expression during brain aging (section 4). The second study, published in the journal *Hippocampus*, explores the effects of neurogenesis blocking on learning and memory at different ages (section 4). Lastly, the third study explores the anatomical and behavioural consequences of TGFβ1 over-expression in the brain during aging (section 5).

We decided to add two appendixes that correspond to a work (Currently under review in the journal *Science*) completed after this thesis and beyond its original scope. Nevertheless, we found useful to make available this work to draw some of our conclusions and future directions.

1. Literature review

1.1 Role of the hippocampus in memory acquisition and storage.

1.1.1 The historical emergence of the hippocampus as a key structure in memory acquisition and storage.

1.1.1.1. H.M. and the medial temporal lobe

The first strong suggestion that the medial temporal lobe (MTL) is a key region in the acquisition and storage of declarative memories came from the case of H.M., a patient studied by Scoville and Milner (1957). H.M. suffered intractable epilepsy and underwent radical experimental surgery to remove his entire medial temporal lobe bilaterally in an effort to remove his epileptic foci. Following the surgery, H.M.'s motor and cognitive abilities, including declarative memories formed prior to the surgery, were intact. His IQ remained above average, and his short-term memory was also intact. However, he suffered complete anterograde amnesia and was unable to form any new episodic memories. In contrast, H.M.'s motor memory remained intact, as he was able to learn and show improvements in motor tasks, although he did not recall performing them (Milner, 1968). Before the observations of H.M. were reported, it was believed that all major cognitive processing in mammals resided in the neocortex. H.M.'s surgery was performed cautiously, and no damage to the neocortex was reported. Instead, the main MTL structures removed from H.M.'s brain included the hippocampus, the entorhinal cortex, and the amygdala, which was confirmed using magnetic resonance imaging (Corkin et al., 1997).

1.1.1.2. Amnesia animal models

After the studies of H.M., the field of memory research focused on reproducing the same patterns of amnesia in animal models. This was carried out by determining the precise effect of lesion size and anatomical location within the MTL under controlled conditions (Eichenbaum, 2002). These investigations revealed a set of conditions required to model MTL amnesia. The animals should have (1) intact sensory, motor, motivational, and general cognitive processes, (2) fully functional short-term

memory, (3) abnormally fast decay of memory after acquisition, and (4) considerable retrograde memory impairment. This means that memories acquired after or recently before the lesion would be lost, but memories formed long before the lesion should remain (Eichenbaum, 2002).

In 1978, twenty years after the observations of H.M., the hippocampus was proposed to function as a cognitive map (O'Keefe and Nadel) in the way that was originally envisioned by Tolman in 1948 (reviewed in Lew, 2011). This proposition came after an extensive review of studies aiming to replicate H.M.'s pattern of amnesia in animal models. Considering broad anatomical, physiological, and behavioural evidence, the authors suggested that the hippocampus is used in the creation and storage of cognitive maps, in which places are represented in terms of relationships among the individual elements of a spatial environment, enabling animals to navigate to locations beyond their immediate field of view. The authors also distinguished between spatial and non-spatial learning, proposing that acquisition of cognitive maps requires a different type of cognitive processing than common habit learning.

With the creation of the 8-arm maze by David Olton in 1976 (1976) and the water maze task by Richard Morris in 1981 (1981), it became clear that animals could learn to navigate to locations using only distal spatial cues. Evidence for the existence of spatial cognitive maps came from observations that rats were able to find the target area after being released from different locations during training; therefore, the same references are equally valid from different points of view, similar to a cartographic map. Morris showed that this behaviour is hippocampal-dependent by lesioning the hippocampus or neocortex of rats and showing that only hippocampal lesions resulted in significant spatial learning impairments (Morris et al., 1982).

1.1.1.3. Alternative proposals

Not all memory researchers agreed with O'Keefe and Nadel's point of view. In 1979, Olton proposed an alternative theory to the cognitive map. Olton considered that the hippocampus is critical when the solution of a problem requires memory for a particular recent experience (Olton, 1979). Olton and colleagues called this type of memory "working memory", defined as the capacity to actively remember information obtained in a single trial and then use that information again at a later time point. In contrast, they used the term "reference memory" to refer to information remembered across trials (Meck et al., 1984), which would be characterized as memory for information that is constant across training (such as the distal cues in the water maze), even though subtle information (like the release point in the water maze) may differ from trial to trial. The two types of memory described by Olton and colleagues were consistent with the theory put forth by Tulving in 1972, which made a distinction between "episodic memory", which is memory for events that belong to specific times and places, and "semantic memory", which is memory for knowledge that is time- and event-independent (Tulving et al., 1972). In conclusion, Olton considered that hippocampus-dependent memory is memory for unique episodes.

The evidence supporting Olton's proposal came from the use of a novel test that he and his colleagues invented called the radial arm maze (Walker and Olton, 1979). In this task, rats were trained to find food in the distal portions of each of the eight arms of the maze. Rats quickly learned which arms they had already visited and thus did not tend to visit the same arm twice. This task was shown to be hippocampus-dependent, as performance was severely impaired if the fornix was transected, leaving the hippocampus unable to communicate with a number of other brain structures (Walker and Olton, 1979). The response of O'Keefe and Nadel to this finding was that orientation within the maze, such as making right or left turns, is not hippocampus-dependent, whereas navigating directly to the place of the reward is dependent on the hippocampus, explaining why fornix transection leads to memory impairment in some but not other versions of the task.

1.1.1.4. Medial temporal lobes in memory processing

However, it was still necessary to show functional evidence of a role for the MTL in humans without surgical blocking. When the technology of functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) became available in the early 1990s, several researchers scanned the brains of human subjects while they were performing memory tasks. Nevertheless these early studies failed to provide definitive evidence of MTL activity during memory tasks, as they could not distinguish between baseline MTL activity and activity specifically induced by the memory task (Martin, 1999).

To overcome this problem, Martin and colleagues measured baseline MTL activity by showing subjects a series of images with no meaning. This allowed them to show a consistent difference between baseline activity and activity specifically induced by a semantic memory task. Their main conclusion was that within the MTL, the hippocampus showed stronger activation than the amygdala or parahippocampal cortices during the recall of a semantic memory (Martin et al., 1997). In another study, strong activation of MTL structures was observed during a memory recollection exercise. The main finding of this study was that different groups of brain structures were activated during recall of episodic versus semantic memories (Maguire and Mummery, 1999).

Furthermore, functional evidence of the presence of spatial cognitive maps in humans came from a study showing activation of the hippocampus during the recall of driving routes by London taxi drivers (Maguire et al., 1997). Follow-up studies also revealed that compared to control subjects, taxi drivers had increased hippocampal volume, presumably due to their extensive construction and use of cognitive maps (Maguire et al., 2000, 2003, 2006). A study in mice found parallel results after extensive water maze training (Lerch et al., 2011b), further demonstrating that only tasks that required the formation of a cognitive map resulted in an increase in hippocampus size.

1.1.1.5. Cellular engagement in hippocampal memory

The body of evidence from amnesic patients, lesion work in animal models, live observation of hippocampal activation, and long-term structural changes supports a very clear engagement of the hippocampus in contextual, episodic, and non-episodic memory. However, these studies rarely shed light on the cellular processing of memory in the hippocampus. A major landmark in the investigation of cellular processing of memory came from the discovery of place cells in the CA1 and CA3 regions of the hippocampus. In an initial series of experiments, O'Keefe and Dovstrosky showed that certain cells in CA1 fire when a rat faces a specific direction, demonstrating that particular sets of cells are active during spatial navigation tasks (O'Keefe and Dostrovsky, 1971). This subpopulation is now known as head direction cells. In later experiments, O'Keefe and colleagues defended the idea that cognitive maps were created at the circuit level by showing the existence of place cells. By recording from electrodes implanted in the hippocampi of freely-moving rats, it was shown that different cells were activated in different places of a radial arm maze. The authors concluded that spatial environments are accurately represented by place fields within the pyramidal layer of the CA1 (O'Keefe, 1976) and, to a lesser extent, the pyramidal layer of CA3 (McNaughton et al., 1983).

1.1.2 Currently-used rodent tasks to assess learning and cognition

Some of the most useful tools to evaluate memory acquisition and storage in the hippocampus are animal cognitive tasks. Two cognitive tasks are discussed here: the spatial version of the Morris water maze and contextual fear conditioning. These tasks reveal unique but complementary information about the role of the hippocampus in learning and memory.

1.1.1.6. Water maze

Mazes are experimental devices typically employed for evaluation of complex spatial memory in rodents. Often, food or water are used as reinforcers to motivate rats to complete the maze (Paul et al., 2009). Thus, the general principle is that animals should learn the location that provides them with safety,

food, or water. However, what is measured is a complex behaviour that not only involves short- and long-term memory but is also regulated by anxiety and sensorimotor abilities (Bats et al., 2001). Therefore, several groups have used modified mazes to evaluate reference memory, working memory, or stress responses (Buresova et al., 1985, Aguilar-Valles et al., 2005). Despite the considerable diversity of mazes developed to evaluate spatial memory, those more commonly used are the Morris water maze (Morris et al., 1982), the Olton radial arm maze (Walker and Olton, 1979), and the Barnes circular maze (Barnes, 1979). In this thesis, the water maze was used extensively to evaluate spatial memory, using different protocols to meet the demands of different experimental objectives.

Richard Morris devised the water maze in 1981 as an alternative to the radial arm maze. It was developed to evaluate the ability of rats to use specific visual cues as proximal and distal references for particular spatial locations (Morris, 1981, Paul et al., 2009). The maze is composed of a circular pool filled with opaque water. Inside the pool there is an escape platform slightly submerged below the surface of the water, rendering it invisible to animals performing the task. The original protocol proposed (Morris, 1981, Morris et al., 1982) included the division of the pool into four quadrants with the hidden platform located in the center of one of the quadrants. An animal is given several training trials during which it is released at different locations along the perimeter of the pool and allowed to search for the platform. If unable to find the escape platform, the animal is then guided to the platform after swimming for a set period of time. The animal gradually learns to locate the platform by using a set of stable distal visual cues, which presumably allows the animal to form a cognitive map of the surrounding spatial environment (Morris, 1981).

The primary way of assessing the animal's memory for the spatial location of the platform is by conducting a probe test. After the training is completed (or between training trials to evaluate acquisition), the platform is removed from the pool. Typically, the percentage of time that the animal spends swimming

in the correct quadrant is used as an index of spatial memory. In addition, the number of times that the animal crosses the location of the platform or the time spent swimming in a circular zone centered on the platform location (Teixeira et al., 2006, Maei et al., 2009) are also used as indices of spatial memory (Blokland et al., 2004, Maei et al., 2009).

Multiple methodological variables that affect water maze performance need to be considered when using this task. One variable is the size of the pool. Different pool dimensions are suggested for rats and mice, and pool size can influence performance even among different strains of mice (van der Staay, 2000, Van Dam et al., 2006). Another variable is the habituation of animals to the sensorimotor demands of the task, which entails brief and controlled swimming trials that do not involve learning of specific spatial locations. Some research groups consider this habituation to be important in preventing unnecessary stress (Morris, 1981) and stimulating search behaviour. Nevertheless, most groups do not use this procedure (D'Hooge and De Deyn, 2001).

Some factors are critical to water maze experimental design. The number of trials and length of inter-trial interval are relevant for the final outcome of the task. For instance, whereas some studies show that 4 to 6 trials per day for 5 days is sufficient to allow animals to reach asymptotic performance, other studies show that up to 10 days of training may be necessary (Bolding and Rudy, 2006). Other important variables include the quantity and quality of visual cues. The optimal shape and number of visual cues remains unclear; some studies suggest that using fewer numbers of cues with more basic shapes improves spatial learning (Lamberty and Gower, 1991). In contrast with the belief that distal cues are necessary for the construction of spatial maps, some researchers posit that navigation in the water maze does not require real spatial mapping but rather simpler strategies, such as navigation based on familiar scenes rather than specific visual cues (Sutherland R. J et al., 1987). Supporting this view, rats were still able to find the

platform after the relative position of the cues was changed with respect to the pool (Hamilton et al., 2007).

Other factors that may influence water maze performance are body weight, physical condition, and age, which may all affect swimming velocity (D'Hooge and De Deyn, 2001). Also, some studies propose that males perform better than females, likely due to physical strength but not to greater spatial navigation abilities (Sherry and Hampson, 1997). Earlier studies showed that differences between males and females vanish with age, as rats older than 6 months do not show sex differences. The authors conclude that this is due to differential maturation rates between sexes (Bucci et al., 1995).

Although the water maze is considered an essential spatial memory task for a variety of animal models, important differences have been found between species and strains. Thigmotaxis, which is the tendency to swim around the perimeter of the pool, is more common in mice than in rats, and it is a factor that is important to consider in the analysis of performance, as it can be related to motivation, motor skills, or the ability to form spatial maps. The tendency for rats to perform better than mice in the water maze is likely related to their better swimming skills, because performance is similar between species in dry tests of spatial navigation (Whishaw and Tomie, 1996).

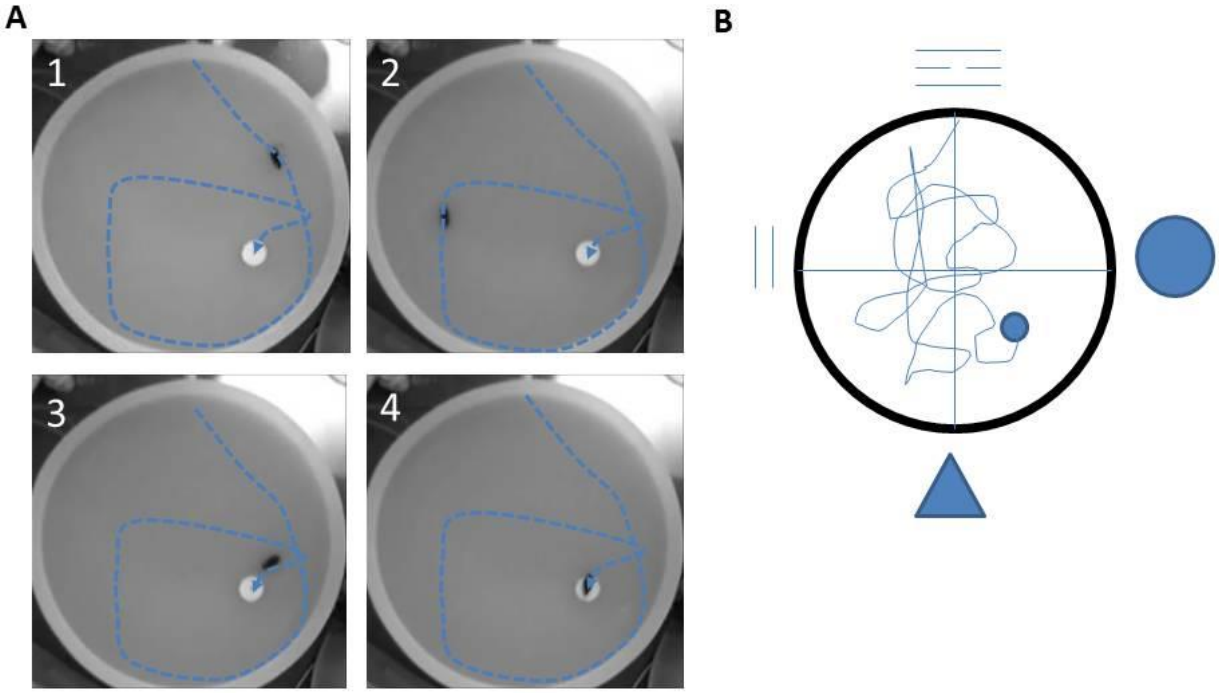


Figure 1. Water maze.

The spatial version of the water maze is a widely used task to evaluate spatial learning and map formation abilities of experimental rodents. (A) The animals can escape from the pool by finding a submerged platform invisible to them as the water is opaque with the addition of paint. The sequence of pictures shows a mouse swimming in different locations of the pool until it finds the platform. (B) The formation of a memory for the spatial location of the platform is contingent on the presence of distal visual cues. The diagram shown here depicts the position of the spatial cues used in our facility. The large circle represents the pool divided in four quadrants, and the location of the platform is shown as a small circle in the center of the southeast quadrant.

1.1.1.7. Contextual fear conditioning

The contextual fear conditioning task has become a commonly used assay for hippocampus-dependent learning (Fanselow, 2000). In a typical conditioning session, a rodent is placed in a chamber, where it naturally explores its new environment. After some minutes, a footshock is delivered (typically in the range of 0.3 to 1.5 mA) lasting between 0.5 and 2 s. The animal initially displays vigorous locomotor activity in response to the shock but subsequently becomes immobile, which is referred to as freezing. Freezing involves the cessation of all movement except for cardiorespiratory movements (Fanselow,

1982). Contextual fear conditioning is considered to be a form of Pavlovian conditioning. Here, the shock is the unconditional stimulus (US)—analogous to the meat powder used in Pavlov's studies—that evokes the unconditioned response (UCR), which is freezing. The conditioned stimuli (CS) are the cues that make up the novel chamber, or the context, where the shock is received, which functions like the bell in Pavlov's classic studies (Douglas, 1972, Fanselow, 2000). In this manner, the spatial context is the CS that comes to elicit the conditioned response (CR) of freezing. When the animal is returned to the chamber, the amount of freezing is used as an index of memory for the context-shock association.

This task has been found to be particularly useful for evaluating hippocampus-dependent context memory. As stated by Sutherland (1989), hippocampus-dependent context memory involves the integration of available elements of a context into a single configural representation. This hypothesis alludes to gestalt theory, in which the entire context can be recognized after sampling a few of its features.

Contextual information is considered hippocampus-dependent. Thus when the context is used as a CS, the associative fear memory should rely on the hippocampus (O'Keefe and Nadel, 1978). Accordingly, rodents with hippocampal lesions should not be able to associate the US and CS when the CS is a context, but they should be able to associate the US and a non-contextual CS. Indeed, rodents with hippocampal lesions show freezing in response to a tone (CS) after the tone was paired with a shock (US) because this memory relies on amygdalar instead of hippocampal circuits (Phillips and LeDoux, 1992). In contrast, when the CS is a context, hippocampal-lesioned rodents show amnesia when tested after surgery (Kim and Fanselow, 1992). Furthermore, when the lesion is made long after training (i.e., one month), no degradation of previously-formed memories is observed, suggesting that the hippocampus is more engaged in the acquisition and organization of contextual memories rather than their long-term storage (Kim and Fanselow, 1992, Anagnostaras et al., 1999).

1.2 Structural, histological, and cytological organization of the hippocampus

1.2.1 Types of cells in the hippocampus

The rodent hippocampus is composed of a vast and intricate network of connections that occupies a considerable portion of the brain. The principal cells of the hippocampus are glutamatergic excitatory cells, which include the pyramidal neurons of the cornu ammonis (CA1, CA2, and CA3 regions) and the granule cells of the granular cell layer of the fascia dentata (dentate gyrus, DG). These different subpopulations of excitatory cells form the trisynaptic circuit, which is considered to be one of the basic memory circuits in the brain.

1.2.1.3 Pyramidal cells

Pyramidal cells were first described by Santiago Ramón y Cajal (1894b). He characterized these cells in mice as having triangular-shaped somas with basal ramified dendrites and large protoplasmic prolongations called apical dendrites, pyramidal neurons are rich in dendritic spines (Bannister and Larkman, 1995). Pyramidal cells are abundant in all cognitive areas of the forebrain, including the neocortex, amygdala, CA regions of the hippocampus, and the subiculum.

A pyramidal neuron receives synaptic inputs on its dendrites, soma, and axon. The soma and axon receive mostly inhibitory GABAergic inputs, whereas the dendrites receive mostly excitatory glutamatergic inputs (Somogyi et al., 1985). Generally, proximal dendrites are innervated by local sources of excitatory input, such as surrounding excitatory cells. Conversely, the distal dendrites receive excitatory inputs from remote areas, such as cortical regions or the thalamus (Cauller and Connors, 1994). The very distinct morphologies of basal and apical dendrites suggest that inputs to these domains might be differentially integrated. One possibility is that distal dendrites are more responsive to

coincidental inputs than are proximal dendrites; another possibility is that distal dendrites control the responsiveness of more proximal dendrites (Larkum et al., 2004).

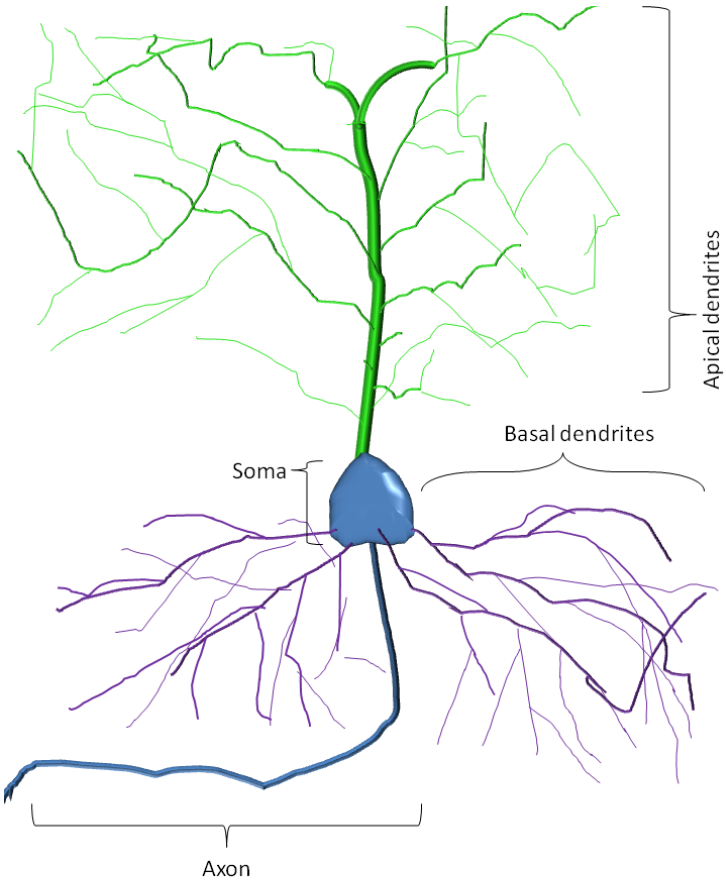


Figure 2. Pyramidal cell.

Pyramidal cells are the principal cell type of CA1, CA2, and CA3 regions of the hippocampus. They contain a triangular-shaped soma and a noticeable cytoplasmic elongation generating the apical dendrites. They also contain a number of independent basal dendrites originating in the lower part of the soma. The axon has its origin at the base of the soma.

1.2.1.2 Granule cells

Granule cells are found in several brain areas, including the olfactory bulb (Scott et al., 1993), cerebellum (Marr, 1969), and the DG. In the DG, they constitute the most abundant type of cells and the main excitatory population of neurons (Hosseini-Sharifabad and Nyengaard, 2007). A granule cell has a round-shaped soma from which normally one or two dendrites extend. The first-order, thick dendrites

divide shortly after originating from the soma, and then subdivide several times into higher orders of dendrites, normally reaching seventh-order or above. All orders of dendrites normally have many dendritic spines. In rodents, granule cells do not have basal dendrites, but they have long axons that can bifurcate abundantly (Frotscher et al., 2000).

Granule cells in the hippocampus have large presynaptic boutons containing several postsynaptic terminals and cytoplasmic protuberances called filopodia (Blaabjerg and Zimmer, 2007). Filopodia form excitatory inputs onto target inhibitory cells. In the hippocampus, filopodia are mostly known for forming connections with CA3-associated inhibitory cells in the *stratum lucidum*, *stratum radiatum* and *stratum lacunare moleculare* (Frotscher, 1989).

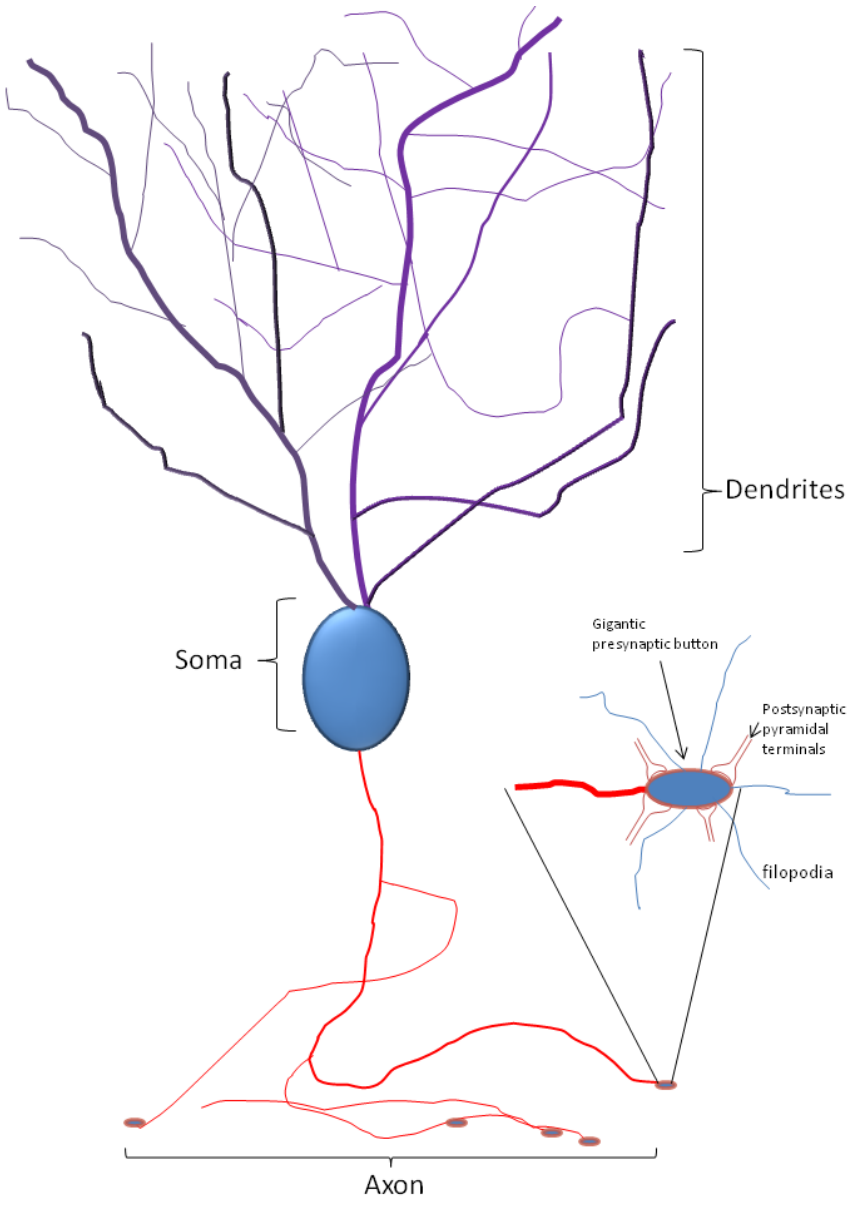


Figure 3. Granule cell

Granule cells, the most abundant neurons in the DG, are composed of small round-shaped somas from which one or more extensively-ramified apical dendrites elongate. An axon protrudes from the basal part of the soma, forming the mossy fibers. The terminals of these axons contain large boutons called large mossy terminals, which connect directly with target cells or by small cytoplasmic extensions called filopodia.

1.2.1.4 Inhibitory neurons

The inhibitory cells of the hippocampus are highly numerous and extensive, projecting to all subareas. This inhibition provided by these cells is critical for tuning and regulating hippocampal activity. Inhibitory cells reside in the main pyramidal and granule cell layers and represent a very large population of neurons in the hippocampus (Buzsaki, 1984). Subpopulations of inhibitory cells vary in terms of morphology and neurochemistry. The majority of these cells are positive for glutamic acid decarboxylase 67 (GAD67) and parvalbumin in the soma and dendrites. Normally, they have a short dendritic arbour without a main dendrite and a very complex and ramified axon that contacts with nearby excitatory cells or other inhibitory cells (McBain and Fisahn, 2001). Some of the cells found in the DG and the *stratum lacunare* are called basket cells because of their unique shape (Seress, 1978) (Lacaille and Schwartzkroin, 1988). The main function of these cells is to maintain excitatory activity within a homeostatic balance and to isolate and tune specific information-relevant inputs from unspecific noise. Furthermore, inhibitory cells play a strong role in certain forms of synaptic plasticity, such as long-term depression (LTD) (Maffei, 2011).

1.2.1.5 Astrocytes

Astrocytes were once thought to be merely the glue of the brain, but recent advances have revealed that astrocytes are critical for vasculature control, immune response, neurotransmitter homeostasis, and synaptic integration of surrounding neurons. The hippocampus is rich in astrocytes; they are dispersed across all subareas, particularly the molecular layer of the DG and the pyramidal and molecular layers of the CA regions, although their presence is limited within the granule cell layer of the DG. Each astrocyte has a defined territory, with no crossing between astrocytic processes, lamellipodia (laminar cytoskeleton prolongations), and filopodia (finger-like cytoskeletal projections). Thus, each astrocyte appears to exert a dynamic influence on a specific area (Colombo et al., 2004,

Hirrlinger et al., 2004). Each astrocyte normally covers every blood vessel crossing its territory, thereby forming a tight blood-brain barrier. Astrocytes also cover several thousands of synapses, thereby regulating glutamate metabolism (Bushong et al., 2002, Bushong et al., 2004).

The heterogeneity of astrocytes inspired great debate, which has now been properly addressed due to the availability of reporter genes. Some cells are considered 'germinal astrocytes', which are GFAP-positive precursors residing in neurogenic areas (Garcia et al., 2004). Recent work with mRNA microarrays has indicated that very few genes are ubiquitously expressed in all astrocytes, whereas many genes are differentially expressed in astrocytes within specific brain regions (Bachoo et al., 2004). Functional studies of the hippocampus have led to the distinction of separate populations of astrocytes with different voltage-dependent current patterns and different responses to glutamate (Steinhauser et al., 1992).

Studies with transgenic mice that express enhanced green fluorescent protein (EGFP) driven by the GFAP promoter have confirmed that the astrocyte population in the hippocampus is heterogeneous (Nolte et al., 2001, Matthias et al., 2003). About half of the astrocytes are rich in GFAP and are characterized by irregular somas with branched processes, low input resistance, very negative resting membrane potential, voltage- and time-independent K+ currents, strong glutamate uptake, and gap-junction coupling. In contrast, other astrocytes are characterized by low GFAP expression, higher input resistance, less negative membrane potential, voltage-dependent K+ and Na+ currents, AMPA receptors, and low glutamate uptake. This latter population is not coupled through gap junctions (Wallraff et al., 2004).

GFAP-poor astrocytes are positive for S100 calcium-binding protein, which is considered to be another astrocyte marker. Some astrocytes are also positive for proteoglycan NG2 (Matthias et al., 2003), a marker believed to be specific to a class of oligodendrocyte progenitor cells. The role of NG2-positive

cells remains elusive; these cells might be transitory cells or they might represent a previously unidentified class of glial cells distinct from oligodendrocytes, microglia, and classically-recognized astrocytes. Localized in both the white and grey matter (Butt et al., 2002), NG2-positive cells receive direct synaptic inputs from neuronal fibers (Bergles et al., 2000). The functional significance of these cells is unknown (Bergles et al., 2000).

As observed *in vitro*, synaptic transmission in the hippocampus is modulated by dynamic changes in the astrocytic coverage of synapses via a highly dynamic partnership between astrocytes and neurons (Hirrlinger et al., 2004, Benediktsson et al., 2005). Astrocytic processes show morphological changes from minute to minute. This structural plasticity is important to brain signalling, as perisynaptic astrocytic processes have surface molecules that influence synaptic transmission. Such molecules include the excitatory amino acid transporters 1 and 2 (EAAT1/2), which transport glutamate and regulate its extracellular concentration (Danbolt, 2001). Furthermore, an outstanding discovery is that astrocytes have communication capabilities. Astrocytes are now considered excitable cells in the sense that, when activated by internal or external signals, they deliver specific messages to neighbouring cells, a type of activity called gliotransmission (Bezzi and Volterra, 2001).

Astrocytes cannot generate action potentials. Their excitation, which is chemically encoded, cannot be demonstrated by regular electrophysiological methods but rather by Ca^{2+} currents. Two forms of astrocyte excitation are well known: one that is generated by chemical signals in neuronal circuits (neuron-dependent excitation) and one that occurs independently of neuronal input (spontaneous excitation). Neuron-dependent excitation of astrocytes is widespread in the hippocampus (for references see (Bezzi and Volterra, 2001, Haydon, 2001, Volterra and Meldolesi, 2005), occurring after fiber stimulation and the release of various transmitters and factors such as glutamate, GABA, acetylcholine,

noradrenaline, dopamine, ATP, nitric oxide, and brain derived neurotrophic factor (BDNF) (Araque et al., 2002, Rose et al., 2003, Zhang et al., 2003, Fellin et al., 2004).

Hippocampal astrocytes discriminate among neuronal inputs of different origins and can integrate concomitant inputs (Perea and Araque, 2005). Hippocampal astrocytes of the *stratum oriens*, which express both glutamatergic and cholinergic inositol 1,4,5-trisphosphate (Ins(1,4,5)P3)-generating receptors, respond to stimulation of Schaffer collaterals (which are glutamatergic) or nerve fibres in the *stratum oriens/alveus* (which are cholinergic and glutamatergic) with Ca^{2+} influx. This response is mediated only by acetylcholine receptors; glutamate is taken up without activating receptors. When released from stimulated Schaffer collaterals, however, glutamate induces mGluR-dependent $[Ca^{2+}]i$ elevation. When Schaffer collaterals and *stratum oriens/alveus* fibres are stimulated simultaneously, the responses do not correspond to the sum of the $Ca^{2+}i$ signals elicited by separate stimulations; instead, they are either larger or smaller depending on the frequency of the stimulation (positive or negative, respectively) (Parri et al., 2001).

Spontaneous excitation of astrocytes can result in the excitation of neighbouring neurons, a finding that overturns the common idea that information is generated only by neurons, traveling through neuronal circuits before reaching glial cells. Nevertheless, $[Ca^{2+}]i$ monitoring of large cell populations in brain slices and the intact brain shows that both neurons and astrocytes are sources of excitation and might operate in coordinated networks (Aguado et al., 2002, Hirase et al., 2004). In the particular case of the DG, it has been determined that astrocytes can effectively regulate neurotransmission between perforant pathway terminals and granule cell dendritic spines. It has been shown that NMDA receptors positioned outside the synaptic cleft are receptive to glutamate released by astrocytes, thereby causing synaptic facilitation (Jourdain et al., 2007). The astrocytes in the molecular layer of the DG are receptive to action potentials in the perforant pathway. Strong Ca^{2+} influx is observed after perforant pathway

stimulation, leading to the release of synaptic-like vesicles from filopodia close to synaptic clefts. This process is considered to be dependent on TNFα (Jourdain et al., 2007, Santello et al., 2011).

1.2.2 Organization and connectivity of the *cornu ammonis*

The principal cells of the hippocampus are found in two large cortical zones stretching across the anterior-posterior axis of the rodent brain. The first zone, called *cornu ammonis* (CA), is composed entirely of pyramidal cells in 3 distinct layers: CA1, CA2 and CA3. These areas are defined by their morphology and their connectivity with other hippocampal areas. The second zone, called the dentate gyrus (DG), encompasses a very large population of glutamatergic granule cells. Although the DG is anatomically distinct from the CA zone and is separated by a meningeal lamina, its function and connectivity are closely related to the rest of the hippocampus and therefore considered to be a part of it.

1.2.2.1 CA1

The CA1 in rodents has three main afferents. The first is the Schaffer collaterals that originate in ipsilateral CA3 pyramidal cells. The second is the commissural fibers that originate in contralateral CA3 pyramidal cells. The third is the perforant pathway that originates mostly from the pyramidal cells of the medial entorhinal cortex (EC) layer V (Amaral and Witter, 1989). In these pathways, most neurotransmission occurs via glutamatergic excitatory synapses. Yet, it is very important to also consider the effect of inhibitory neurotransmission in the circuitry organization of CA1 (Knowles and Schwartzkroin, 1981, Lacaille, 1991, Li et al., 1992, Bernard and Wheal, 1994).

The major destinations of efferent pathways from the CA1 layer are the subiculum and EC, and also the septal nucleus, amygdala, other hippocampal structures, and the olfactory bulb. The connections to the subiculum and EC provide indirect information to numerous sensory, temporal, and associational

neocortical modules, as well as to the thalamus and mammillary bodies (Björklund et al., 1987, Saunders et al., 1988, van Groen and Wyss, 1990, Traub and Miles, 1991).

1.2.2.2 CA2

The CA2 region is small in rodents and therefore has been poorly investigated. Most researchers who study hippocampal circuitry consider CA2 to be part of the CA3. Although these two areas share the same type of pyramidal cells, they do not share the *stratum lucidum* that is characteristic of CA3, indicating that the main source of input to CA2 is not the DG. In recent years, the CA2 region has garnered more attention, and details of its special characteristics have been discovered. For example, CA2 is resistant to temporal epilepsy (Sloviter, 1983) and also has a possible role in the onset of schizophrenia (Benes et al., 1998). Possibly the most interesting characteristic of the CA2 region is that although its pyramidal cells sustain similar basal levels of activity as their CA1 homologues, they do not show elicited long-term potentiation (LTP). This is due to a large difference between CA2 and the rest of the CA areas in Ca^{2+} metabolism, rendering elicitation of LTP in the CA2 impossible. Such unique characteristics of the CA2 suggest a functional role in memory processing that is distinct from that of CA1 and CA3 (Zhao et al., 2007).

1.2.2.3 CA3

Pyramidal cells of the CA3 receive their main afferents from DG mossy fibers, which provide 90% of excitatory input (Amaral and Witter, 1989), and the perforant pathway directly from the EC. Both types of afferent axons establish connections with both basal and apical dendrites of CA3 pyramidal cells and a large number of interneurons. The main efferents of the CA3 are the Schaffer collaterals, which are excitatory inputs that target nearby CA3 pyramidal cells and CA1 pyramidal cells (Swanson et al., 1978). In addition to the Schaffer collateral projections, the CA3 also sends both excitatory and inhibitory projections to the lateral septal nucleus (Knowles, 1992).

1.2.2.4 Organization and connectivity of the DG

The DG contains the largest population of excitatory cells in the hippocampus, and it is the entry gate for most of the information pathways that are processed and sorted in the hippocampus. Physical and cellular barriers prevent the flow of significant amounts of information from the DG to areas outside the hippocampus. However, the circuitry of the DG is complex, involving inhibitory neurons of different types and excitatory neurons other than granule cells.

The rodent DG granule cell layer receives inputs from many sources. CA3 cells proximal to the DG project to the inner third of the molecular layer and establish connections with granule cell dendrites (Claiborne et al., 1986, Li et al., 1994). This strongly suggests the existence of a feedback loop between the DG and the CA3. The perforant pathway from the EC is responsible for the majority of the excitatory input to the outer two thirds of the molecular cell layer, forming extensive excitatory synapses onto DG granule cells. The rodent EC receives inputs from several structures, including the olfactory bulb, anterior olfactory nucleus, and piriform cortex (Amaral, 1993, Witter, 1993). Another major source of excitatory input comes from the perirhinal cortex, with content from auditory, visual, polysensory, autonomic, and limbic association cortices (Witter et al., 1989). The perforant pathway arises mostly from layer II of the EC with all—or almost all—layer II cells contributing. These cells vary morphologically and include pyramidal cells, stellate cells, and multipolar cells (Schwartz and Coleman, 1981, Ruth et al., 1982, 1988). More sparse projections can be found from layers IV and VI of the EC to the outer two thirds of the molecular layer (Kohler, 1985).

The mossy fiber axons of granule cells project to CA3. They also have a system of collaterals in the hilus. Most of these collaterals are confined to the hilus, but a subpopulation also projects to the molecular layer of the DG, where they establish connections with granule cell dendrites (Cavazos et al., 1992). The large presynaptic terminals of granule cells in CA3 are easily recognizable and have been

reported to contact a variety of cell types that include all types of inhibitory interneurons and principal cells. Electrophysiological studies have shown that DG granule cells can activate the entire range of mossy excitatory and inhibitory cells in the hilar area as well as all CA3 components (Patton and McNaughton, 1995).

The granule cell layer of the DG is the only forebrain structure which continuously adds new principal cells during adulthood (Altman and Das, 1965, Altman and Bayer, 1990). The cells that continuously divide and ultimately generate neurons that mature and integrate into the circuitry are found in the basal part of the granule cell layer, and the region that contains a mix of progenitor cells and immature neurons is called the sub-granular zone (Kuhn et al., 1996). The entire process of proliferation of new cells, migration through the granule cell layer, maturation, and circuit integration during adulthood is called adult hippocampal neurogenesis. To abbreviate, it will be referred to as "neurogenesis" throughout the remainder of this thesis.

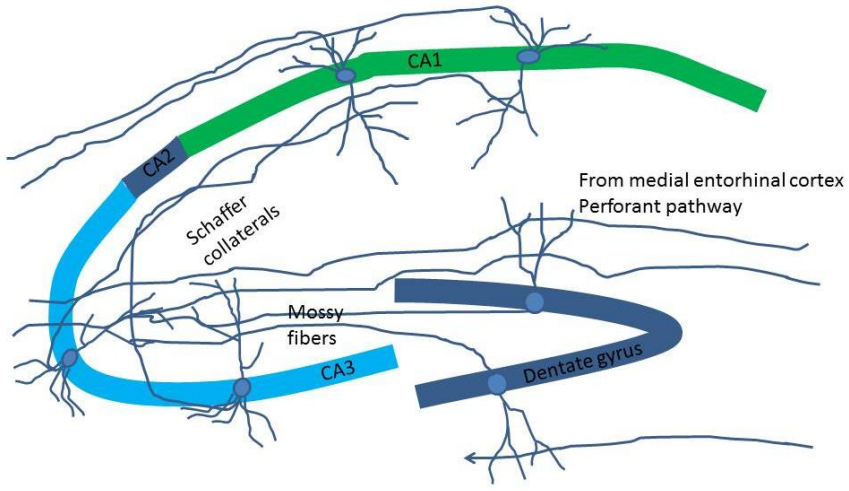


Figure 4. Hippocampal connectivity

The main source of input to the hippocampus comes from the perforant pathway from the medial EC. The perforant pathway connects with dendrites on granule cells in the DG and pyramidal cells in the CA3. Granule cells almost exclusively project to targets in CA3 by way of the mossy fibers, and CA3 pyramidal cells project to other CA3 cells and CA1 cells by way of the Schaffer collaterals.

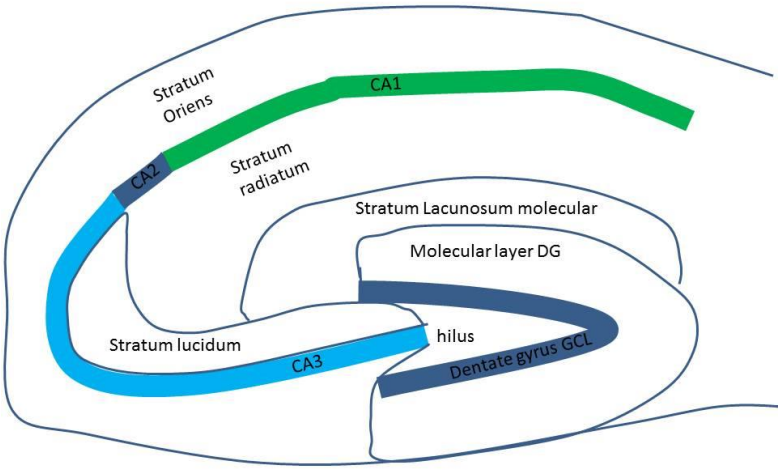


Figure 5. Hippocampal layers

The hippocampus is formed by several layers containing different cell populations. The principal cells are organized in the CA1, CA2, and CA3 regions and the granule cell layer (GCL) of the DG. Outside the CA areas, hosting their basal projections ,is the stratum oriens. Inside the CA areas, hosting their apical projections, is the stratum radiate. Hosting the mossy terminals around CA3 is the stratum lucidum, and hosting the dendrites of the DG is the molecular layer of the DG. The stratum lacunosum molecular is between the DG and CA3 and contains independent populations of inhibitory cells.

1.3 Role of the dentate gyrus in spatial navigation and other types of memory

The dentate gyrus (DG) is a structure exclusively found in the hippocampal formation of mammals, containing approximately half of the total number of hippocampal cells (Hosseini-Sharifabad and Nyengaard, 2007). In recent years, evidence has suggested that the DG is critical for memory formation and storage. The DG receives inputs from the entorhinal cortex and projects predominately to the CA3. Current computational models suggest that the DG sparsely encodes inputs to the CA3, thereby reducing overlapping information and increasing the resolution of the content (Kesner, 2007). Thus, the DG helps properly organize memories in time and space. Consequently, the DG has been recognized as a structure that makes important contributions to spatial memory encoding and retrieval (Aimone et al., 2011).

1.3.1 Role of the dentate gyrus in pattern separation

The DG receives multiple inputs that include olfactory, visual, auditory, and somatosensory information from the perirhinal and lateral entorhinal cortices. The DG also receives inputs from topographically-organized grid cells that are found in the medial entorhinal cortex (Hafting et al., 2005). In an experiment testing rats' ability to detect the spatial locations of novel objects, blocking sensory input to the DG resulted in impairments in both novelty detection and spatial location memory, whereas blocking topographic inputs affected only spatial location memory. Therefore, the authors suggest that the DG uses conjunctive encoding of visual objects and spatial information to provide conspicuous spatial representations (Hunsaker et al., 2007). According to this hypothesis, one of the main memory processing roles of the hippocampus is to differentiate similar spatial events from each other. This process is referred to as pattern separation

of episodic information, whereby similar spatial events are disambiguated and possible interference is reduced (Kesner, 2007).

The model proposed by Rolls states that pattern separation should be facilitated by sparse connectivity between DG granule cells and CA3 pyramidal cells via the mossy fibers. This sparse connectivity results in a low probability of any two CA3 neurons receiving synaptic input from the same subset of DG granule cells. The mossy fibers are essential during learning and influence the firing of CA3 neurons according to their distribution (Rolls, 1996). The granule cells in the DG are thought to act as a competitive learning network with reduced redundancy and sparse orthogonal outputs to the CA3 (O'Reilly and McClelland, 1994). Some particular characteristics of mossy fiber terminals may promote pattern separation. For instance, mossy fiber synapses are very large and located close to the soma of pyramidal CA3 neurons, making it likely that these terminals are very efficient in triggering activation of CA3 cells. On the other hand, projections from layer II of the entorhinal cortex establish contacts with the apical dendrites of the CA3 pyramidal cells within the stratum lacunosum. This area is located far from the soma, and therefore these connections are less likely to efficiently trigger action potentials (Rolls, 1996).

Another important characteristic of the DG that likely promotes pattern separation is that the frequency of granule cell firing is very low, thereby limiting the number of synapses onto any CA3 pyramidal cell that are active at any given time. This sparse firing should result in less noise and hence more accurate representations of spatial or contextual memories (Jung and McNaughton, 1993). Consider two discrete populations of DG granule cells. If a context activates one of these populations, the downstream activation will be restricted to a small number of CA3 pyramidal cells. If a different context activates the other population, the chances of overlapping activation of CA3 pyramidal cells will be low, therefore similar, but different contexts can be

discriminated. Considering all of these characteristics, disruption of DG function should lead to spatial deficits due to increased information overlap or similarities in representations. Thus, remembering a specific location in a radial arm maze or water maze, or remembering a particular context where shock was received, should be severely impaired (Kesner, 2007).

1.3.2 Dentate gyrus lesions impair episodic memory

Lesions specifically targeting the DG produce disruptions in spatial memory similar to those observed following complete hippocampal blocking. Lesions produced by colchicine, an anti-microtubule polymerization drug that induces semi-specific DG lesions (specifically kills granule neurons, but also toxic for CA1 and CA3 causing some levels of cell death), have deleterious effects on memory in the radial arm maze and the passive avoidance task (Walsh et al., 1986, Tilson et al., 1987). In the case of the water maze, the semi-specific deletion of the DG with colchicine produced a dramatic performance deficit in the water maze (Sutherland et al., 1983) and in a spatial reference memory task (Nanry et al., 1989, Xavier et al., 1999).

Jeltsch and colleagues (2001) also report memory disruption after DG lesions. In a version of the radial arm maze without a spatial component, the colchicine-induced disruption in performance was dose-dependent and attributed to collateral damage of CA1 pyramidal neurons and mossy cells. However, in a version of the maze with a spatial component, the colchicine-induced disruption in performance was complete and attributed to the large lesion in the DG. A similar effect was found for contextual fear conditioning; when rats received DG or CA1 lesions, they showed considerable deficits in memory retention, but when they received a CA3 lesion, they showed no disruption (Lee and Kesner, 2004a).

After these findings two questions remained: (1) Is pattern separation dependent only on the DG or it is dependent on both the DG and CA3? (2) Is pattern separation dependent on differences

in topological distributions or metric differences? To answer the latter question, Poucet (1989) developed the chess board maze to test the ability of rats to recognize the movement of a specific object or a change in the spatial distribution of several objects following lesions to different hippocampal areas. Consistent with the studies of Lee and Kesner (2004a, b), rats with DG lesions showed difficulty detecting metric differences in the arrangement of objects, whereas rats with CA1 lesions showed difficulty detecting topological changes in the arrangement of the objects (Goodrich-Hunsaker et al., 2005). These studies suggest that there is a subregional division of functionality within the hippocampus. Specifically, the CA1 encodes information on object location, whereas the DG encodes information on the precise distance between objects.

1.3.3 Relationship of the dentate gyrus with the CA3

The primary axonal target of the DG is the CA3. Therefore is important to determine if the connectivity between the two areas plays a role in memory. If the CA3 is where the actual representations of separated patterns are allocated, then orthogonally-separated ensembles of CA3 cells should be activated in response to similar spatial patterns. Tanila (1999) showed that the CA3 layer displayed orthogonal ensembles of activated cells in response to similar stimuli, and that the experience of two different but similarly designed spaces created perfectly orthogonal representations in the CA3 of rats. A similar study showed that the CA3 differentiates between two environments with distinct firing rates, whereas CA1 place cells maintain comparable responses to the two environments (Leutgeb et al., 2004). Visualizing the immediate early gene *arc* as a marker of neural activation with compartmentalized fluorescent *in situ* hybridization (catFISH), the response of CA1 place cells to similar environments overlapped, but there was almost no overlap in the CA3 (Guzowski et al., 2001). Computationally, each different environment could be represented as a different chart, and if those charts are sufficiently

orthogonal, they could operate independently (Stringer et al., 2004). This system has been found to be optimal; other mathematical analyses showed that the sparseness of the pattern separation carried out by the DG and the orthogonal representations in CA3 could easily explain the strong dynamism and flexibility of the system (Cerasti and Treves, 2010).

1.3.4 Encoding, retention, or retrieval?

A controversial question is whether the pattern separation process involving the DG and CA3 works preferentially for encoding or also for retention and retrieval. In a study using the Hebb-Williams maze, DG lesions impaired memory encoding within training days but not between training days, suggesting that the lesions did not affect memory retrieval (Lee and Kesner, 2004b). Further data obtained from the same group corroborated this conclusion, adding that ipsilateral lesions of both the DG and CA3 affected neither spatial encoding nor retrieval, but when each structure was lesioned in a different hemisphere, there was a deficit in encoding but not retrieval. These findings show that the connectivity between the two structures is key for memory acquisition but not for retrieval (Jerman et al., 2006).

Similarly, in the water maze, transection of the mossy fibers resulted in impairments in encoding but not retrieval (Lassalle et al., 2000). It is important to clarify that there are memory tasks in which the DG and CA3 have dissociated functionalities. Animals with lesions of the DG were able to learn a place-preference task and an odour place task, but animals with lesions of the CA3 failed to perform either task, indicating that the CA3 but not the DG is critically involved in associative learning (Gilbert and Kesner, 2003). However, as previously mentioned, DG lesions effectively disrupt water maze learning (Sutherland et al., 1983, Nanry et al., 1989, Lassalle et al., 2000).

1.3.5 Electrophysiological evidence of dentate gyrus participation in spatial navigation.

Since its discovery in the 1970's, long-term potentiation (LTP), or long term enhancement of evoked excitatory postsynaptic potentials (Lomo, 1971b, a), has been regarded as an ideal candidate for the physiological substrate of hippocampal memory formation. The finding of LTP in the DG after stimulation of the perforant pathway in anesthetised rabbits strongly suggested that LTP is a phenomenon that occurs *in vivo* in the DG (Bliss and Lomo, 1973). As LTP is impaired after perforant pathway disruption, this form of synaptic plasticity is consistent with a classic Hebbian mechanism, and a few years after its discovery, LTP was suggested to be the physiological phenomenon responsible for creating associative memories (Levy and Steward, 1979). However, there are many technical difficulties associated with determining whether LTP is involved in memory formation (Skelton et al., 1985). Indeed, the link between LTP and behavioural changes remains elusive, and in the case of the DG, only correlative evidence exists.

Some studies support a relationship between LTP dysfunction in the DG and spatial memory deficits. Knockout mice that lack the tyrosine kinase receptor "Fyn" showed disruptions in LTP formation without alterations in synaptic transmission or short-term synaptic facilitation (Grant et al., 1992). These mice also showed a deficit in ability to form spatial memories. Furthermore, induction of LTP can block the subsequent formation of spatial memories by means of charge overdrive. In support of this proposition, a considerable impairment in water maze performance was observed in mice following induction of LTP by high frequency stimulation of the perforant pathway, although a recovery in spatial learning was observed after some weeks, and memories formed previously to LTP induction were not altered (Grant et al., 1992). However, Morris and colleagues failed to replicate these results, finding no correlation between LTP

induction and spatial learning deficits but rather a small positive correlation between LTP and memory retrieval (Jeffery and Morris, 1993).

During the aging process, a decline in memory could be related to impairments in hippocampal function, as some aged rats perform as poorly in memory tasks as do young rats with hippocampus lesions. Aged animals with impaired memory also show rapid decay of LTP and structurally defective axo-dendritic connections in the third layer of the molecular DG, suggesting defective connectivity between the perforant pathway and the DG (deToledo-Morrell et al., 1988). Other evidence suggesting a relationship between LTP in the DG and spatial learning is the observation that after water maze learning, there is a transient increase in glutamate release around perforant pathway-DG synapses, similar to that observed after LTP induction in anesthetised rats and hippocampal slices (Richter-Levin et al., 1995).

1.3.6 Immediate early gene expression in the dentate gyrus

A major advance in the study of memory is the observation that long-term memory storage requires the synthesis of new proteins, particularly proteins that work as transcription factors and can regulate the expression of structural or synaptic proteins. Some of these proteins are expressed only after neuron activation. These so-called immediate early genes (IEGs) are transcribed after a period of activity, and their expression is likely related to memory formation or retrieval.

Seizures result in massive release of glutamate and, as a consequence, general activation of the DG. The IEG c-Fos is expressed in a large percentage of DG cells during seizures (Dragunow and Robertson, 1987a, b, Morgan et al., 1987, Kee et al., 2007a). In another experiment, the mRNA of Fos as well as other IEGs like Zif-268 and Jun was observed in DG cells after high-frequency stimulation aimed to elicit LTP (Cole et al., 1989). Contrarily, other evidence suggests that the activation of IEGs is not correlated with long-term enhancement of excitatory postsynaptic

potentials, and therefore this gene expression could be either not necessary or even opposed to this enhancement (Schreiber et al., 1991).

In awake rats, LTP induction enhanced the expression of Jun and c-Fos in the DG; conversely, blockade of LTP by NMDA receptor antagonists impaired the expression of these IEGs. Consistent with these results, other groups report that different types of stimulation have different effects on IEG expression, with fast expression of Zif-268 and slower but consistent expression of Fos and Jun (Demmer et al., 1993, Worley et al., 1993, Steward et al., 2007). An interesting finding is that IEGs expressed in response to LTP induction in the DG do not decline due to aging, suggesting that the memory decline normally associated with aging is not due to the lack of responsiveness of these transcription factors (Lanahan et al., 1997). In contrast, if mice show amyloid deposition and severe cognitive decline, a strong deficit is also observed in the expression of IEGs in the DG following an object recognition task (Steward et al., 2007).

Some questions about the specificity of IEGs remain. Hippocampal neurons can fire during memory processing but also in response to specific spatial locations, as has been specifically observed in CA1. To determine whether the expression of IEGs corresponds to learning or just "placing", the septum of mice was removed, thus interfering with learning and LTP formation but not with location-firing. In this situation, no Arc expression was observed in the CA1, therefore the authors concluded that Arc expression corresponds only to spatial learning (Miyashita et al., 2009).

1.3.7 Summary

Behavioural evidence suggests that DG lesions impair specific aspects of spatial memory in rodents, such as encoding of episodic memories, metric localization, and pattern separation. Anatomically, there is a strong basis for these specific roles of the DG, including both strong DG-

CA3 connectivity and a sparseness and separation of inputs from the DG to the CA3. The relevance of this DG-CA3 relationship has also been shown experimentally, as disruption of communication between the DG and CA3 leads to spatial memory impairment. Several studies also present computational models supporting this theory and, finally, strong physiological evidence of LTP formation and IEG expression in the DG confirms the importance of the DG in spatial memory.

1.4 Structure and cellular function of transforming growth factor beta1 (TGFβ1).

Transforming growth factor beta-1 (TGFβ1) was first detected in hepatic tissue and named due to its presence in transformed fibroblasts (de Larco and Todaro, 1978). Soon after its discovery, the protein was observed to have at least three subtypes and a variety of tertiary alternative structures and to exist in homologues forms throughout the animal kingdom. TGFβ1 is strongly involved in development and immune system regulation.

1.4.1 TGFβ1 structure, family, and evolution

1.4.1.1 TGFB1 history

In 1978, sarcoma virus-transformed mouse fibroblasts were found to secrete polypeptide growth factors. These factors were observed to stimulate the division of cells in monolayer cultures and also the formation of growing colonies. In spectrometric analysis, three peaks of activity were identified at molecular weights 7000, 12,000, and 25,000 kDa. All three molecular species compete for membrane epidermal growth factor (EGF) receptors but do not react to EGF antibodies, thus leading to the discovering of a new family of growth factors with similar biological activity to EGF (de Larco and Todaro, 1978). In further studies, these growth factors were named transforming growth factors, and two were isolated: TGFα and TGFβ. Despite their similarities, the two growth factors are not homologues and do not share a common origin. Only TGFα actually binds to the EGF receptor (Roberts et al., 1980, Todaro et al., 1980, Todaro et al., 1981, Roberts et al., 1982, Lei and Rao, 1992).

1.4.1.2 TGFβ1 structure

TGFβ1 proteins belong to a diverse family whose members are structurally similar and share a similar biosynthetic pathway. These proteins are secreted as mature peptides that form hetero-

or mono-dimers. The synthesis of each monomer starts with a large pre-pro-protein that, after cleavage of the NH2 terminal, is cleaved by a furin-type protein convertase. This generates a C-terminal fragment that will be secreted after its bonding to another monomer, forming either a mono-dimer or hetero-dimer via disulphide binds between 7 to 9 cysteine residues (*see references in* (Herpin et al., 2004)). All family members of TGFβ1 have a common conserved αhelix and six βsheets that together comprise two fingers (Daopin et al., 1992, Schlunegger et al., 1992, Schlunegger and Grutter, 1992).

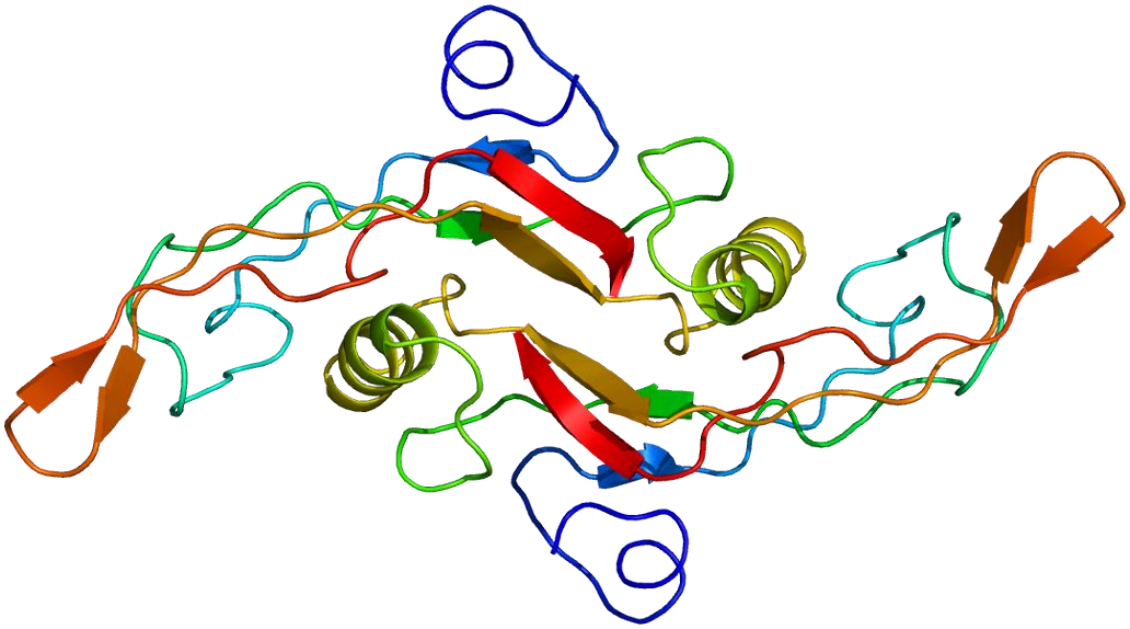


Figure 1. TGFβ1 protein.

Three-dimensional structure representation of TGFβ1. Notice that in each monomer there is a single α helix and six βsheets (Taken from the free source Wikimedia commons) (Hinck et al., 1996).

1.4.1.3 TGFβ1 superfamily

TGFβ1 belongs to a superfamily that includes hundreds of members in all animal species. Forty-five TGFβ1 related proteins have been found in humans, and most have orthologous proteins in all vertebrates and a number of invertebrates. TGFβ1 proteins found in invertebrates include the

family located in the decapentaplegic locus in *Drosophila*, which shows significant homology with vertebrate members of the TGFβ1 family (Padgett et al., 1987). This suggests that the TGFβ1 superfamily has a very early ancestor in evolution and a widespread occurrence of signalling factors in practically all metazoans, from *poriphera* (Suga et al., 1999) and *nematoda* (Lin and Wang, 1992) to large mammals (Derynck et al., 1985).

The growth factors that belong to the TGFβ1 family are subdivided into four subfamilies that have consistent, conserved functions in development and immunoregulation in both invertebrates and vertebrates. The first group is the decapentaplegic-Vg1 related (DVR) family that includes invertebrate BMPs (bone morphogenetic proteins), which are known for their involvement in bone formation and enlargement (Wang et al., 1988, Wozney et al., 1988). In mammals, more than 15 members of this family have been described, with all of them strongly involved in development (Hogan, 1996). The second group is the GDFs (growth and differentiation factors), present in both vertebrates and invertebrates, which are key regulators of cell differentiation in embryogenesis and adult tissue (McPherron et al., 1997).

The third and fourth groups are the activins and inhibins, which have important endocrine and paracrine roles in mammals. These two factors are mostly known for their regulation of FSH (follicle stimulating hormone) secretion in the anterior pituitary gland (Ying, 1987). As all other members of the TGFβ1 superfamily, activin and inhibin exert strong paracrine and autocrine regulation over cell growth, differentiation, and death (McDowell and Gurdon, 1999). It is important to note that these two proteins are not encoded by different genes. Rather, they are encoded by the inhibinβ gene, with different homo- or hetero-dimers resulting in different types of functional activins and inhibins. Homologous proteins have also been found in invertebrates and studied in *Drosophila* (Kutty et al., 1998).

TGFβ1 protein subfamily members are widely-spread multifunctional factors. Some of the most well-known roles of TGFβ1 are regulation of development (Kimelman and Kirschner, 1987), tissue repair and remodelling (Roberts et al., 1986), hematopoiesis (Ohta et al., 1987), and immunosuppression and anti-inflammation (Gamble and Vadas, 1988). Five isoforms of TGFβ1 have been characterized in vertebrates: TGFβ1, TGFβ2, and TGFβ3, which are found in mammals, and TGFβ4 and TGFβ5, which are found in birds (Choi et al., 1999) and amphibians (Kondaiah et al., 2000). A TGFβ1 gene was also described in fish from the *Salmonidae* family (Hardie et al., 1998). All TGFβ1 genes are homologous proteins that are believed to come from an ancestral gene duplication in older vertebrates (Herpin et al., 2004).

1.4.2 TGFβ1 signalling pathway

1.4.2.1 TGFβ1 receptors

TGFβ1 receptors are widespread serine/threonine kinase transmembrane receptors (Mathews and Vale, 1991). These receptors are divided into two subcategories, I and II, that are not related; both subcategories have activin, BMP, and TGFβ receptors (Herpin et al., 2004). Another subcategory of receptors, the betaglycan type II receptors, does not have intrinsic signalling functions, but regulates the binding of ligands to active receptors (Cheifetz et al., 1987). Both subtypes of active receptors are found throughout evolution. These receptors have a high degree of sequence conservation from arthropods and molluscs to higher order vertebrates (ten Dijke et al., 1993, Arora et al., 1995, Letsou et al., 1995, Marques et al., 2002). Through their highly conserved intracellular domains, they phosphorylate a specific class of transduction proteins, the SMADs. In mammals, there is a wide distribution of ALK1 (activin-like receptor type

I), ALK2, BMPR1, TβR1, and TβRII receptors, and some invertebrates also have Daf-1 receptors (Georgi et al., 1990).

1.4.2.2 Transduction effectors of the TGFβ1 family

1.4.2.2.1 SMAD transduction pathway

Transduction effectors of the TGFβ1 family are proteins known as SMADs. They were originally called *mad* in *Drosophila* and *sma* in *C. elegans* (Raftery et al., 1995, Savage et al., 1996), before the become known as SMAD. After the discovery of this family of transductors, 8 homologues were detected in mammals (Graff et al., 1996, Hoodless et al., 1996, Liu et al., 1996). SMADs are the main intracellular component of the TGFβ1 transduction pathway. Surface receptors phosphorylate regulatory SMADs (R-SMADs) after nuclear translocation these proteins become involved in gene transcription. SMAD1, SMAD5 and SMAD8 are responsive to BMP, and SMAD2 and SMAD3 are responsive to TGFβ1 receptors. Another functional type of SMAD is the common-partner SMAD (co-SMAD), as SMAD4. Co-SMAD dimerize with R-SMADS before nuclear translocation and work together as transcription factor. The last type are the inhibitory SMADS (I-SMADs), which are SMAD6 and 7 (Shi and Massague, 2003). I-SMADs dimerize with R-SMADS inhibiting the activation and nuclear translocation of these proteins. SMADs can be found in either the cytoplasm or the nucleus, although R-SMADS tend to be localized in the cytoplasm more than co-SMADS, and I-SMADS are more homogenously distributed (Pierreux et al., 2000, Watanabe et al., 2000).

Once a TGFβ1 receptor is activated, SMAD complexes form and accumulate in the nucleus. This happens through an active export process that is regulated by a nucleoporin complex. When R-SMADS are dephosphorylated, they return to the cytoplasm where they are phosphorylated again if the receptor is still active. Pathway termination is mediated by I-SMADS,

the most studied of which is SMAD7. The I-SMADs form dimers with R-SMADs and label them for polyubiquitination by the protein Arkadia, an E3-ubiquitin ligase (Koinuma et al., 2003, Miyazono and Koinuma, 2011) leading them to degradation.

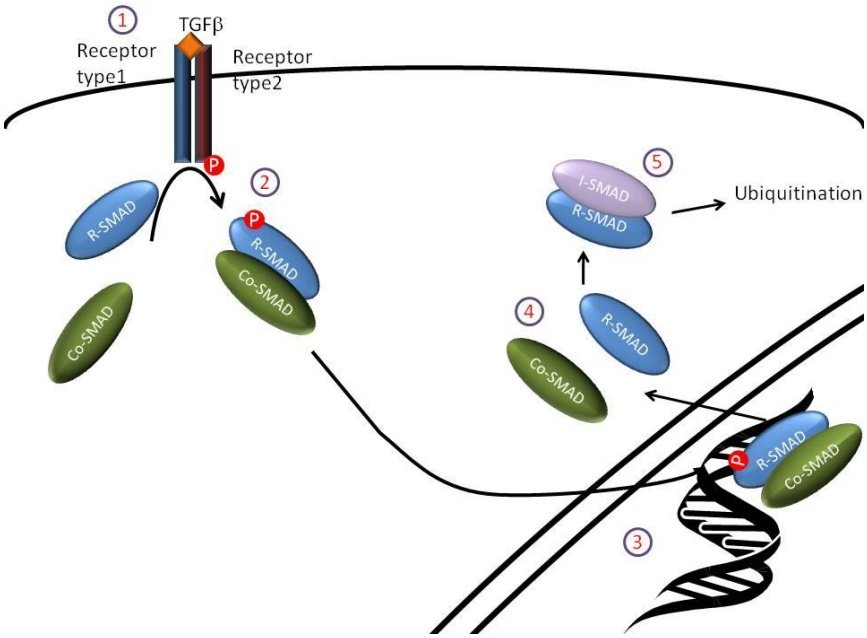


Figure 2. SMAD signalling pathway.

SMAD proteins are a homologous group of factors whose activity enhances or represses the transcription of specific target genes. (1) When the ligand reaches a receptor, a heterodimer is formed with a different receptor type, and transphosphorylation occurs. (2) This process ends with the activation of an R-SMAD, which forms a heterodimer with a co-SMAD. (3) The activated complex is shuttled to the nucleus where it activates or represses gene expression. (4) The complex is eventually desphosphorylated and returns to the cytoplasm, where the cycle either restarts or (5) the SMAD proteins are ubiquitinated and destroyed in a reaction mediated by an I-SMAD.

1.4.3.2.2 Other signalling pathways

In addition to the SMAD signalling pathway, after activation by its ligand, TβRII and TβRI receptors can activate also other signalling pathways including ERK-MAPK (Mu et al., 2012) and the FAS c-Jun N-terminal kinases (JNK) dependent proapoptotic protein Death-associated protein 6 (DAXX) (Perlman et al., 2001). Binding of TGFβ1 induces the formation of a homodimer or a

heterodimer with the TβRI and TβRII receptor. TβRI is a tyrosine kinase receptor and TβRII is mostly a threonine/serine kinase receptor. The relative abundance of these receptors in any tissue determines the degree to which the SMAD pathway is activated (viaTβRI) vs. the ERK-MAPK pathway (via TβRII) (Chin et al., 1999a). TGFβ1 activation of the ERK pathway results in the activation of p38MAPK as TβRs are inhibited by SMAD7. p38MAPK coadyuvates gene transcription within the AP1 complex. This pathway up-regulates expression of p53, which leads to apoptotic cell death in epithelial or liver tissue (Zhang et al., 2006). ERK kinase is also activated by the TβRII, this pathway activates PI3K and the mammalian target of rapamycin (mTOR). This pathway strongly affects proliferation and apoptosis (Lamouille and Derynck, 2007, 2011) and is known to be active in response to Aβ deposition (An et al., 2003).

DAXX is a transduction protein commonly activated by the Fas receptor. After activation DAXX activates the JNK pathway leading to cell death (Yang et al., 1997). However, more recently it was discovered that TGFβ1 can also activate DAXX. This leads to activation of the JNK pathway, which enhances transcription of p53 and triggers apoptosis (Perlman et al., 2001). This mechanism is best characterized as part of the anti-tumoral characteristic of TGFβ1, however, it is also relevant for neurodegeneration in the brain. Resident microglia cells become active as part of the inflammatory response. However, in chronic neurodegerative disease active microglia can negatively impact brain structure (see 1.5.1). Recent findings in DAXX function in the brain suggest that this signalling molecule generates an important control over active microglia survival, ablating them in an apoptotic process controlled by Ste-20-like kinase 1 (MST1), an apoptotic transcription factor (Yun et al., 2011). This mechanism could represent an alternative way in which TGFβ1 protects against neurodegeneration, but more research in this signalling pathways is necessary.

1.4.3 Basic biological effects of TGFβ1

1.4.3.1 TGFβ1 ligands regulate cell cycle.

TGFβ1 regulate the cell cycle through actions of the SMAD proteins. The mechanism of these actions could be related to the regulation of the cyclin protein system (Keski-Oja and Moses, 1987). Some cyclin-dependent kinases (CDKs) have been identified as targets of SMAD transcription. An example is p21Cip1, a small peptide that responds in stromal cell culture to addition of TGFβ1 in the media by binding and inhibiting CDKs, thereby preventing them from triggering cell proliferation (Zhou et al., 2003). This inhibitory peptide has also been found to prevent proliferation in glial cells and has been described in glioblastoma lines as leading to the arrest of the cell cycle following stimulation by TGFβ1 and transcription by SMAD proteins (Seoane et al., 2004). A similar peptide, cyclin-E-dependent p15Ink4B, which inhibits CDK4/6, is known to be responsive to SMAD transcription after TGFβ1stimulation in lung epithelial cells. This transcription leads to cell cycle arrest during the G1 stage by induction of TGFβ1 ligands. However, this also requires the presence of other co-adjuvants like Ink4 Cdk inhibitor and Cip/Kip (Reynisdottir et al., 1995).

1.4.3.2 Involvement of TGFβ1 family members in behaviour

Contrasting with the strong body of evidence relating TGFβ1 to development and immunity, there are very few studies relating it to behaviour. Some studies suggest that TGFB1 superfamily members could be involved in cognitive functions in both invertebrates and mammals. The first of these studies reported that when *Aplysia californica* was treated with recombinant TGFβ1 in the pleural-pedal ganglia, excitatory postsynaptic potentials elicited from sensory

neurons increased, therefore facilitating the elicitation of the gill-withdrawal reflex. This effect was found to be co-dependent on serotonergic transmission (Zhang et al., 1997). Further examination of this effect revealed that TGFβ1 strongly down-regulated the distribution of synapsin congregating in the dendritic processes of sensory neurons, apparently facilitating synaptic transmission (Chin et al., 2002). Another phenomenon associated with synaptic facilitation was the outgrowing of dendritic process after chronic TGFβ1 treatment in Aplysia motor cells (Wainwright et al., 2002). Similar effects of TGFβ1 have not been observed in mammals, although one study reports that the addition of TGFβ2 to mouse hippocampal slices resulted in a minor increase in excitatory postsynaptic currents. This mild effect is associated with CREB phosphorylation (Fukushima et al., 2007). Recent data, also suggest that through p38MAPK activation, TGFβ1 can lead to CREB phosphorylation and nuclear transduction. As CREB is a key protein in memory regulation, this data open the door to future pursuits in the direct influence of TGFβs in behaviour regulation. However, this data comes from immune cells is still not known if the same mechanism applies in the brain (Jang et al., 2011, Huang et al., 2012).

Compared to TGFβ1, activin is a stronger candidate for regulating behaviour. It has been proposed that this endocrine factor may facilitate memory and exert other behavioural effects in mammals. Activin mRNA increases after LTP induction in neurons (Inokuchi et al., 1996), and this is followed by increases in the number of synaptic contacts and the dimensions of dendritic spines (Shoji-Kasai et al., 2007). This finding suggests that activin may modulate behavior by facilitating the maintenance of long-term memories. This hypothesis is supported by a study showing that activin antagonists disrupt long-term memory (Ageta et al., 2010).

1.5 TGFβ1 in the diseased brain

TGFβ1 is present in the human brain as well as in the brains of all vertebrates (Lindholm et al., 1992). Homologous proteins are also present in invertebrate animals (Burt and Law, 1994, Chin et al., 1999b, Herpin et al., 2004). TGFβ1 is secreted by neurons and glia and has receptors on both cell types (da Cunha and Vitkovic, 1992, Lefebvre et al., 1992). The signalling effectors of TGFβ1—TGRβI receptors and the family of the cytoplasmic signalling proteins SMADs—are also found in the brain (Lin et al., 2005). Similar to many cytokines, TGFβ1 is regulated in pathological situations. The role of TGFβ1 in cognition or behaviour remains largely unknown.

1.5.1 TGFβ1 as an anti-inflammatory factor in the human and rodent brain

Under basal conditions, TGFβ1 in the mouse brain is scarce, and some research groups have failed to detect it using *in situ* hybridization (Wilcox and Derynck, 1988). However, it has been cloned from the rat hippocampus (Nichols and Finch, 1991), where it is down-regulated by treatment with the anti-inflammatory agent corticosterone. It has also been reported that after a cortical lesion, there is significant increase in the amount of TGFβ1 mRNA surrounding a scar, mostly produced by macrophages and activated microglia, although certain amounts are also produced by astrocytes (Lindholm et al., 1992).

1.5.1.1 TGFβ1 as microglial regulator.

Inflammatory responses in the central nervous system (CNS) are involved in the pathological development of several degenerative diseases, and TGFβ1 plays an important role as an anti-

inflammatory factor. The key histological component of inflammatory responses in neurodegenerative diseases is microglial cells. The inflammatory process is believed to play an important role in the pathology of several diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), prion diseases (TSEs), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). The inflammatory process is mediated by active microglia, which are cells of hematologic origin, but residents of brain parenchyma. These cells usually respond to tissue damage and remove damaged or dead cells by phagocytosis. The chronic activation of microglia may be a major cause of neuronal damage through the release of pro-inflammatory cytokines, reactive oxygen species, proteinases, and complement proteins. Therefore, regulation of microglia activation is considered an important strategy of neurodegenerative disease therapy (Dheen et al., 2007).

Several authors have reported that TGFβ1 is an important regulator of microglial activation. The general consensus is that TGFβ1 acts as an inhibitor of microglial activation both *in vitro* and *in vivo*, as opposed to tumor necrosis factor α (TNF α) and macrophage/granulocyte colony stimulation factor (GM-CSF)(von Zahn et al., 1997). It is suggested that the molecular mechanism of this inactivation is inhibition of the transcription factor class II transactivator (CIITA), which is a key factor in the antigen presentation process that leads to microglial activation and phagocytic activity (Hailer et al., 1998, O'Keefe et al., 1999). Other work shows that TGFβ1 also regulates the major histocompatibility complex I (MHC I) in microglia (Milner and Campbell, 2003).

The process by which TGFβ1 inhibits microglia activation is by down-regulation of cyclooxygenase-2 (COX-2). COX-2 is an inducible inflammatory enzyme that is a common target of non-steroidal anti-inflammatory drugs (NSAIDs) (e.g., aspirin). This protein stimulates oxidatory burst and apoptosis by prostaglandin secretion. Some inflammatory cytokines like Interleukin-1 (Il-1) and interferon gamma (IFN-γ) activate the expression of COX-2 in microglia, leading these cells to

reach an active state. Although it is not clear whether TGFβ1 inhibits COX-2 expression directly, it does interfere with the ability of inflammatory cytokines to promote COX-2 expression. This phenomenon is believed to be caused by the TGFβ1 signalling pathway interfering with other cytokine expression (Basu et al., 2002, Herrera-Molina and von Bernhardi, 2005). Another process by which TGFβ1 can block microglia activity is down-regulation of the expression of integrins, which are molecules fundamental for the signalling and adhesion required by microglia cells to become active and display their ameboid-like morphology (Milner and Campbell, 2003).

TGFβ1 induces apoptosis of microglia cells. It is proposed that the TGFβ1-induced apoptosis is a process independent of the members of the BCL-2 family. It is also proposed that a family of caspase inhibitors, the rat inhibitor-of-apoptosis protein-1 (RIAP-1) and RIAP-3, are inhibited by TGFβ1 signalling, therefore causing microglial death (Xiao et al., 1997, Jung et al., 2003). Other data confirm an influence of TGFβ1 on microglia but report that TGFβ1 exerts protection against the death of microglial cells in culture (Kim et al., 2004).

1.5.1.2 TGFβ1 role in astrogliosis.

Astrocytes respond to different forms of CNS insult, including increase of TGFβ1 levels, through a process known as astrogliosis. During astrogliosis, glial cells grow in size, project numerous new processes, and become reactive to inflammatory insult. Substantial progress has been made in determining the functions and mechanisms of astrogliosis and identifying the roles of astrocytes in CNS pathology. Reactive astrogliosis is not an all-or-nothing phenomenon, but can be observed along a gradient of changes that occur in a context-dependent manner (Sofroniew, 2009). These changes vary from alterations in gene expression to cell hypertrophy and long-lasting scar formation with rearrangement of tissue structure (Sofroniew, 2009, Sofroniew and Vinters).

A key indicator of astrocyte activation is the increase in expression and accumulation of the intermediate filament glial fibrillary acidic protein (GFAP) (Sofroniew and Vinters, 2010). Treatment of astrocytes *in vitro* with TGFβ1 has been shown to produce little morphological change but significantly increases GFAP mRNA and protein levels (Reilly et al., 1998). Furthermore, it is suggested that young neurons, mostly during the embryonic period, are prompted to secrete TGFβ1 that induces GFAP expression in astrocytes through the signalling pathway triggered by their own membrane TGFβ1-RII. TGFβ1 secreted by an astrocyte would autoregulate the expression of GFAP. Neurons at different developmental stages and from different regions can exert dissimilar effects on the expression of GFAP in astrocytes; for example, cultures from neocortical origin are more susceptible to expressing GFAP in response to TGFβ1 (de Sampaio e Spohr et al., 2002, Sousa Vde et al., 2004).

Another effect of TGFβ1 on astrocytes is an increase in glutamatergic toxicity in co-cultures with neurons (Brown, 1999). It has been suggested that under the influence of TGFβ1, astrocytes decrease their capability of metabolizing glutamate and therefore induce excitotoxicity in neurons. This effect was demonstrated by effectively blocking cell death with the use of NMDA receptor antagonists. On the other hand, in co-culture with the neural cell line GT1-7, astrocytes mediate neuron survival through a process dependent on AP-1 and c-Jun kinase (Dhandapani et al., 2003).

TGFβ1 blocks neuronal cell death by a process mediated by astrocytes. A key discovery of the relationship between TGFβ1 and astrocytes during the inflammation process is that TGFβ1 is able to induce the serpin family member plasminogen activator inhibitor (PAI-1). PAI-1 is only secreted by astrocytes (Docagne et al., 1999) and can stop apoptotic cell death in the surrounding neurons through an inhibitory interaction with the tissue plasminogen activator (t-PA), the trigger for a proteolytic

cascade that precedes apoptotic cell death in the hippocampus (Wang et al., 1998). This system, known as the PAI-1/tPA axis, is medically relevant as it prevents the damage caused by stroke (Tsirka, 1997).

1.5.2 TGFβ1 expression and function in neurodegenerative diseases

For approximately two decades, it has been known that TGFβ1 is differentially expressed in human neurodegenerative disease and animal disease models. The expression and possible relevance of TGFβ1 has been described for many common neurodegenerative diseases, from acute diseases like stroke to chronic disease like PD, ALS, MS, HD, AD, and TSE. Even congenital diseases like Down syndrome (DS) involve regulation of TGFβ1.

1.5.2.1 TGFβ1 in multiple sclerosis

TGFβ1 is known to be a key regulator in multiple sclerosis (MS), although the exact nature of its participation is not well understood. MS is a neurodegenerative disease with an etiology entirely related to immune system regulation and, therefore, it is important to understand the role of TGFβ1 in this brain pathology. The most commonly described roles of TGFβ1 in mammals are those involved in immune system regulation, and it has been widely considered an anti-inflammatory cytokine. Its receptors are commonly found in most cells of the immune system, and several immune functions have been directly related to TGFβ1 expression (Mirshafiey and Mohsenzadegan, 2009). TGFβ1 signalling is involved in the inhibition of cytolytic processes and T cell differentiation and activation; these cells are known for their role during the demyelination process, which is important in MS pathology.

There is no clear evidence of the precise role for TGFβ1 during MS onset. As previously discussed, TGFβ1 regulates proliferation and activation of macrophages, microglia, and T cells. However, these cells important for oligodendrocytes' myelination, a process dependent on growth

factor secretion. There is evidence that the secretion of TGFβ1 by these immune cells may be leading this process (Diemel et al., 2003). Some work has been done with a mouse model of MS known as experimental allergic encephalitis (EAE). Treatment with an anti-TGFβ1 antibody aggravates the severity of the disease in these mice, and treatment with TGFβ1 alleviates disease symptoms (Johns et al., 1991, Racke et al., 1991).

On the other hand, it has been reported that TGFβ1 in the CNS may be critical in promoting MS and EAE. TGFβ1 and its receptors are expressed in CNS inflammatory lesions of MS patients and in various animal models of the disease, which suggests a role for this cytokine in the MS disease process. The recent identification of TGFβ1 as a crucial factor for the differentiation of TH17, an IL-17-producing T cell subset that is believed to be autoreactive and disease-promoting, suggests a pathogenic role of TGFβ1 in EAE (Luo et al., 2007, Xiao et al., 2007).

1.5.2.2 TGFβ1 in amyotrophic lateral sclerosis

TGFβ1 also is present in the development of other scleroses that attack the CNS. Amyotrophic lateral sclerosis (ALS) is a disease characterized by degenerating spinal cord motor neurons and their associated white matter and motor cortex. There seems to be a direct relationship between the progression of ALS and levels of TGFβ1 in patients' plasma(Houi et al., 2002). The measurement of TGFβ1 in patients with different degrees of disease progression has revealed a direct correlation between the stage of the disease and TGFβ1 titers (Katsuno et al., 2011).

Other groups have reported the same correlation between ALS stage and TGFβ1 levels in cerebrospinal fluid (CSF) (Ilzecka et al., 2002, Hensley et al., 2003). Furthermore, high levels of TGFβ1 were found in superoxide dismutase 1 (SOD1) over-expressing mice -a model of ALS-. In this mouse model, high levels of TGFβ1 protein were also found in the spinal cord (Hensley et al., 2003). Despite this body of evidence, the role of TGFβ1 in ALS remains elusive. During the progression of

the disease, severe inflammation occurs in response to the massive death of nervous tissue; therefore, the over-expression of TGFβ1 could be a homeostatic response. In ALS mouse models there is astrogliosis, and this could also be related to an elevation of TGFβ1 levels (Deniselle et al., 1999).

1.5.2.3 TGFβ1 in Parkinson's disease

Parkinson's disease (PD) is a chronic neurodegenerative pathology that is characterized by the massive death of dopaminergic neurons of the nigrostriatal pathway, which compromises fine voluntary movements. TGFβ1 is elevated in dopaminergic striatal regions as well as the CSF of PD patients compared to normal individuals (Mogi et al., 1994, Mogi et al., 1995). In the case of PD, it is possible that TGFβ1 exacerbates the neurodegenerative process. When added in high levels to mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the neurodegeneration process increases, and the same happens when it is over-expressed with a viral vector in the midbrain of healthy mice, suggesting that TGFβ1 can be a risk factor for PD development (Sanchez-Capelo et al., 2003). Conversely, other members of the TGFβ1 family have been found to be protective and drug targets for PD treatment, such as TGFβ2 and TGFβ3 (Lin et al., 1993, Tomac et al., 1995).

1.5.2.4 TGFβ1 in Alzheimer's disease.

One of the most studied roles of TGFβ1 is in Alzheimer's disease (AD), and while its participation in the disease is widely accepted, its specific role remains elusive. When compared to normal brains, TGFβ1 mRNA was increased 3-fold in AD patient brains post-mortem, and levels of TGFβ1 were strongly correlated with the degree of cerebral amyloid angiopathy (CAA) (Wyss-Coray et al., 1997). The authors of this study concluded that TGFβ1 may be important in AD amyloidogenesis. Later, it was found that the CSF of AD patients contains increased levels of TGFβ1 protein compared to normal individuals (114 vs. 83 pg/ml) (Zetterberg et al., 2004). This finding in humans is consistent with the

observation that amyloid deposition in human amyloid precursor protein (hAPP) over-expressing mice induces TGFβ1 secretion in astrocytes and neurons (Apelt and Schliebs, 2001). Also, some researchers have suggested a possible polymorphism in TGFβ1 as a risk factor for the development of AD, but the results of these studies were either negative (Araria-Goumidi et al., 2002, Hamaguchi et al., 2005) or inconclusive (Luedecking et al., 2000).

1.5.2.5 TGFβ1 in prion disease

TSE is caused by transmissible misfolded proteins capable to self-replicate. Misfolded proteins result in cell death, which causes the spongiform encephalopathy. TGFβ1 is up-regulated during prion disease, and some scientists consider this cytokine to be potentially responsible for the pathology of this disease (Stoeck et al., 2006). Prion disease became well known in the 1990s because of several cases of "mad cow disease", and the Creutzfeldt-Jakob version of the disease was found to attack cows and humans. The disease is poorly understood and shows such a slow progression that it is difficult to detect and track properly. A murine model of this disease has been studied, and it has commonly been observed that the disease causes an atypical inflammation in the brain characterized by microgliosis and astrogliosis and the absence of proinflammatory cytokines IL-1β and IL-6. Instead of the interleukins, cytokines TNF-α and TGFβ1 are over-expressed. It is not known if these cytokines take the lead role in the inflammation process or have an anti-inflammatory effect (Araria-Goumidi et al., 2002). This has led to the idea that TGFβ1 could be at least partially responsible for the common symptoms of this disease. In fact, a study suggests that TGFβ1 is responsible for the amyloid deposition in neurons after prion infection and replication. A common feature in all models of prion disease is that microglia appear to decrease the effectiveness of prions. As previously discussed, TGFβ1 is a strong controller of microglia activation and survival and might prevent microglia from clearing the amyloid depositions (Baker et al., 1999).

1.5.2.6 TGFβ1 in Huntington's disease

In contrast to other neurodegenerative diseases, TGFβ1 is down-regulated in Huntington's disease (HD). Both in symptomatic and asymptomatic patients, the levels of TGFβ1 in plasma are low. These reduced levels were also observed in mouse models of HD in both plasma and cortical neurons. When astrocytes were cultured and transfected with the mutated *huntingtin* gene (HTT), they also showed reduced levels of TGFβ1 mRNA and protein. Together, these data suggest that TGFβ1 expression is inhibited in this disease, likely by the same mutation that causes the disease. The most likely consequence of this odd TGFβ1 behaviour is the acceleration of the neurodegenerative process (Battaglia et al.).

Table 1. TGFβ1 and neurodegenerative disease

Involvement of TGFβ1 in neurodegenerative diseases		
Pathology	*TGFβ1 secretion*	*Proposed role of TGFβ1*
Multiple sclerosis	↑	Preventing microglia to destroy myelin; symptomatology alleviation (Johns et al., 1991, Racke et al., 1991)
Amyotrophic lateral sclerosis	↑	Enhancement of astrogliosis (Deniselle et al., 1999)
Parkinson's disease	↑	TGFβ1 could exacerbate symptomology associated with MPTP onset of Parkinsonism (Sanchez-Capelo et al., 2003). TGFβ1 family members could cooperate in the prevention of nigrostriatal neurons apoptosis (Lin et al., 1993, Tomac et al., 1995).
Alzheimer's disease	↑	Increased CAA pathology (Wyss-Coray et al., 1997) clearance of Aβ deposition (Wyss-Coray et al., 2001)
Prion disease	↑	Inactivation of microglia and prevention of Aβ clearance (Baker et al., 1999).
Huntington's chorea	↓	Decreased by mutant Huntingtin expression, decrease exacerbates symptomatology (Battaglia et al., 2010)

1.5.3 TGFβ1 as an anti-neurodegenerative factor.

There is ample evidence that TGFβ1 is regulated in the brain in response to a variety of pathological conditions. A common characteristic of all neurodegenerative diseases is the high number of dead neurons found in every case, albeit in different brain regions. *In vitro* studies show that TGFβ1 regulates microglia activity, thereby preventing death of both astrocytes and neurons. It remains to be determined whether this phenomenon also occurs *in vivo*.

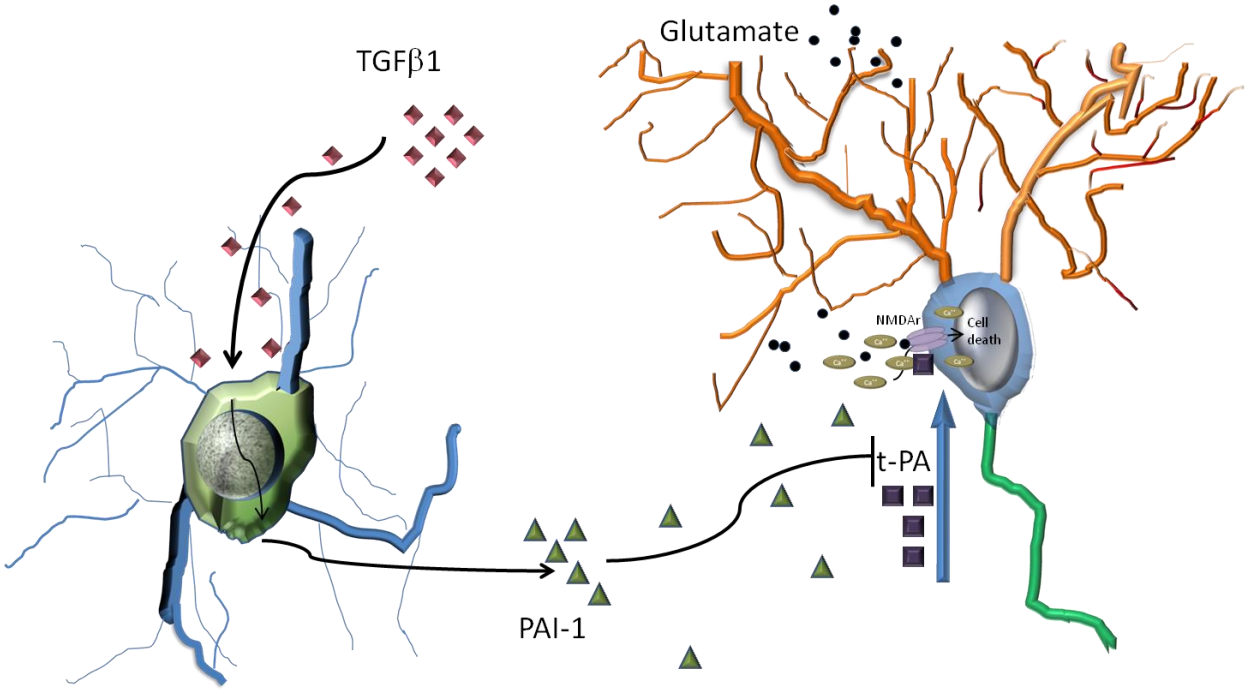
1.5.3.1 TGFβ1 response to stroke and the proposed role of PAI-1

1.5.3.1.1 Stroke

TGFβ1 is secreted in the brain after acute stroke, and many studies have shown that TGFβ1 prevents neuronal death in a process mediated by astrocytes and the factor PAI-1 (plasminogen activator inhibitor-1) (Henrich-Noack et al., 1996, Buisson et al., 2003, Kim and Lee, 2006). When TGFβ1 expression was analyzed in post-ischemic rats following a double vessel occlusion, it was found that TGFβ1 mRNA was dramatically elevated in the hippocampus for at least 21 days after surgery (Lehrmann et al., 1995). When the peptide was injected directly into the ventricles or hippocampus in post-ischemic rats, a strong neuroprotective effect in the hippocampus was observed. Nonetheless, a higher dose of TGFβ1 failed to produce the same improvement (Henrich-Noack et al., 1996). It is also notable that the expression of TGFβ1 following the vascular accident mostly came from activated microglia and macrophages, thus suggesting that the secretion could be regulating the immune reaction to some extent (Lehrmann et al., 1998).

Whether the regulation exerted by TGFβ1 in the brain after stroke is positive or negative was tested by injecting a TGFβ1 antagonist (TGFβ1 receptor II in a soluble inactive form) directly into the affected area in rats. The result of these experiments was a 3.5 fold increase in the size of the damaged brain area after the stroke. Aiming to understand these results, and using the same antagonist, the

researchers discovered that the apoptosis prevented by TGFβ1 is elicited by NMDA receptor-associated excitotoxicity in cultured cells (Ruocco et al., 1999). In humans, certain polymorphisms of



TGFβ1 were found to constitute a risk factor for stroke and vascular dementia (Kim and Lee, 2006).

After activation of the TGFβ1 signalling pathway, astrocytes (left) release the antiproteolitic factor PAI-1, which inhibits the activity of t-PA. When not inhibited, t-PA cleaves the NR1 subunit of NMDA receptors, allowing a high current of calcium to enter the cell after glutamate stimulation and triggering cell death (Buisson et al., 2003).

1.5.3.2 Role of PAI-1

TGFβ1 directly triggers the transcription of PAI-1 in cultured cells by downstream regulation of TGFβ1 signalling cascade transcription factors (Westerhausen et al., 1991). As PAI-1 is an inhibitor of t-PA-induced apoptosis, it is a strong candidate for regulating the protective effects of TGFβ1.
Figure 6. Regulation of the PAI-1 axis

Actually, TGFβ1 protects neurons *in vitro* from NMDA-, AMPA-, and kainite-induced apoptosis in a manner that is also dependent on PAI-1 but only in the presence of astrocytes. When only neurons are

present, TGFβ1 can even be a pro-apoptotic growth factor (Buisson et al., 1998). Recombinant t-PA has been used as the only FDA-approved drug to remove embolus after stroke (Buisson et al., 2003). However, t-PA exerts apoptosis in neurons in a process that is dependent on NMDA receptor-mediated calcium influx. As part of its proteolytic activity, t-PA cleaves the NR1 NMDA receptor subunit, facilitating calcium permeability and therefore excitotoxicity (Nicole et al., 2001). This can create the problem of increased neuronal death parallel to the embolus clearance. By blocking t-PA action via proteolysis inhibitors, PAI-1 could effectively block ischemia-related apoptosis caused by excitotoxicity (Wang et al., 1998, Nicole et al., 2001, Buisson et al., 2003).

Experiments performed in ischemic brains showed that PAI-1 is released in response to TGFβ1 secretion after stroke injury. TGFβ1 is only expressed in astrocytes and in parallel to t-PA, suggesting that the regulation of the t-PA/PAI-1 axis is the most probable way in which TGFβ1 promotes neural survival after a vascular accident (Docagne et al., 1999, Lin et al., 2006). However, the transduction pathway involved in this regulation remains mysterious (Buisson et al., 2003). Despite the lack of understanding its activity, it is now clear that the regulation of the t-PA/PAI-1 axis, and particularly the PAI-1 protective effect, is critical for the control of damage produced by cerebral ischemia. In fact, with the development of a t-PA and PAI-1 knockout mouse, it became clear that the absence of t-PA increased the size of the lesion, and the presence of PAI-1 considerably reduced the amount of damaged tissue (Nagai et al., 1999).

1.5.4 Neurodegeneration in the TGFβ1$^{-/-}$ mouse

The first effective genetic blocking of TGFβ1 was reported in 1992. From a DNA library from 129/SvJ mice, a targeting vector was designed that disrupted the mature peptide but not the promoter or precursor components. The purified construct was electroporated to ES D3 cells, and after a subcloning procedure, ES D3 cells positive for the deleterious TGFβ1 allele were injected into

blastocystes and later into pseudopregnant mice (Shull et al., 1992). Although the heterozygous mice were viable, their homozygous littermates had a nearly 0% survival rate after one month of age. Post-mortem analysis showed persistent conjunctivitis and gross inflammation of the digestive tract as well as severe cardiac failure. Some inflammation was observed in the brain, but not in all animals (Shull et al., 1992). Perhaps the most relevant finding of this study was that the primary cause of death was the focal influx of inflammatory cells, specifically lymphocytes and neutrophils, causing tissue damage and sometimes organ failure. Other researchers obtained similar results with a different knockout mice with the same deletion (Shull et al., 1992, Kulkarni et al., 1993).

It is also important to add that common pro-inflammatory cytokines including INF-γ TNF-α and C-C motif chemokine 3 (CCL3, formerly known as macrophage inflammatory protein 1α (MIP-1α)), were also elevated in TGFβ1$^{-/-}$ mice (Shull et al., 1992). In further studies with the same mice, it was observed that as much as 50% of the TGFβ1$^{-/-}$ mice were dead before birth. These fetuses were small and had necrotic areas, but the most serious problem was clearly the low levels of red blood cells and vascularization in extraembryonic tissue, defective yolk sac, endothelium differentiation, and flawed capilar tube formation (Dickson et al., 1995). Surprisingly, crossing these mice with the NIH genetic background prevented fetal death (Bonyadi et al., 1997).

A detailed study of the TGFβ1$^{-/-}$ mouse brain confirmed some basic assumptions derived from the analysis of TGFβ1 in neurodegenerative diseases and stroke. In this study, TGFβ1$^{-/-}$ mice were able to reach 21 days of age. At this point in development, they showed typical markers of neurodegeneration, mainly pronounced neuronal death not related to development and a severe increase in active microglia (Brionne et al., 2003). When primary cell cultures were established with neurons extracted from these mice, similar results were found, most notably a severe decrease of their viability that was effectively rescued with the addition of TGFβ1 (Brionne et al., 2003). TGFβ1$^{-/+}$ mice

had a normal life span and showed a milder neurodegenerative phenotype, but their response to excitotoxic injury was poor, with larger numbers of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) positive cells. This result confirmed previous research considering TGFβ1 as an active anti-neurodegenerative factor whose expression can down-regulate the effects of excitotoxic lesions and prevent inflammation in the brain under basal conditions (Brionne et al., 2003).

1.5.5 TGFβ1 over-expression models

TGFβ1over-expressing mice show several organ impairments, both in the periphery and CNS. However, some beneficial effects have also been found. Over-expression of TGFβ1 has been performed in different organs, such as the mammary glands where ductal development was severely impaired (Jhappan et al., 1993, Pierce et al., 1993) and the epidermis where the skin did not develop properly and death occurred 24 hours after birth (Sellheyer et al., 1993).

In 1995, the creation of a mouse line over-expressing porcine TGFβ1 driven by the GFAP promoter was reported. In this model, the expression of TGFβ1 was limited to the CNS, mimicking some disease-typical expression of TGFβ1 by astrocytes. The original idea was to reproduce the over-expression state of the cytokine found in common diseases and assess its possible role within them (Wyss-Coray et al., 1995). These mice had a hydrocephalic phenotype, characterized by consistent enlargement of the lateral ventricles and increased lamination of the blood vessels. As expected, the researchers found detectable levels of TGFβ1 mRNA from porcine origin in the brain of young transgenic mice. They also found a 2-fold increase of murine TGFβ1 mRNA in heterozygous mice and a 6-fold increase in homozygous mice. Using double immunostaining and primary cultures, it was shown that this excessive TGFβ1 expression was coming from the astrocytes in which the porcine insert had been secreted (Wyss-Coray et al., 1995).

TGFβ1 has been observed to enhance the expression of some extracellular matrix proteins in the brain (Tada et al., 1994, Kanaji et al., 1997). In TGFβ1 over-expressing mice, this finding was confirmed by the analysis of the extracellular matrix proteins fibronectin and laminin, whose mRNAs were increased 2-fold in heterozygous mice and 3-fold in homozygous mice. This explains the hydrocephalic phenotype found in the mice, considering that a thicker basal lamina would prevent the proper outflow of CSF (Wyss-Coray et al., 1995). Since the relationship between amyloid deposition and TGFβ1 secretion is not understood (Wyss-Coray et al., 1997), it would be of interest to determine the response of AD animal models to chronic over-expression of TGFβ1.

A cross between mice over-expressing TGFβ1 and mutated hAPP showed prevalent amyloid deposition around blood vessels and meninges. Also, higher levels of TGFβ1 expression occur in extreme cases of cerebral amyloid angiopathy in human brains (Wyss-Coray et al., 1997). Other studies show that in TGFβ1 over-expressing mice, the thickening of the endothelium is accompanied by aberrant morphology, with the formation of pilli in the vessels light and the formation of abnormal endothelial cells with condensed chromatin (Wyss-Coray et al., 2000). A previous study showed that despite the strong vascular deposition of Aβ, the chronic over-expression of TGFβ1 in hAPP over-expressing mice leads to a clearance of parenchymal deposition in the neocortex and hippocampus (Wyss-Coray et al., 2001). *In vitro* experiments suggest that this clearance is carried out by microglial activation and astrogliosis. The authors suppose that thickened basal lamina in blood vessels is a good seed for amyloid deposition, making it easier to drag to the lymph nodes to be cleared. Microglia could also be clearing Aβ deposits in neuritis, preventing the characteristic atrophy that could lead to cognitive impairment (Wyss-Coray et al., 2001).

Another characteristic of TGFβ1 overexpressing mice is reduced brain perfusion, a vascular phenotype similar to those of Alzheimer's disease (Gaertner et al., 2005). These abnormalities affect

brain blood flow, which negatively correlates with the levels of TGFβ1 on each brain. This decrease in blood flow also negatively correlates with the levels of thioflavin-S staining found in blood vessels, a common marker of Alzheimer's disease expressed in endothelium (Gaertner et al., 2005). Further analysis, showed that brain vasculature of TGFβ1 overexpressing mice have a reduced vessel reactivity, and more rigidity as is partially explained with higher levels of collagen IV in endothelium. Pia madre also shows a pathological thickness increase (Nicolakakis et al., 2011). All these data may explain the observed reduction in glucose uptake in TGFβ1 overexpressing mice (Gaertner et al., 2005). However, these vasculature deficiencies do not affect whisker responsiveness, cholinergic terminals in the hippocampus or spatial memory impairment (Nicolakakis et al., 2011).

Table 2. TGFβ1 over-expression effects

Modifications observed after TGFβ1 over-expression in the mouse brain	
Hydrocephalus	↑
ECM: Fibronectin	↑
ECM: Laminin	↑
ECM: Perlecan	↑
ECM: Capilar endothelial thickness	↑
Aβ deposition in capilars (crossed with hAPP mice)	↑
Aβ deposition in parenchyma and neuritis (Crossed with hAPP mice)	↓
Cell death after excitotoxicity (Kainate systemic induction)	↓
Pia madre tickness	↑
Blood vessels responsivness	↓
Glucose uptake	↓
Thioflavin-S staining in blood vessels	↑

1.6 Adult neurogenesis: basic concepts and techniques

Since the neuron theory was fully established by Santiago Ramón y Cajal, it was long thought that no new neurons are generated in the adult brain (Stahnisch and Nitsch, 2002). This misconception lasted for about sixty years, until evidence of the formation of newborn neurons in the mammalian olfactory bulbs were discovered by tritiated thymidine labelling (Altman and Das, 1965, 1966, 1967, Altman, 1969). Despite the strength of the technique used, this series of discoveries was broadly ignored for a long time, and it was not until the 1990s that this fact was generally accepted in the scientific community. Since then, adult neurogenesis has been studied in a wide variety of mammals (Kaplan and Hinds, 1977, Bayer et al., 1982) and birds (Goldman and Nottebohm, 1983).

1.6.1 Anatomy and timeline of adult neurogenesis

1.6.1.1 From stem cells to fully mature, functional neurons in the hippocampus

Neural stem cells (NSCs) are a cell type that is capable of long-term self-renewal and generation of different cells lineages. Identities of adult NSCs, which are believed to be largely quiescent *in vivo*, are currently under debate (Duan et al., 2008). The most accepted idea based on genetic tracing, pharmacological blocking, and morphological and immunohistochemical analysis, is that adult NSCs in the subgranular zone (SGZ) of the hippocampus are astrocyte-like cells expressing glial fibrillary acidic protein (GFAP) and exhibiting certain radial glial properties, similar to type B cells in the SVZ (Alvarez-Buylla and Lim, 2004). In the adult SGZ, a subpopulation of cells that express sex-determining region Y-box 2 (Sox2) and give rise to more $Sox2^{+}$ cells as well as neurons and astrocytes has been suggested to be adult NSCs (Suh et al., 2007). Although not definitive, this study provided evidence for the self-renewal and multipotency

of NSCs in the SGZ. It has been suggested that distinct cells in the adult SGZ can serve as NSCs under normal conditions or following dramatic injuries, similar to what has been observed in the SVZ (Zhang et al., 2007).

Continuous neurogenesis in the SGZ throughout adult life implies a sustained maintenance of an adult NSC population, which may be supported by several molecular mechanisms. Hedgehog signalling occurs in adult NSCs (Ahn and Joyner, 2005) and appears to be required for both the establishment and maintenance of proper NSC pools in the adult SGZ. An interesting finding is that expression of leukemia inhibitory factor (LIF) promotes the self-renewal of adult NSCs and also prevents their differentiation, leading to an expansion of the NSC pool in the adult SGZ but not an increase in neurogenesis (Bauer and Patterson, 2006).

During adult hippocampal neurogenesis, NSCs undergo different stages with proliferative capabilities, including NSCs proper, transient amplifying progenitors, and neuroblasts (Kempermann et al., 2004). A variety of physiological, pathological, and pharmacological stimuli have been shown to regulate SGZ cell proliferation during adulthood, such as physical exercise (van Praag et al., 1999b), seizures (Madsen et al., 2000), ischemia (Kee et al., 2001), and antidepressant treatment (Malberg et al., 2000). Marker immunolabelling has shown which cells are responsive to these stimuli. For example, seizures enhance proliferation of doublecortin (DCX)-positive neuroblasts but not nestin-positive progenitors (Jessberger et al., 2005), whereas selective inhibitors of serotonin reuptake target both nestin-positive progenitors and DCX-positive neuroblasts (Encinas et al., 2006). Growth factors, such as fibroblast growth factors (FGFs) (Palmer et al., 1995), epidermal growth factor (EGF) (Kuhn et al., 1997), and TGFβ1 (Buckwalter et al., 2006), also regulate cell proliferation. Neurotransmitters, such as GABA (Tozuka et al., 2005), also regulate adult neurogenesis.

Neural progenitor cells in the adult DG follow a precise maturational pathway from proliferation to neuronal maturation and functional incorporation. The whole process takes between 4 and 6 weeks according to studies using retrovirus labelling or immediate early expression to mark integration (Zhao et al., 2006). This time course of maturation is similar to that observed during initial hippocampus development (Esposito et al., 2005). The neuronal phenotype appears within the first days after proliferation. These immature neurons show small action potentials and are spatially restricted to the SGZ (Ambrogini et al., 2004). They do not have afferent synaptic contacts and display a high membrane resistance, which indicates a low number of ion channels (Ge et al., 2006).

One week post-mitosis, adult-generated neurons are localized in the inner granule cell layer. They do not have dendrite spines, and their dendritic arbours are limited to the granule cell layer (Ambrogini et al., 2004). By the third week, newborn neurons start to receive excitatory afferents and display repetitive action potentials with high frequency. They also show a lower voltage threshold for action potential elicitation and easily show LTP (Zhao et al., 2006). Detailed morphological analysis shows that dendritic spine formation is detectable at about 16 days (Zhao and others 2006). At this time point, is possible to activate the new neurons with kainate and observe Fos, zif268, or Homer1A expression in reponse (Jessberger and Kempermann, 2003); the same is observed after tetanic stimulation of the perforant pathway (Bruel-Jungerman et al., 2005).

Complete maturation of newborn neurons occurs 4 weeks after mitosis. This corresponds with the expression of GABAergic afferents and spiny dendritic arbours, which are fully branched and reach the most distal parts of the molecular layer (Esposito et al., 2005). The growth of axonal projections is characterized anatomically. Normally, at 17 days post-mitosis the axons reach their targets in the CA3 (Hastings and Gould, 1999). However, more detailed retrovirus work has shown

that some axons appear 10 days post-mitosis and continue to grow from days 16 to 56. It is interesting that axons are developed and functional before the formation of the first dendritic spines, similar to what occurs in the developing hippocampus (Jones et al., 2003, Zhao et al., 2006).

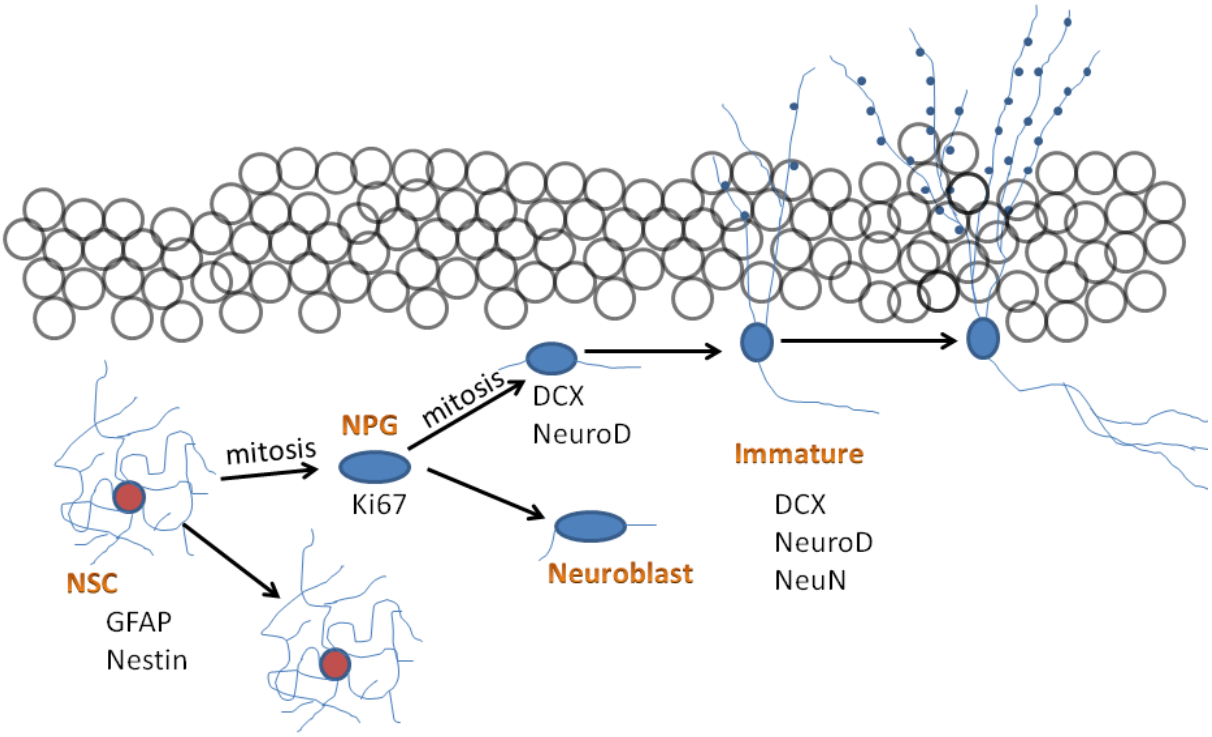


Figure 7. Proliferation in the dentate gyrus.

A population of neural stem cells (NSCs) persist in the SGZ of the DG. After mitosis, these cells generate neural progenitors that, after further division, form neuroblasts. If these cells survive, they can become immature granule neurons and finally mature into functional granule cells.

1.6.2 Methods to track newborn cells in the dentate gyrus.

1.6.2.2 Tritiated thymidine

It was by the method of using tritiated thymidine ([^{3}H]T) to reveal newborn cells by autoradiography that mitotic activity in the DG of rodents and other mammals was first detected (Baver and Altman, 1975). [^{3}H]T incorporates easily into DNA during the S phase of the cell cycle

without interfering with DNA stability. It also stays active for a very long time (tritium half-life is 12.5 years), and autoradiographic detection is very reliable without the possibility of false positives. Using this method, it was demonstrated that mature granule cells are generated in the rat DG not only during the first weeks of life but also during adulthood. By observing their ultrastructure, it was clarified that a month after injection, the [^{3}H]T-positive cells are actually neurons (Kaplan and Hinds, 1977). [^{3}H]T labelling has some important disadvantages including its price and exposure to radiation. However, in studies of new species or new brain areas in which neurogenesis has not previously been observed, [^{3}H]T is still in standard use because of its reliability (Harman et al., 2003).

1.6.2.3 Bromodeoxyuridine (BrdU)

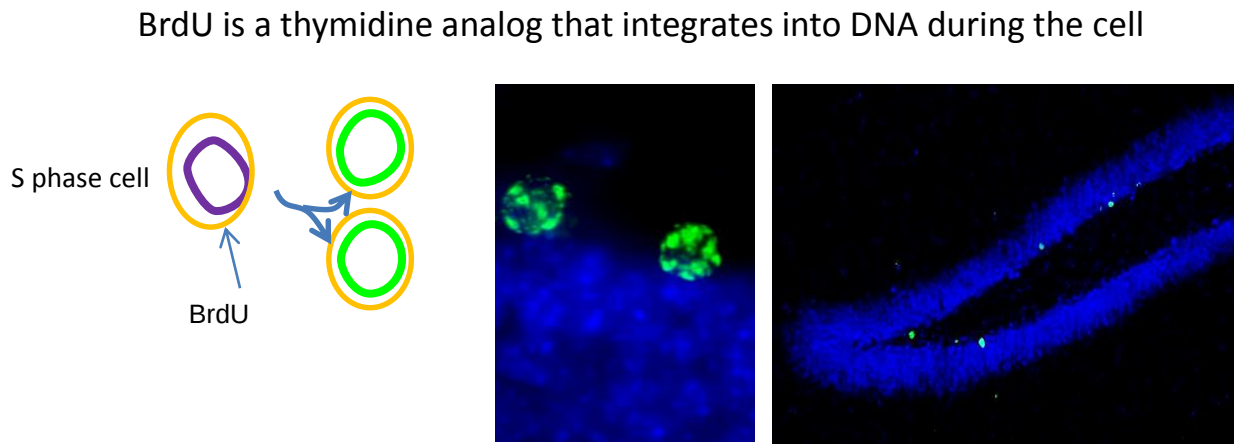
We used Bromodeoxyuridine in this thesis as it is a very reliable marker of mitosis in a time curse, being especially useful to detect the timepoint in which the studied neurons were born. Although it has been known since the 1950s that the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) incorporates into the DNA of living cells (Eidinoff et al., 1959), it was not until the 1990s that it became a widely used tracer for *in situ* analysis of cytogenesis with the use of specifically-generated anti-BrdU antibodies (Gratzner et al., 1976). The first report of the systematic use of BrdU to track adult neurogenesis was published in 1988 (Miller and Nowakowski). BrdU and other related analogs (i.e., IdU, CldU), can be used to mark cell proliferation, maturation, and survival, with even the possibility of using them concurrently when proper antibodies are available (Hayes and Nowakowski, 2002).

Nevertheless, BrdU and other nucleotide analogs have some limitations. To detect BrdU, tissue fixation is required, and therefore is not possible to use them in living cells or tissue. Another problem is that the amount of BrdU incorporated from a single injection is diluted to levels difficult

to detect after several rounds of cell division, as usually happens in the DG (Hayes and Nowakowski, 2002). An additional problem of using nucleotide analogs is that they are also incorporated into damaged DNA undergoing repair, although at a shorter time scale than during replication (Selden et al., 1993). Also, is known that some cells re-enter the cell cycle as a prelude to apoptosis leading to BrdU-positive apoptotic cells; this is reported after acute stroke in cortical neurons (Kuan et al., 2004).

Figure 8. BrdU labelling.

Labelling new neurons using BrdU



After treating animals with BrdU, the thymidine analog is incorporated into the DNA of mitotic cells. In the D,G it will be found in neural progenitors as well as immature and mature granule cells. After tissue fixation, BrdU can be observed by immunolabelling.

1.6.2.2 Endogenous markers.

Some endogenously expressed proteins are excellent markers for analyzing adult hippocampal neurogenesis. Some are specific to newborn neurons and therefore reliable markers of neurogenic activity (Ming and Song, 2005).

1.6.2.2.1 Ki67, marker of proliferation.

This marker is very specific and reliable to detect proliferation activity in the Dentate gyrus and we use is as a standard choice to track proliferation changes through the aging process. The Ki67 protein has become the gold standard for detection of cell proliferation in all tissue. Ki67 was discovered in the city of Kiel in Germany after a research group tried to generate specific monoclonal antibodies against nuclear fractions of highly replicating hocking cells (the number 67 comes from the number of the plate in which the antibody was generated) (Gerdes et al., 1983). This protein is expressed in the nucleus during the S phase of the cell cycle and on the surface of

chromosomes during mitosis. In G_0 cells, this marker is totally absent (Scholzen and Gerdes, 2000). The role of Ki67 is not completely understood, but it is known to be strongly associated with rRNA during proliferation (Bullwinkel et al., 2006). Several studies have used Ki67 immunolabelling to detect replicative activity in the adult DG, revealing 2000 to 5000 replicating cells at any given time in the adult mouse DG (Almgren et al., 2007). .

1.6.2.2.2 Doublecortin, marker of immature neurons.

Doublecortin (DCX) is a microtubule-associated protein that promotes microtubule bundling (Horesh et al., 1999). Its name comes from the syndrome of double cortex (*Lissencephaly*), in which immature neurons are incapable of migration (Caspi et al., 2000). DCX is expressed soon after the establishment of neuroblasts and is down-regulated until the entire dendritic arbour is assembled. In the DG, DCX expression is detected between 2 days and 3 weeks after replication (Brown et al., 2003). We used this marker as a standard method to detect and quantify immature neurons and mossy fibers in the dentate gyrus. We present data using this protein in appendixes I and II.

1.6.2.3 Transgenic reporter mice.

In recent years, genetic and cell culture technology have allowed the creation of transgenic reporter mice that allow visualization of neurogenesis in different ways. The advantage of using reporter mice to study hippocampal neurogenesis is that there is no need for exogenous invasive markers or endogenous markers that are restricted to only certain stages of neuronal development (Ming and Song, 2005). Several transgenic mice have been designed to express certain reporter genes driven by specific promoters. For example, adult mice expressing GFP under the control of the regulatory regions of the *nestin* gene show labelling of neural progenitors and immature

neurons (Yamaguchi et al., 2000). Because the expression of the reporter gene is transient, it is not possible to track the same population of cells along their maturation process.

More recent designs use a Cre recombinase system, which allows for permanent expression of the reporter gene in adult-generated cells in a time-specific manner. *Cre* recombinase (Cre) is a Type I topoisomerase that catalyzes site-specific recombination of DNA between *loxP* sites. The *loxP* recognition element is a 34 base pair (bp) sequence composed of two 13 bp inverted repeats flanking an 8 bp spacer region that confers directionality. Recombination products are dependent on the location and relative orientation of the loxP sites (Akagi et al., 1997).

A commonly used inducible Cre recombinase system (used in several neurogenesis studies and in this thesis) is *Cre-ER*, or Cre activated by an estrogen receptor. In this system, an intraperitoneal injection of the estrogen receptor antagonist tamoxifen (TMX) causes the excision of the floxed site, removing a stop codon that inactivates the expression of a reporter gene (*LacZ* and *GFP* are most commonly used) (Hayashi and McMahon, 2002). After recombination, the reporter gene is permanently expressed in all the lineage cells (Lagace et al., 2007).

We extensively used this marker in section 3 of this thesis. The staining of *lacZ* allow us to determine the phenotype and maturation stages of the newborn neurons. This method has a high yield of tagged cells; as opposed to BrdU (as the labelling occurs continuously and is not circumscribed to an injection time) we found this especially advantageous to calculate the rates of newborn neurons included in memory circuits.

1.6.2.4 Retroviral labelling.

Retroviruses are useful tools in neurogenesis research because they infect only replicating cells and can integrate reporter genes in the genome of the host cells (which are expressed permanently by the host cell and progeny). Retroviruses were first discovered in the 1920s (Rubin, 2011) and later characterized as a new viral type with a capsid containing RNA instead of DNA. The viral RNA is transcribed by a reverse transcriptase to generate DNA, which is incorporated into the nuclear genome. Retroviruses only label replicating cells because incorporation of the viral DNA into the nuclear genome only occurs when the nuclear membrane is dispersed during mitosis (Zhao and Elder, 2005). Retroviruses used as research tools are depleted in some structural proteins, preventing them from generating new virions. The genes are generally substituted by beta-galactosidase (from the *lacZ* gene) or more recently by *gfp* gene (green fluorescent protein) (Sanes, 1989).

Retroviral technology has been used in neuroscience to trace neural linages and fates and to transform progenitors and manipulate their progeny (Luskin, 1994). Retroviruses have been used to infect hippocampal progenitor cells, and it has been demonstrated that virus-labelled cells can also be labelled with Ki67, DCX, and NCAM (Seki et al., 2007). In distinction from endogenous markers, retrovirus-expressed GFP is present the whole lifespan of the infected cell and progeny, allowing for the visualization of morphological changes during the development from progenitor cells to fully mature granule neurons (Zhao et al., 2006). In addition, GFP expression provides more accurate and reliable definition of morphological details of adult-born cells and can be used for axonal tracing and analysis of the large mossy terminals (Toni et al., 2008). We have used this tool for morphological analysis of large mossy terminals in CA3 as currently this is the better method for morphological quantifications on newborn cells. The results are presented in Appendix I.

1.7 Adult neurogenesis: potential role in memory

Santiago Ramón y Cajal noticed common anatomical features between the DG and the olfactory bulbs. Besides the obvious presence of granule cells with similar morphology, he noticed that the layer assemblies were similar in these otherwise unrelated structures(Cajal, 1894a). However, as both structures are strongly related to certain types of memory acquisition and storage, it was later proposed that newborn neurons in the adult DG and olfactory bulbs could play specific roles in memory function (Gould et al., 1999, Alonso et al., 2006). Regarding the olfactory bulbs, there is some consensus about the function of newborn neurons in cognitive integration (Moreno et al., 2009, Valley et al., 2009, Sultan et al., 2010). The role of neurogenesis in hippocampus-dependent learning and memory is more controversial, with evidence both in favour and against the possibility that adult-born neurons play an important role in episodic memory function.

1.7.1 Newborn neurons in the hippocampus and their contribution to memory

1.7.1.1 Initial evidence for a role of hippocampal adult neurogenesis in memory regulation

The first evidence suggesting involvement of hippocampal adult-born neurons in memory was found using the hippocampus-dependent trace eye-blink conditioning paradigm. In this task, neurogenesis in the SGZ was up-regulated after conditioning. In contrast, in a non-hippocampus-dependent version of the task, an increase in neurogenesis was not observed (Gould et al., 1999). A similar effect was found for a spatial version of the Morris water maze, in which a 2-fold increase in neurogenesis was observed after training (Gould et al., 1999). The authors proposed that this increase in neurogenesis could be a response to a demand for newborn cells in the face of cognitive challenges. Therefore, newborn neurons could serve an adaptive function in hippocampus-dependent cognitive tasks.

Reducing hippocampal neurogenesis interferes with hippocampal learning. After reducing neurogenesis with the anti-mitotic DNA methylation agent methylazoxymethanol acetate (MAM), a deficit was observed in trace eye-blink conditioning. This learning deficit was not observed when the drug was administered for only one week or when proliferation rates recovered following cessation of the drug administration (Shors et al., 2001). Following this key finding, a major debate over the importance of newborn neurons in memory acquisition began. Some authors found that the relationship between reductions in adult neurogenesis and learning impairments was merely correlative. Also, there was no way to discard negative systemic effects of MAM as a plausible explanation of the findings (Macklis, 2001). Other study, using the same MAM administration protocol, found no impairment in other hippocampus-dependent tasks such as contextual fear conditioning and the water maze (Shors et al., 2002). Other recent study have not shown increased neurogenesis after extensive spatial water maze training (Lerch et al., 2011b).

1.7.1.2 Evidence that increasing neurogenesis positively affects learning and memory

Early experiments showed that environmental enrichment (Young et al., 1999) and exercise (van Praag et al., 1999b) increase hippocampal neurogenesis. Therefore, these tools have been used to explore the effects of increasing neurogenesis in memory function. it was suggested that increased neurogenesis levels could positively affect learning and memory in hippocampus-dependent tasks. As predicted, initial studies showed that environment enrichment led to an increase in the survival of newborn cells and facilitated spatial learning in the water maze (Nilsson et al., 1999, van Praag et al., 1999a).

Exercise also improves LTP elicitation, possibly via increased adult neurogenesis (Farmer et al., 2004). Nonetheless, other studies showed an effect of exercise not on spatial memory

retention but only on swimming path length during training trials, which is a measurement that is known to be conflated with the use of alternative non-spatial strategies (van Praag et al., 1999a, Van der Borght et al., 2007). Moreover, exercise or environmental enrichment can improve memory formation in several ways that do not involve the hippocampus but rather the frontal lobes (Ratey and Loehr, 2011). Therefore, the effects of exercise and enrichment on hippocampal neurogenesis and other brain processes needs to be clarified.

Besides the evidence early published, the involvement of neurogenesis on enrichment-induced cognitive improvement is contested (Lazic, 2012b). Although one study suggests that suppresion of neurogenesis with radiation can block improvements in cognition induced by environmental enrichment (Wojtowicz et al., 2008), this effect could easily be associated with side effects of radiation as neuro-inflammation and microglia activation (Wojtowicz, 2006). Another study using a similar protocol but with the drug MAM found that running-induced improvement in cognition was not successfully blocked by neurogenesis reduction (Meshi et al., 2006). An experimental approach in which neurogenesis was reduced by depleting serotonergic neurotransmission to the DG showed that the beneficial effects of enrichment on performance in a hippocampus-dependent task were not directly linked to hippocampal neurogenesis (Ueda et al., 2005).

Antidepressant treatment is widely used as a tool for neurogenesis enhancement, although this method does not result in cognitive improvements. A possible limitation of the use of antidepressant therapy to enhance neurogenesis is that antidepressant drugs increase cell proliferation but not survival (Malberg et al., 2000). However, some memory-rescuing effects of antidepressant therapy have been reported in pathological conditions like models of AD (Dong et al., 2004), radiation-related cognition impairment (Akyurek et al., 2008), and chemotherapy

treatment (ElBeltagy et al., 2010), in which neurogenesis is impaired. Nonetheless, there has not been any direct evidence of healthy individuals showing improvements in cognition as a result of antidepressant treatment.

Recently, a number of authors have suggested that hippocampal neurogenesis could have a role in pattern separation and completion, a processes that is thought to rely on the DG. In the adult DG, there is a mixture of mature and immature granule cells. According to Aimone and colleagues (2011), computational models show that mature granule cells could encode selected contextual information with high precision, whereas immature cells could encode much more information, but with lower precision. According to this model, these two cell populations would be able to separate and complete contexts in a more efficient manner than only mature neurons could.

Some recent studies have tested Aimone and colleagues' hypothesis using a running paradigm. In these studies, running enhanced proliferation rates in the hippocampus and facilitated pattern separation in mice (Creer et al., 2010). However, a recent study showed that the behavioural effect described by Creer and colleagues correspond to a poorly analyzed correlation analysis that does provide evidence for causality of neurogenesis over the behaviour explored (Lazic, 2012b). Another approach to testing this hypothesis was the generation of an inducible Bax knockout mouse. In these mice, the expression of Bax in newborn neurons is inhibited. As Bax is a Bcl-2 family member that positively regulates programmed cell death, the new cells lacking this protein are not able to undergo apoptosis. As a consequence, Bax knockout mice show larger numbers of immature granule neurons compared to their wild type littermates. Behaviourally, the knockout mice showed enhanced performance in a contextual fear pattern separation task (Sahay et al., 2011). These results, however, contrast with the idea that apoptosis is needed for proper turnover

in the DG, it has been proven before that apoptosis in the DG result in memory impairment (Dupret et al., 2007). The group that originally described Bax as essential to programmed cell death in the hippocampus, reported that after knocking out this gene, the number of granule cells grew disproportionally, resulting in several noticeable morphological abnormalities (Sun et al., 2004). This apparent contradiction has not been clarified.

1.7.1.3 Evidence that suppression of adult neurogenesis negatively affects learning and memory

Most studies providing evidence that hippocampal neurogenesis is involved in cognition have used various neurogenesis suppresion methods. One notable observation is that aged animals show a dramatic reduction in hippocampal neurogenesis (Kuhn et al., 1996). Therefore, the age-related decline in neurogenesis has been used as a tool to test the relationship between neurogenesis and memory. This approach has been used in two studies testing the cognitive performance of aged rats in the spatial water maze. They found that shorter latencies to reach the goal quadrant were associated with higher levels of newborn neurons (Drapeau et al., 2003, Wati et al., 2006). In contrast, other report showed no correlation between neurogenesis levels and water maze performance in aged rats (Merrill et al., 2003). In accordance to the analysis of Lazic and Colleagues (2012b), this study also failed to show a proper correlation as it excluded young rats with high levels of neurogenesis from the data pool; therefore, the correlation in aged animals can be explained by a relation of neurogenesis senescence and general health or mood decay, which independently can explain the memory results.

Another tool for studying hippocampus neurogenesis and memory has been irradiation. Subjecting the hippocampus of rats to convergent x-ray beams efficiently induces apoptosis in proliferating cells (Peissner et al., 1999, Mizumatsu et al., 2003). An initial study reported that rats that received targeted irradiation of the DG showed poor retention in a hippocampus-dependent place

recognition task but no impairment in the water maze or a novel object recognition task (Madsen et al., 2003). Other studies showed impaired performance in the water maze following neurogenesis blocking by irradiation. These studies demonstrated that the largest effect of irradiation appears when animals are trained several weeks after the treatment and not just immediately before or after irradiation, suggesting that the age of the new neurons could be critical in achieving this particular impairment (Snyder et al., 2005, Saxe et al., 2006). Another study showed that irradiation is also effective at impairing contextual fear memory but ineffective when the shock is paired with a discrete cue such as a tone, suggesting that the effect of irradiation is specific to the hippocampus-dependent memory (Winocur et al., 2006).

A new generation of studies has used transgenic approaches to endogenously down-regulate neurogenesis, a technique that is considered less invasive. However, the use of these tools has also not provided a conclusive answer on the roles of neurogenesis in memory. The temporal depletion of the orphan receptor TLX resulted in a large decrease in adult hippocampal neurogenesis that was associated with impaired spatial memory but normal contextual fear conditioning (Zhang et al., 2008). Another study in which cyclin D1, a critical factor for cell cycle regulation in the DG (Kowalczyk et al., 2004), was depleted, no impairment in spatial learning or contextual fear conditioning was observed. Conversely, this study showed that performance in an olfactory task was impaired (Jaholkowski et al., 2009). In other work, the blocking of newborn cells in inducible diphtheria toxin receptor-expressing mice led to a spatial memory deficit in the Barnes maze one week later (Imayoshi et al., 2008, Clelland et al., 2009). Although these methods are less invasive and more controllable, they still fail to show consistency across studies.

Other suppresion-type experiments have used more complex memory paradigms. For example, a recent study showed that the reduction of neurogenesis using temozolomide (TMZ), an anti-replicative drug used in cancer treatment (Newlands et al., 1992), is not associated with an impairment during water

maze probe tests but rather during reversal learning, when mice learn a new platform location after the initial training (Garthe et al., 2009). Also, in models using irradiation and lentiviral-driven *wnt* suppresion, mice showed poor performance in different pattern separation tasks, including a radial arm separation task and a touch screen separation task (Clelland et al., 2009).

Remote memory is known to rely partly on the hippocampus and cortical regions (Anagnostaras et al., 1999). Although the role of newborn neurons in remote memory has not been extensively studied, a few studies have explored the issue and have suggested new and interesting roles for hippocampal neurogenesis in this type of memory. A recent study showed that suppresion of neurogenesis renders remote memory more hippocampus-dependent, whereas increases in neurogenesis accelerate the export of memory to cortical modules. Therefore, the authors concluded that a possible function of newborn hippocampal neurons is to facilitate memory relocation to cortical areas and hippocampal clearance (Kitamura et al., 2009). This work supports the hypothesis of Feng and colleagues (2001), who suggested a role of newborn neurons in clearing memories stored in the hippocampus. They showed that wild type mice showed poorer recall of a remote contextual memory after three weeks of running. Conversely, presenilin-1 knockout mice, which have impaired adult neurogenesis, showed normal recall after three weeks.

Table 3. Hippocampal neurogenesis and memory

Manipulation	Animal	Effect on neurogenesis	Task	Outcome	Reference
Studies involving reduction of hippocampal neurogenesis					
MAM	Wistar rats	↓80%	Trace eye-blink conditioning	Impairment	(Shors et al., 2001)

MAM	Wistar rats	↓80%	Contextual fear conditioning, Water maze	No effect	(Shors et al., 2002)
Aging, 21 months	F344 rats	↓70%	Water maze	No correlation between neurogenesis and memory	(Merrill et al., 2003)
Aging, 16 months	F344 rats	↓50%	Water maze	Correlation between and neurogenesis and memory	(Drapeau et al., 2003)
Irradiation	Long Evans rats	↓80%	Contextual fear conditioning	Impairment after 14 days	(Snyder et al., 2005)
Exercise/irradiation	C57BL/6 mice	↑ 80% exercise ↓90% irradiation	Water maze	Memory improvement with exercise, no effect with radiation	(Meshi et al., 2006)
Irradiation	Wistar rats	↓>90%	Non-matching-to-sample (NMTS)	Impairment with context, no effect with CS	(Winocur et al., 2006)
Depletion of NSCs expressing diphtheria toxin receptor	C57BL/6 mice	↓?	Barnes maze	Impairment	(Imayoshi et al., 2008)
Irradiation /exercise	Wistar rats	↓ 90%	Contextual fear conditioning	Impairment	(Wojtowicz et al., 2008)
Depletion of TLX receptor in NSCs	C57BL/6 mice	↓>85%	Contextual fear conditioning, water maze	No effect in contextual fear conditioning, impairment in water maze	(Zhang et al., 2008)
Irradiation	C57BL/6 mice	↓90%	Pattern separation using radial maze and touchscreen	Impairment	(Clelland et al., 2009)

Temozolomide	C57BL/6 mice	↓90%	Water maze	Impairment in reversal learning	(Garthe et al., 2009)
Depletion of cyclinD1	C57BL/6 mice	↓>90%	Contextual fear conditioning, water maze	No effect (but impairment in olfactory recognition task)	(Jaholkowski et al., 2009)
Studies involving increase of hippocampal neurogenesis					
Enrichment	Wistar rats	↑ 100%	Water maze	Improvement	(Nilsson et al., 1999)
Enrichment/exercise	C57BL/6 mice	↑254%	Water maze	Improvement	(van Praag et al., 1999a)
Enrichment	C57BL/6 mice	↑45%	Water maze	Rescue after stroke	(Young et al., 1999)
Enrichment/exercise	C57BL/6 mice	↑ 100%	Y-maze	Improvement	(Van der Borght et al., 2007)
Enrichment/exercise	C57BL/6 mice	↑ 100%	Contextual fear pattern separation	Improvement	(Creer et al., 2010)
Apoptosis interruption by Bax inactivation	C57BL/6 mice	↑ 70%	Contextual fear pattern separation	Improvement	(Sahay et al., 2011)

1.7.2 Hippocampal neurogenesis and memory at the cellular level.

In considering the role of hippocampal adult neurogenesis in memory function, it is important to aknowledge the possibility of newborn neurons becoming integrated within memory-supporting circuits. The first suggestion of newborn neuron function within memory circuits was that of Aimone and colleagues (2006). They hypothesized that immature neurons, which are more excitable and prone to generate LTP than are fully mature neurons, are better for storing relevant information about the timing of events. This hypothesis received strong support, and researchers

have begun to use cellular imaging techniques combined with behaviour to assess the contribution of newborn cells in the DG in memory traces.

The possibility of newborn neurons becoming integrated within memory circuits is now well established. An early study showed that the immediate early gene *arc* is more likely to be expressed in newborn cells as a result of exploratory behaviour, compared to mature granule neurons (Ramirez-Amaya et al., 2006). A comprehensive study suggested the idea of preferential recruitment of adult-generated cells over developmentally-generated cells in the DG using the immediate early gene *c-fos* and the water maze (Kee et al., 2007a, b).

These two studies showed that the recruitment of newborn cells only occurs when these cells are fully mature, and therefore they would be unlikely to encode time-sensitive memories as hypothesized by Aimone (2006). Conversely, other study showed a special proclivity for newborn cells to become active when learning is reinforced a month after the original training, which occurred when the cells were only 10 days old (Trouche et al., 2009). Although these results could support Aimone's hypothesis that the recruitment of cells that were new and very excitable at the moment of training would enforce the time contextualization of the stored episode, there is only indirect evidence of this engagement. Nonetheless, considering that newborn neurons can establish functional connections with CA3 target cells only 15 days after mitosis, it is unlikely that the role proposed by Aimone (use of birth date to mark the timeline of memories) is related to direct memory circuit engagement (Toni et al., 2008).

A recent study suggest that granule cells have a period during which they are more likely to be activated and, after that, their probability of engagement in memory trace is minimal, after this period the granule cells would be considered "retired" (Alme et al., 2010). In rats close to one year old, only neurons born during adulthood are engaged in memory circuits (Sandoval et al.,

2011). Even newborn cells in senescent rodents can be recruited in learning circuits and preferentially incorporated (despite their very small numbers), which would require more new cells as the majority of granule cells retire and the neurogenesis rate at that age is very low (Marrone et al., 2011). This body of evidence is also supported by a study showing that both neurogenesis and apoptosis in the DG are required for spatial learning (Dupret et al., 2007). Conversely, these studies contrast with the fact that granule neurons born during infancy and adulthood have equivalent probabilities of integration into spatial and contextual memory circuits (Stone et al., 2011).

2 Objectives and hypothesis

The dentate gyrus of the hippocampus has received much attention in recent years for being implicated in different types of memory. This structure plays a role in context recognition and spatial memory. The dentate gyrus is also one of only two brain structures that continuously adds neurons during juvenility and into adulthood. How does adult neurogenesis affect cognitive processing in the dentate gyrus? Much contrasting information has been published about the potential importance of adult neurogenesis in memory encoding and retrieval. A definitive answer has not yet been reached.

In our first study, we evaluated whether an age-related decrease in neurogenesis, a TGFβ1-induced down-regulation of neurogenesis, or a running-induced increase in neurogenesis modifies the proportion of newborn neurons that integrate into memory circuits. **Our first hypothesis was that if newborn neurons are functionally distinct, after neurogenesis manipulations there would be a change in the percentage of newborn neurons recruited into memory circuits as a fixed proportion of newborn neurons will be necessary for optimal performance of memory circuits. Our alternative hypothesis, that newborn neurons are functionally indistinct to their developmentally-generated neurons, would be proven if after manipulations newborn neurons are recruited at similar rates to memory circuits, but the number of those neurons belonging to memory circuits changes proportionally to the change on neurogenesis.**

In the second study, we dissected the role of adult-born neurons across the lifespan by evaluating the effect of ablating hippocampal neurogenesis on spatial memory in different ages of healthy wild-type mice. **If ablating neurogenesis affects spatial memory in middle-aged mice,**

we would conclude that the depletion of adult-born neurons leads to deficits in memory function. On the contrary, if ablating neurogenesis affects spatial memory in juvenile mice not fully developed, we would conclude that newborn cells play a role in sculpting dentate gyrus morphology and that their importance relies in their contribution to the structural integrity of the dentate gyrus and not in their unique functional qualities.

Although TGFβ1 is widely known to be present in the adult brain, its role in memory is poorly understood. The most well recognized role of this cytokine is its function in the physiological response of the brain to different insults. Both brain and plasma levels of TGFβ1 are increased in AD. Whether this expression is beneficial or detrimental to brain function is still not clear and is currently under study in humans and animal models. Some of the most pronounced physiological effects of TGFβ1 occur in the dentate gyrus. TGFβ1 is known for its regulation of cell proliferation and apoptosis; in the adult dentate gyrus, TGFβ1 is a strong down-regulator of proliferation. Nothing is known, however, regarding the role of TGFβ1 in mammal behaviour. **We hypothesized that TGFβ1 affects the integrity of the dentate gyrus and hippocampal circuits, which should be reflected during learning and memory in hippocampal-associated tasks.**

3. Mature adult-born neurons are incorporated into memory circuits as a function of their availability.

3.1 Abstract

In the dentate gyrus (DG) of the hippocampus, levels of adult neurogenesis decline dramatically with age, and are modulated by a number of genetic and environmental factors. How does the availability of new neurons influence their functional integration into hippocampal memory networks? Here two models make contrasting predictions. The first possibility is that the contribution of new neurons is simply proportional to availability or supply of new neurons. For example, when new neurons are scarce, fewer will be integrated into memory networks in the DG. Conversely, when there is a relative abundance of new neurons, larger numbers are integrated into memory networks in the DG. An alternative possibility is suggested by the view that new neurons make a unique contribution to hippocampal memory. Because this contribution is critical, there is selective pressure to maintain a fixed proportion of new neurons in any given trace, independent of their relative abundance. Indeed, according to this model, memories formed with suboptimal levels of adult generated neurons will be compromised. To distinguish between these two models, we evaluated how recruitment rates vary as a function of the availability of new neurons, using aging and transgenic approaches to reduce neurogenesis, and using running to increase it, evaluating recruitment in two hippocampal-dependant tasks by using histological markers for neurogenesis and granule neuron activation. The data indicate that the proportion of new cells incorporated into memory circuits is proportional to their availability. The data suggest that new neurons generated during adulthood are functionally equivalent to pre-existing granule neurons.

3.2 Introduction

During adulthood, there is continuous addition of new neurons to the DG (Ming and Song, 2005, Zhao et al., 2008). Similar to developmentally generated dentate granule cells (DGCs) adult generated DGCs are thought to eventually contribute to the formation of hippocampus-dependent memory (Shors et al., 2001, Deng et al., 2010). While adult generated DGCs may eventually develop similar cellular phenotypes to developmentally generated DGCs (Laplagne et al., 2006), adult generated DGCs differ transiently from their mature granule cells neighbors. Between two and four weeks post-mitotic, the threshold for inducing LTP in new neurons is reduced (Schmidt-Hieber et al., 2004), and from four to six weeks, their LTP magnitude increases (Ge et al., 2007). These physiological characteristics might make these newborn neurons functionally distinct and give new neurons a transient advantage for integration into hippocampal memory networks, thus making new neurons more likely to become part of memory circuits.

These characteristics of the adult DGCs suggest that they may add some unique component to hippocampal memory networks. If true, there should be selective pressure to maintain a fixed proportion of adult DGCs within memory circuits that would make adult DGCs functionally distinct. Conversely, DGCs could have recruitment rates equivalent to developmentally generated DGCs; this would imply that these new neurons are added to memory circuits at the same rate as the developmentally generated neurons. This would mean that these two populations, development and adult generated neurons, are functionally indistinct. Previously this question has been addressed using immunohistochemical approaches to estimate integration rates of DGCs into hippocampal networks (Ramirez-Amaya et al., 2006, Kee et al., 2007b, a, Tashiro et al., 2007). In these studies, proliferation markers were used to label adult generated DGCs and expression of

immediate early genes (IEGs such as c-Fos or Arc) was examined after memory testing. Because IEG expression is regulated by neuronal activity (Guzowski et al., 2005), they are appropriate markers to estimate the proportion of adult generated DGCs that are integrated into hippocampal memory networks.

Using this approach, we recently showed that both developmental and adult generated DGCs are integrated at equivalent rates (Stone et al., 2010). In this study we manipulated levels of neurogenesis to asses whether this could change their relative recruitment rates. We used different proliferation markers such as BrdU (Kee et al., 2007b) and Nestin-Cre^{ERT2}-LacZ reporter (Lagace et al., 2007, Imayoshi et al., 2008) to visualize new neurons, together with the proteins of IEGs *Arc* and *c-Fos* (Kee et al., 2007b, Stone et al., 2010) to visualize neurons in the memory trace. We found that new neurons are recruited to the memory trace in the same proportion as developmentally-generated neurons, regardless whether the levels of adult neurogenesis was increased or decreased. However, the numbers of adult generated DGCs that become part of memory circuits increase when these cells are more abundant and decrease with lower levels of proliferation. Therefore, in this study we show that adult generated DGCs are functionally indistinct from pre-existing DGCs, and are recruited to the memory trace in higher or lower amounts according to their availability.

Figure9R.3.1 two models of newborn neuron circuit recruiment

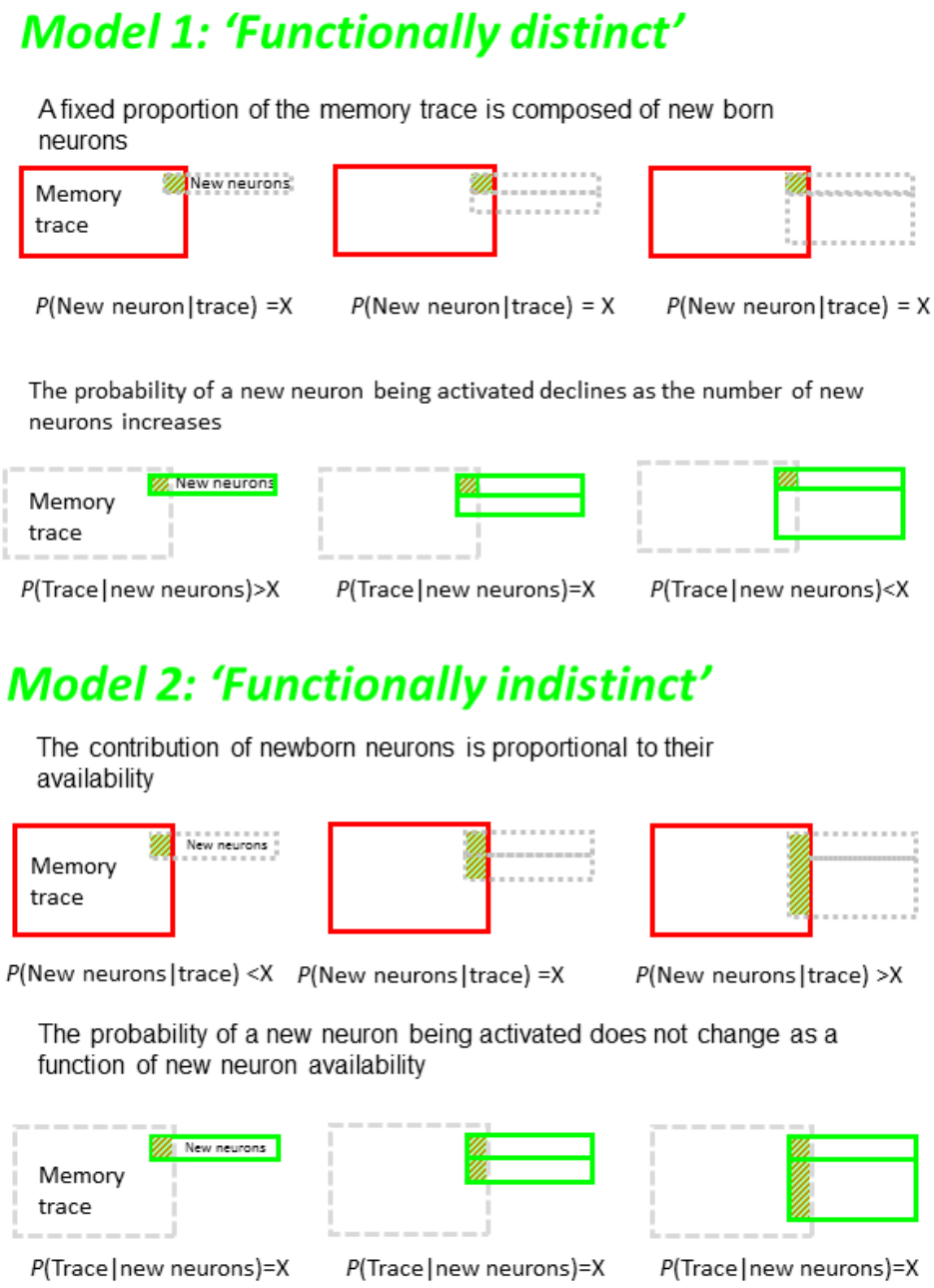


Figure R.3.1 *Schematic depiction of the two hypotheses: In the 'functionally distinct' model, a fixed part of the memory trace (red square) is always composed of newborn neurons (yellow area). However, the percentage of the total population of new neurons (green) that belongs to the given memory trace varies with changes in the total population, being the highest with the less newborn neurons (as in normal aging or transgenic models) and the lowest in cases with a high component of newborn cells (as in running). In the 'functionally indistinct' model, in the memory trace, the proportion of newborn neurons is variable and it depends on the variations in the amount of cells that are added to the system which uses them, as they become available.*

3.3 Materials and methods

3.3.1 Mice

Male WT offspring from a cross between C57Bl/6NTacfBr [C57B6] and 129Svev [129] mice (Taconic, NY) were used in these experiments. All mice were bred in our colony at The Hospital for Sick Children, and maintained on a 12 h light/dark cycle with free access to food and water. Behavioural procedures were conducted during the light phase of the cycle, blind to the treatment condition of the mouse and according to protocols approved by the Animal Care Committee at The Hospital for Sick Children.

Nestin-Cre^{ERT2} (Nestin^{+}) mice. The Nestin^{+} mice express tamoxifen (TAM)-inducible Cre recombinase under the control of a Nestin promoter, and have been previously described (Imayoshi et al., 2008). The line of Nestin^{+} mice we used in our experiments corresponds to line 47 in Imayoshi et al. (Imayoshi et al., 2008), and have highest recombination efficiency in the subgranular zone of the hippocampus.

Rosa-LacZ mice. The Rosa-LacZ reporter mice have been previously described (Zambrowicz et al., 1997). The gene encoding LacZ is under the control of the ubiquitous Rosa26 locus promoter, but expression of the LacZ transgene is dependent on the Cre recombinase-mediated removal of a transcriptional STOP cassette. All lines were maintained on a C57BL/6 background (Taconic Farms, Germantown, NY). Genotypes were determined by PCR analysis of tail DNA samples as previously described (Zambrowicz et al., 1997, Baldi et al., 2005, Imayoshi et al., 2008). All of the transgenic mice were kept heterozygote to avoid possible complications by over-expressing Cre-recombinase (Forni et al., 2006) or loss of the Rosa allele (Zambrowicz et al., 1997).

TGFβ1 Mice. Mice overexpressing TGFβ1 driven by a GFAP promoter (corresponding to line T64) were used to decrease the rates of proliferation in the subgranular zone (Wyss-Coray et al., 1995, Wyss-Coray et al., 1997, Wyss-Coray et al., 2000, Wyss-Coray et al., 2001, Buckwalter et al., 2006). These mice were crossed with the *Nestin-Cre^{ERT2}* mice and the *Rosa-LacZ mice* in order to label the production of newborn neurons. Double transgenic mice, *Nestin-Cre^{ERT2}* x Rosa-LacZ were used as littermate controls, and triple positive mice TGFβ1 x *Nestin-Cre^{ERT2}* x Rosa-LacZ.

3.3.2 Water maze apparatus and procedures

The apparatus and behavioral procedures have been previously described (Teixeira et al., 2006). Behavioral testing was conducted in a circular water maze tank (120 cm in diameter, 50 cm deep), located in a dimly-lit room. The pool was filled to a depth of 40 cm with water made opaque by adding white, non-toxic paint. Water temperature was maintained at 28 ± 1 °C using a heating pad beneath the pool. A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface, in a fixed position in one quadrant. The pool was surrounded by curtains, at least 1 m from the perimeter of the pool. The curtains were white with distinct cues painted on them.

Prior to commencing training, mice were individually handled for 2 min each day over 5 consecutive days. Mice were trained over 5 days. On each training day, mice received six training trials (presented in 2 blocks of consecutive 3 trials; inter-block interval was ~ 1 h; inter-trial interval was ~15 s). On each trial, they were placed into the pool, facing the wall, in one of 4 start locations. The order of these start locations was pseudo-randomly varied throughout training. The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on a given trial, the experimenter guided the mouse onto the platform. Following the completion of training, spatial memory was assessed in a series of 3 probe tests with an inter-test interval of approximately 3 min. In this test, the platform was removed from the pool, and the

mouse was allowed 60 s to search for it.

Behavioral data from training and probe tests were acquired and analyzed using an automated tracking system (Actimetrics, Wilmette, IL). In probe tests, we quantified performance in two ways. First, we measured the amount of time mice searched the target zone (20 cm radius, centered on the location of the platform during training vs. the average of three other equivalent zones in other areas of the pool. These zones each represent approximately 11% of the total pool surface. Second, we represented probe test performance as a heat map (or density plot), with hot colors corresponding to areas of the pool that were more frequently visited.

3.3.3 Contextual fear conditioning apparatus and procedures

The apparatus and behavioral procedures have been previously described (Wang et al., 2009). Contextual fear conditioning experiments were conducted in a windowless room containing 4 conditioning chambers. Each conditioning context consisted of a stainless steel conditioning chamber (31 cm × 24 cm × 21 cm; Med Associates, St. Albans, VT), containing a stainless steel shock-grid floor. Shock grid bars (diameter 3.2 mm) were spaced 7.9 mm apart. The grid floor was positioned over a stainless-steel drop- pan, which was lightly cleaned with 70% ethyl alcohol to provide a background odor. The front, top, and back of the chamber were made of clear acrylic and the two sides made of modular aluminum. Mouse freezing behavior was monitored via four overhead cameras. Freezing was assessed using an automated scoring system (Actimetrics, Wilmette, IL), which digitized the video signal at 4 Hz and compared movement frame by frame to determine the amount of freezing.

During training, mice were placed in the context for 5 min and were presented with 3 unsignaled footshocks (0.5 mA, 2 s, 1 min apart) starting at 2 min. Following the last footshock

mice remained in the context for an additional minute, and then were returned to their home cage. On the next day, mice were placed back into the same context for a total of 5 min and freezing was monitored.

3.3.4 Tissue handling and preparation for stereology

Ninety min following the completion of behavioral testing, mice were anesthetized with chloral hydrate 4% and perfused transcardially with PBS and then 4% paraformaldehyde (PFA). Brains were removed, fixed overnight in PFA and then transferred to 30% sucrose solution and stored at 4 °C. Fifty μm coronal cryostat sections were cut beginning from a random starting point anterior to the hippocampus beginning and continued along the entire anterior-posterior extent of the DG. Sections were kept in sequential order and maintained free-floating in PBS. A 1⁄4 section sampling fraction was used to create 4 sets (each containing sections at 200 μm intervals) for use in immunohistochemical staining. Accordingly, each set comprised a systematic random sample representative of the entire DG for use in quantification analyses. Additional sets were stored in a PBS solution containing 50% glycerol and 10% ethylene glycol to keep at -20°C for later processing.

3.3.5 Immunohistochemistry

For BrdU/Fos staining, the BrdU antigen was unmasked by incubating the sections in 1 N HCl at 45°C for 30 min. Incubation for 48 h at 4°C was performed using primary antibodies against Fos (rabbit anti-Fos polyclonal antibody; 1:1000; Calbiochem, San Diego, CA) and BrdU (rat anti-BrdU monoclonal antibody; 1:500; Accurate Chemicals, Westbury, NY). Secondary antibody staining with Alexa-488 goat anti-rat and Alexa-568 goat anti-rabbit (1:500; Molecular

Probes, Eugene, OR) was carried out for 2 h at room temperature. Antibodies were diluted in blocking solution containing 2% goat serum, 2.5% bovine serum albumin, and 0.3% Triton X-100 dissolved in PBS. Sections were mounted on slides (VWR, West Chester, PA) with Permafluor anti-fade medium (Lipshaw Immunon, Pittsburgh, PA).

The following primary antibodies were used: rabbit polyclonal anti-LacZ (1:6000; Molecular Probes, OR) and anti-Arc (1:5000; Molecular Probes, OR). All sections were treated with 3% hydrogen peroxide. Sections were then incubated overnight with LacZ primary antibody and then for 60 min with Anti-Rabbit conjugated Alexa-488 (1:750; Invitrogen, OR). After extensive washes, anti-Rabbit block IgG was used (1:100, Jackson Immuno-search, PA). Then sections incubated O/N in anti-Arc antibody, and then for 60 min with Goat Anti-Rabbit biotinylated (1:1000 Jackson Immuno-search, PA) and amplified using Vectastain Elite ABC kit (Vector Laboratories, CA) finally Arc+ cells were visualized using tyramide signal amplification. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) 1:20,000 to mark cells nuclei. Sections were mounted on gelatin coated slides using Permafluor anti-fade medium (Thermo scientific, CA).

3.3.6 Imaging

Data and images were acquired using either a Nikon Eclipse 80i, Olympus BX61 epifluorescent microscope, or Zeiss LSM710 confocal microscope. Analysis for cell counting used Stereoinvestigator 9.1 (MBF bioscience, VT). To calculate cell-number, cell-density or proportion of double-positive cells we used 1/4 systematic section sampling fractions covering the entire anterior-posterior extent of the DG. We estimated the total number of LacZ-labeled cells following TAM treatment using the optical fractionator method on the Olympus BX61 epifluorescence microscope using a 60X, 1.45 N.A objective and a motorized XYZ stage

attached to a computer with Stereoinvestigator 9.1 (MBF bioscience) (Hosseini-Sharifabad and Nyengaard, 2007). A random systematic sampling was used for these stereological analyses (section interval of 1/4, grid size of 250 × 250 µm, 2D counting frame of 90 × 90 µm using disectors of 30 µm in thickness). Tissue thickness measured in each counting frame was used to estimate the total number of $LacZ^+$ cells in the entire DG. Conditions were optimized to obtain a Gundersen coefficient of error below 0.05 (Gundersen et al., 1999) .

3.3.7 Running

In each cage designated as running cage, in order to increase neurogenesis levels, we allowed mice access to running wheels, a single low profile wireless running wheel (Med Associates, MA) was placed. The running wheels stayed in the cage during the entire experiment duration except for bedding and battery change. Wheel rotation was wirelessly tracked 24 hrs. /day and the total distance traveled recorded every 2 hrs. Another set of mice cages from the same litters were used as home cage animals, without running wheels.

3.3.8 Statistical analyses

Behavioral and cell counting data were evaluated using parametric analysis of variances or t-tests, where appropriate. For relationships between two variables, linear regression was used, and group differences between ssociated slopes were compared with ANCOVA analysis. This method tests the effect of a categorical factor on a dependent variable (probability of colocalization in a cell population) while controlling for the effect of a continuous co-variable (number of new-born cells) (Zar, 2010 Chapter 18). Statistics were performed using STATISTICA 8.0 software (StatSoft Inc, OK).

3.3.9 Probability notation

To denote the probability of new born cells to either being recruited to memory circuits or the probability of an active cell to be new-born cell, we used conditional probability notation. According to the definition of Kolmogorov, $P(A|B) = \frac{P(A \cap B)}{P(B)}$ where given two events *A* and *B* the conditional probability of *A* with a defined *B,* is the quotient of the joint probability of *A* and *B*, and the frequency of *B* (Gillies, 2000). For data visualization purposes we present our results in percentages. In our work we define probability of recruitment as the joint occurrence of a cell belonging to both the new-born cell population and the memory trace cells population, divided by the total population of new-born cells. The probability of contribution is the occurrence of a cell to be new-born and being part of the memory trace divided by the total population of cells that belong to the memory trace.

3.3.10 Drugs

Tamoxifen (TMX) treatment was used to induce Cre recombination in Nestin-Cre^{ER2T} mice, to promote the constant expression of LacZ in nestin progeny cells. Mice were treated with TMX, as previously described (Lagace et al., 2007). Briefly, TMX (Sigma, IL) was dissolved in 10% ethanol (Sigma, MO) and suspended in sunflower seed oil (Sigma, MO) (Lagace et al., 2007). At 8 weeks of age, mice received 5 daily injections (180 mg/kg; i.p.) across consecutive days.

BrdU administration was used to label mitotic cells in the dentate gyrus. 5-bromo-2'-deoxyuridine (BrdU; Sigma, MO) was dissolved in 0.15 M phosphate-buffered saline (PBS) and heated to 50–60 °C, at a concentration of 20 mg/ml Animals received 200 mg/kg of BrdU per injection i.p (Kee et al., 2007a).

3.4 Results

3.4.10 Aging decreases the Nestin-LacZ labeled granule cells, but does not create a deficit in spatial memory or increase the proportion of newborn neurons in spatial memory circuits.

Aging is associated with a decrease in the levels of adult neurogenesis in the hippocampus (Kuhn et al., 1996, Drapeau et al., 2003, Lazic, 2012a). Here we assessed the rates of activation (Arc+) on newborn hippocampal cells in young (2 month old) and middle-aged (6 month old) mice induced by recall of a spatial memory following intensive training in the water maze (Fig. R.3.2 A). To visualize newly generated cells we used a transgenic reporter line (Nestin-CreER^{T2} x RosaLacZ) where TMX treatment leads to the permanent expression of the LacZ reporter gene in progenitor cells and their progeny (Imayoshi et al., 2008). To identify activated neurons, we visualized the activity-regulated gene, Arc (Guzowski et al., 2001).

Young and middle-aged reporter mice were injected with tamoxifen (TMX), and 6 weeks later were trained in the hidden platform version of the water maze. There were no differences in the latency to find the platform between young and middle-aged animals ($F_{4, 160} = 0.62$ $P > 0.05$ age x training days) (R.3.2 B). Twenty-four hours after the completion of training, we removed the platform and tested the mice in a 60 s probe test. The time spent by the mice in area 20 cm zone (centered on the former platform location) vs. equivalent zones in the other three quadrants of the pool was used to assess spatial memory, the probe test was repeated three times in order to maximize the expression of IEGs (Kee et al., 2007a). No memory difference between ages was detected ($F_{1,31} = 2.94$, $P > 0.05$ Zone x Age interaction) and both groups showed significant discrimination, spending more time in target vs. other areas of the pool (Young $t_{16} = 3.54$, $P < 0.01$ and Middle-aged $t_{16} = 2.81$, $P < 0.05$). (R.3.2 C,D).

Following the probe tests, we found sparse expression of Arc in the DG, consistent with previous literature (Chawla et al., 2005, Kee et al., 2007b, Stone et al., 2010) with about 0.5% of the total granule cell population expressing Arc. Moreover, we observed similar levels of Arc in young and middle-aged groups (t_{12}=0.43, $P > 0.05$). Consistent with previous results showing age-related declines in neurogenesis, the number of LacZ+ cells was significantly lower in the middle-aged group compared with the young animals (t_{12}=3.99, $P < 0.01$) (R.3.2 E-G). A subset of the observed cells was double labeled with both LacZ and Arc immunostaining. First, we computed the probability of Arc expression in LacZ+ cells (P[Arc|LacZ]) (proportion of newborn neurons activated after memory test) and found that this was similar in young and middle-aged mice (t_{12} = 0.78, $P > 0.05$). However, as neurogenesis (LacZ+ cells) declined with age, the likelihood that an activated granule cell was a newborn neuron (i.e., P[LacZ|Arc]) (Proportion of the memory trace composed by newborn neurons) declined as well (t_{12} = 4.61, $P < 0.001$)(R.3.2.H,I).

These results suggest that probability that newly generated cells are recruited into hippocampal memory traces supporting a water maze memory is similar both in young and middle-aged adults even though these mice have very different levels of neurogenesis. This suggests that the relative scarcity of recently born neurons in older mice is not compensated by increase in their recruitment, as the levels of newly generated neurons decrease, their inclusion in the memory networks decreases too. Together, these data suggest that newly generated neurons and their neighbors form an indistinct population when activated during spatial memory.

Figure10R.3.2

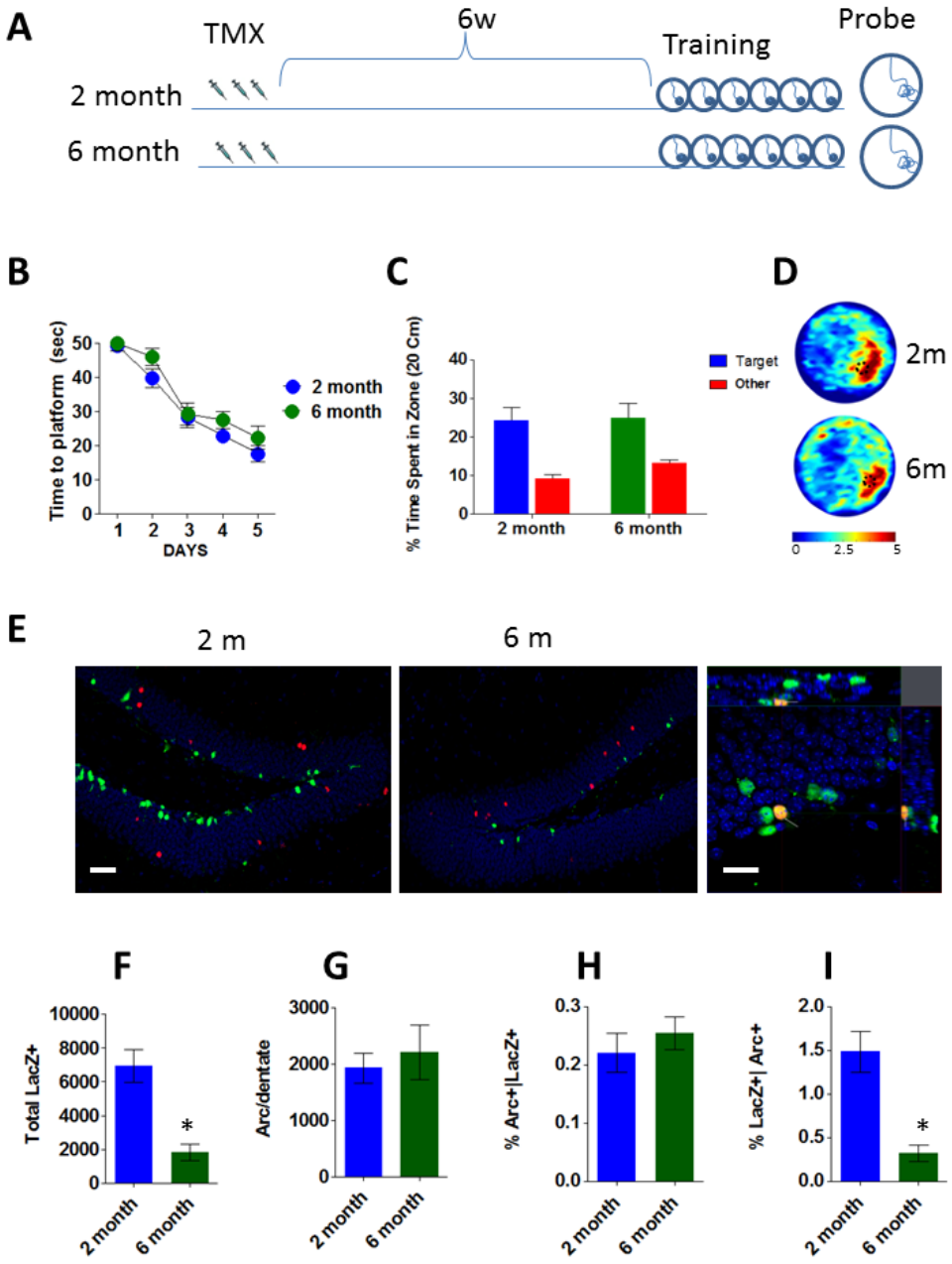


Figure R.3.2 *Spatial memory activation of newborn cells comparing young and middle-aged groups. A) Schematic of timeline used in the experiment. B) Both young and middle-aged Nestin mice equally learned the location of the hidden platform. C) When tested a day after the last trial, both groups persistently searched for the platform in the target zone compared to the three equivalent other zones. D) A plot of the each group's search during the probe test in a density map shows the pattern of search in the right southeast platform position; scale shows the crossings in a 5x5 area. E-G) Both LacZ+ (green) and Arc+ (red) cells are expressed in the dentate gyrus of both age groups (Scale bar 50µm), but with a decrease in LacZ+ cells (*, P<0.01), but comparable and Arc+ cells. A subset of those cells colocalize as shown in higher magnification (Scale bar 20µm). H) The proportion of LacZ+ cells activated is similar in both groups. I) The proportion of Arc+ cell that are also LacZ+ cells is significantly less in the older group (*, P<0.01).*

3.4.11 Over-expression of the Transforming-growth factor beta-1 (TGFβ1) dramatically reduces neural Nestin-progeny; but it does not create a deficit in contextual memory and does not increase the proportion of newborn neurons in memory circuits.

TGFβ1 is an endogenous cytokine secreted by neurons and astrocytes (da Cunha and Vitkovic, 1992), and its anti-proliferative effects are known to affect also hippocampal neurogenesis in adult mice when overexpressed (Buckwalter et al., 2006). Here, we assessed the rates of activation of newborn hippocampal neurons in adult mice with strong neurogenesis impairment. To visualize newly generated cells and have an efficient knock down on newborn cells, we crossed a TGFβ1 over-expressing transgenic mice (GFAP promoter driven) (Wyss-Coray et al., 1995, Wyss-Coray et al., 2001) with the transgenic reporter line (Nestin-CreER^{T2} x RosaLacZ) generating triple transgenic mice (TGFβ1 x Nestin-CreER^{T2} x RosaLacZ). Treatment with TMX successfully induced recombination and permanent expression of LacZ in progenitor cells and their progeny (Fig. R.3.2 A). As in the previous experiment, we identified activated neurons with Arc.

Triple transgenic and only Nestin-CreER^{T2} x RosaLacZ were injected with TMX, and 6 weeks later were place in a fear conditioning chamber and trained to associate the context with a foot-shock. 24 hours later, animals were placed in the boxes with no shock and showed robust freezing with no differences between TGFβ1 transgenic and wild types freezing percentages (t_{12} =0.17, P>0.05) (R.3.3 D).

After testing, we found sparse expression of Arc at equivalent rates in both TGFβ1 transgenic and wild types (t_8=0.42 P>0.05). Consistent with previous results showing neurogenesis decline in TGFβ1 mice, LacZ+ decreased significantly in the TGFβ1 mice compared with their wild type littermates (t_8=6.21, P<0.001)(R.3.3.B,E,F). A subset of the observed cells was double labeled. First, we computed the probability of Arc expression in LacZ+ cells (P[Arc|LacZ]) and found that this probability was similar in both TGFβ1 mice compared to their wild type littermates (t_8=0.41,

P>0.05). However, a strong decline in neurogenesis (LacZ+ cells) was observed in TGFβ1overexpressing mice, the likelihood that an activated granule cell was a newborn neuron (*P*[LacZ|Arc]) declined too (t_8= 6.46, *P*<0.001)(R.3.3. C,G,H).

These results suggest that the probability of newly generated cells being recruited into hippocampal memory traces supporting a context memory is similar in both wild type and TGFβ1 transgenic animals with severe neurogenesis reduction. This finding supports the idea that scarcity of recently born neurons in young animals with neurogenesis suppresion is not compensated by increase in their recruitment. On the contrary, as the levels of newly generated neurons decrease, their inclusion in the memory networks decreases too. Together, these data suggest that newly generated neurons and their neighbors form an indistinct population when activated during spatial memory. In addition, neither TGFβ1 overexpression in young animals, nor the severe reduction in neurogenesis, has an effect in contextual learning and retrieval.

Figure11R.3.3

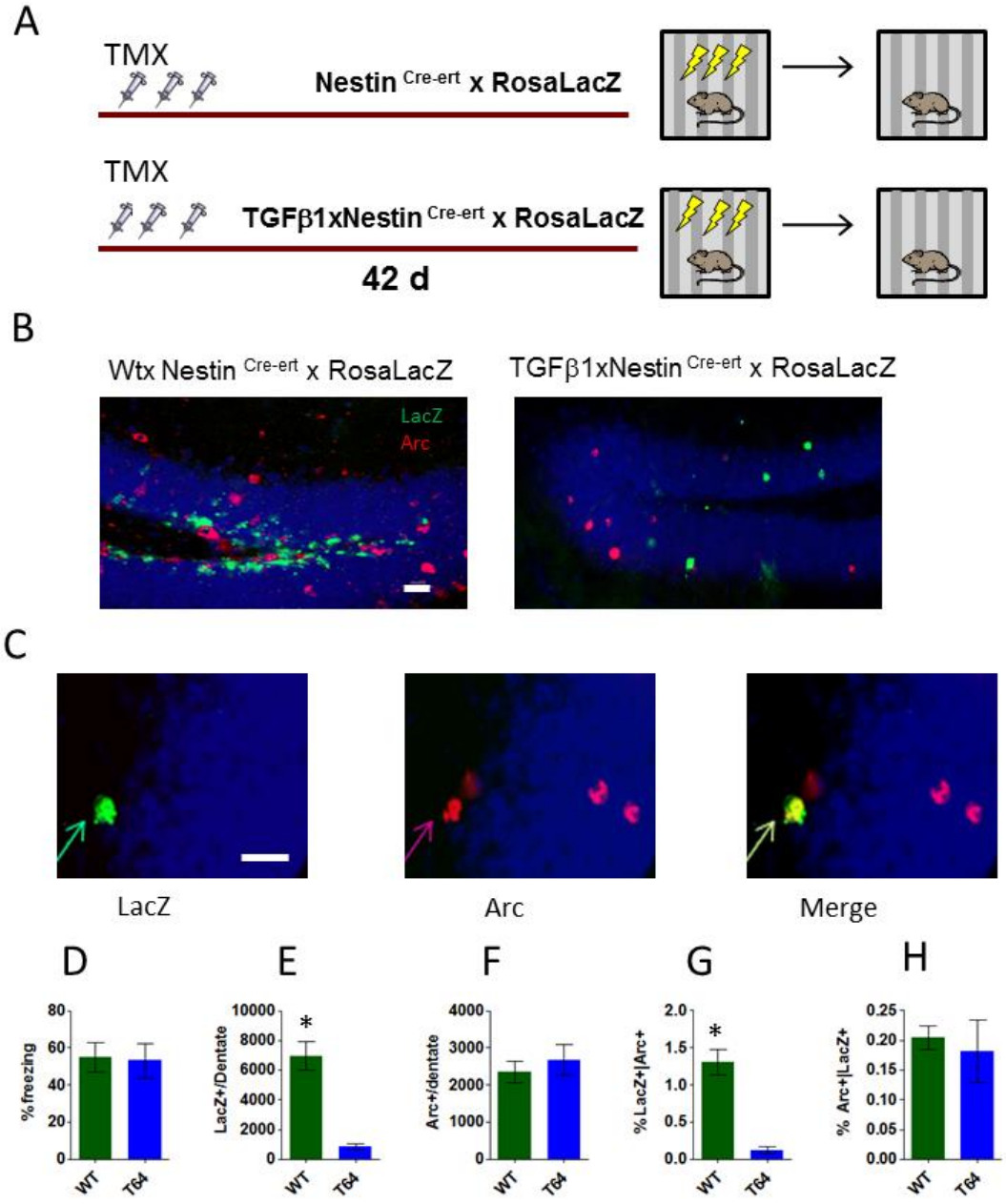


Figure R.3.3 *Genetic suppresion of neurogenesis also minimizes the participation of newborn cells in memory circuits. A) A cohort of mice that over-express TGFβ1 and LacZ in Nestin progeny, or only LacZ, were treated with tamoxifen (TMX) and trained after 42 days with three shocks in a contextual chamber, the memory levels were tested next day by placing animals in chamber without shocking. B, F) There is a strong decay in LacZ+ cells numbers in the TGFβ1 mice (* P<0.001). C) A subset of cells in both groups expressed both LacZ and Arc. D) The levels of freezing on the test day are identical in both groups, revealing no memory deficit in TGFβ1 mice. F) The quantifications of Arc+ cells show similar memory trace between genotypes. G) Proportion of LacZ+ cells that express Arc is similar between groups. I) Reduction in the proportion of Arc+ cells that also express LacZ (*, P<0.001).*

3.4.12 Voluntary running increases Nestin-LacZ labeled granule cells, but does not increase contextual memory or change the proportion of newborn neurons in memory circuits.

Voluntary running efficiently increases the levels of adult neurogenesis in the hippocampus (van Praag et al., 1999a, van Praag et al., 1999b). Here we assessed the rates of activation induced by recall of a contextual memory after foot-shock conditioning on newborn hippocampal cells in sedentary and voluntary running mice. To visualize newly generated cells, we used a transgenic reporter line (Nestin-CreER^{T2} x RosaLacZ) where TAM treatment leads to the permanent expression of the LacZ reporter gene in progenitor cells and their progeny (Imayoshi et al., 2008). To identify activated neurons, we visualized the activity-regulated gene, Arc (Guzowski et al., 2001).

Sedentary and running reporter mice were injected with TMX and group placed in cages with free access to running wheels. Six weeks after, mice were placed in contextual fear chamber to associate a foot-shock with the context. They were tested 24 hours later in the same context and the freezing percentage used as memory index (Fig. R.3.4 A). No difference in contextual memory was observed between sedentary and runner mice ($t_{17} = 0.76$, $P > 0.05$) (Fig. R.3.4 B).

Following the probe tests, we found sparse expression of Arc in the DG, consistent with previous literature (Chawla et al., 2005, Kee et al., 2007b, Stone et al., 2010) and no differences in levels of expression was observed between sedentary and runner mice were found (t_{13}=0.52, P>0.05). Consistent with previous results showing running increase in neurogenesis, LacZ+ cells increased significantly by 2-fold in runners compared with the sedentary animals ($t_{13} = 2.91$, $P <$ 0.05) (Fig. R.3.4 C-D). A subset of the observed cells was double labeled. First, we computed the probability of Arc expression in LacZ+ cells (P[Arc|LacZ]) and found that this was similar in sedentary and running animals (t_{13}=0.03, P>0.05). However, as neurogenesis (LacZ+ cells)

increased 2-fold with running, the likelihood that an activated granule cell was a newborn neuron (*P*[LacZ|Arc]) duplicated as well (t_{12}=3.017, *P*<0.01)((Fig. R.3.4 G-I).

These results suggest that probability of newly generated cells to be recruited into hippocampal memory traces supporting a contextual fear memory is similar in both sedentary and running mice. This implies that the relative increase of recently born neurons in running mice is not compensated by a decrease in their recruitment. Conversely, as the levels of newly generated neurons increase, their inclusion in the memory networks increases at the same proportion. Together, these data again suggest that newly generated neurons and their neighbors form an indistinct population when activated during contextual memory. In addition, running and increasing neurogenesis before training, does not affect contextual memory outcome.

Figure12R.3.4

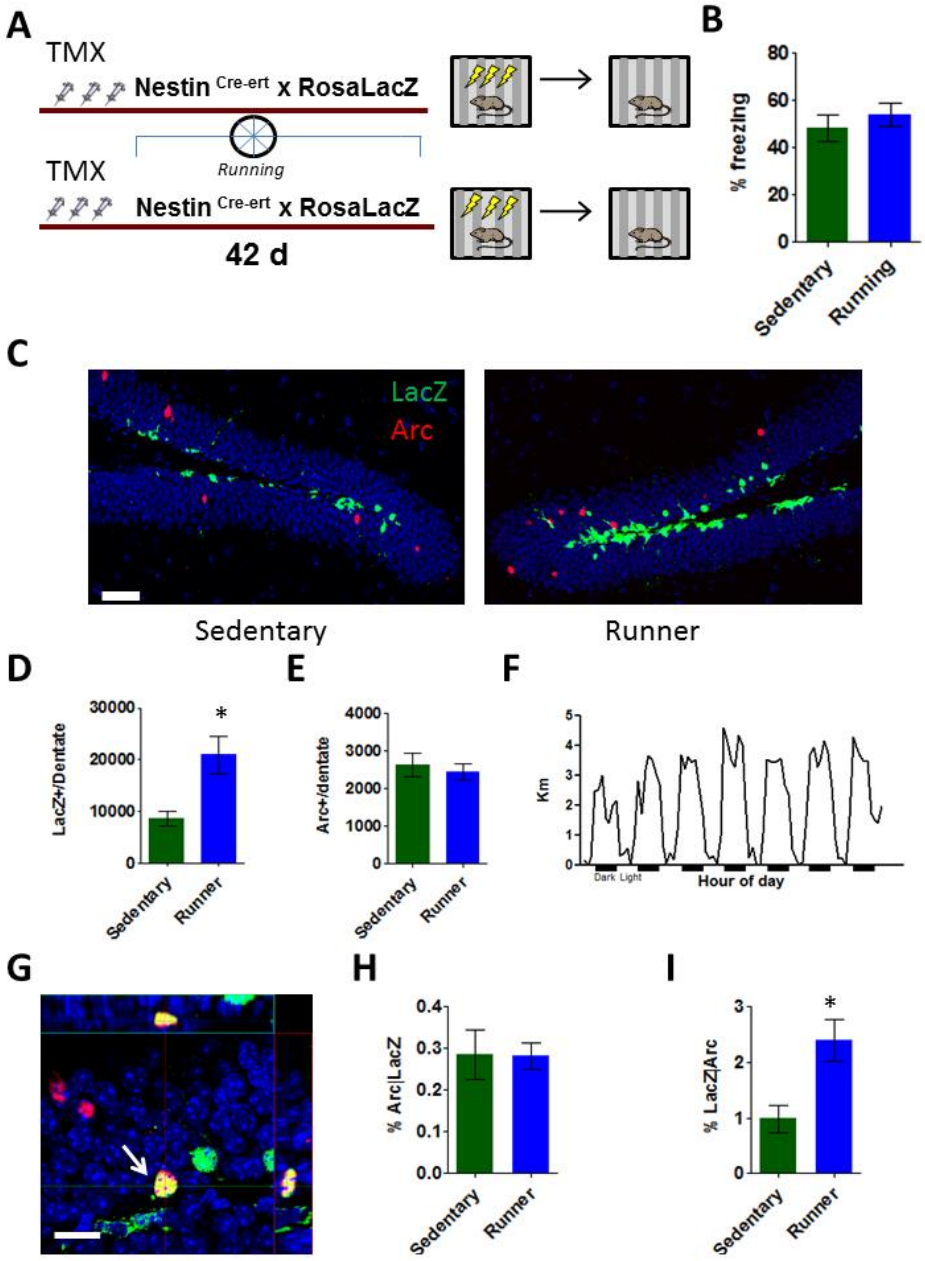


Figure R.3.4. Running increase of neurogenesis, but does not change the ratios of activated newborn cells in memory circuits. A) Schematic of timeline used in the experiment. A cohort of mice with free access to a running wheel mice and express LacZ in Nestin progeny, and a sedentary group that also express LacZ, were treated with tamoxifen (TMX) and trained after 42 days with three shocks in a contextual chamber. The memory levels were tested the next day by placing animals in chamber without shocking. F) Mice had 24hr access to a running wheel preferentially running during dark hours, for several kilometers per day. B) Running did not increase the levels of retention and those are similar to sedentary animals. C,D,E) A 2-fold increase in the number of LacZ+ cells was observed in the running mice (, P<0.01) while Arc+ cells were equivalent in both groups (Scale bar 50µm). G) A subset of cells in both groups expressed both LacZ and Arc (Scale bar 20mµ). H) Proportion of LacZ+ cells that express Arc+ is similar between groups. I) There is an increase in the proportion of Arc+ that also express LacZ (*, P<0.01).*

3.4.13 Increasing neurogenesis by voluntary exercise does not affect recruitment of newborn neurons, but increases the contribution of new granule cells to the spatial memory trace.

Voluntary aerobic exercise increases hippocampal neurogenesis levels in large amounts, and has been reported to enhance spatial memory (van Praag et al., 1999a, van Praag et al., 1999b). Here we assessed the rates of activation on newborn hippocampal cells in sedentary and running mice induced by recall of a spatial memory following intensive training in the water maze. To identify newly generated cells, we injected them with the thymidine analogue BrdU. To identify activated neurons, we used the activity-regulated gene c-Fos (Kee et al., 2007a). Mice began running at 7 weeks of age and showed a consistent rhythmic pattern of running, with peaks of activity during their active dark phase, and lower activity during the light phase of the light-dark cycle, a finding consistent with the active phases of the rodent's circadian rhythm. The mice in the cage ran about 6 km per day (Fig. R.3.5 A,B).

Sedentary and running mice were treated with BrdU to mark newborn cells, and 6 weeks later, the mice were trained in the hidden platform version of the water maze. There were no differences in the latency to find the platform between sedentary and running animals during training ($F_{4, 132}$ =1.63, P>0.05) (Fig. R.3.5 C). Twenty-four hours after the completion of training, we removed the platform and tested the mice in a 60 s probe test. The time spent by the mice in area 20 cm zone (centered on the former platform location) vs. equivalent zones in the other three quadrants of the pool was used to assess spatial memory. Both groups showed significant discrimination, spending more time in target vs. other areas (Sedentary t_{15} =6.27, P<0.001; Running t_{18}=8.84 P<0.001) but when comparing the time spent in target zone between groups, no difference was found (t_{33}= 1.25, P>0.05) (Fig. R.3.5 D,E).

As we previously reported, following the probe tests we found sparse expression of c-Fos in the DG, consistent with previous data (Kee et al., 2007a, Stone et al., 2010) with about 0.5% of

the total granule cell population expressing c-Fos. Moreover, we observed similar counts of c-Fos sedentary and running groups ($t_{18} = 0.67$, $P > 0.05$). Consistent with previous results, BrdU+ cells increased 3-fold in the running group compared with the sedentary animals ($t_{18} = 5.32$, $P < 0.001$)(Fig. R.3.6 A,C-D). A subset of the observed cells were double labeled. First, we computed the probability of c-Fos expression in BrdU+ cells (P[c-Fos|BrdU]) and found that this was similar in young and middle-aged mice ($t_{18} = 0.03$, $P > 0.05$). However, as neurogenesis increased, the likelihood that an activated granule cell was a newborn neuron (P[BrdU|c-Fos]) increased 3-fold as well ($t_{18} = 3.44$, $P < 0.01$) (Fig. R.3.6 B,E,F).

These results suggest that the probability of newly generated cells being recruited into hippocampal memory traces supporting a water maze memory is similar both in runners with high neurogenesis levels and in sedentary mice with regular levels of neurogenesis. This indicates that a strong increase of recently born neurons running mice is not compensated by a decrease, as a way to compensate their proportion in memory supporting circuits. Conversely, as the levels of newly generated neurons increase, their inclusion in the memory networks increases too. Together, these data suggest that newly generated neurons and their neighbors form an indistinct population when activated during spatial memory. It also shows that a strong increase in neurogenesis itself does not drive increases in memory performance.

Figure13R.3.5

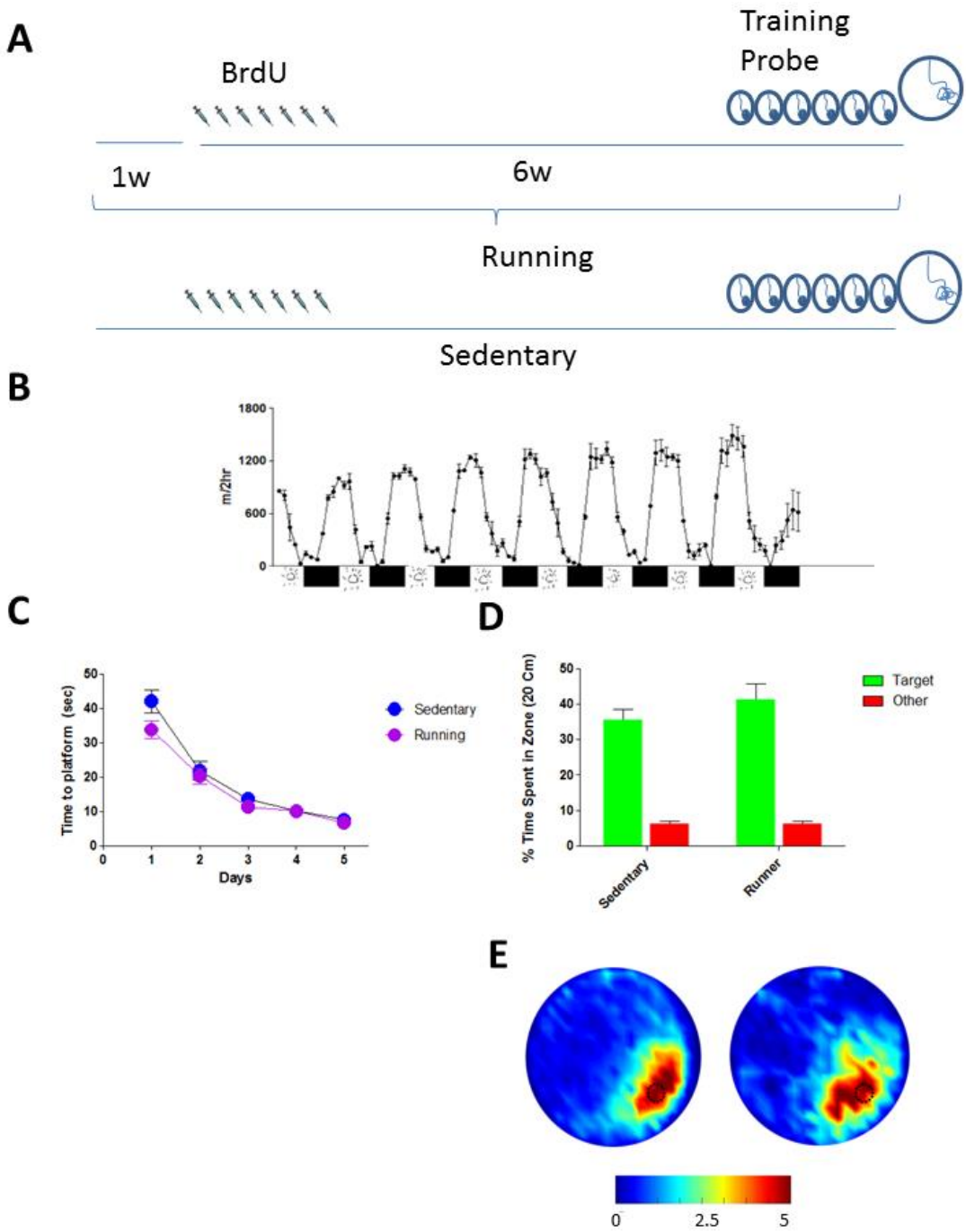


Figure R.3.4 *Running increases neurogenesis but does not enhance spatial memory. A) Schematic of timeline used in the experiment. After one week of running, both sedentary and running mice were treated with BrdU for seven days. After six weeks of free running, they were trained during five days with six trials per day to find a hidden platform in the water maze, and the next day they were probed without platform. B) Trace of the running during a week, showing preferential running during dark hours. C) Both runners and sedentary mice similarly learned the platform location. D, E) Both groups showed a strong preference for the target zone of the maze during the probe, which also can be observed in the density plots with a color scale representing the number of visits of a 5 x 5cm area.*

Figure14R.3.6

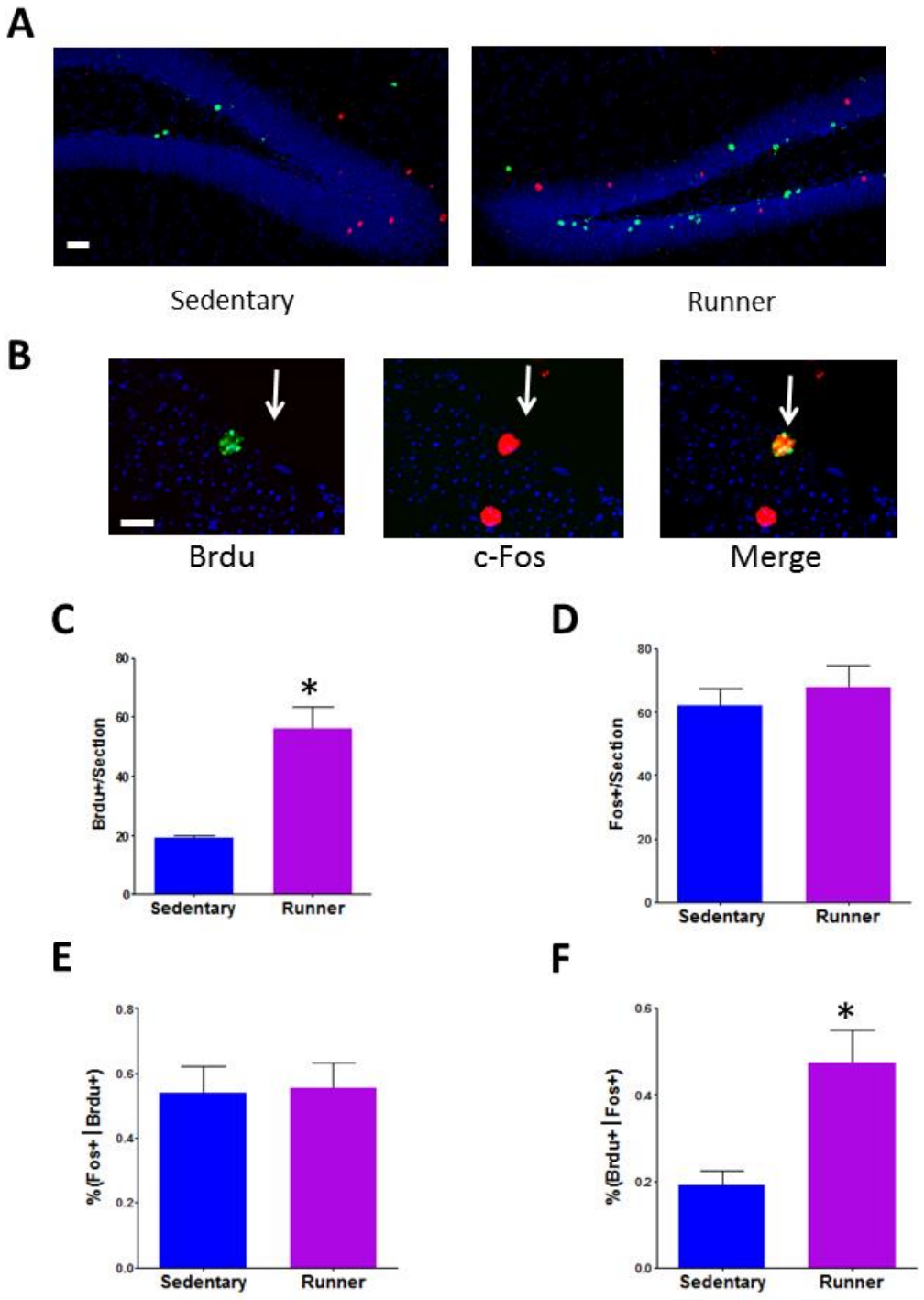


Figure R.3.4 *A) running induced increase in neurogenesis does not change the ratio of activated newborn cells in a memory circuit. A,C,D) Running increases BrdU labeling 3-fold after 6 weeks (*, P <0.001) while c-Fos numbers are constant across groups after activation (Scale bar 50µm). B) A subset of granule cells were stained for BrdU or c-Fos as shown in the representative picture (Scale bar 20µm). E) Proportion of BrdU+ cells that express c-Fos is similar between groups. I) There is an increase in the proportion of BrdU+ that also express c-Fos (*, P<0.01).*

3.4.14 The age-associated neurogenesis decline does not exerts influence over the recruitment rates of newborn cells, while the newborn cells contribution to memory circuits declines with aging.

Hippocampal neurogenesis continuously decreases in adult animals becoming very scarce in middle-aged ones. We wanted to take advantage of this continuous decay on neurogenesis to assess the proportional changes in newborn neurons recruitment and participation in memory circuits across ages and constant neurogenesis decline.

We started BrdU treatment in a group of 2 month-old mice, 6 month-old mice and 10 month-old mice. After 6 weeks, some of these mice were directly used for histology without any learning (control) and the rest were trained in the spatial water maze and probed (Fig. R.3.7 A). We found that when comparing the levels of c-Fos expression in the dentate gyrus of all the groups, there was no difference between the three ages analyzed ($F_{2, 104} = 0.61$, $P > 0.05$) but there was a significant increase in the three groups after water maze training ($F_{1, 104} = 21.29$, $P < 0.001$)(R.3.8 D). Quantification of BrdU positive cells indicated an age-related decline in neurogenesis rates similar in both water maze treated animals or home cage controls ($F_{2, 91} = 11.88$, $P < 0.001$) (Fig. R.3.8 B,C).

Animals trained in the water maze showed no differences in the latency to find the platform between the three ages tested ($F_{8, 364} = 1.6$, $P > 0.05$). 24 hours after the completion of training, we removed the platform and tested the mice in a 60 s probe test. The ages showed significant discrimination, spending more time in target vs. other areas (2-month $t_{29} = 6.43$, $P < 0.001$; 6-month old $t_{30} = 7.89$, $P < 0.001$; and 10-month old $t_{32} = 7.65$, $P < 0.001$) also no memory difference between ages was detected (no % in target zone x Age interaction; $F_{2, 91} = 0.6$, $P > 0.05$) (Fig. R.3.7 C,D).

A subset of the observed cells were double labeled with both BrdU and c-Fos. First, we computed the probability of c-Fos expression in BrdU+ cells (*P*[c-Fos|BrdU]) (proportion of active new neurons) and found that this was similar in the three age groups ($F_{2,91} = 0.07$, $P > 0.05$). However, as neurogenesis (BrdU+ cells) declined with age, the likelihood that an activated granule cell was a newborn neuron (proportion of the memory trace composed by newborn neurons) (*P*[BrdU|c-Fos]) declined as well ($F_{2, 91} = 4.95$, $P < 0.01$) (Fig. R.3.8 E,F).

When analyzing the relationship between the levels of neurogenesis during aging and the probability of newborn cells being incorporated into memory circuits (*P*[c-Fos|BrdU]), we did not find any correlation overall or in any of the three age groups (2 month-old Pearson r = 0.02, $P > 0.05$; 6 month-old r = 0.04, $P > 0.05$; 10 month-old r = 0.06, $P > 0.05$; overall experiment r= 0.001 $P > 0.05$)(Fig. R.3.8 H). Nevertheless, the likelihood that an activated granule cell was a newborn neuron (*P*[BrdU|c-Fos]) strongly correlates with the declining levels of neurogenesis, in each group and the overall result (2 month-old Pearson r = 0.57, $P < 0.01$; 6 month-old r = 0.41, $P < 0.05$; 10 month-old r = 0.44, $P < 0.05$ and overall r = 0.54, $P < 0.001$). When the resulting slopes are compared, no difference is found between them showing a constant effect due to neurogenesis decline which could be interpreted as a causal-effect of neurogenesis levels ($F_{2,87} = 1.28$, $P > 0.05$)(Fig. R.3.8 I).

Spatial memory retention also does not show any decay due to neurogenesis decline. When levels of neurogenesis were compared with time spent in target zone during memory test, no correlation between memory and newborn cells was found (2 month-old Pearson r = 0.0005, $P > 0.05$; 6 month-old r = 0.11 $P > 0.05$; 10 month-old r = 0.002, $P > 0.05$; overall experiment r = 0.02, $P > 0.05$) (Fig. R.3.8 G).

These results suggest that the probability of newly generated cells being recruited into hippocampal memory traces supporting a water maze memory is similar in the three age groups.

This suggests that the relative scarcity of recently born neurons in older mice is not compensated by an increase in their recruitment. Conversely, as the levels of newly generated neurons decrease, their inclusion in the memory networks decreases too. This conclusion is corroborated by a strong correlation between the decaying neurogenesis and rates of contribution. Together, these data suggest that newly generated neurons and their neighbors form an indistinct population when activated during spatial memory.

Figure15R.3.7

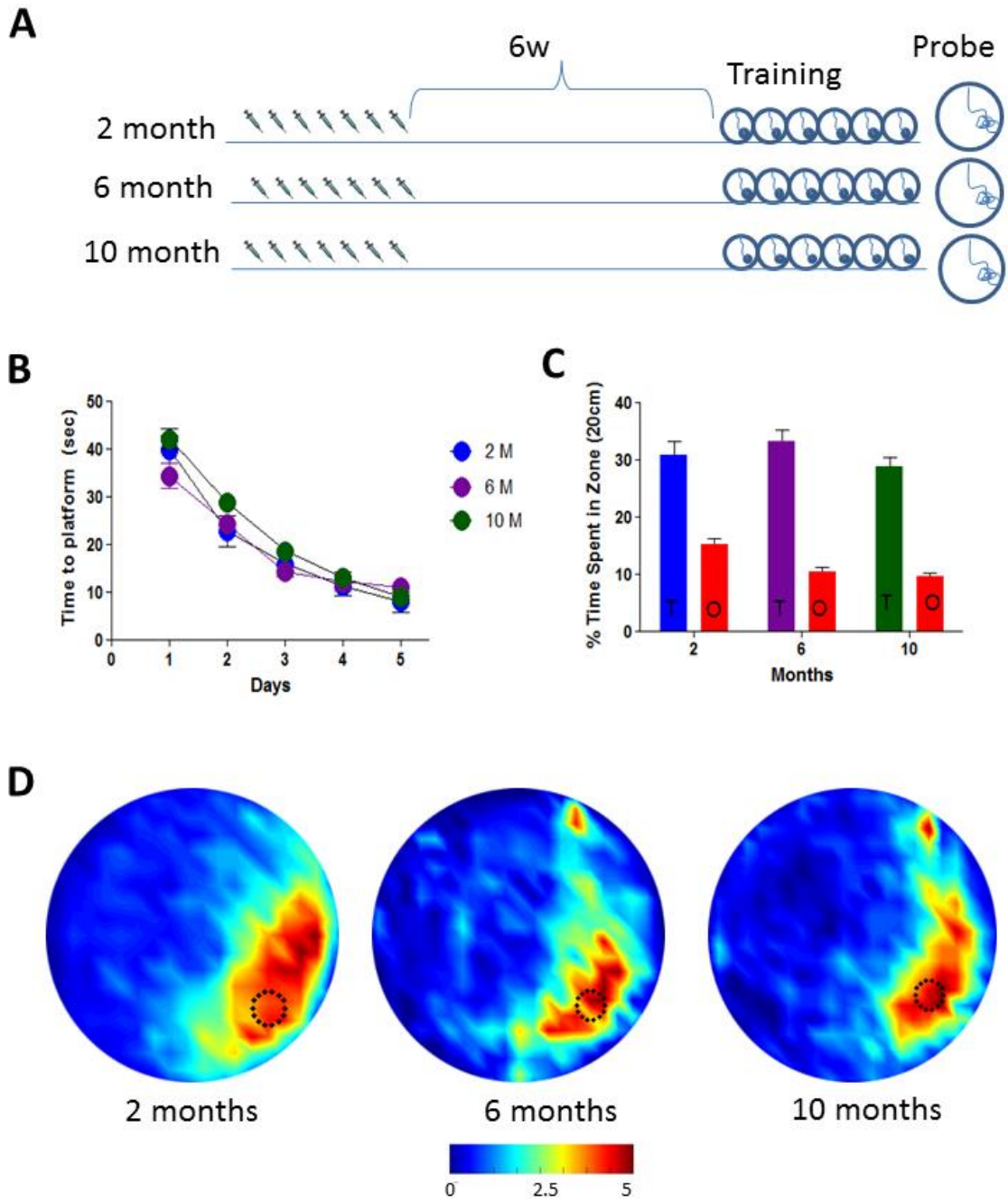


Figure R.3.7. *Aging is a natural method for evaluating the impact of decreasing neurogenesis in the hippocampal networks organization. A) Schematic of timeline used in the experiment. At 2, 6 and 10 months old, mice received injections of BrdU for 7 days. After 6 weeks, they were trained for 5 days, then were given a probe test. B) The three aging groups learned equally during the 5 days of training. C) After training, they were given a probe test in which the three groups showed a consistent bias for the target zone vs. the other three equivalent zones. D) Density plots show the most visited areas with a color scale representing the number of visits of a 5 x 5cm area.*

Figure 16R.3.8

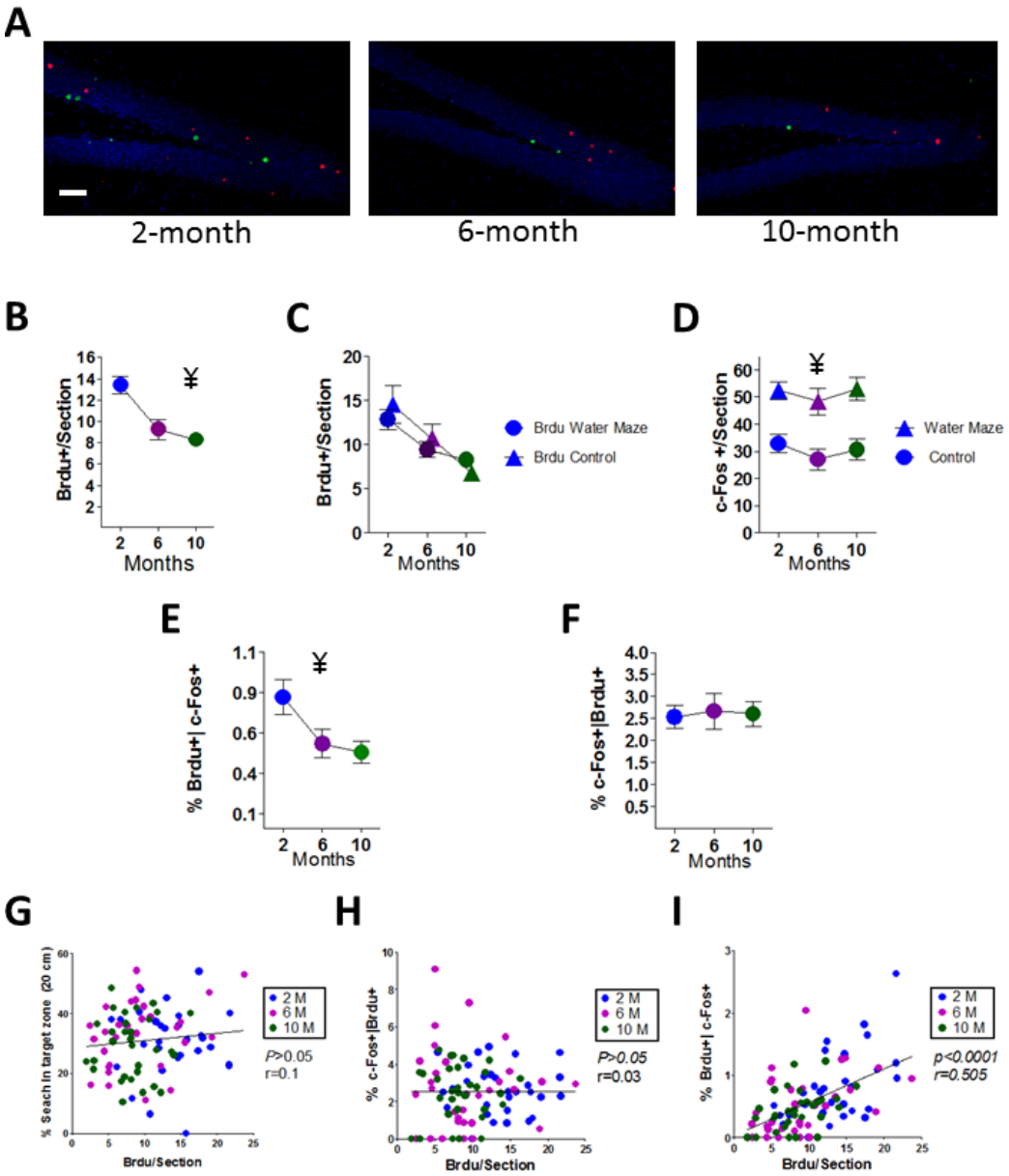


Figure R.3.8 ***Aging does not influence in the proportion of newborn cells recruited into the memory circuits. However, it does affect the proportion of the memory trace composed of newly generated cells. A) A decrease of BrdU (green) positive cells occurred, but there was not an evident decrease in Fos+ expression related to aging in the three age groups (Scale bar 50µm). B,C) The levels of neurogenesis decrease with aging and did not change with training relative to the home cage condition. D) Fos+ cells are similar in the three ages analyzed after probe test; however, in the three ages, they differ from home cage controls. E) The proportion of the memory trace (Fos+ cells) composed with newborn neurons (BrdU+ cells) decreased in the two aged groups vs. the younger group. F) The proportion of newborn cells (BrdU+ cells) that are activated (Fos+ cells), does not change between the three aging groups. G) Memory levels are plotted against the levels of BrdU+ cells; we do not observe a correlation between the two factors. H) Proportion of c-Fos+ cells within the BrdU+ cell do not show correlation when compared with neurogenesis levels. I) When rates of active cells (Fos+) composed by newly generated cells (BrdU+) are plotted against the total levels of neurogenesis, we observe a direct correlation between the two factors. ¥ denotes aging main effect or training effect (P <0.01).***

3.5 Discussion

Neurons generated in the adult hippocampus show transiently higher plasticity (increased LTP magnitude, and lower threshold for induction of LTP) (Schmidt-Hieber et al., 2004, Ge et al., 2007). Given this distinctive physiology it has been proposed that new neurons make distinct contribution to hippocampal memory function (Aimone et al., 2009, Aimone et al., 2011). In particular, their hyper-plasticity might make them more likely than their developmentally generated neighbors to be recruited into memory-supporting circuits. In the present study, we tested this hypothesis by looking at the rates of recruitment of adult generated DGCs in memory circuits.

If adult generated cells constitute a distinct population within the memory circuits, there might be a selective pressure towards maintaining a fixed proportion in a given memory trace. Alternatively, if adult generated DGCs and their neighbours are functionally indistinct, the proportion of memory trace composed by adult generated neurons should correspond to the levels of neurogenesis. To test these two alternative hypotheses, we used naturalistic and transgenic strategies to manipulate levels of adult neurogenesis. We have found that fluctuating neurogenesis levels do not influence the likelihood of newborn neurons being recruited into memory circuits. Rather, the proportion of the memory trace composed by adult generated neurons changes as a function of the availability of newborn neurons. Therefore, all mature granule cells in the dentate gyrus, whether born developmentally or during adulthood, appear to be functionally indistinct.

While adult-generated DGCs exhibit transiently higher levels of plasticity (between 2-6 weeks of age), eventually they display similar electrophysiological characteristics and connectivity compared to their developmentally generated neighbor DGCs (Laplagne et al., 2006, Laplagne et al., 2007). Other data shows that despite their initially higher levels of plasticity, newborn neurons

are not maximally recruited until they are 6 weeks of age (Kee et al., 2007b), the time point at which we did our evaluation. Therefore, when new neurons are highly plastic, they do not integrate into memory circuits. Instead, integration occurs when they show similar characteristics than their neighbours.

The idea that adult-generated neurons are functionally indistinct from their developmentally-generated neighbors has consequences for interpreting neurogenesis suppresion studies. For example, while some studies find that suppresion of newborn neurons impairs formation of hippocampus-dependent memories (Snyder et al., 2005, Winocur et al., 2006, Zhang et al., 2008), many other studies find no relation (Shors et al., 2002, Merrill et al., 2003, Jaholkowski et al., 2009). The lack of effects of pre-training suppresion (see also Chapter 7), suggests that memories can be formed in the absence of new neurons, most likely because functionally indistinct neurons can substitute. Where there are effects, this may be because the tasks are more cognitively taxing, and therefore more sensitive at detecting neurogenesis-related phenotypes. Alternatively, side-effects of the manipulations used to depress neurogenesis may impede learning in non-specific ways (e.g., irradiation sickness).

A second implication of the idea that new neurons –once they reach six weeks of age- are functionally equivalent to previously generated DGCs—is that there should not be any relation between levels of neurogenesis and memory performance. Indeed, consistent with some other studies (Merrill et al., 2003), we show that there is no correlation between spatial memory and neurogenesis levels. None of the three age groups we used in this study showed correlations between memory and neurogenesis, suggesting there is no causal relationship. In contrast, some previous studies reported significant correlations between neurogenesis and memory. However, these studies used all the groups together to calculate the correlation coefficient (Aizawa et al.,

2009, Creer et al., 2010). Therefore, their results can be explained by causes independent of neurogenesis, as consequences of other differences between their experimental groups.

We found that levels of neurogenesis correspond with the number of new cells incorporated into memory circuits. Therefore, these findings show that with declining neurogenesis levels, there is not a compensatory mechanism keeping a fixed proportion of new neurons within the memory trace, which would maintain the circuit functionality. Moreover, when newborn neurons decrease, there is also a decline in their use, without compromising memory efficiency.

DGCs generated during adulthood are functionally equivalent to their developmentally generated neighbours. This functional equivalence facilitates their possible use in regenerating therapy, as they would be able to substitute loss of neurons generated early in life. As newly generated neurons are functionally equivalent to their neighbours, they would not be expected to improve memory capabilities on their own. However, newly generated neurons may increase the capacity of the dentate gyrus by generating a new substrate for encoding. The addition of new functional neurons to the circuit also leads to a restructuring of hippocampal circuits, suggesting that the addition of new neurons may destabilize already stored memories. This idea has led to the suggestion that neurogenesis may modulate forgetting (Feng et al., 2001, Meltzer et al., 2005) or memory clearance (Kitamura et al., 2009).

4. The effect of suppressing hippocampal neurogenesis on spatial learning changes across the lifespan.

4.2 Abstract

Reducing hippocampal neurogenesis sometimes, but not always, disrupts hippocampus-dependent learning and memory. Here, we tested whether animal age, which regulates rate of hippocampal neurogenesis, is a factor that influences whether deficits in spatial learning are observed after reduction of neurogenesis. We found that suppressing the generation of new hippocampal neurons via treatment with temozolomide, an anti-proliferation agent, impaired learning the location of a hidden platform in the water maze in juvenile mice (1-2 months old) but not in adult mice (2-3 months old) or middle-aged mice (11-12 months old). These findings suggest that during juvenility, suppression of neurogenesis may alter hippocampal development, whereas during adulthood and aging, pre-existing neurons may compensate for the lack of new hippocampal neurons.

4.3 Introduction

The dentate gyrus of the hippocampus is a brain region in which new neurons continue to be generated throughout the lifespan (Christie and Cameron, 2006, Ming and Song, 2011). Due to the critical role of the hippocampus in cognitive function (Eichenbaum, 2004a, Wang and Morris, 2010b), the potential contribution of newly-generated neurons to learning and memory has been the focus of numerous studies (reviewed in Deng et al., 2010). These studies, however, have produced conflicting results, with some reporting that reduction of neurogenesis impairs hippocampus-dependent learning and memory (e.g., Winocur et al., 2006, Imayoshi et al., 2008, Garthe et al., 2009) and others reporting that reduction of neurogenesis has little or no effect (e.g., Meshi et al., 2006, Hernandez-Rabaza et al., 2009, Jaholkowski et al., 2009). Whether a disruption in learning and memory is observed following a reduction in hippocampal neurogenesis is likely determined by an interplay of factors, including the number of neurons targeted (Ko et al., 2009), the maturational stage of the targeted neurons at the time of testing (Farioli-Vecchioli et al., 2008), the type of behavioral task used to assess learning and memory (Shors et al., 2002), and whether the targeted neurons are removed before or after learning (Arruda-Carvalho et al., 2011).

Age is a strong regulator of hippocampal neurogenesis (Lazarov et al., 2010) and thus is another factor that may influence whether the suppression of neurogenesis produces impairments in learning and memory. After the initial formation of the dentate gyrus during embryonic and early postnatal periods (Altman and Bayer, 1990), the continued generation of new neurons declines steadily with age, with relatively high rates of neurogenesis in juvenility, moderate rates in adulthood, and low rates in old age (Seki and Arai, 1995, Kuhn et al., 1996, Ben Abdallah et al., 2010). Here, we predicted that the effect of reducing neurogenesis on spatial learning would be more pronounced in younger animals, when large numbers of new neurons are being generated

and incorporated into developing hippocampal circuits. We suppressed neurogenesis in juvenile (1-2 months old), adult (2-3 months old) and middle-aged (11-12 months old) mice by treatment with temozolomide (TMZ), which alkylates the DNA of dividing cells thereby leading to apoptotic cell death. We found that TMZ treatment impaired learning to locate a hidden platform in the water maze in juvenile mice but not in adult or middle-aged mice, suggesting that the likelihood of detecting disruptions in learning and memory after suppression of neurogenesis depends on animal age.

4.4 Materials and methods

4.4.1 Mice.

Mice (129Svev × C57Bl/6, F1 cross) were bred at The Hospital for Sick Children and kept on a 12 hr light/dark cycle (lights on at 0700 hrs) with free access to food and water. All procedures were approved by the Animal Care Committee at The Hospital for Sick Children.

4.4.2 TMZ treatment

Starting at 1 (juvenile), 2 (adult), or 11 (middle-aged) months of age, mice were treated with TMZ using a previously established protocol (Garthe et al., 2009). Mice were given 4 rounds of TMZ (Sigma); each round occurred one week apart and consisted of one injection per day (25 mg/kg, i.p.) for 3 consecutive days (juvenile n = 16 (4M, 12F), adult n = 17 (4M, 13F), middle-aged n = 14 (5M, 9F)). Control mice were injected with vehicle (10% DMSO in 0.9% saline) on an identical schedule (juvenile n = 15 (3M, 12F), adult n = 16 (4M, 12F), middle-aged n = 13 (5M, 8F)). TMZ treatment has been found to transiently reduce leukocyte number (i.e., 1 day after treatment), but otherwise has no negative effects on general health, locomotor skill, or exploratory activity in mice (Garthe et al., 2009).

4.4.5 Water maze.

Four weeks after the start of TMZ or vehicle treatment, mice were trained in the hidden platform version of the Morris water maze (Morris, 1981). A circular plastic pool (120 cm diameter, 50 cm height) was filled with water (~26º C) to a depth of 40 cm. Water was made opaque by the addition of nontoxic paint. A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface in the center of one of the pool quadrants. The pool

was surrounded by curtains, located at least 1 m from the pool wall, that were painted with distinct geometric cues. Mice received 3 training trials per day for 6 days. Trials started when mice were released into the pool, facing the wall, from one of 4 possible points. A different release point was used for each trial on each day, and the order of release points varied pseudorandomly across days. Trials ended when mice reached the platform or 60 s elapsed. If a mouse failed to find the platform, it was guided by the experimenter. Before training on days 1, 3, and 5, mice received a 60-s probe test with the platform removed from the pool. A final probe test was given on day 7. Swim paths were recorded by an overhead video camera and tracked using automated software (Watermaze 3.0, Actimetrics). The dependent measure during training was latency to reach the platform. The dependent measures during probe tests were number of crosses over the platform location (10 cm diameter), percentage of time spent in a circular zone (15 cm radius) centered on the platform location, average proximity to the platform location (Gallagher et al., 1993), and percentage of time engaged in thigmotaxic behavior (swimming within 5 cm of the pool wall). Density plots depicting areas of the pool visited more frequently during the probe tests were generated using a custom program (Matlab 2010, Mathworks) developed in our laboratory.

4.4.6 BrdU injection and immunohistochemistry

One day following the last TMZ or vehicle injection, mice received a single injection of 5-bromo-2'-deoxyuridine (BrdU, 200 mg/kg, i.p., Sigma) dissolved in phosphate-buffered saline (PBS). Twelve days later, mice were anesthetized with chloral hydrate and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA). Brains were postfixed overnight in PFA and transferred to 30% sucrose. Coronal sections (40 µm) were cut along the entire anterior-posterior extent of the dentate gyrus using a cryostat. Sections were treated with 1 N HCl at 45º C for 30 min, 1% H_20_2 for 15 min, and 0.2 M glycine in PBS for 10 min. To quench age-related

autoflourescence, sections were also treated with 0.15 mM CuSO4 for 1 h (Schnell et al., 1999). Sections were incubated with the primary antibody (rat anti-BrdU, 1:1000, Accurate Chemicals) overnight and the secondary antibody (Alexa-488 goat anti-rat, 1:1000, Invitrogen) for 2 h. Antibodies were diluted in blocking solution containing 2.5% bovine serum albumin, 5% goat serum, and 0.3% Triton X-100 dissolved in PBS. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:10000, Sigma) and mounted on slides with Permafluor anti-fade medium (ThermoScientific). Quantification of $BrdU^+$ cells was performed for all middle-aged mice and a random subset of juvenile (TMZ $n = 6$, vehicle $n = 6$) and adult (TMZ $n = 7$, vehicle $n = 6$) mice using a Nikon epifluorescent microscope. $BrdU^+$ cells in the subgranular zone were manually counted in every fourth section, and an estimate of the total number of $BrdU^+$ cells per dentate gyrus was obtained by multiplying the number of cells counted by 4.

4.4.7 Statistical analysis

BrdU data were analyzed using unpaired *t*-tests. Behavioral data were initially analyzed using ANOVA with *Age* (juvenile, adult, middle-aged) and *Treatment* (vehicle, TMZ) as between-subject factors and *Day* as a within-subject factor. Due to the presence of higher-order interactions, separate ANOVAs with *Treatment* as a between-subject factor and *Day* as a within-subject factor were performed within each *Age*, followed by unpaired *t*-tests or Tukey's post-hoc tests. Because both male and female mice were used, we initially included *Sex* as a factor in the ANOVAs; no significant effects involving *Sex* were found, therefore this factor was dropped from analysis.

4.5 Results

As expected (Seki and Arai, 1995, Kuhn et al., 1996, Ben Abdallah et al., 2010), juvenile mice had more new hippocampal neurons compared to adult mice, and middle-aged mice showed a marked decline in hippocampal neurogenesis (Fig. 1B-D). Despite these age differences,

treatment with TMZ (Fig. 1A) effectively suppressed neurogenesis in all age groups (juvenile: t_{10}=6.14, p<.001, adult: t_{11}=7.14, p<.001, middle-aged: t_{27}=16.96, p<.001), reducing the number of BrdU$^+$ cells in the subgranular zone of the dentate gyrus by 70-80% (Fig. 1B-D).

Figure R.4.1 Aging and TMZ treatment decrease levels of neurogenesis

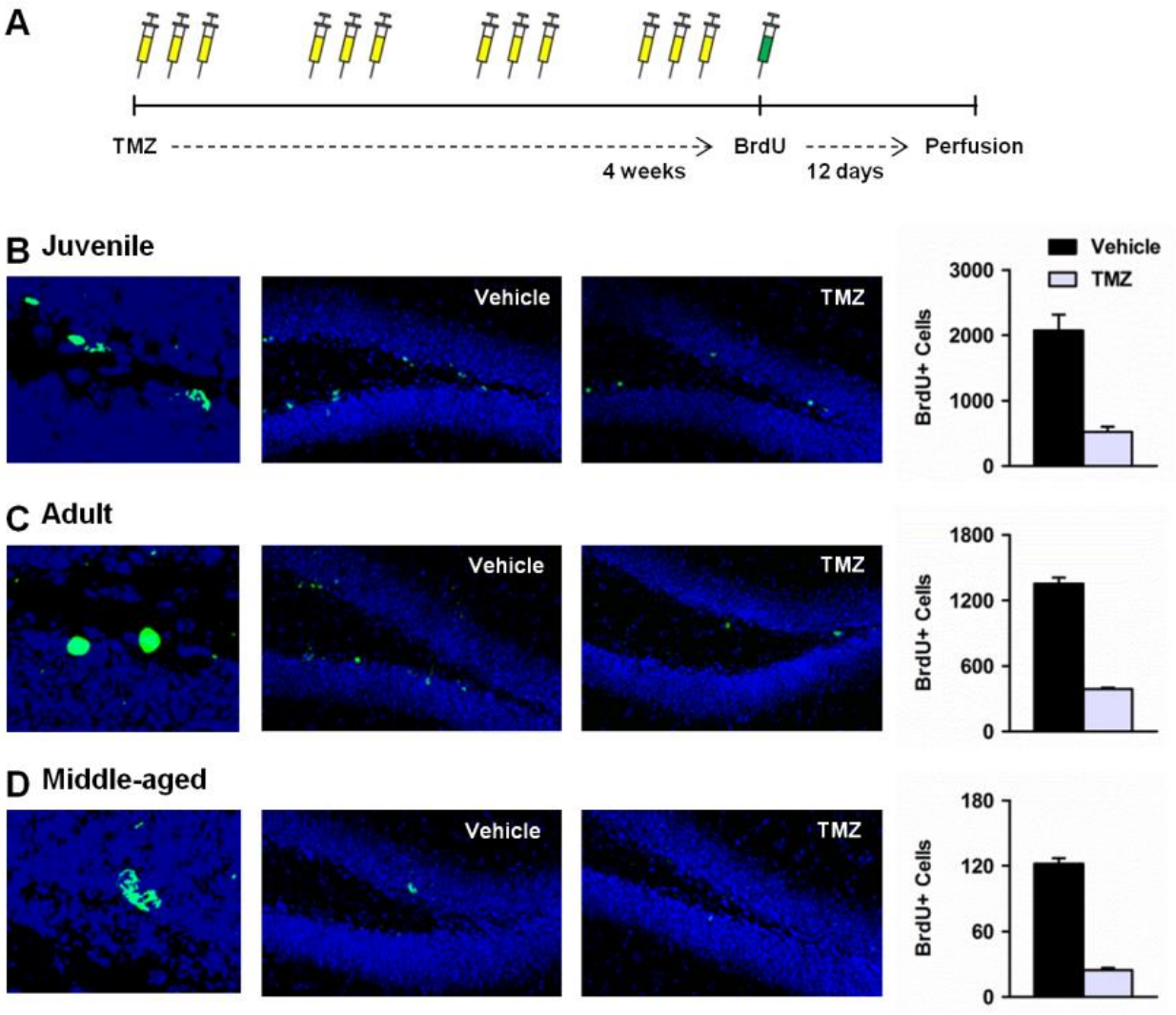


Figure R.4.1 *TMZ treatment. Four weeks of TMZ treatment (**A**) reduced the number of BrdU$^+$ cells in the subgranular zone of the dentate gyrus in (**B**) juvenile, (**C**) adult, and (**D**) middle-aged mice. Left panels: high-magnification image of BrdU$^+$ cells; middle panels: low-magnification image of BrdU$^+$ cells in vehicle-treated mice; right panels: low-magnification image of BrdU$^+$ cells in TMZ-treated mice.*

Following TMZ treatment, juvenile (Fig. 2A), adult (Fig. 3A), and middle-aged (Fig. 4A) mice were trained to locate a hidden platform in the water maze. To assess the progression of spatial learning, no-platform probe tests were conducted at regular intervals throughout training (Days 1, 3, 5, and 7). Overall, we found differences among age groups in water maze performance (*Age* main effect, latency: $F_{2,85}$=2.85, p<.001, crosses: $F_{2,85}$=18.46, p<.001, zone: $F_{2,85}$=13.12, p<.001, proximity: $F_{2,85}$=5.92, p=.004, speed: $F_{2,85}$=6.70, p=.002; *Age* × *Day* interaction, crosses: $F_{6,255}$=2.42, p=.027, zone: $F_{6,255}$=2.70, p=.015, proximity: $F_{6,255}$=3.12, p=.006, speed: $F_{6,255}$=3.72, p=.001). Consistent with the age-related impairment in spatial learning reported in previous studies (Barnes, 1979, Gallagher et al., 1993), we found that middle-aged mice performed worse than juvenile and adult mice (middle-aged vs. juvenile, latency: p=.003, crosses: p<.001, zone: p=.029, speed: p=.001; middle-aged vs. adult, latency: p<.001, crosses: p<.001, zone: p<.001, proximity: p=.049). We also found that adult mice performed better than juvenile mice (latency: p=.022, zone: p=.034, proximity: p=.004). Furthermore, although all groups of mice showed improvements in performance across days (*Day* main effect, latency: $F_{5,425}$=82.82, p<.001, crosses: $F_{3,255}$=101.68, p<.001, zone: $F_{3,255}$=107.91, p<.001, proximity, $F_{3,255}$=104.99, p<.001, thigmotaxis: $F_{3,255}$=299.60, p<.001, speed: $F_{3,255}$=4.66, p=.003), TMZ-treated mice generally showed poorer performance compared to vehicle-treated mice (*Treatment* main effect, crosses: $F_{1,85}$=5.77, p=.018, zone: $F_{1,85}$=4.47, p=.037; *Treatment* × *Age* × *Day* interaction, thigmotaxis: $F_{6,255}$=2.83, p=.011).

Analysis of performance within each age group, however, revealed that the effect of TMZ treatment on spatial learning was more pronounced in juvenile mice compared to adult and middle-aged mice. Among juveniles, TMZ-treated mice learned the platform location more slowly than

vehicle-treated mice, crossing the platform location fewer times (Fig. 2C; *Treatment* main effect, $F_{1,29}$=6.33, p=.018; *Treatment* × *Day* interaction, $F_{3,87}$=3.15, p=.029; day 5, t_{29}=2.90, p=.007), spending less time in a zone centered on the platform location (Fig. 2D; *Treatment* × *Day* interaction, $F_{3,87}$=2.87, p=.041; day 5, t_{29}=2.90, p=.007), and swimming further from the platform location (Fig. 2E; *Treatment* main effect, $F_{1,29}$=5.49, p=.026). Interestingly, TMZ-treated mice also spent more time swimming along the walls of the pool (Fig. 2F; *Treatment* × *Day* interaction, $F_{3,87}$=2.84, p=.042; day 3, t_{29}=2.05, p=.050), suggesting difficulty in shifting from thigmotaxis to the use of spatial strategies to locate the hidden platform (Garthe et al., 2009). Both groups, however, showed improvements in performance across days (*Day* main effect, latency: $F_{5,145}$=29.37, p<.001, crosses: $F_{3,87}$=31.94, p<.001, zone: $F_{3,87}$=36.40, p<.001, proximity: $F_{3,87}$=17.53, p<.001, thigmotaxis: $F_{3,87}$=54.57, p<.001). Swim speed increased across days (Fig. 2G; *Day* main effect, $F_{3,87}$=6.05, p<.001), but no differences between groups were observed, indicating that TMZ did not disrupt motor function.

Figure18R.4.2 Neurogenesis suppresion impairs memory in juvenile mice

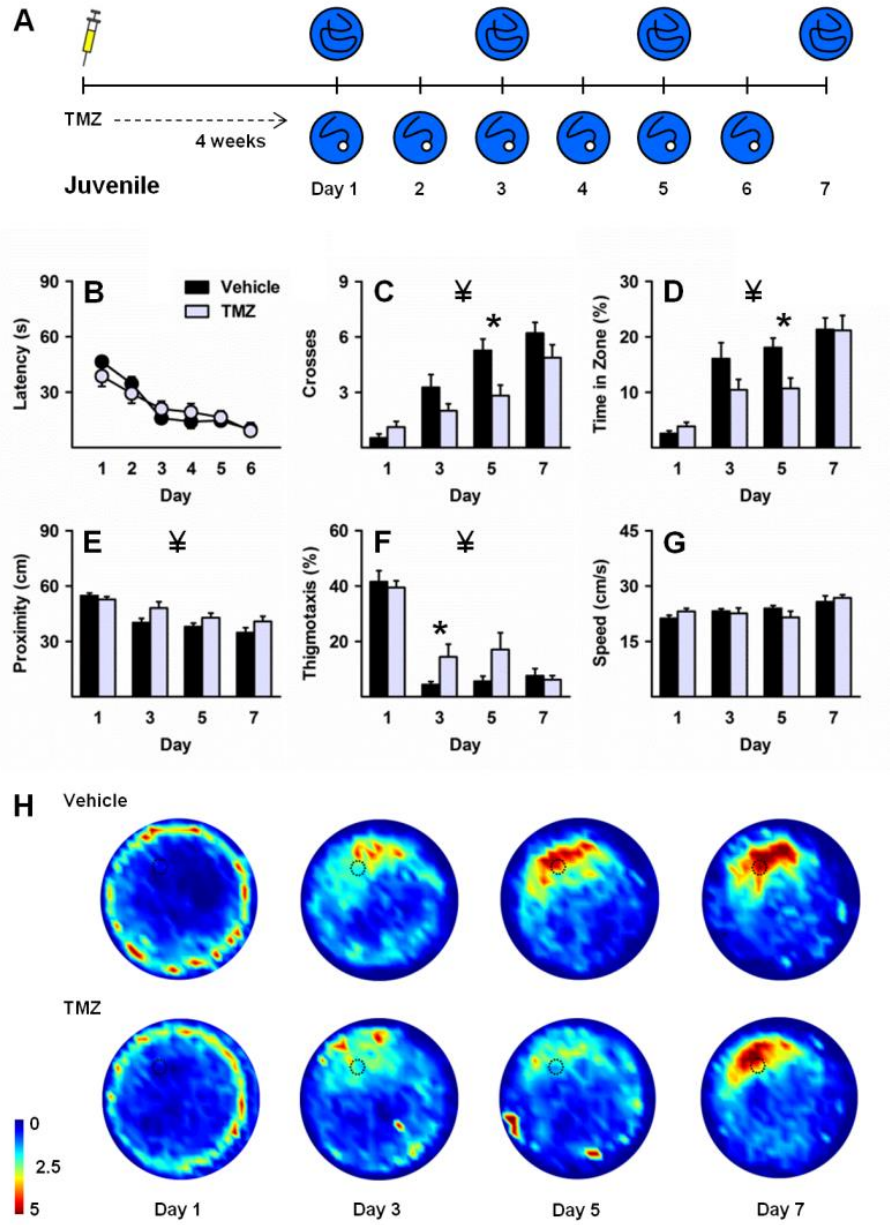


Figure R.2.3*. Juvenile mice. After TMZ treatment (**A**), juvenile mice were trained to locate a hidden platform in the water maze (bottom row of circles), with no-platform probe tests conducted at regular intervals throughout training (top row of circles). (**B**) There was no difference between groups in latency to reach the platform during training. During probe tests, however, TMZ-treated mice showed less precise searching compared to vehicle-treated mice considering (**C**) number of platform crosses, (**D**) time spent in a circular zone centered on the platform location, (**E**) average proximity to the platform, and (**F**) time engaged in thigmotaxic behavior. (**G**) Both groups showed equivalent swim speed. (**H**) Density plots showing where mice concentrated their search for the platform during the probe tests, with color scale representing average number of visits per mouse per 5 × 5 cm area. ¥ denotes Treatment main effect or Treatment × Day interaction, p < .05. * denotes TMZ vs. vehicle, p < 0 .05.*

Figure R.4.3 Neurogenesis suppresion does not impair memory in adult mice

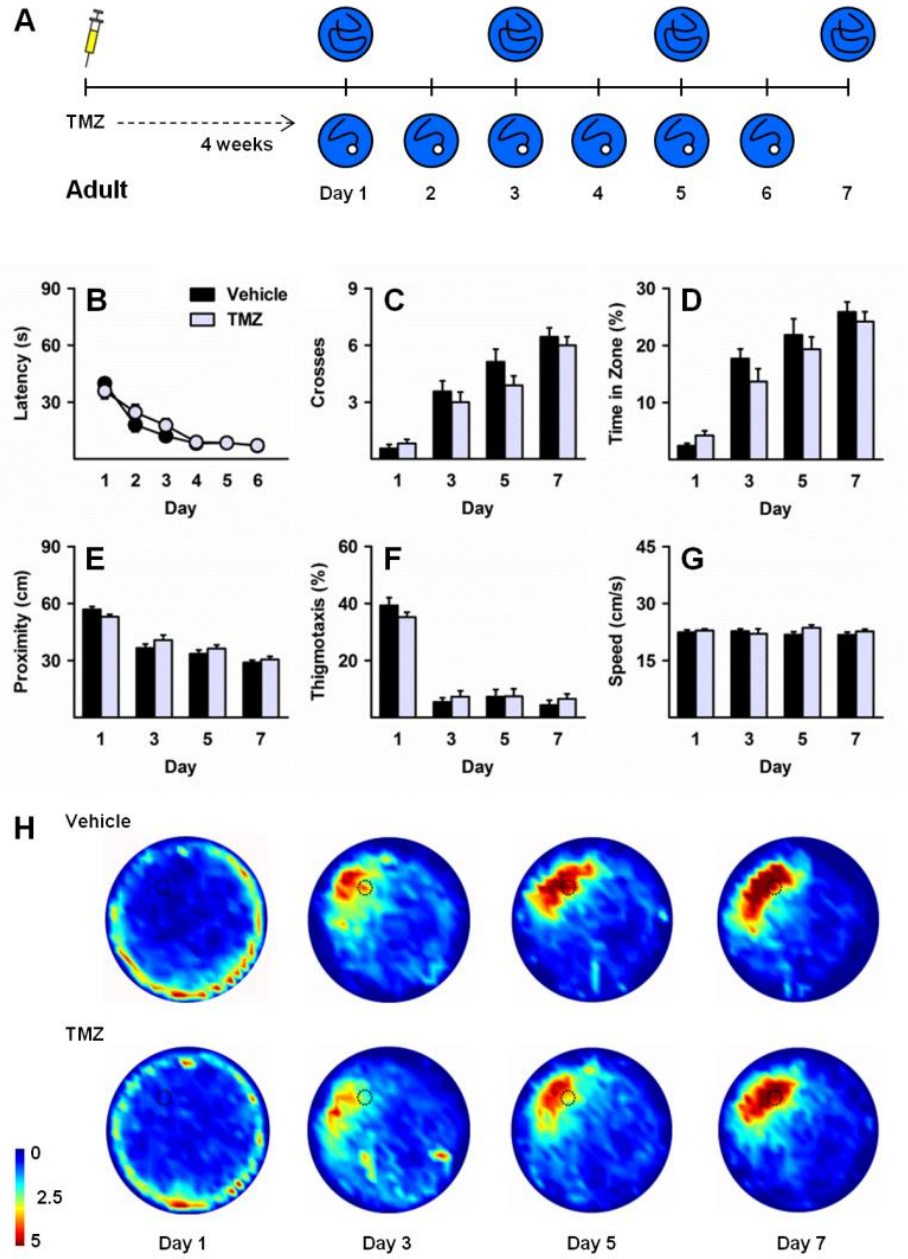


Figure R.2.3*. Adult mice. After TMZ treatment (**A**), adult mice were trained to locate a hidden platform in the water maze (bottom row of circles), with no-platform probe tests given at regular intervals during training (top row of circles). There were no differences between TMZ- and vehicle-treated groups in (**B**) latency to reach the platform during training, (**C-F**) measures of search precision during the probe tests, or (**G**) swim speed. (**H**) Density plots showing where mice concentrated their search for the platform during the probe tests, with color scale representing average number of visits per mouse per 5 × 5 cm area.*

In contrast to juvenile mice, we found no significant effect of TMZ treatment on spatial learning in adult mice. Both TMZ- and vehicle-treated mice showed improvements in performance across days (Fig. 3B-F; *Day* main effect, latency: $F_{5,155}$=46.90, p<.001, crosses: $F_{3,93}$=49.65, p<.001, zone: $F_{3,93}$=51.39, p<.001, proximity: $F_{3,93}$=94.02, p<.001, thigmotaxis: $F_{3,93}$=176.92, p<.001) and equivalent swim speed (Fig. 3G).

Likewise, we found no effect of TMZ treatment on spatial learning in middle-aged mice. Both TMZ- and vehicle-treated mice showed improvements in performance across days (Fig. 4B-F; *Day* main effect, latency: $F_{5,125}$=17.22, p<.001, crosses: $F_{3,75}$=27.30, p<.001, zone: $F_{3,75}$=31.62, p<.001, proximity: $F_{3,75}$=27.56, p<.001, thigmotaxis: $F_{3,75}$=167.80, p<.001) and equivalent swim speed (Fig. 4G).

Figure20R.4.4 Neurogenesis suppresion does not impair memory in middle-aged mice

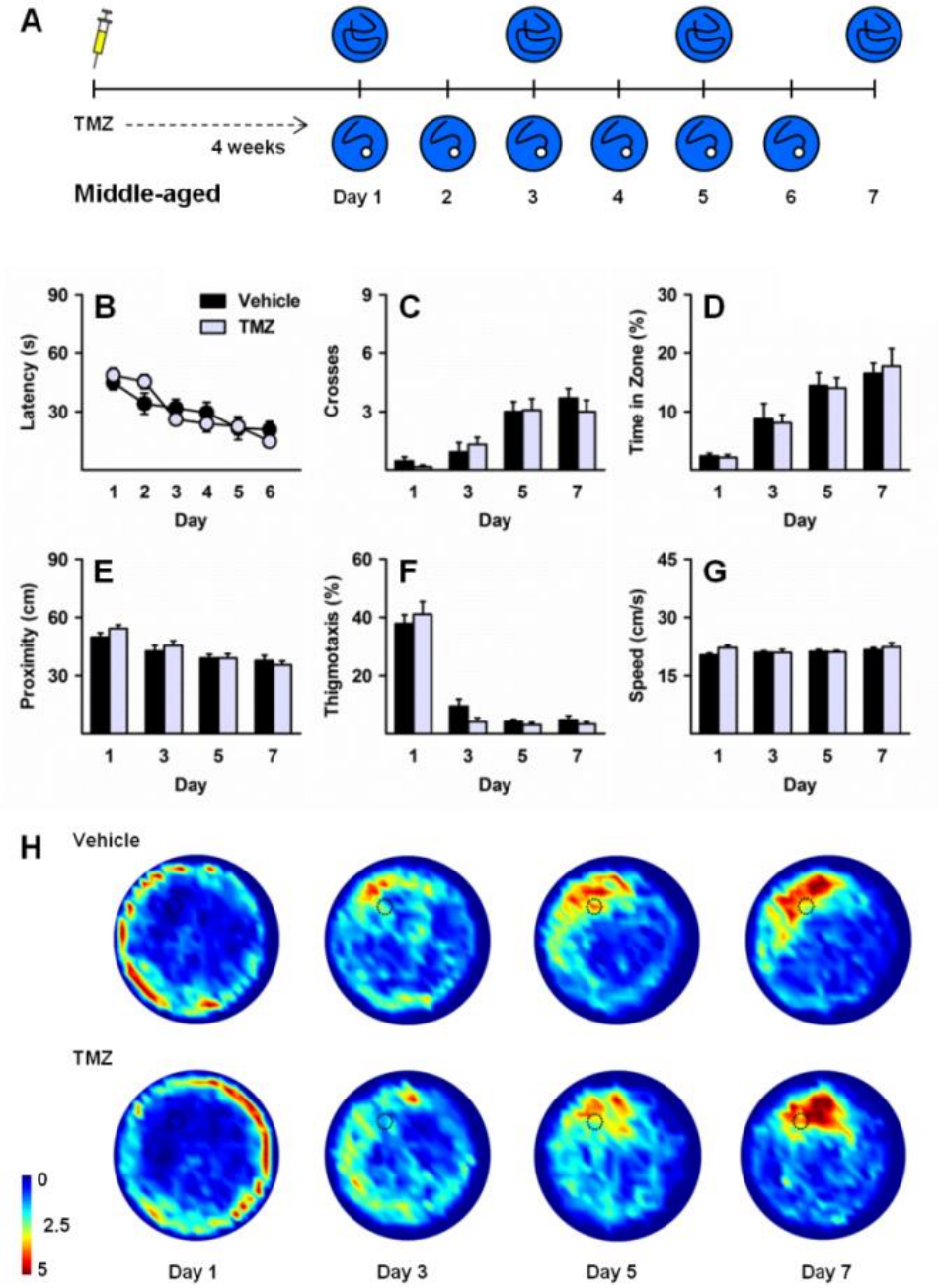


Figure 4*. Middle-aged mice. After TMZ treatment (**A**), middle-aged mice were trained to locate a hidden platform in the water maze (bottom row of circles), with no-platform probe tests given at regular intervals during training (top row of circles). There were no differences between TMZ- and vehicle-treated groups in (**B**) latency to reach the platform during training, (**C**-**F**) measures of search precision during the probe tests, or (**G**) swim speed. (**H**) Density plots showing where mice concentrated their search for the platform during the probe tests, with color scale representing average number of visits per mouse per 5 × 5 cm area.*

4.6 Discussion

We tested whether the effect of suppressing hippocampal neurogenesis on spatial learning depends on the age of the animal. Although treatment with TMZ caused a similar proportional reduction in dentate gyrus neurogenesis in juvenile, adult, and middle-aged mice, we found that this reduction of neurogenesis impaired learning of a hidden platform location in the water maze only in juvenile mice and not in adult or middle-aged mice. Similar to a recent report that decreasing neurogenesis using a transgenic approach altered social behavior in juvenile mice but not in adult mice (Wei et al., 2011), these findings indicate that age is a factor that may influence whether the experimental reduction of neurogenesis affects behavior. Specifically, our results suggest that the younger the animal, the more likely that suppression of neurogenesis will produce behavioral disruptions.

The spatial learning impairment in juvenile mice after suppression of neurogenesis could be attributed to the relatively large number of new neurons affected and/or the impact of this missing population of neurons on the ongoing development of hippocampal circuitry. Although all age groups exhibited a similar proportional reduction (approximately 75%) in neurogenesis in the dentate gyrus after TMZ treatment, the high baseline rate of neurogenesis among juveniles meant that this age group showed the largest decrease in terms of the absolute number of cells (i.e., a 1.5- and 10-fold greater decrease in number of BrdU+ cells compared to adult and middle-aged mice, respectively). Moreover, this relatively large reduction of neurogenesis occurred against the backdrop of continuing structural and functional maturation of the hippocampus (Bachevalier and Beauregard, 1993, Dumas, 2005), with ongoing changes in dendritic arborization (Rahimi and Claiborne, 2007), neurotransmission (Nurse and Lacaille, 1999), and spatial firing (Araque et al., 2002) taking place during the first two postnatal months. Treatments that reduce neurogenesis

during juvenility, therefore, may not only affect a disproportionately large number of new neurons but also alter the initial formation of hippocampal circuitry, leading to observable disruptions in hippocampus-dependent behaviors.

Consistent with some previous studies showing no effect of reducing hippocampal neurogenesis on water maze performance in adult rodents (Shors et al., 2002, Madsen et al., 2003, Raber et al., 2004, Meshi et al., 2006, Jaholkowski et al., 2009), we found no spatial learning impairments in adult or middle-aged mice following neurogenesis suppression. Although adult-generated hippocampal neurons transiently show a lower threshold and higher magnitude of synaptic plasticity during their growth (Schmidt-Hieber et al., 2004, Ge et al., 2007), they ultimately converge with their developmentally-generated neighbors in terms of electrophysiological properties (Esposito et al., 2005, Ge et al., 2007), morphology (Laplagne et al., 2006), connectivity (Laplagne et al., 2006, Toni et al., 2008), and integration into circuits supporting behavior (Stone et al., 2011). This structural and functional similarity between developmentally- and adult-generated neurons may permit pre-existing neurons to compensate for the lack of newly-generated neurons in the adult brain, without an obvious decrement in learning ability. Once the hippocampus has fully matured, therefore, it may be relatively resilient to perturbations of neurogenesis, which may account for the mild or even nonexistent disruptions in learning and memory after reduction of neurogenesis during adulthood (reviewed in Deng et al., 2010).

As animals enter old age, they display declines in both cognitive ability (Barnes, 1979, Gallagher et al., 1993) and hippocampal neurogenesis (Kuhn et al., 1996, Heine et al., 2004), prompting the idea that age-related cognitive deficits may result from a failure in the generation of new hippocampal neurons. This possibility is supported by correlative studies reporting

relationships between low rates of hippocampal neurogenesis and poor spatial learning abilities among aged rodents (Drapeau et al., 2003, Driscoll et al., 2006) (but see Bizon and Gallagher, 2003, Merrill et al., 2003, Bizon et al., 2004) and an experimental study showing that running-induced enhancements in hippocampal neurogenesis are associated with improvements in spatial learning in aged rodents (van Praag et al., 2005). Therefore, it might be expected that the suppression of hippocampal neurogenesis in aged animals would produce impairments in learning and memory. However, in this study, we observed no disruption in spatial learning after reduction of neurogenesis in middle-aged mice. This suggests that age-related cognitive deficits are not caused by fading hippocampal neurogenesis but rather by other neurobiological factors that may co-vary with neurogenesis level.

5. Chronic over-expression of TGFβ1 alters hippocampal structure and causes learning deficits

5.1 Abstract

The cytokine transforming growth factor β1 (TGFβ1) is chronically upregulated in several neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jacob disease, amyotrophic lateral sclerosis and multiple sclerosis, and following stroke. While previous studies have shown that TGFβ1 may be neuroprotective, chronic exposure to elevated levels of this cytokine may contribute to disease pathology on its own. In order to study the effects of chronic exposure to TGFβ1 in isolation we used transgenic mice that over-express a constitutively active porcine TGFβ1 in astrocytes. We found that TGFβ1 over-expression altered brain structure, with the pronounced volumetric increases localized to the hippocampus. Within the dentate gyrus of the hippocampus, increases in granule cell number and astrocyte size were responsible for volumetric expansion, with the increased granule cell number primarily related to a marked reduction in death of new granule cells generated in adulthood. Finally, these cumulative changes in dentate gyrus micro- and macrostructure were associated with the age-dependent emergence of spatial learning deficits in TGFβ1 over-expressing mice. Together, our data indicate that chronic upregulation of TGFβ1 negatively impacts hippocampal structure and, even in the absence of disease, impairs hippocampus-dependent learning.

5.2 Introduction

Transforming growth factor-β1 (TGFβ1) is a widely expressed cytokine that regulates a wide range of biologic processes during development and adulthood (Massague, 2012). Within the brain it is secreted by neurons and glia, and, similar to many other cytokines it is chronically up-regulated in many neurodegenerative conditions including Alzheimer's disease (Apelt and Schliebs, 2001, Zetterberg et al., 2004), amyotrophic lateral sclerosis (Hensley et al., 2003), multiple sclerosis (Link et al., 1994), Parkinson's disease (Mogi et al., 1994, Mogi et al., 1995), prion diseases (Baker et al., 1999) and stroke (Henrich-Noack et al., 1996, Buisson et al., 2003). In these diseases, up-regulation of TGFβ1 is observed within the affected brain areas, as well as cerebrospinal fluid and plasma.

TGFβ1 is neuroprotective, and therefore up-regulation in a disease state may oppose or slow neurodegeneration (Buckwalter et al., 2006, Tesseur and Wyss-Coray, 2006). For example, in mouse models over-expression of TGFβ1 reduces cell death induced by acute or chronic neuronal insults (Brionne et al., 2003). Conversely, genetic deletion of TGFβ1 is associated with increased neurodegeneration both *in vivo* and *in vitro* (Brionne et al., 2003). Similarly, over-expression of TGFβ1 attenuates cell death during ischemia, leading to a reduction in lesion size, whereas inhibiting TGFβ1 signaling during ischemia results in a 3-fold increase in lesion size (Ruocco et al., 1999).

While these data suggest that TGFβ1 plays a neuroprotective role in disease, opposing or slowing neurodegeneration, the long-term consequences of chronic TGFβ1 on brain structure and function are not known. Here we address this question using a mouse line where over-expression of a porcine TGFβ1 is driven by the astrocytic promoter, glial fibrillary acidic protein (GFAP)

(Wyss-Coray et al., 1995, Wyss-Coray et al., 1997). Using these mice, we found that chronic over-expression of TGFβ1 was associated with an age-dependent increase in the size of the hippocampus. This increase was most pronounced in the dentate gyrus of the hippocampus, where both neuron number and astrocyte size correlated with increased volume measured by magnetic resonance imaging. While adult neurogenesis was reduced in the dentate gyrus, a profound reduction in apoptotic cell death appeared to be responsible for increased number of dentate neurons and associated volumetric changes. These changes in dentate gyrus structure were associated with an age-dependent emergence of spatial learning impairments in the water maze.

5.3 Materials and methods

5.3.1 .Mice

Male and female offspring were derived from a cross between wild-type C57Bl/6 (B6) female mice (Taconic) and heterozygous male TGFβ1 mice where over-expression of a porcine TGFβ1 is driven by a GFAP promoter (Wyss-Coray et al., 1995, Wyss-Coray et al., 1997, Buckwalter et al., 2006). The TGFβ1 mice were maintained on a B6 background (>10 generations). All mice were bred in our colony at The Hospital for Sick Children, and maintained on a 12 h light/dark cycle with free access to food and water. Behavioral procedures were conducted during the light phase of the cycle by an experimenter blind to the genotype of the animals. All procedures were approved by the Animal Care Committee at The Hospital for Sick Children.

5.3.2 Western blot

Transgenic mice and their WT littermates were sacrificed and their brains flash frozen in isopentane (Sigma) at -75 °C (WT [2 month-old], n = 4; WT [12 month-old], n = 4; TGFβ1 [2 month-old], n = 4; TGFβ1 [12 month-old], n = 5). Frozen cortical and hippocampal tissues were dissected and homogenized in RIPA buffer containing 1% Nonidet P-40, 0.1% Sodium dodecyl sulphate, 0.5% Deoxycholic acid and protease inhibitors (Sigma). Protein concentrations were assayed by the Bradford method (Bio-Rad Laboratories). Protein samples (30–60 µg) were fractionated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% skim milk powder in TBS-T (0.1% Tween 20, 1 mM Tris-HCl pH 8.0 and 150 mM NaCl) (Sigma). The primary antibody used was anti TGF-β1 (1:2,000, Cell Sciences) with HRP-conjugated Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) (1:5000, Abcam) as loading control. The blots were incubated with a HRP-conjugated secondary antibody (goat anti-rabbit 1:10,000) and the bands detected with enhanced chemiluminescence (GE Healthcare).

5.3.3 Magnetic Resonance Imaging

Another cohort of mice was used for magnetic resonance imaging (MRI) (WT [2 month-old], $n = 6$; WT [12 month-old], $n = 12$; TGFβ1 [2 month-old], $n = 6$; TGFβ1 [12 month-old], $n = 12$). Specimen preparation, imaging and analysis were performed as described previously (Lerch et al., 2011a). Briefly, mice were anaesthetized with chloral hydrate (400 mg/kg, i.p.), and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA, at 4° C). Bodies, along with the skin, lower jaw, ears, and the cartilaginous nose tip were removed. The remaining skull structures containing the brain were allowed to postfix in 4% PFA at 4 °C for 12 h. Following a washout period of 5 days in PBS and 0.01% sodium azide at 15 °C, the skulls were transferred to a PBS and 2 mM ProHance® (Bracco Diagnostics Inc., Princeton, NJ) solution for at least 7 days at 15 °C prior to imaging 12-21 days post-mortem.

A multi-channel 7.0 T MRI scanner (Varian Inc., Palo Alto, CA) with a 6 cm inner bore diameter insert gradient was used to acquire anatomical images of brains. Brains were imaged within skulls in order to minimize geometric distortion. Before imaging, the samples were removed from the contrast agent solution, blotted and placed into plastic tubes (13 mm in diameter) filled with a proton-free susceptibility-matching fluid (Fluorinert FC-77, 3 MCorp., St. Paul, MN). Three custom-built, solenoid coils (14 mm in diameter, 18.3 cm in length) with over wound ends were used to image three brains in parallel. Parameters used in the scans were optimized for grey/white matter contrast: a T2-weighted, 3D fast spin-echo sequence with 6 echoes, with TR/TE = 325/32 ms, four averages, field-of-view 14 × 14 × 25 mm3, and matrix size = 432 × 432 × 780 giving an

image with 32 μm isotropic voxels. Geometric distortion due to position of the three coils inside the magnet was calibrated using a precision machined MR phantom. After MRI scanning, brains were washed with PBS and stored in 10% formalin for later immunohistochemistry.

We used an image registration-based approach to assess anatomical differences related to age and genotype. Image registration finds a smooth spatial transformation that best aligns one image to another such that corresponding anatomical features are superimposed. We used an automated intensity-based group-wise registration approach (Lerch et al., 2011a) to align all brains in the study into a common coordinate system, yielding an average image of the 36 MRI scans. To quantify the deformation that brought the images into alignment with the population average and provide a summary of how they differ, the Jacobian determinants of the deformation fields were then calculated as measures of volume difference at each voxel. Significant volume differences were then calculated in two ways. First, an anatomical atlas comprised of 64 distinct structures (Dorr et al., 2008) was warped onto the population average and was used to compute volumes of each structure. Second, individual voxel differences between genotypes were then calculated by comparing Jacobian determinants at each voxel. After MRI scanning, brains were washed with PBS and stored in 10% formalin for their use in NeuN and GFAP immunohistochemistry.

5.3.4 Tissue handling and preparation for analysis

Mice were anesthetized with chloral hydrate 4% and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed, fixed overnight in PFA and then transferred to 30% sucrose solution. Fifty μm coronal sections were cut using a cryostat along the entire antero-posterior extent of the hippocampus. Sections were kept in sequential order and maintained free-floating in PBS. A 1⁄6 section sampling fraction was used to create 6 sets (each containing sections at 300 μm intervals) for use in immunohistochemical

staining. Accordingly, each set comprised a systematic random sample representative of the entire hippocampus for use in quantification analyses. Additional sets were stored in a PBS solution containing 50% glycerol and 10% ethylene glycol for later processing.

5.3.5 BrdU administration

5-bromo-2'-deoxyuridine (BrdU; Sigma, MO) was dissolved in 0.15 M PBS and heated to 50–60 °C, at a concentration of 20 mg/ml. Animals received 200 mg/kg of BrdU per i.p. injection (Kee et al., 2007a). At P60, mice either received four BrdU injections at intervals of 6 h (WT, n = 4; TGFβ1, n = 4) and were perfused 24 h later or 2 BrdU injections/day for 5 days (interval 12 h) (WT, n = 5; TGFβ1, n = 6) and were perfused 25 d later (at P85). A third group of mice were received a single injection at P10 (WT, n = 4; TGFβ1, n = 5) and were perfused at P85.

5.3.6 Immunohistochemistry

Mice were anesthetized with chloral hydrate and perfused transcardially with PNS followed by 4% PFA. Brains were removed, fixed overnight in PFA and then transferred to 30% sucrose solution. Fifty µm coronal sections were cut using a cryostat along the entire antero-posterior extent of the hippocampus. Sections were kept in sequential order and maintained free-floating in PBS. A 1⁄6 section sampling fraction was used to create 6 sets (each containing sections at 300 µm intervals) for use in immunohistochemical staining. Accordingly, each set comprised a systematic random sample representative of the entire hippocampus for use in quantification analyses. Additional sets were stored in a PBS solution containing 50% glycerol and 10% ethylene glycol for later processing.

For BrdU staining, antigen unmasking was performed by incubating the sections in 1 N HCl at 45°C for 30 min. Sections were incubated with a primary antibody (rat anti-Brdu 1:1000, Accuarate Antibodies, N.Y) for 48h at 4°C, and then a secondary antibody (goat anti-rat Alexa-

488 1:1000, Invitrogen, CA) for 2 h at room temperature. Antibodies were diluted in blocking solution containing 2% goat serum, 2.5% bovine serum albumin, and 0.3% Triton X-100 dissolved in PBS. Sections were mounted on slides (VWR, West Chester, PA) with Permafluor anti-fade medium (Lipshaw Immunon, Pittsburgh, PA).

For the other antigens analyzed, antigen unmasking was performed in a steamer for 40 min in a 0.01M citrate solution with 0.01% Tween 20. They detected GFAP (mouse monoclonal 1:2500; Cell Signaling Technology, MA) (WT [2 month-old], n = 4; WT [12 month-old], n = 6; TGFβ1 [2 month-old], n = 6; TGFβ1 [12 month-old], n = 4) NeuN (mouse monoclonal 1:1000; Millipore, MA) (WT [2 month-old], n = 6; WT [12 month-old], n = 5; TGFβ1 [2 month-old], n = 5; TGFβ1 [12 month-old], n = 5) or Ki67 (rabbit polyclonal 1:2500, Abcam) ([P10]: WT, n = 5, TGFβ1, n = 4; [P60]: WT, n = 4, TGFβ1, n = 5; [P150]: WT, n = 6, TGFβ1, n = 5; P360 [WT], n = 6, TGFβ1, n = 4). Signals were amplified and visualized using Vectastain Elite ABC kit (Vector Laboratories) with Diaminobenzidine (DAB) (Sigma) as a chromogen. Sections were mounted on gelatin coated slides, stained with Harris hematoxylin (Sigma) for nuclear visualization, and coverslipped using crystal mount (EM).

TUNEL staining was carried out using the DeadEnd Tunel kit® (Promega) with minor modifications to manufacturer instructions to detect apoptotic cell death in P60 mice (WT, n = 6, TGFβ1, n = 7).

5.3.7 Golgi-Cox staining

Mice were perfused with PBS and whole brains were removed and impregnated with Golgi-Cox solution using the FD Rapid Golgi Stain Kit (FD neurotechnologies). After 3 weeks, coronal 120 µm sections were cut in 30% sucrose solution using a vibratome (Leica). Sections

were stored in the dark, mounted on gelatin coated slides, developed with 1% NH_4OH, fixed with Kodak fixative, and then coverslipped.

5.3.8 Imaging

Data and images were acquired using Nikon Eclipse 80i an Olympus BX61 epifluorescent microscopes, or a Zeiss LSM710 confocal microscope. Cell counting and astrocyte morphology analysis was conducted using Stereoinvestigator 9.1 (MBF bioscience). We estimated the total number of NeuN and GFAP positive cells using the optical fractionator method on the Olympus BX61 microscope using a 60X, 1.45 N.A. objective and a motorized XYZ stage attached to a computer (Hosseini-Sharifabad and Nyengaard, 2007). Random systematic sampling was used for these stereological analyses (section interval of 1/6, grid size of 250 × 500 µm, 2D counting frame of 30 µm × 30 µm [NeuN] or 90 µm x 90 µm [GFAP] using z-axis dissectors 15 µm deep). These parameters were used to estimate the total number of cells in the entire DG, CA1 or CA3. Conditions were optimized to obtain a Gundersen coefficient of error below 0.05 (Gundersen et al., 1999). For Ki67, BrdU and TUNEL quantifications, the total number of positive cells in a 1/6 fraction was counted, and multiplied by six times to provide an estimate of the total number of Ki67 and TUNEL positive cells. For BrdU, the average number of cells per section is reported.

5.3.9 Water maze apparatus and procedures

The apparatus and behavioral procedures have been previously described (Teixeira et al., 2006). Behavioral testing was conducted in a circular water maze tank (120 cm in diameter, 50 cm deep), located in a dimly-lit room. The pool was filled to a depth of 40 cm with water made opaque by adding white, non-toxic paint. Water temperature was maintained at 28 ± 1 °C using a heating pad beneath the pool. A circular escape platform (10 cm diameter) was submerged 0.5 cm below

the water surface in a fixed position. The pool was surrounded by curtains, at least 1 m from the perimeter of the pool. The curtains were white with distinct cues painted on them.

Prior to training, mice were individually handled for 2 min each day over 5 consecutive days. The complete water maze training protocol consisted of 3 consecutive trials per day for eight days. On each trial, the mouse was placed into the pool, facing the wall, in one of 4 start locations. The order of these start locations was randomly varied throughout training but similar for all animals. The trial was completed once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on a given trial, the experimenter guided the mouse onto the platform. At the end of each trial, mice were allowed 15 s to rest atop the platform before beginning the next trial. Spatial memory was assessed during probe tests by removing the platform from the pool, and allowing the mouse swim for 60 s. Mice were given a probe test before training on days 1, 3, 5,7, and on day 9.

Behavioral data from training and the probe tests were acquired and analyzed using an automated tracking system (Actimetrics, IL). For the probe tests, we quantified performance in two ways. First, we measured the amount of time mice spent in the target zone (20 cm radius, centered on the location of the platform during training). This zone represents approximately 11% of the total pool surface. Second, we represented probe test performance as a heat map (or density plot), with hot colors corresponding to areas of the pool that were more frequently visited. During probe tests, we additionally recorded swim speed and thigmotaxis (the percentage of total time that mice swam within 5 cm of the pool walls).

5.3.10 Statistical analyses

Behavioral, MRI and cell counting data were evaluated using parametric ANOVAs or t-tests,

where appropriate. Multiple comparisons were accounted controlled for in the MRI brain volume analysis by applying the Bonferroni correction to the structure volume comparisons and the False Discovery Rate (1% FDR; (Genovese et al., 2002)) for the voxel wise whole brain comparisons.

5.4 Results

5.4.1 Levels of TGFβ1 over-expression are stable with age

Using an anti-porcine TGFβ1 antibody we first evaluated transgenic TGFβ1 expression in 2 and 12 month-old TGFβ1 and WT littermate control mice. Analysis of homogenates from hippocampus and cortex indicated that the transgene was robustly expressed, and expression levels did not change with age (hippocampus [t_7 = 1.40, P > 0.05]; cortex [t_7 = 1.40, P > 0.05]). As expected, no transgene signal was detected in WT mice (**Fig. R.5.1**).

Figure R215.1 Levels of TGFβ1 over-expression are stable with age

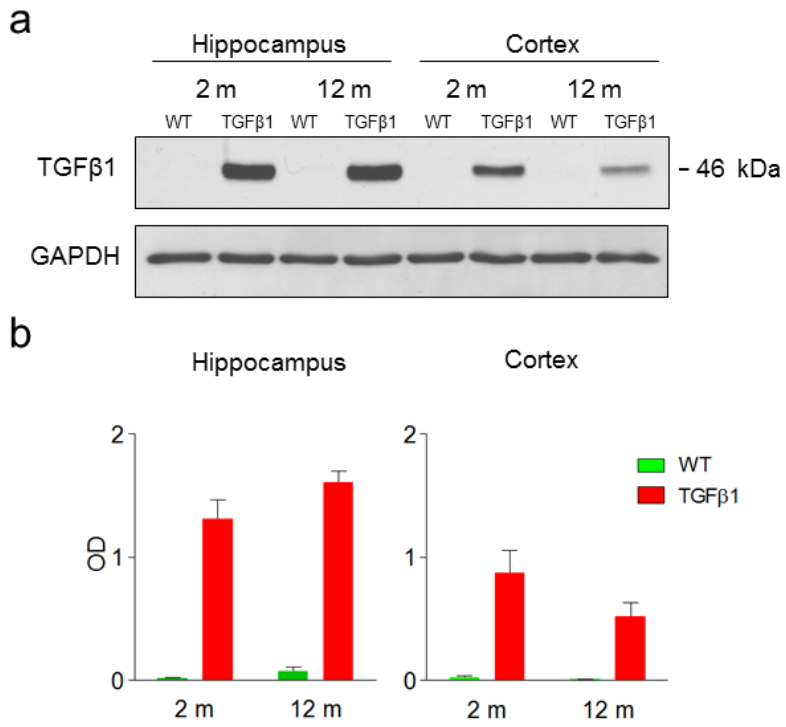


Figure R.5.1 *Expression of porcine TGFβ1 in hippocampus and cortex.* ***a****. Representative western blot of TGFβ1 precursor peptide (46 kDa) in hippocampal and cortical tissue from young (2 month-old) and aged (12-month old) TGFβ1 mice and their WT littermates.* ***b****. Ratio of optical densities (OD) of TGFβ1 vs. GAPDH (loading control) bands for TGFβ1 mice and their WT littermates. Levels of TGFβ1 did not change with age in hippocampus or cortex.*

5.4.2 TGFβ1 over-expression produces age-dependent macroscopic changes in brain structure

Using high resolution, 3D magnetic resonance imaging we next evaluated the long-term consequences of TGFβ1 over-expression on brain structure. Brains from young (2 month-old) and old (12 month-old) control and TGFβ1 mice were scanned and digitized using a multichannel 7.0 Tesla MRI scanner (**Fig. R.5.2a**). Unbiased, computer-generated atlases were then created for all MR scans, which were then deformed into exact alignment with one another. Subsequent quantitative volume differences of individual mouse neuroanatomical features were determined using this composite brain atlas (Dorr et al., 2008). In aged, 12 month-old TGFβ1 mice, these analyses confirmed that TGFβ1 over-expression was associated with enlargement of the 3rd and lateral ventricles, as previously reported (Wyss-Coray et al., 1995). In addition to hydrocephalus, total brain volume was increased in TGFβ1 mice at both ages (genotype main effect: $F_{1,31} = 29.82$, $P < 0.01$) (**Fig. R.5.2b**). The volume of many forebrain, midbrain, and hindbrain regions was increased, and the most pronounced changes were observed in hippocampal CA3 and dentate gyrus (both granule cell and molecular layers) regions (**Fig. R.5.2c**). In contrast, similar volumetric changes were not observed in young, 2 month-old TGFβ1 mice.

Figure22 R.5.2 Macroscopic changes produced by TGFβ1 overexpression

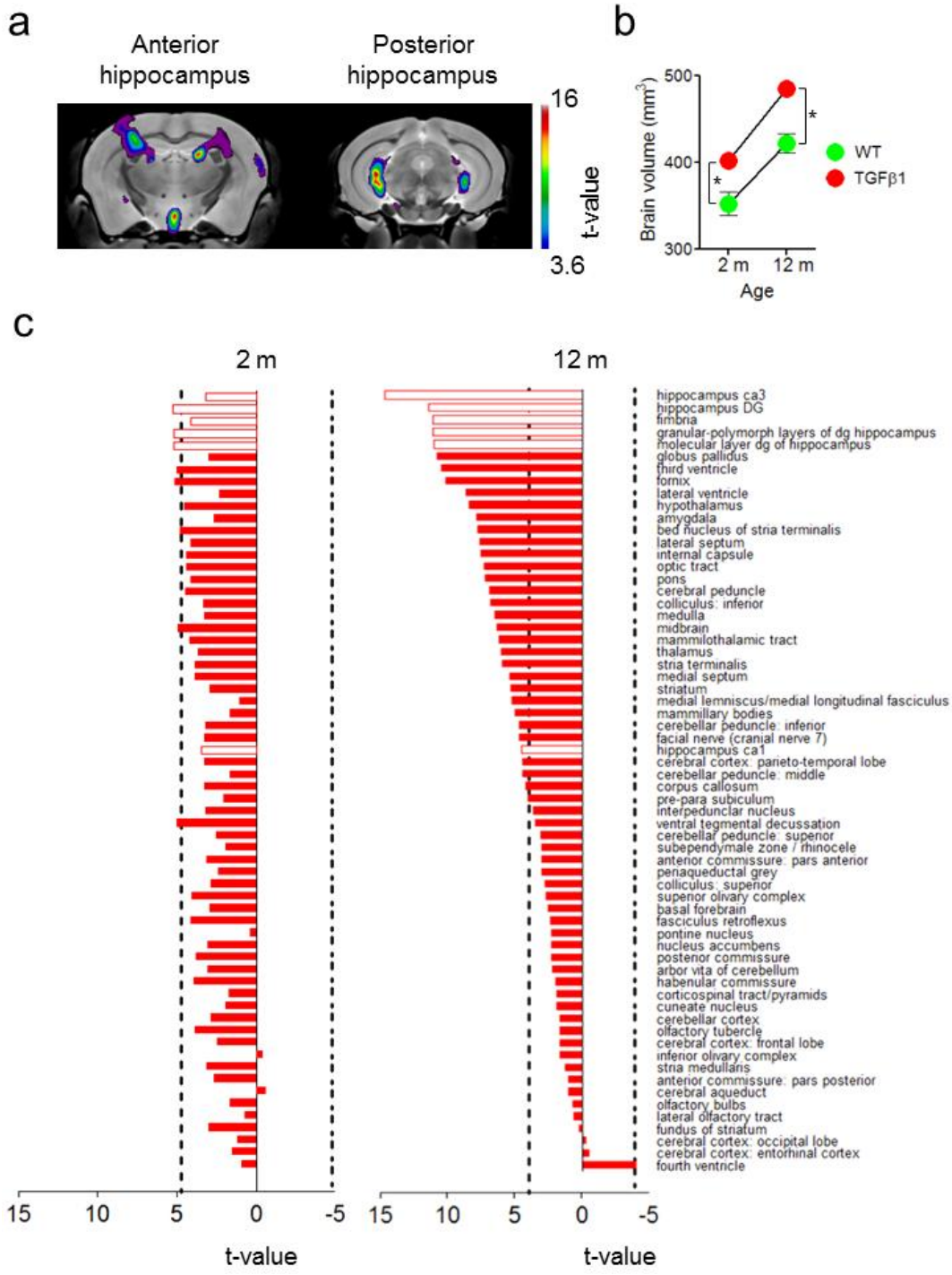


Figure R.5.2 *Volumetric changes in brain structure measured with MRI following TGFβ1 over-expression.* ***a.*** *Coronal slices shows voxel wise increases in volume in aged TGFβ1 mice localized to the anterior and posterior hippocampi (1% FDR). The color scale indicates the significance of the difference by Student-t value.* ***b.*** *The volume of the whole brain is larger in TGFβ1 mice, and the difference increases with age. The effect size of volumetric changes both in young (**c**) and aged (**d**) TGFβ1 mice are shown for each of the 64 brain structures. The effect size is shown by Student-t values where the critical value for P < 0.05 is shown after Bonferroni correction (hatched lines). Hippocampus CA3, granule cell layer of the dentate gyrus and molecular cell layer of the dentate gyrus are structures*

with volume increases in both aged and young mice. Thirty six structures in the aged group of animals are larger than those of their WT littermates. Nine of those structures are also larger in the young transgenic mice, including the subdivisions of the dentate gyrus.

5.4.3 TGFβ1 over-expression is associated with age-dependent increases in astrocyte size in dentate gyrus

The MRI analyses revealed that chronic over-expression of TGFβ1 mice led to age-dependent, macroscopic changes in brain structure. These were especially pronounced in the hippocampus, and, in particular, the molecular and granule cell layers of the dentate gyrus. As the molecular layer is predominantly composed of astrocytes, and previous studies have shown that TGFβ1 activates astroglia, leading to increased branching and soma size (Reilly et al., 1998, de Sampaio e Spohr et al., 2002, Sousa Vde et al., 2004), we next asked whether changes in astrocyte number and/or morphology could account for increased molecular layer volume in aged, TGFβ1 mice. To do this we cut sections from the same MRI-scanned brains and stained for the astrocytic marker GFAP (**Fig. R.5.3a**). While overall numbers of astrocytes did not vary with age or genotype (no significant genotype or age × genotype interactions; data not shown), we observed age-dependent changes in astrocyte size in TGFβ1 mice. Both astrocyte volume (significant age × genotype interaction: $F_{1,16} = 7.90$, $P < 0.05$; **Fig. R.5.3b**) and total surface area (significant age × genotype interaction: $F_{1,16} = 11.92$, $P < 0.01$; **Fig. R.5.3c**) were increased in aged TGFβ1 mice, suggesting that age-dependent astrocytic growth may account for increased molecular layer volume in TGFβ1 mice. Consistent with this, within animals there was a high degree of correspondence between MRI-measured molecular layer volume and immunohistochemistry-based estimates of astrocyte volume (Regression analysis for MRI- and immunohistochemistry-based measures for all 4 groups: Pearson's $r = 0.65$, $P < 0.005$) (**Fig. R.5.3d**). This within-animal correspondence suggests that changes in astrocyte size contribute significantly to increased molecular layer volume in TGFβ1 mice.

Figure 23R.5.3 TGFβ1 over-expression increases astrocytic size in the dentate gyrus.

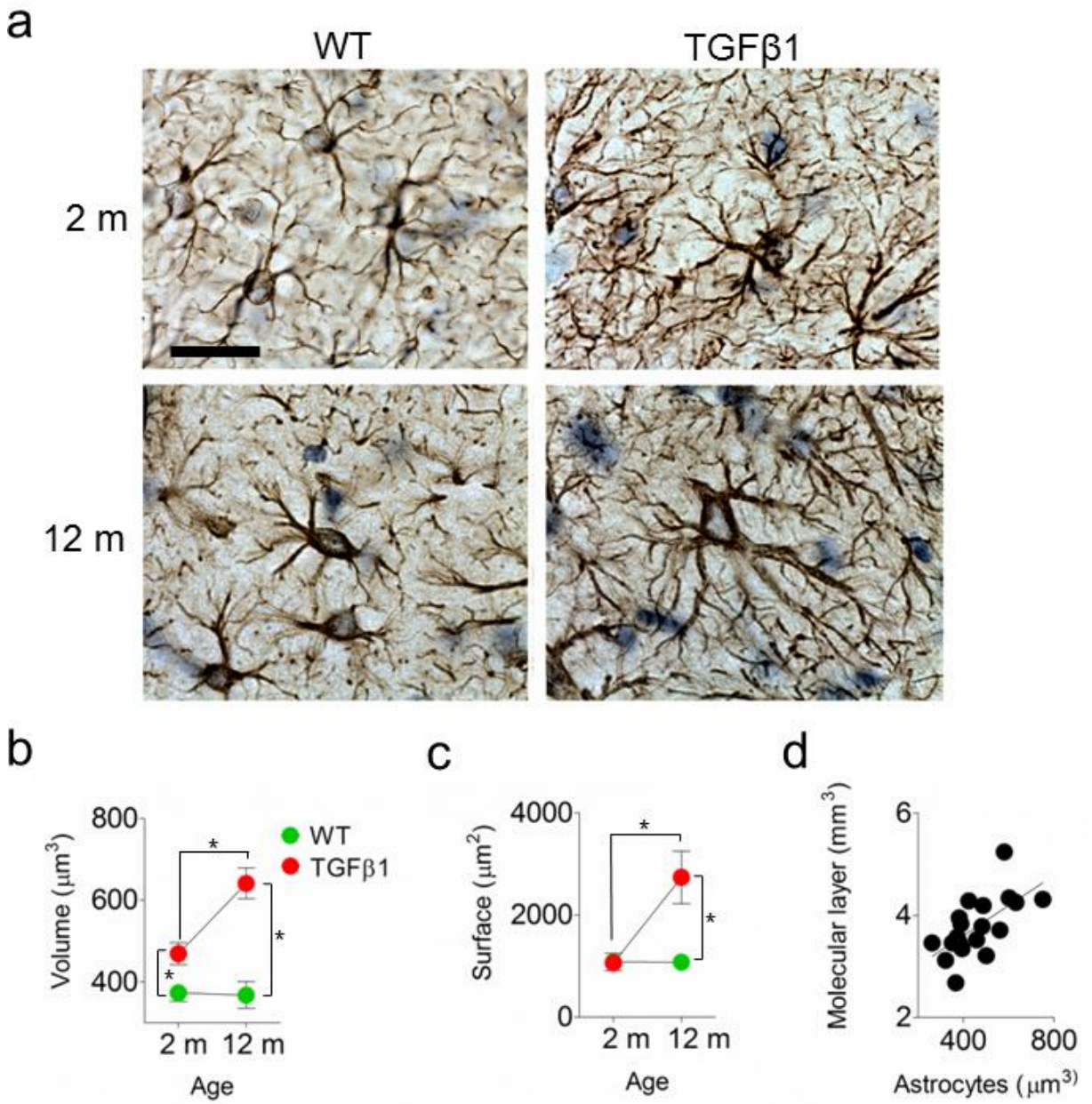


Figure R.5.3 *Age-dependent changes in astrocyte morphology following TGFβ1 over-expression.* ***a****. Representative images of astrocytes in the dentate gyrus in young (2 m) and aged (12 m) WT and TGFβ1 mice. Scale bar = 20 μm.* ***b****. Astrocytes were enlarged in TGFβ1 mice compared to WT littermate controls.* ***c****. Surface area was increased in aged TGFβ1 mice. d. MRI-measured molecular layer volume correlated with immunohistochemical estimates of astrocyte volume in young and old TGFβ1 mice.*

5.4.4 TGFβ1 over-expression is associated with age-dependent increases in neuron number in dentate gyrus

The MRI analyses also revealed age-dependent increases in the volume of the dentate granule cell layer in aged, TGFβ1 mice. As the granule layer is almost exclusively composed of neurons (and very few astrocytes), this raises the possibility that chronic over-expression of TGFβ1 leads to a net increase in the number of dentate granule cells. To address this we cut sections from the same MRI-scanned brains and stained for the mature, neuronal marker NeuN. Stereological quantification revealed age-dependent increases in numbers of $NeuN^+$ cells in TGFβ1 mice in the dentate granule cell layer (significant age × genotype interaction: $F_{1,16} = 18.31$, $P < 0.001$): Whereas in young WT and TGFβ1 mice there were similar numbers of $NeuN^+$ cells, in aged mice, there were 1.4 fold more $NeuN^+$ cells in TGFβ1 mice (**Fig. R.5.4a**). In contrast, we found no evidence for increased neuron number in non-neurogenic regions of the hippocampus, CA1 and CA3 regions (no significant genotype or age × genotype interactions; **Fig. R.5.4b-c**). These findings suggest the possibility that a net increase in the number of dentate granule cells accounts for the observed, MRI-measured increase in dentate granule cell layer volume. To directly examine this, we next asked to what extent do MRI-measured granule cell layer volume and immunohistochemistry-based estimates of neuron number correspond across mice. Similar to the analysis of astrocytes in the molecular layer, we found that within animals there was a high degree of correspondence between MRI-measured granule cell layer volume and immunohistochemistry-based estimates of neuron number (Regression analysis for MRI- and immunohistochemistry-based measures for all 4 groups: Pearson's $r = 0.64$, $P < 0.01$) (**Fig. R.5.4d**). This within-animal correspondence suggests that a net increase in neuron number accounts, in part, for increased dentate granule cell layer volume in aged, TGFβ1 mice.

Figure 24R.5.4 TGFβ1 over-expression increases neuron numbers in the dentate gyrus.

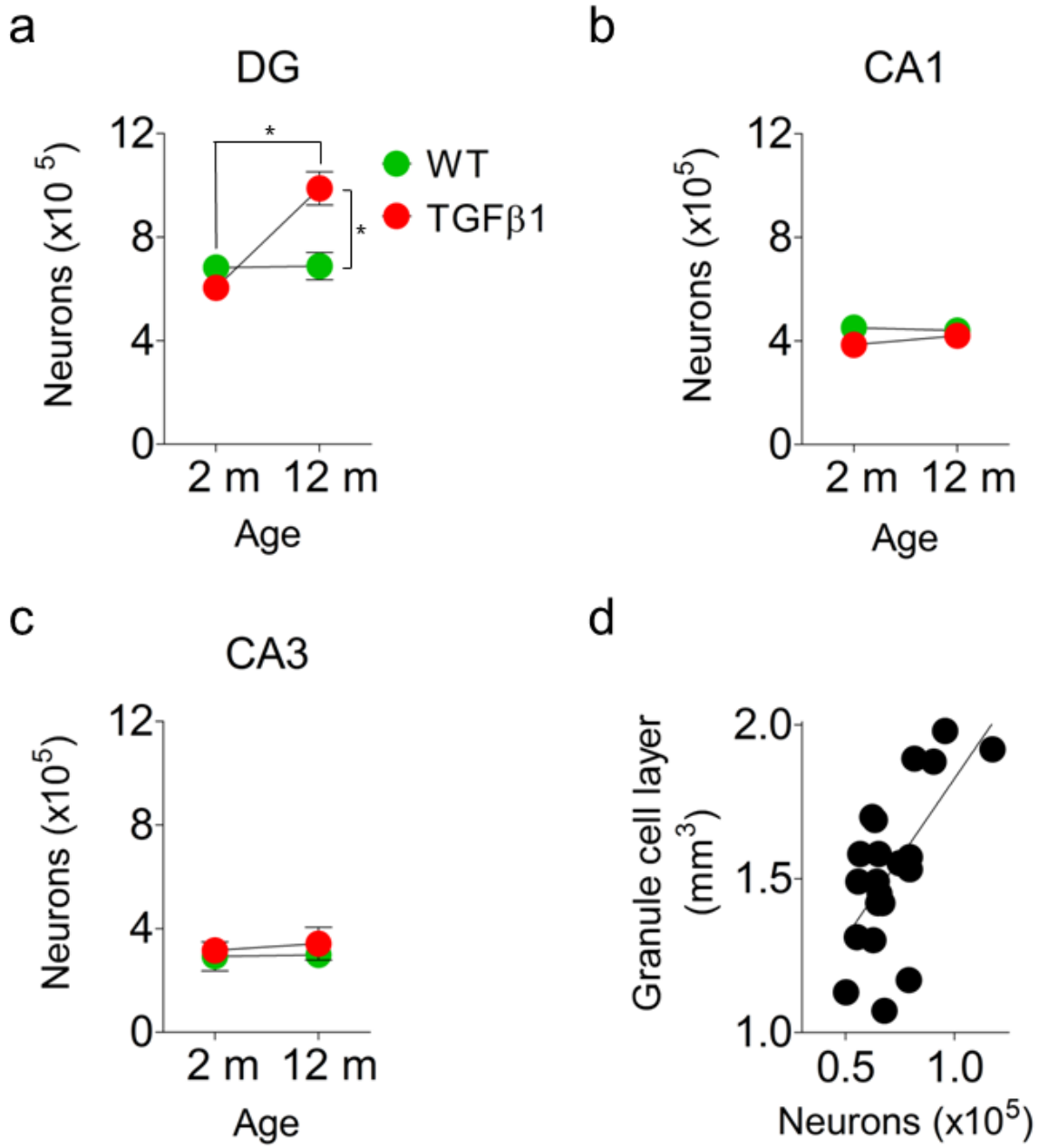


Figure R.5.4 *Age-dependent changes in granule cell number following TGFβ1 over-expression. Neurons in in young (2 m) and old (12 m) WT and TGFβ1 mice in (**a**) DG, (**b**) CA1 and (**c**) CA3. **d**. MRI-measured granule cell layer volume correlated with immunohistochemical estimates of granule cell number in young and old TGFβ1 mice.*

5.4.5 TGFβ1 over-expression alters granule cell morphology and dentate gyrus neuroanatomy

Our experiments indicate that TGFβ1 over-expression led an increase in the number of dentate granule cells. We next asked whether this increase in neuron number altered overall dentate gyrus neuroanatomy and dentate granule cell morphology in TGFβ1 mice. To examine gross dentate gyrus neuroanatomy, we inspected nissl-stained sections through the anterior-posterior extent of the hippocampus. Strikingly, we found a consistent, age-dependent change in the shape of dentate gyrus granule cell layer (**Fig. R.5.5a**). In normal mice, the dentate gyrus comprises an upper and lower blade, joined by a single, acute fold. In contrast, in aged TGFβ1 mice there were typically additional folds in the upper blade. To quantify this we measured the 'caliper' distance (van der Worp et al., 2001) between the upper and lower blades in TGFβ1 and littermate control mice. Consistent with additional folds in the upper blade, we found that TGFβ1 over-expression increased the inter-blade distance, and this was more pronounced in aged, TGFβ1 mice (significant age × genotype interaction: $F_{1,97} = 4.09$, $P < 0.05$) (**Fig. R.5.5b**).

To examine the morphology of dentate granule cells, we next impregnated a separate set of brains using the Golgi-Cox method and several indices of dendritic complexity were computed in TGFβ1 compared to WT mice. At both ages we found that TGFβ1 over-expression reduced dendritic complexity of granule cells (**Fig. R.5.5c)**. Sholl analysis revealed that there was a reduction in total dendritic length in both young ($F_{1,52} = 34.54$, $P < 0.001$) and aged TGFβ1 mice ($F_{1,60} = 6.58$, $P < 0.05$) (**Fig. R.5.5d-e**). Consistent with this there was a reduction in total number of intersections in both young ($F_{1,51} = 10.99$, $P < 0.01$) and aged TGFβ1 mice ($F_{1,60} = 11.52$, $P < 0.01$) (**Fig. R.5.5f-g**). An identical pattern of results was found using alternate measures of complexity, such as density of bifurcation nodes (data not shown). In addition to reduced complexity, granule cells in TGFβ1 mice had less expansive dendritic arbors (**Fig. R.5.5h**). This

reduction was most pronounced in aged TGFβ1 mice (significant age × genotype interaction: $F_{1,16}$ = 7.90, P < 0.05; **Fig. R.5.5i**). Together, these analyses reveal that TGFβ1 over-expression is associated with age-dependent changes in the micro- as well as the macrostructure of the dentate gyrus. In particular, increased granule cell folding and reduced granule cell complexity may represent consequences of neuronal over-population of the granule cell layer.

Figure 25R.5.5 TGFβ1over-expression alters neuromorphology of neurons in the DG.

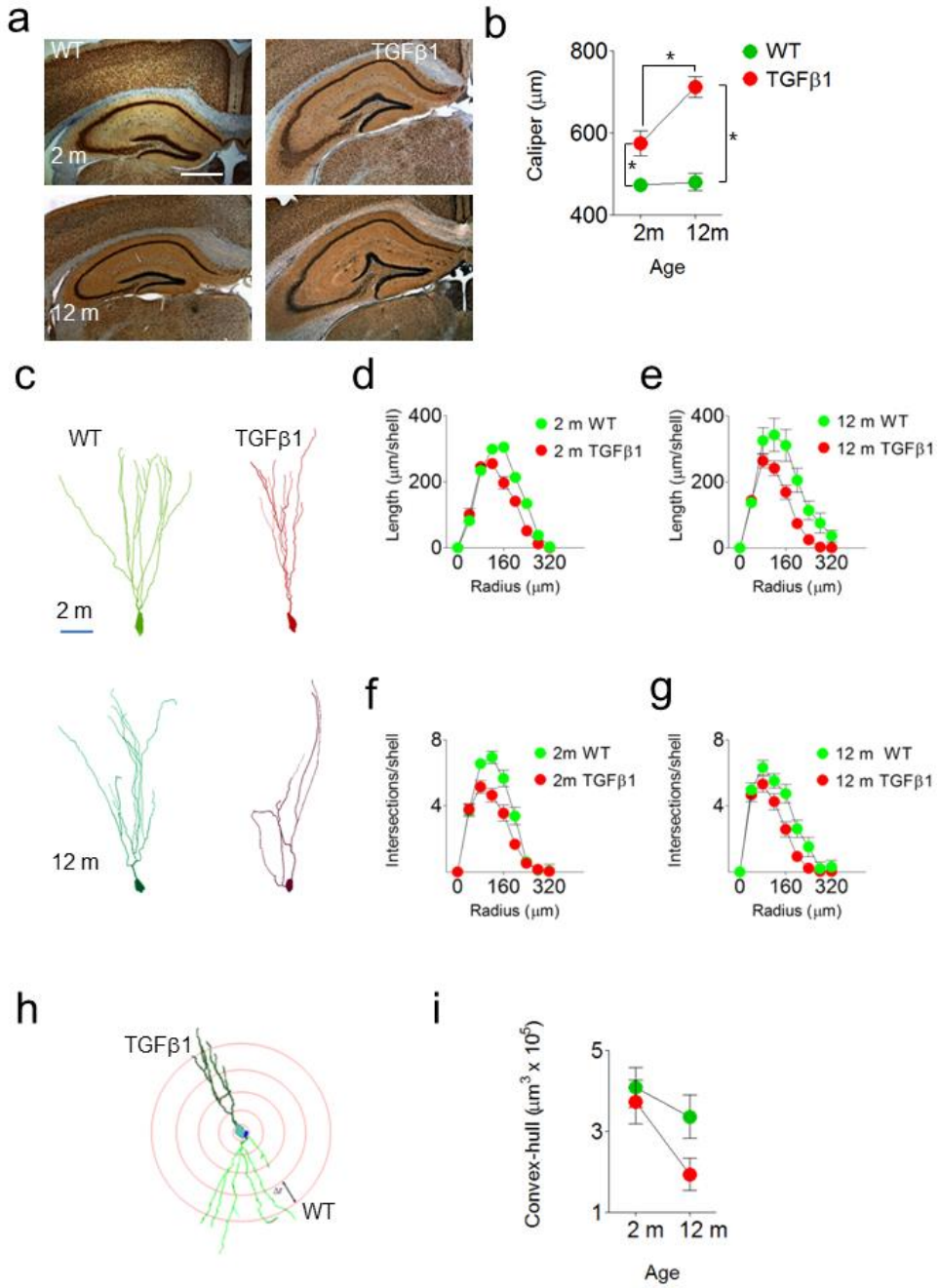


Figure R.1.5. *Altered dentate gyrus anatomy following TGFβ1 over-expression.* ***a****. Abnormal blade folding in aged TGFβ1 mice. In normal mice, the dentate gyrus comprises an upper and lower blade, joined by a single, acute fold. In contrast, there were typically additional folds in the upper blade in aged TGFβ1 mice. Scale bar = 100 μm.* ***b****. The caliper distance between the upper and lower blades is increased in young (2 m) and aged (12 m) TGFβ1 mice.* ***c****. representative Golgi-Cox impregnated granule cells in young (2 m) and aged (12 m) WT and TGFβ1 mice. Scale bar = 20 μm. Sholl analysis indicates that number of intersections (**d-e**) and total dendritic length (**f-g**) is reduced in young and aged TGFβ1 mice.* ***h****. Representative reconstructions of granule cells illustrating less expansive dendritic arbor in aged TGFβ1 mice.* ***i****. Quantification of reduced less expansive dendritic arbor in in aged TGFβ1 mice using Convex-hull method.*

5.4.6 Increased neuron number is associated with reduced apoptotic cell death/reduced neuronal turnover in DG

Our analyses reveal that TGFβ1 over-expression leads to age-dependent increases in neuron number in the dentate gyrus. As the dentate gyrus is one of two brain regions where neurogenesis persists throughout life (Zhao et al., 2008), this raises the possibility that hippocampal neurogenesis is altered in these mice. Consistent with this, previous studies have shown that TGFβ1 modulates adult neurogenesis in the hippocampus (Buckwalter et al., 2006). However, paradoxically, TGFβ1 over-expression resulted in a reduction, rather than increase, in proliferation (Buckwalter et al., 2006). An alternative possibility is that TGFβ1 over-expression reduces death of newborn cells, and therefore compensates for reduced levels adult neurogenesis. Consistent with this, in neuronal cultures exogenous application TGFβ1 is neuroprotective (Buisson et al., 1998, Ruocco et al., 1999, Dhandapani et al., 2003), and similar effects have been observed *in vivo* (Brionne et al., 2003).

To address these questions, we first examined levels of proliferation in the dentate by quantifying expression of Ki67 in the dentate gyrus (**Fig. R.5.6a-b**). Ki67 is a cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (Kee et al., 2002). At P10, Ki67 expression was equivalent in WT and TGFβ1 mice ($t_8 = 0.32$, $P > 0.05$), suggesting that postnatal neurogenesis is unaffected by TGFβ1 over-expression. At P60 (or 2 months), Ki67 expression was reduced in TGFβ1 mice ($t_7 = 3.28$, $P < 0.05$), and this reduction was even more pronounced at later time-points (5 months [$t_9 = 5.21$, $P < 0.001$], 12 months [$t_8 = 3.43$, $P < 0.01$]). Therefore, consistent with previous reports (Buckwalter et al., 2006), these data indicate that chronic over-expression of TGFβ1 leads to reduced neurogenesis in adulthood.

Next, to evaluate whether apoptotic cell death in the dentate gyrus is reduced in adult, TGFβ1 mice we stained for TUNEL, a marker of programmed cell death (**Fig. R.5.6c**). Strikingly,

TUNEL staining was reduced by approximately 4-fold in 2 month-old TGFβ1 mice (t_{11} = 3.19, P < 0.001), suggesting that apoptotic cell death is reduced by TGFβ1 over-expression (**Fig. R.5.6d**). Since TUNEL staining in both WT and TGFβ1 mice was largely limited to the subgranular zone, the neuroproliferative region of the dentate gyrus, this suggests that fewer newborn cells are dying in adult TGFβ1 mice. To address this more directly we next injected 2 month-old WT and TGFβ1 mice with the proliferation marker BrdU and waited either 1 or 45 days. Consistent with our Ki67 data, there was an approximate 50% reduction in BrdU labeling in TGFβ1 mice at the 1 d survival delay (t_6 = 5.63, P < 0.01) (**Fig. R.5.6e**), indicating that adult neurogenesis is reduced. However, after 45 d equivalent numbers of BrdU-labeled cells were found in WT and TGFβ1 mice (t_9 = 0.50, P > 0.05) (**Fig. R.5.6f**). Consistent with the TUNEL data, these results suggest that a reduction in cell death masks a reduction in levels of neurogenesis in adulthood. In contrast, there was no difference in the long-term survival rates of cells generated post-natally (at P10; t_7 = 0.38, P > 0.05) (**Fig. R.5.6g**).

Figure 26R.5.6 TGFβ1 over-expressions modifies neurogenesis and turnover in DG.

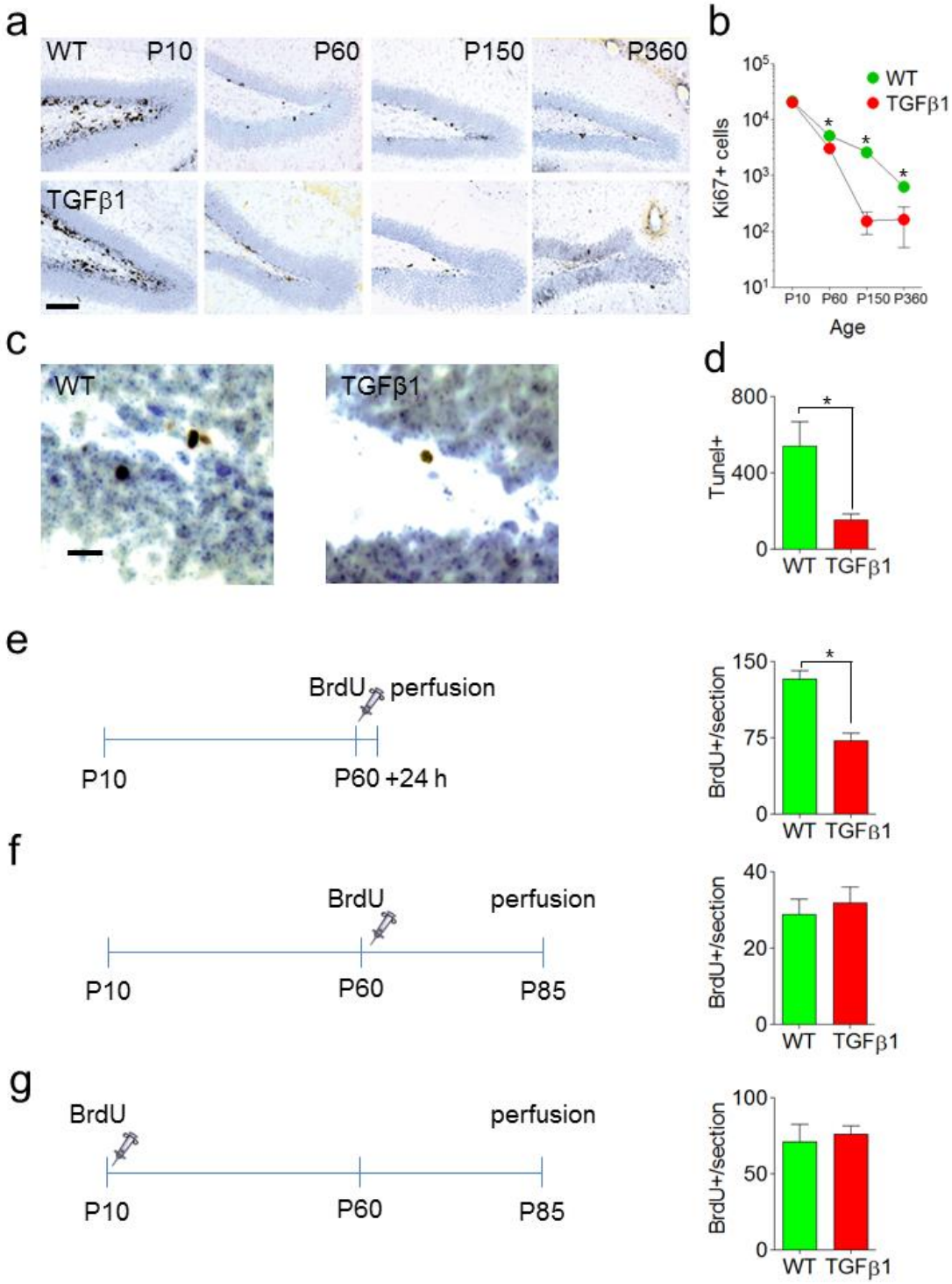


***Figure R.1.6**. Reduced adult neurogenesis and reduced apoptotic cell death following TGFβ1 over-expression**. a**. Representative sections stained for proliferation marker Ki67 in DG in P10-360 WT and TGFβ1 mice. Scale bar = 50 µm. **b**. Number of Ki67⁺ cells is reduced in TGFβ1 mice from P60 on, reflecting reduced proliferation in DG. **c**. Representative images of TUNEL⁺ cells in DG WT and TGFβ1 mice at P60. Scale bar = 20 µm. **d**. TUNEL⁺ cells were reduced approximately 4-fold in TGFβ1 mice. **e-g**. Experimental scheme for BrdU injections (left) and resulting numbers of BrdU⁺ cells in DG in WT and TGFβ1 mice (right).*

5.4.7 TGFβ1 over-expression is associated with age-dependent deficits in spatial learning

Our experiments indicate that TGFβ1 over-expression produced age-dependent changes in brain anatomy both at the macro- (i.e., brain region) and micro (e.g., neuronal and astocytic morphology) scales. These changes were especially pronounced in the hippocampus, a region that plays an important role in learning and memory, and is impacted in memory disorders including Alzheimer's disease (Cook, 1979). Therefore, to evaluate whether these changes in structure impacted hippocampal function, we trained both young (2 month-old) and old (12 month-old) control and TGFβ1 mice in the water maze. We chose a hidden version of the task where both spatial memory acquisition and expression depend on the hippocampus (Teixeira et al., 2006). Mice were trained with 3 trials per day for 8 days (**Fig. R.5.7a**). While escape latencies declined in all groups during training (significant main effect of training: $F_{5,553} = 31.95$, $P < 0.001$), latencies were longer in aged, TGFβ1 mice (significant age × genotype interaction: $F_{1,79} = 7.05$, $P < 0.01$), suggesting that chronic over-expression of TGFβ1 impairs spatial learning (**Fig. R.5.7b**).

Throughout the course of training, spatial bias was additionally assessed with a series of probe tests. In three groups (young WT, young TGFβ1 and aged WT), the selectivity of searching in these probe tests increased over time: Mice in these groups spent more time searching the target vs. other zone in the probe tests on days 5, 7 and 9 (planned comparisons for time spent in target vs. other; all *P*s < 0.05). In contrast, aged TGFβ1 mice did not search selectively in the target zone in any of the probe tests (planned comparisons for probe tests on days 1, 3, 5, 7, and 9 contrasting time spent in target vs. other; all *P*s > 0.05) (**Fig. R.5.7c**). An ANOVA examining time spent in target zone with age and genotype as between subject variables revealed a significant age × genotype × day interaction ($F_{1,79} = 6.87$, $P < 0.05$) (**Fig. R.5.7d**), confirming that chronic over-

expression of TGFβ1 leads to spatial learning deficits in aged mice. A similar pattern of results was obtained using alternate metrics of water maze probe test performance (data not shown). These age-dependent deficits in TGFβ1 mice were not due to non-specific effects on motor function, as swim speed in the probe tests was equivalent in all groups (no main effects of age, genotype or significant age × genotype interaction; **Fig. R.5.7e**). Additionally, age-dependent deficits in TGFβ1 do not appear to be associated with an increased prevalence of non-spatial search strategies. At the beginning of training, naïve mice tend to spend more time searching near the walls of the pool. While we found that thigmotaxic behavior was more prevalent in aged vs. young groups (significant main effect of age: $F_{1,79} = 11.57$, $P < 0.05$), it was not selectively affected in aged, TGFβ1 mice (no significant age × genotype or significant age × genotype × day interactions; **Fig. R.5.7f**). Together, these data indicate that chronic over-expression leads to age-dependent deficits in spatial learning.

Figure27R.5.7 TGFβ1 over-expression impairs spatial learning in aged mice.

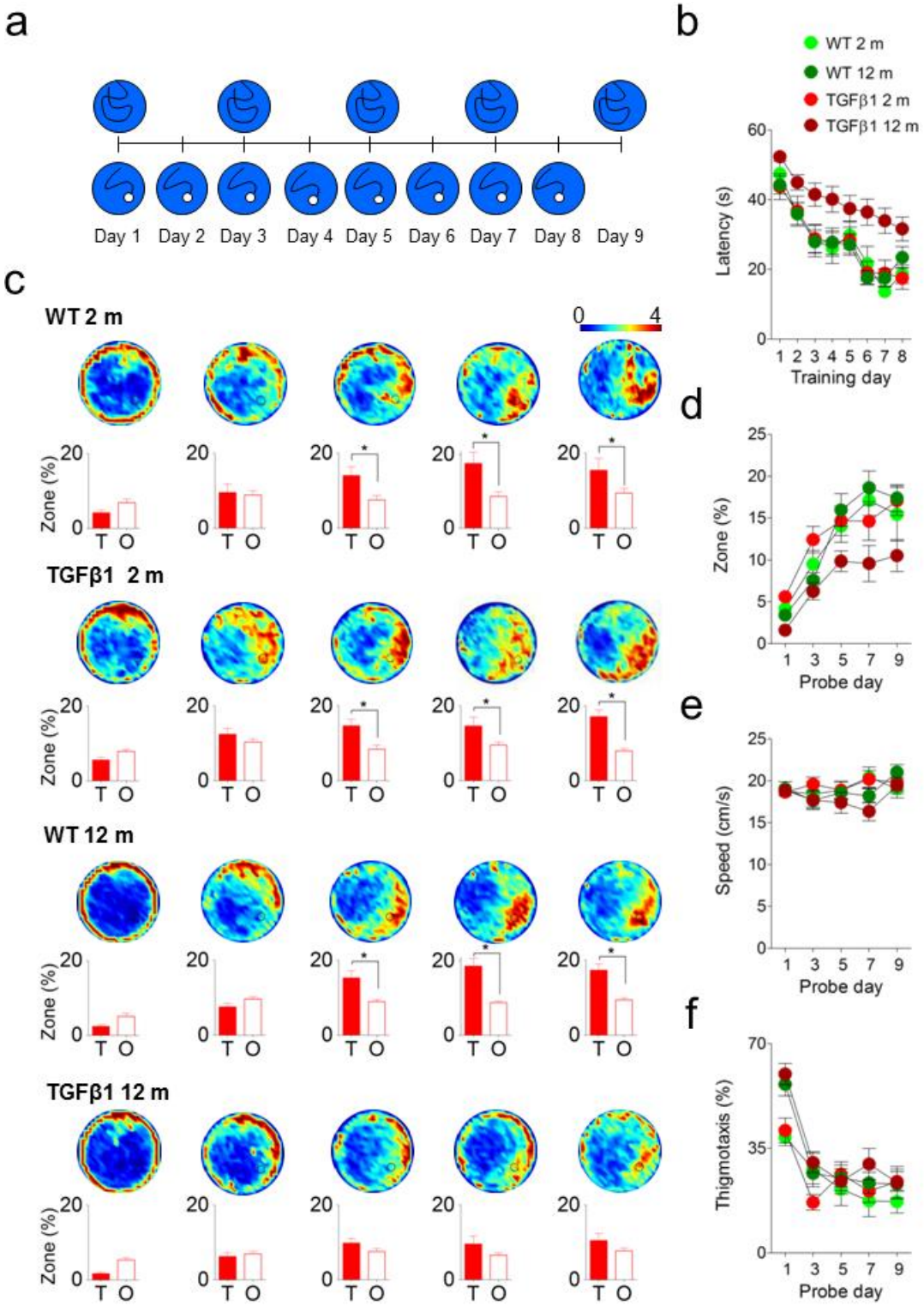


Figure R.5.7 *Emergence of spatial learning deficits in TGFβ1 mice.* ***a****. Experimental design for water maze.* ***b****. Latency to reach escape platform in young (2 m) and aged (12 m) WT and TGFβ1 mice during training.* ***c****. Density plots showing where mice concentrated their search for the platform during the probe tests on days 1, 3, 5, 7 and 9,*

with color scale representing average number of visits per mouse per 5 × 5 cm area (upper). Shown below density plots are quantified data for time spent searching target (T) zone vs. other (O) zones in each of the probe tests. ***d-f****. During probe tests, (****d****) time spent in target zone, (****e****) swim speed, and (****f****) thigmotaxic behavior for young and aged WT and TGFβ1 mice.*

5.5 Discussion

Under non-pathological conditions, TGFβ1 expression is relatively scarce (Nichols and Finch, 1991, Lindholm et al., 1992). However, it is rapidly and transiently up-regulated in response to brain injury, and is chronically up-regulated in many degenerative conditions including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis and prion diseases such as Creutzfeldt-Jakob disease (Pratt and McPherson, 1997). To evaluate how chronic up-regulation of TGFβ1 impacts brain structure and function we examined young (2 month-old) and aged (12 month-old) transgenic mice over-expressing a constitutively activated porcine TGFβ1 in astrocytes. Our MRI analyses revealed changes in the volume of many brain regions, including the hippocampus. These changes were particularly pronounced in the dentate gyrus, with increases in granule cell number and astrocyte size in the granule cell and molecular layers, respectively, accounting for increased dentate gyrus volume. While adult neurogenesis was roughly halved in the dentate gyrus, there was an approximate four-fold reduction in apoptotic cell death, consistent with the neuroprotective role of TGFβ1 as well as age-dependent increases in granule cell number. Finally, these micro- and macroscopic level changes in dentate gyrus structure were associated with deficient hippocampus-dependent learning in the water maze.

We used high resolution MRI to systematically track global changes in brain structure. Our analyses indicated that chronic up-regulation of TGFβ1 alone led to profound changes in brain structure. There was an overall increase in brain volume, an increase that is driven in part by ventricular enlargement (or hydrocephalus) as has been previously reported in TGFβ1 over-expressing mice (Wyss-Coray et al., 1995). However, we additionally identified volumetric increases in a number of forebrain structures. These included the parietal cortex, medial and lateral septum, amygdala and the mammillary bodies, many of which represent brain regions that play

central roles in memory and cognition (Aggleton and Brown, 2006). However, the most pronounced volumetric changes were localized to the hippocampus, and in particular the dentate gyrus. Within the dentate gyrus, histological analyses revealed increased neuron number in the granule cell layer and astrocyte size. These changes in microstructure following TGFβ1 over-expression appeared to be partly responsible for changes in macrostructure since variation in neuron number and astrocyte size accounted for ~45% and 41% of the variance of MRI-measured dentate volume, respectively. Furthermore, dentate granule cells had less dendritic complexity, and, in particular, had less expansive arborization. This latter phenotype might reflect neuronal 'overcrowding' in aged, TGFβ1 over-expressing mice.

The dentate gyrus is one of two regions in the adult brain where neurogenesis persists into adulthood (Zhao et al., 2008). While there was a net increase in total granule cells in the DG in TGFβ1 over-expressing mice, paradoxically we found that levels of adult neurogenesis were reduced by roughly half (see also (Buckwalter et al., 2006)). Instead, it appears that a reduction in apoptotic cell death of newly-generated dentate granule cells is responsible for the net increase in neuron number in TGFβ1 over-expressing mice since we found a roughly 4-fold reduction in TUNEL-positive cells in the DG. As these $TUNEL^{+}$ cells were localized to the SGZ, this suggests that TGFβ1 over-expression leads to an increase in granule cell number by selectively inhibiting apoptotic cell death of newly-generated cells. There are a number of different mechanisms by which this may occur. For example, the neuroprotective effects of TGFβ1 may be mediated by upregulation of the type I plasminogen activator inhibitor (PAI-1) in astrocytes (Buisson et al., 1998, Docagne et al., 1999). Previous studies have shown that in response to increased TGFβ1 levels, astrocytes secrete PAI-1 which inhibits NMDA-mediated excitotoxicity. An alternative possibility is that increased TGFβ1 levels reduce apoptotic cell death via activation of anti-

apoptotic ERK/BAD signaling in neurons (Nunez and del Peso, 1998, Zhu et al., 2001, Zhu et al., 2002). Finally, TGFβ1 also activates the death-associated protein 6 (DAXX). This intracellular signalling molecule can promote apoptosis through activation of the transcription factor p53. However it also can blocks apoptosis by a pathway not well characterized (Michaelson et al., 1999). This pathway has been only described to affect active microglia (Yun et al., 2011), but further studies are needed to assess its relevance in dentate gyrus adult neurogenesis.

The hippocampus, including the dentate gyrus, regulates memory formation (Eichenbaum, 2004b). Consistent with the micro- and macroscale alterations in dentate architecture we found that TGFβ1 over-expressing mice had impaired spatial learning in a hippocampus-dependent version of the water maze, where mice were trained to locate a hidden platform in a fixed location. Like some (but not all) of the structural alterations, impairments in this spatial learning task emerged in an age-dependent manner: Relative to WT littermate controls, only aged (12 month-old), and not young (2 month-old) TGFβ1 over-expressing mice were slower in locating the platform during training and in developing a spatial bias for the trained platform location in probe tests. The delayed emergence of deficits suggests that the impact of TGFβ1 overexpression is cumulative. A number of circuit- and cellular level alterations might contribute to these learning deficits. First, the relative quiescence of dentate granule cells ensures that the dentate gyrus relays a sparse code onto target cells in the CA3, and this sparsification is thought to be critical for memory formation and, especially, pattern separating (Treves and Rolls, 1994, Treves et al., 2008). Increasing the number of dentate granule cells may therefore compromise sparsification and the ability of DG-CA3 to pattern separate. Second, cell death in the DG appears be necessary for normal dentate function, and therefore reduced cell death in TGFβ1 over-expressing mice may contribute to impairments in hippocampal dependent learning. For example, pharmacological or

genetic inhibition of cell death in the DG impairs spatial learning (Dupret et al., 2007, Kim et al., 2009, Lee et al., 2012). Moreover, as attenuated cell death is coupled with a reduction in adult neurogenesis, neuronal turnover in the dentate is greatly attenuated in TGFβ1 over-expressing mice. Recent models have proposed that the turnover of neurons in the dentate gyrus may play an important role in memory regulation (Meltzer et al., 2005, Inokuchi, 2011), and therefore reduced turnover in TGFβ1 over-expressing mice may contribute to impairments in hippocampal dependent learning. Fourth, TGFβ signaling pathways modulate synaptic plasticity in the adult brain (Krieglstein et al., 2011), including in particular the maintenance of persistent late-phase LTP in CA1 (Ageta et al., 2010). Therefore, dysregulation of hippocampal TGFβ1 levels may impair synaptic plasticity required for the formation of spatial memories.

Recent published data indicates that there are a number of vasculature deficiencies in the TGFβ1 over-expressing mice (Gaertner et al., 2005). These include an increase in thickness and collaged protein abundance, and these changes might also contribute to volumetric increases detected in MRI. Lack of glucose uptake and hypo-perfusion are some other vascular-related phenotypes that have been observed in this mice. However, non-hippocampal dependent behaviour like the responsiveness of whiskers, which is typically modified with vascular changes, are not impaired (Ginsberg et al., 1987). Since no other behavioural deficits have been observed in this mice and there are no spatial learning deficits in young mice (when vasculature changes should be present), we suggest that vasculature deficiencies are not causally-related to the spatial learning impairments observed in aged mice. Rather these impairments are more likely due hippocampal structural changes (Nicolakakis et al., 2011).

Conclusion. Here we examined the impact of chronic up-regulation TGFβ1 in the absence of disease. Our results identified macroscale, microscale changes and associated with this, deficits

in hippocampal dependent learning. Together these structure-function changes suggest that while TGFβ1 may be neuroprotective, chronic up-regulation may contribute to disease pathology itself.

6. General Discussion

6.1. Summary

In our first study, we showed that adult-generated granule neurons in the dentate gyrus (DG) are functionally indistinct from those generated during development. In the last decade, researchers proposed that optimal DG functionality is achieved by the cooperation of developmentally-generated neurons and adult-generated neurons with the enhanced plasticity. This view is supported by theoretical models showing that two classes of neurons with different types of responsiveness and synaptic facilitation are necessary to support the recognition of different patterns, thereby allowing for the construction of accurate cognitive maps and context representations (Aimone et al., 2006, 2009, 2011). Experimental data also support these ideas, such as the observation that newly-generated neurons are preferentially activated over developmentally-generated neurons *in vivo* (Ramirez-Amaya et al., 2006, Kee et al., 2007b). It has even been proposed that developmentally-generated neurons eventually stop participating in memory formation, and therefore newly-generated neurons become necessary to form new spatial and contextual memories (Alme et al., 2010). However, other studies showed that adult-generated neurons have the same probability of incorporation into memory circuits as their developmentally-generated neighbours (Stone et al., 2011).

To approach the problem of the functional distinctiveness versus equivalence of adult-generated neurons, we increased or decreased proliferation rates in the DG. We hypothesized that if newly-generated neurons are necessary to properly encode new contexts and spatial maps, a fixed proportion of newly-generated and previously-generated neurons should be maintained. Nevertheless, we found that the proportion of newly-generated to previously-generated neurons was not fixed but rather dependent on the abundance of newly-generated neurons. When the number of newly-generated neurons was decreased, thereby reducing their proportion, this failed to register a difference in circuit size (i.e. numbers of c-Fos

positive neurons) or memory acquisition and retention. When the number of newly-generated neurons was increased, thereby increasing their proportion, there was again no change in circuit size or memory function. This data together, shows that a fixed proportion of newborn neurons is not necessary for memory circuits to perform optimally.

In our second study, we found that the suppression of DG neurogenesis at different ages had different effects on memory. Although the blocking of new neurons is commonly used to evaluate the roles of neurogenesis in different types of memory, this methodology has provided mixed results (see section1.7 for references). As we found that neurons generated during adulthood are functionally equivalent to those of development origin, we hypothesized that the effect of neurogenesis blocking on memory would be contingent on the number of new neurons that are suppresed. Indeed, we detected memory impairment only in juvenile mice, in which proliferation rates are two-fold higher than in young adults and nine-fold higher than in middle-aged mice.

Different possible explanations arise from these results, including a possible compensation for the missing neurons in older mice, a possible disruption of DG development in juvenile mice, and possible age-dependent side effects. As only a moderate number of new neurons were missing in the young adult and middle-aged mice, it is possible that the hippocampus compensates for the missing neurons by an alternate route, thereby preventing negative consequences of neuron blocking. As the blocking occurred prior to training, no cells already engaged in memory circuitry were lost. Therefore, an extra recruitment of previously-generated granule cells could compensate for the missing newly-generated cells, although this would be more of a challenge in juvenile mice in which large numbers of cells were missing. It is also possible that at one month of age, the DG has still not reached a fully functional number of neurons, as it continues to generate large quantities of granule cells. The functionality of the DG depends on a large number of densely-packed principal neurons and a sparseness of their firing (Rolls, 1996). A smaller DG

with fewer neurons will have less sparseness for the same memory representation and therefore be less functional. The last and least likely possibility is that there were side effects of temozolomide that primarily impacted juvenile mice. However, we regularly monitored body weight during treatment without detecting a decrease, and we found no problems in swimming speed. Clinical studies in humans show that if properly administered, temozolomide has minimal side effects in adults and middle-aged individuals (Freyschlag et al., 2011) as well as children and adolescents (Ruggiero et al., 2006).

In our first two studies, we established that newly-generated neurons are functionally equivalent to their previously-generated neighbors and observed that blocking of new neurons impairs memory only when they are generated in large quantities. An important remaining question is whether a disruption in the continuous turnover of new neurons, which is common in the adult DG, would lead to an excessive number of neurons and eventual dysfunction of memory circuitry. Therefore, in our third study, we used a transgenic mouse that over-expresses TGFβ1 selectively in the brain, disrupting not only neurogenesis but also the capability of the DG to self-deplete excess newly-generated cells.

When TGFβ1 was over-expressed in the brain, we observed many morphological changes, including an abnormally large DG that was partially due to a constant increase in the number of granule cells during aging, consistent with previous findings that TGFβ1 blocks apoptotic cell death (see references in Buisson et al., 2003). A possible consequence of this disruption in neuron turnover is the survival of ill-shapen or poorly-connected neurons with small dendritic arbours that would normally be removed before reaching maturity. Also, mature granule cells that have been damaged may not be removed but instead remain in the structure occupying space and resources. An excess number of granule cells could result in a lack of space for proper dendritic growth, consistent with the deteriorated dendritic branching revealed by our Golgi-Cox staining. These morphological abnormalities may lead to an underperformance of hippocampal circuits and, consequently, memory impairment. We found that aged TGFβ1 mice showed a 40% increase

in the number of DG granule cells, whereas the number of postsynaptic targets and CA3 pyramidal cells was the same as young mice. This disequilibrium may lead to excessive noise within the circuitry and an inability to separate stimuli in the CA3, thereby the creation or recall of inaccurate contextual representations.

These structural and functional changes in the DG of aged TGFβ1 mice were consistently associated with deficits in learning and memory. Aged TGFβ1 mice did not efficiently learn the location of a hidden platform in the water maze, suggesting that neurons were unable to properly process input signals. The observed retention deficit could have been due either to impaired encoding or an inability of the circuitry to store previously-acquired information. Normal swim speed and thigmotaxis implies that mice were capable of performing the task but incapable of solving it via the generation of accurate navigational maps.

After establishing that newly-generated neurons are functionally equivalent to their previously-generated neighbours, we observed that both reducing large numbers of new cells and preventing their turnover results in memory impairment. Therefore, memory is critically dependent on the maintenance of optimal DG structure and function within certain equilibrium parameters. The interruption of proliferation leads to memory impairments in younger animals, but the blockade of neuron turnover in older animals, which may occur in response to disease, also poses major problems to memory function.

6.2 Is hippocampal neurogenesis important for memory?

We observed that the suppression of hippocampal neurogenesis during juvenility, a time when new neurons are in abundance, disrupts spatial learning. During young adulthood and middle age, however, suppression of neurogenesis did not affect learning. In our first experiment, we observed that there is not a specific pressure to use newly-generated neurons in memory circuits, but that they are used according to their availability. This implies that what determines the involvement of new DG neurons in memory is

their number at any given time, not qualities like their transient plasticity, which has recently tended to be the most predominant view. Thus, the most likely explanation for why we observed neurogenesis suppression-induced memory impairments in juveniles but not in older animals is that as levels of hippocampal neurogenesis naturally decline, previously-generated granule cells and other mechanisms could substitute for the lack of new neurons, thereby preventing disruptions in memory.

In contrast to a previous study (Drapeau et al., 2003), we did not observe a correlation between behaviour and neurogenesis level across age. In general, six and ten month-old mice showed no performance deficits in the water maze despite a sharp decrease in number of new neurons. However, when we used measures of precision in locating the platform, such as number of platform crossings, middle-aged mice performed more poorly than juvenile or young adult mice. According to some current theories, the precision of spatial cognitive maps relies strongly on the DG (Kesner, 2007). Therefore, the acute precision of spatial maps may depend on the flexibility afforded by a larger number of granule cells, but, overall, age-related differences in task performance may be difficult to detect.

The literature on adult hippocampal neurogenesis provides contradictory information regarding the role of adult-generated neurons in memory. Sometimes natural or experimental reductions in the number of new neurons are associated with memory deficits, but other times memory is unaffected. In general, there is no consensus on which types of memory are affected by neurogenesis blocking or which blocking method is most effective. Most reported deficits are in hippocampus-dependent memory function, supporting the idea that the optimal function of hippocampal circuitry requires the plasticity of adult-born neurons. Neurogenesis blocking-induced disruption of hippocampus-dependent memories can be revealed using many tests: trace eye-blink conditioning, the hidden platform version of the water maze, the Barnes maze, contextual fear conditioning, and various pattern recognition tasks. Nevertheless, the degree of hippocampus-dependency varies by test. For example, contextual fear conditioning also depends

on the amygdala, and spatial navigation tasks also depend on the entorhinal cortex and frontal lobes. This means that performance in these tests requires very different circuit structures and mechanisms to compensate for the decreased plasticity resulting from the loss of newborn neurons in the DG. However, some key differences among previous studies can also be associated with differences in strains and ages of rats and mice, which should produce significant variation in basal levels of neurogenesis among studies. In addition, the quality of housing and level of enrichment can also produce changes in basal neurogenesis levels and thereby influence experimental results.

Another possibility is that we did not observe actual effects of depleting neurogenesis but rather side effects of the suppression technique. Most suppression techniques rely on drugs or procedures that potentially have effects that are not specific to the proliferation zone of the DG. One of the first drugs used to reduce neurogenesis was the anti-mitotic drug MAM (Shors et al., 2001, Shors et al., 2002), which can impair locomotion and cause weight loss. Therefore, the memory deficits in these experiments are probably not due to the lack of neurogenesis but rather poor animal health (Dupret et al., 2005). However, other anti-mitotic drugs are currently used that satisfactorily block neurogenesis with minimal secondary effects. Temozolomide, which was used in this thesis, is one of the drugs that, when used carefully, has an acceptable side effect profile (Garthe et al., 2009). Another blocking method is focal irradiation (Snyder et al., 2005), which has been criticized for causing blood vessel damage, cranial nerve damage, and mild brain inflammation (Rogers, 2012). If the irradiation is focal (using a shield to protect the non-targeted areas of the body) and controlled in intensity it is possible to avoid side effects with trial-and-error optimization (Wojtowicz, 2006).

Other common methods for ablating neurogenesis take advantage of genetically modified mice that lack a cell cycle regulator such as Cyclin D (Jaholkowski et al., 2009) or in which blocking of progenitor cells can be induced (Singer et al., 2009). These approaches are precise and effective, but can

also have side effects when the transgenes must be activated using drugs such as tamoxifen or ganciclovir. Transgenes also can cause abnormal development if they are not inducible but permanently expressed. Overall, the reported side effects of these methods are not extensive enough to explain the consistent decline in hippocampus-dependent memory observed across studies. Moreover, because side effects differ from method to method but the results are similar, it is likely that the observed memory impairments are consequences of reduced hippocampal neurogenesis and not nonspecific effects.

Adult-generated neurons integrate into memory circuits in the same way as developmentally-generated neurons. The peak integration of new granule cells into spatial memory circuits occurs about six weeks after mitosis, with rate of integration remaining constant in the following weeks (Kee et al., 2007b, Stone et al., 2011). These findings are consistent with evidence that new granule cells eventually converge with their developmentally-generated neighbours in terms of electrophysiological properties and connectivity (Laplagne et al., 2006). The importance of adult neurogenesis in memory function is further established when memory is impaired after the post-training removal or silencing of new neurons. In particular, post-training blocking impairs both spatial memory and context discrimination, but pre-training blocking has no effect, probably due to compensation (Arruda-Carvalho et al., 2011, Gu et al., 2012). These reports confirm that adult-generated neurons actively participate in memory formation, and this is the most likely reason why we observe memory impairment after depletion of new neurons. However, the target of the depletion has to be a large population of new neurons, such as that which exists in juvenile animals, to prevent compensatory mechanisms from rescuing behavioural deficits.

6.3 How does hippocampal neurogenesis regulate memory?

Adult-generated neurons are temporarily different from developmentally-generated neurons, but the two populations ultimately show equivalent physiology and morphology. The process of maturation is

similar for the two populations. New neurons show higher levels of excitability during the first weeks but eventually appear identical to the rest of the granule cells in the DG (Laplagne et al., 2006). Once new neurons reach maturity, their dendritic arbourization is equivalent in size and shape to that of previously-generated neurons, and spine morphology and density is also equivalent (Esposito et al., 2005). Axons of granule cells can reach different neural populations, such as the hilar inhibitory neurons or mossy pyramidal neurons. Nonetheless, the ultimate target for granule cell axons is the stratum lucidum of CA3, where they form synapses with both pyramidal and inhibitory neurons. Adult-generated neurons have exactly the same pattern of anatomical connectivity as their developmentally-generated neighbours (Esposito et al., 2005).

The addition of new neurons during adulthood necessarily alters the morphology of the DG. After mitosis, neuroblasts start to migrate within the granule cell layer. During migration, new cells can separate previously adjacent neurons and compete with older neurons for space and perforant connections from the entorhinal cortex. Also, new neurons increase the load of axons innervating the stratum lucidum of CA3, resulting in competition for postsynaptic space in order to form thorny synapses. New synapses are not only formed adjacent to older synapses but may also lead to the displacement and posterior atrophy of older synapses (Toni et al., 2008). The addition of new excitatory neurons to an established system may require an expanded coverage of inhibitory neurons to down-regulate and tune excitatory inputs. It may also require the presence of more or larger astrocytes to maintain metabolic and neurotransmitter equilibrium. Overall, many structural changes are associated with the addition of new neurons to the DG, and this should ultimately affect the morphology and network organization of the whole hippocampal structure (Meltzer et al., 2005).

Early studies show that blocked apoptosis in genetically modified mice disrupts normal nervous system development (Kuida et al., 1998). The same principle may apply to adult-generated neurons in the

DG. Apoptosis in the DG affects immature neurons more than mature neurons. Only 10% of new cells in the DG survive until full maturation, and when mice are one year old, their DG contains approximately 6% more neurons than in early adulthood (Lagace et al., 2007). The blocking of apoptosis in the adult brain leads to deformities and a loss of function (Kuhn et al., 2005, Kim et al., 2009). From this point of view, apoptosis in the adult DG can be considered a mechanism of morphological plasticity.

In our third study, we found that TGFβ1 overexpression blocked apoptosis in the DG. This is consistent with previous studies (Brionne et al., 2003, Buckwalter et al., 2006) showing that TGFβ1 blocks excitotoxicity-induced apoptosis by preventing the secretion of PAI-1 by astrocytes (See figure 6 in section 1.5) or Caspase-5 down-regulation by an MAPK/ERK pathway. The immediate consequence is that the survival of neurons exceeds the drop in their proliferation. Therefore, the net increase in number of granule cells at one year of age is about 40% instead of the previously reported 6%. Neuron turnover, which is the continuous addition and subtraction of cells, can be critical for the proper function of the DG. When it is disrupted, by reducing both proliferation and cell death, spatial learning is impaired (Dupret et al., 2007). This may occur because apoptosis is critical for removing dysfunctional cells—those that failed to migrate into an optimal position to establish post-synaptic connections with the perforant pathway and pre-synaptic connections in the CA3. These dysfunctional neurons use up valuable resources, take up valuable space in an already crowded brain structure, and can make useless connections that generate noise or create homeostatic unbalance between excitation and inhibition (Meltzer et al., 2005, Imielski et al., 2012).

The activity of neurons in the DG is normally sparse, with only 1-2% of neurons involved in any given memory trace, making it unlikely that a CA3 pyramidal neuron receives inputs from two different granule cells at the same time. This orthogonalization of inputs is considered to be essential for pattern separation and the creation of precise cognitive maps (Rolls, 1996). The blockade of apoptosis, which

causes an excess of granule cells in the DG, can result in CA3 pyramidal neurons receiving simultaneous inputs from more than one granule cell, leading to defective memory processing. In middle-aged TGFβ1 mice that showed a spatial memory deficit, we did not observe any change in the number of CA3 or CA1 pyramidal neurons. We also did not observe problems with swimming speed, thigmotaxis (tendency to swim near walls), or searching behavior during training. The transgenic mice failed to locate the platform using spatial cues, suggesting a problem with DG-CA3 connectivity. These results are consistent with previous reports that reduction of apoptosis in the DG is associated with learning and memory impairment (Kim et al., 2009, Lee et al., 2012).

6.4 Why is neurogenesis in the dentate gyrus?

The DG is different from all other brain areas linked to episodic-like memory in terms of its principal neuron type, its structural organization, and most strikingly, its capability to partially self-renew during adulthood. We established that newly-generated neurons are functionally indistinct from previously-generated neurons, but we also found that a large depletion or addition of new neurons impairs functionality. These results show that hippocampal neurogenesis is important for learning and memory, but why does this process occur in the DG but no other areas involved in memory processing, such as the amygdala, neocortex, or other layers of the hippocampus? The answer may lie in the role that the hippocampus plays in memory function, automatically encoding all episodic-like memories but also clearing most encoded memories as they are exported and consolidated in cortical modules. Because the hippocampus encodes continuously and automatically, many memories of low relevance are allocated to this structure, necessitating a mechanism to filter excess information (Wang and Morris, 2010a). The DG is constantly remodelling, which may generate constant interference and weakening of previously-encoded memories. Also, the constant addition and renewal of synapses may facilitate encoding of new memories without interference from previously-encoded traces.

This proposal was first formulated in the early 2000s when increasing adult neurogenesis via enrichment was found to promote clearance of hippocampus-dependent memories (Feng et al., 2001). However, this idea did not resonate within the field of study. The idea that neurogenesis was involved in memory consolidation was later supported by a set of clever experiments performed by Kitamura and colleagues (2009). Their work, however, only pointed toward a role for new neurons in consolidation without addressing memory clearance. When we increased neurogenesis through exercise or chemical treatments after formation of contextual fear or spatial memories in adult mice, we observed a consistent degradation of those pre-existing memories. Therefore, we propose that increasing hippocampal proliferation during the critical period of memory consolidation can clear hippocampus-dependent memories (Appendixes I and II).

A functional advantage of memory clearance is that the hippocampus can always be capable of encoding new memories, even if they conflict with previously-acquired information. We found that enhancing neurogenesis for a month after training mice in the hidden platform version of the water maze led to a deficit in memory for the original platform location. On the other hand, after retraining mice with the platform in a new location, mice with enhanced neurogenesis learned the new location more quickly. Conversely, mice with normal levels of neurogenesis did not learn optimally and showed no spatial bias toward the new location (Appendix I). These findings show that hippocampus-dependent learning is facilitated by the clearance of previous memories and that new neurons appear to be a key factor in this process. That memory erasure facilitates new learning is a significant clue toward understanding the adaptive role of adult hippocampal neurogenesis.

Morris and colleagues (Morris et al., 2003, Wang and Morris, 2010a) propose that the molecular mechanism known as synaptic tagging is responsible for the rapid encoding and subsequent clearance or consolidation of memories. According to this theory, rapidly-synthesized proteins tag those synapses

involved in the memory trace. These tagged synapses can later generate strong synaptic plasticity even in response to weak stimulation if it occurs within a short time window, after which the proteins and mRNAs that make up the tag are removed from the synapses. This synaptic tagging model only explains memory clearance within a few hours after learning. Our proposed model may be complementary to this model, as it takes at least two weeks for new neurons to migrate and mature enough to disrupt previously-acquired memories. Furthermore, the competition between newer and older synapses and the eventual replacement of older synapses could function in tandem with the synaptic tagging model, as non-tagged synapses could be weaker and more prone to replacement by new synapses from adult-generated neurons.

In the three studies described in this thesis, we observed that levels of hippocampal neurogenesis change across the lifespan, with perturbations of proliferation having more of an impact in earlier stages of life. On the other hand, post-learning blockade of neurogenesis during infancy prolongs the retention of a hippocampus-dependent context memory, with an individual-level correlation between proliferation and memory persistence (Appendix I). Infant rodents, like human children, present a difficulty in long-term memory retention called "infantile amnesia" (Josselyn and Frankland, 2012). Our findings suggest that high levels of neurogenesis during infancy can explain poor memory consolidation. The accelerated decay of memories during infancy may be important for the development of personality and cognitive function (Mulhern et al., 2005)

Several studies have related hippocampal neurogenesis with affective disorders such as stress, anxiety, and depression (see references in Dranovsky and Hen, 2006). The consensus is that stress and chronic depression reduce hippocampal proliferation and that anxiolytic and antidepressant treatments rescue neurogenesis. It is not clear if a decrease in neurogenesis triggers the observed clinical symptoms. If a decrease of neurogenesis prevents the proper clearance of memories, however, it may reduce the

extinction of stressful stimuli (Pan et al., 2012) and substitution with positive stimuli that can overcome observed symptomatology.

The possibility that neurogenesis is altered in AD is vague. Both animal models and human post-mortem studies yield conflicting evidence, with reports of both increases and decreases in neurogenesis rates. Our studies show that the influence of TGFβ1 alone can lead to both a decrease in proliferation and an increase in survival. This duality of TGFβ1 effects may explain the conflicting evidence. Currently, it is unknown whether an influence of TGFβ1 on neurogenesis is a reason for the severe memory deficits in AD.

7. Future directions

7.1 Participation of new neurons in memory circuits

We demonstrated a functional equivalence between adult-generated and developmentally-generated granule cells in the DG, but only in regard to their contribution to memory acquisition. An outstanding question is whether adult-generated neurons continue to contribute to memories after their initial acquisition. Although a recent study suggested that adult-generated neurons contribute to memory consolidation (Kitamura et al., 2009), and we showed that the manipulation of adult neurogenesis impacts long-term memory stability (Appendix I and II), the role of adult-generated neurons in remote memory remains largely unexplored, and their engagement in circuits supporting remote memoty is not known.

We found that a post-learning increase in neurogenesis destabilized memory for what was learned, suggesting that new neurons play an active role in forgetting. However, this increase in neurogenesis also facilitated new learning, possibly via the destabilization and removal of previously-acquired memories. To confirm this view, is necessary to explor the recruitment of newborn neurons in memory during distal memory recall.The ability of new neurons to actively promote forgetting, therefore, may be relevant to the treatment of post-traumatic stress disorder or other psychiatric conditions.

In the experiments described in this thesis, we examined the role of hippocampal neurogenesis in learning and memory in juvenile, adult, and middle-aged mice. Nonetheless, we also have evidence that neurogenesis impacts memory function in infant mice. In a previous study from our lab (Stone et al., 2011), we found that neurons generated during infancy participate in memory formation during adulthood, but we did not examine the function of these neurons earlier

in life. The question of whether elevated numbers of new neurons lead to forgetting in young mice remains to be addressed at the cellular level of analysis, which would further our understanding of the effects of chemotherapy and irradiation in children with brain tumors.

7.2 Age-dependent memory deficits following neurogenesis blocking.

We found that the blocking of neurogenesis during juvenility impairs learning and memory, probably due to the large number of new neurons present at this age. Also, previous studies show that cognitive performance in different species of wild mice may be proportional to the number of granule cells present (Biegler et al., 2001, Patil et al., 2011). Together, these findings suggest that a suboptimal availability of DG granule cells can have negative consequences for hippocampus-dependent memory function. The analysis of possible correlations between total numbers of granule cells and performance in memory tasks would determine the number of granule cells that are necessary to sustain optimal memory circuitry. This information could also be used to predict the age at which it is safe to disrupt neurogenesis without risking possible cognitive dysfunction.

Although we examined the impact of our experimental manipulations on the absolute number of new granule cells in the DG, an understanding of how neurogenesis blocking affects the overall organization and connectivity of the hippocampus may also be meaningful. Future studies could examine the effect of neurogenesis blocking on the arborisation of dendrites and spines on remaining granule cells, the extension of mossy fibers into the CA3, and the number of large mossy terminals, as well as how these parameters might relate to memory function. In addition to neurons, the adult DG also generates new astrocytes, which play a major role in the regulation of excitatory neuron metabolism and activity. There are currently no studies that have

investigated a potential role of astrocytogenesis in memory, but this might also be an important topic to explore in the light of blocking studies. Ultimately, electrophysiological studies could reveal how the blocking of neurogenesis may affect connectivity and plasticity in the hippocampus in general and the CA3 and CA1 in particular.

7.2 Role of TGFβ1 in memory and disease.

In our third study, we determined that the main effect of chronic over-expression of TGFβ1 on spatial memory was related to a profound disruption of cellular proliferation and death within the adult DG. This is consistent with previous literature showing that both disruptions of neurogenesis-related cell turnover (Dupret et al., 2007) and overgrowth of the DG (Kim et al., 2009, Lee et al., 2012) lead to disruption of hippocampus-dependent memory function.

We hypothesize that the uncontrolled expansion of the DG granule cell population disrupts connectivity among hippocampal subregions, which in turn impairs hippocampus-dependent memory. If this is the case, then the blocking of neurogenesis in TGFβ1 over-expressing mice should rescue memory function by driving the granule cell number back to normal. This potential finding would not only show that TGFβ1 over-expression impairs memory by disrupting cellular turnover but would also suggest that an equilibrium between the numbers of granule cells and other principal neuron types in the hippocampus is necessary for optimal memory function.

Another future research aim could be to dissociate the role of TGFβ1 in AD-related cognitive impairment. TGFβ1 expression commences in early stages and continues throughout the course of the disease. However, several other pathologies associated with AD, such as neuron loss and damage to membrane receptors, could account for the observed memory deficits. To determine

whether TGFβ1 has a specific role in AD-related memory impairments, mouse models of AD could be crossed with TGFβ1 over-expressing mice, allowing for the pharmacological inhibition of TGFβ1 (Laping et al., 2002) or rescue of AD symptoms (Schwarz et al., 2012) to evaluate their differential effects on memory.

Although we observed that chronic over-expression of TGFβ1 impairs hippocampal neurogenesis and hippocampus-dependent memory, no studies have examined whether TGFβ1 has a role in memory function under homeostatic conditions. A difficulty in studying TGFβ1 under homeostatic conditions is the short duration of TGFβ1 protein stability in the brain. Therefore, a different method of analysis such as RT-PCR is necessary to measure changes in TGFβ1 expression after evoked conditions such as learning, exercise, or sleep. If learning, sleep, or other activities that regulate hippocampal neurogenesis also regulate TGFβ1, this would provide evidence that TGFβ1 is involved in hippocampal plasticity. Future studies could utilize specific antagonists of TGFβ1 or the SMAD transduction pathway to further understand whether TGFβ1 regulates neurogenesis under homeostatic conditions.

8. Conclusion

Together, the body of evidence presented in this thesis shows that the dentate gyrus of the hippocampus, with its unique capacity to generate neurons throughout the lifespan, has an important role in episodic-like memory, particularly spatial memory. Although the specific relevance of the dentate gyrus in memory processing remains mysterious, our findings suggest that its malfunction can sensitively affect hippocampus-dependent memory function. Our study of the chronic over-expression of the TGFβ1 underscores this point by demonstrating that affecting neurogenesis in the dentate gyrus impairs memory. Also, the importance of hippocampal neurogenesis depends on the particular life stage, with neurogenesis being more critical at juvenility than at older ages. However, at any developmental stage or with any experimental manipulation, newly-generated neurons appear to be functionally indistinct from their previously-generated neighbours. Our findings suggest that the relevance of hippocampal neurogenesis depends on age and pathological status. This information can have important implications for mental health in the context of neurodegenerative diseases and developmental brain abnormalities.

9. References

Ageta H, Ikegami S, Miura M, Masuda M, Migishima R, Hino T, Takashima N, Murayama A, Sugino H, Setou M, Kida S, Yokoyama M, Hasegawa Y, Tsuchida K, Aosaki T, Inokuchi K (2010) Activin plays a key role in the maintenance of long-term memory and late-LTP. Learn Mem 17:176-185.

Aggleton JP, Brown MW (2006) Interleaving brain systems for episodic and recognition memory. Trends Cogn Sci 10:455-463.

Aguado F, Espinosa-Parrilla JF, Carmona MA, Soriano E (2002) Neuronal activity regulates correlated network properties of spontaneous calcium transients in astrocytes in situ. J Neurosci 22:9430-9444.

Aguilar-Valles A, Sanchez E, de Gortari P, Balderas I, Ramirez-Amaya V, Bermudez-Rattoni F, Joseph-Bravo P (2005) Analysis of the stress response in rats trained in the water-maze: differential expression of corticotropin-releasing hormone, CRH-R1, glucocorticoid receptors and brain-derived neurotrophic factor in limbic regions. Neuroendocrinology 82:306-319.

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437:894-897.

Aimone JB, Deng W, Gage FH (2011) Resolving new memories: a critical look at the dentate gyrus, adult neurogenesis, and pattern separation. Neuron 70:589-596.

Aimone JB, Wiles J, Gage FH (2006) Potential role for adult neurogenesis in the encoding of time in new memories. Nat Neurosci 9:723-727.

Aimone JB, Wiles J, Gage FH (2009) Computational influence of adult neurogenesis on memory encoding. Neuron 61:187-202.

Aizawa K, Ageyama N, Yokoyama C, Hisatsune T (2009) Age-dependent alteration in hippocampal neurogenesis correlates with learning performance of macaque monkeys. Exp Anim 58:403-407.

Akagi K, Sandig V, Vooijs M, Van der Valk M, Giovannini M, Strauss M, Berns A (1997) Cre-mediated somatic site-specific recombination in mice. Nucleic Acids Res 25:1766-1773.

Akyurek S, Senturk V, Oncu B, Ozyigit G, Yilmaz S, Gokce SC (2008) The effect of tianeptine in the prevention of radiation-induced neurocognitive impairment. Med Hypotheses 71:930-932.

Alme CB, Buzzetti RA, Marrone DF, Leutgeb JK, Chawla MK, Schaner MJ, Bohanick JD, Khoboko T, Leutgeb S, Moser EI, Moser MB, McNaughton BL, Barnes CA (2010) Hippocampal granule cells opt for early retirement. Hippocampus 20:1109-1123.

Almgren M, Persson AS, Fenghua C, Witgen BM, Schalling M, Nyengaard JR, Lavebratt C (2007) Lack of potassium channel induces proliferation and survival causing increased neurogenesis and two-fold hippocampus enlargement. Hippocampus 17:292-304.

Alonso M, Viollet C, Gabellec MM, Meas-Yedid V, Olivo-Marin JC, Lledo PM (2006) Olfactory discrimination learning increases the survival of adult-born neurons in the olfactory bulb. J Neurosci 26:10508-10513.

Altman J (1969) Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol 137:433-457.

Altman J, Bayer SA (1990) Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. J Comp Neurol 301:365-381.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Altman J, Das GD (1966) Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J Comp Neurol 126:337-389.

Altman J, Das GD (1967) Postnatal neurogenesis in the guinea-pig. Nature 214:1098-1101.

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41:683-686.

Amaral DG (1993) Emerging principles of intrinsic hippocampal organization. Curr Opin Neurobiol 3:225-229.

Amaral DG, Witter MP (1989) The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neuroscience 31:571-591.

Ambrogini P, Lattanzi D, Ciuffoli S, Agostini D, Bertini L, Stocchi V, Santi S, Cuppini R (2004) Morpho-functional characterization of neuronal cells at different stages of maturation in granule cell layer of adult rat dentate gyrus. Brain Res 1017:21-31.

An WL, Cowburn RF, Li L, Braak H, Alafuzoff I, Iqbal K, Iqbal IG, Winblad B, Pei JJ (2003) Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. Am J Pathol 163:591-607.

Anagnostaras SG, Maren S, Fanselow MS (1999) Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. J Neurosci 19:1106-1114.

Apelt J, Schliebs R (2001) Beta-amyloid-induced glial expression of both pro- and anti-inflammatory cytokines in cerebral cortex of aged transgenic Tg2576 mice with Alzheimer plaque pathology. Brain Res 894:21-30.

Araque A, Martin ED, Perea G, Arellano JI, Buno W (2002) Synaptically released acetylcholine evokes Ca2+ elevations in astrocytes in hippocampal slices. J Neurosci 22:2443-2450.

Araria-Goumidi L, Lambert JC, Mann DM, Lendon C, Frigard B, Iwatsubo T, Cottel D, Amouyel P, Chartier-Harlin MC (2002) Association study of three polymorphisms of TGF-beta1 gene with Alzheimer's disease. J Neurol Neurosurg Psychiatry 73:62-64.

Arora K, Dai H, Kazuko SG, Jamal J, O'Connor MB, Letsou A, Warrior R (1995) The Drosophila schnurri gene acts in the Dpp/TGF beta signaling pathway and encodes a transcription factor homologous to the human MBP family. Cell 81:781-790.

Arruda-Carvalho M, Sakaguchi M, Akers KG, Josselyn SA, Frankland PW (2011) Posttraining ablation of adult-generated neurons degrades previously acquired memories. J Neurosci 31:15113-15127.

Bachevalier J, Beauregard M (1993) Maturation of medial temporal lobe memory functions in rodents, monkeys, and humans. Hippocampus 3:191-202.

Bachoo RM, Kim RS, Ligon KL, Maher EA, Brennan C, Billings N, Chan S, Li C, Rowitch DH, Wong WH, DePinho RA (2004) Molecular diversity of astrocytes with implications for neurological disorders. Proc Natl Acad Sci U S A 101:8384-8389.

Baker CA, Lu ZY, Zaitsev I, Manuelidis L (1999) Microglial activation varies in different models of Creutzfeldt-Jakob disease. J Virol 73:5089-5097.

Baldi E, Efoudebe M, Lorenzini CA, Bucherelli C (2005) Spatial navigation in the Morris water maze: working and long lasting reference memories. Neurosci Lett 378:176-180.

Bannister NJ, Larkman AU (1995) Dendritic morphology of CA1 pyramidal neurones from the rat hippocampus: I. Branching patterns. J Comp Neurol 360:150-160.

Barnes CA (1979) Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. J Comp Physiol Psychol 93:74-104.

Basu A, Krady JK, Enterline JR, Levison SW (2002) Transforming growth factor beta1 prevents IL-1beta-induced microglial activation, whereas TNFalpha- and IL-6-stimulated activation are not antagonized. Glia 40:109-120.

Bats S, Thoumas JL, Lordi B, Tonon MC, Lalonde R, Caston J (2001) The effects of a mild stressor on spontaneous alternation in mice. Behav Brain Res 118:11-15.

Battaglia G, Cannella M, Riozzi B, Orobello S, Maat-Schieman ML, Aronica E, Busceti CL, Ciarmiello A, Alberti S, Amico E, Sassone J, Sipione S, Bruno V, Frati L, Nicoletti F, Squitieri F (2010) Early defect of transforming growth factor beta-1 formation in Huntington's disease. J Cell Mol Med.

Bauer S, Patterson PH (2006) Leukemia inhibitory factor promotes neural stem cell self-renewal in the adult brain. J Neurosci 26:12089-12099.

Baver SA, Altman J (1975) Radiation-induced interference with postnatal hippocampal cytogenesis in rats and its long-term effects on the acquisition of neurons and glia. J Comp Neurol 163:1-19.

Bayer SA, Yackel JW, Puri PS (1982) Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science 216:890-892.

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

Benediktsson AM, Schachtele SJ, Green SH, Dailey ME (2005) Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures. J Neurosci Methods 141:41-53.

Benes FM, Kwok EW, Vincent SL, Todtenkopf MS (1998) A reduction of nonpyramidal cells in sector CA2 of schizophrenics and manic depressives. Biol Psychiatry 44:88-97.

Bergles DE, Roberts JD, Somogyi P, Jahr CE (2000) Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. Nature 405:187-191.

Bernard C, Wheal HV (1994) Model of local connectivity patterns in CA3 and CA1 areas of the hippocampus. Hippocampus 4:497-529.

Bezzi P, Volterra A (2001) A neuron-glia signalling network in the active brain. Curr Opin Neurobiol 11:387-394.

Biegler R, McGregor A, Krebs JR, Healy SD (2001) A larger hippocampus is associated with longer-lasting spatial memory. Proc Natl Acad Sci U S A 98:6941-6944.

Bizon JL, Gallagher M (2003) Production of new cells in the rat dentate gyrus over the lifespan: relation to cognitive decline. Eur J Neurosci 18:215-219.

Bizon JL, Lee HJ, Gallagher M (2004) Neurogenesis in a rat model of age-related cognitive decline. Aging Cell 3:227-234.

Björklund A, Hökfelt T, Swanson LM (1987) Integrated systems of the CNS. Amsterdam ; New York

New York, NY: Elsevier

Blaabjerg M, Zimmer J (2007) The dentate mossy fibers: structural organization, development and plasticity. Prog Brain Res 163:85-107.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 232:331-356.

Blokland A, Geraerts E, Been M (2004) A detailed analysis of rats' spatial memory in a probe trial of a Morris task. Behav Brain Res 154:71-75.

Bolding K, Rudy JW (2006) Place learning in the Morris water task: making the memory stick. Learn Mem 13:278-286.

Bonyadi M, Rusholme SA, Cousins FM, Su HC, Biron CA, Farrall M, Akhurst RJ (1997) Mapping of a major genetic modifier of embryonic lethality in TGF beta 1 knockout mice. Nat Genet 15:207-211.

Brionne TC, Tesseur I, Masliah E, Wyss-Coray T (2003) Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain. Neuron 40:1133-1145.

Brown DR (1999) Dependence of neurones on astrocytes in a coculture system renders neurones sensitive to transforming growth factor beta1-induced glutamate toxicity. J Neurochem 72:943-953.

Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1-10.

Bruel-Jungerman E, Laroche S, Rampon C (2005) New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. Eur J Neurosci 21:513-521.

Bucci DJ, Chiba AA, Gallagher M (1995) Spatial learning in male and female Long-Evans rats. Behav Neurosci 109:180-183.

Buckwalter MS, Yamane M, Coleman BS, Ormerod BK, Chin JT, Palmer T, Wyss-Coray T (2006) Chronically increased transforming growth factor-beta1 strongly inhibits hippocampal neurogenesis in aged mice. Am J Pathol 169:154-164.

Buisson A, Lesne S, Docagne F, Ali C, Nicole O, MacKenzie ET, Vivien D (2003) Transforming growth factor-beta and ischemic brain injury. Cell Mol Neurobiol 23:539-550.

Buisson A, Nicole O, Docagne F, Sartelet H, Mackenzie ET, Vivien D (1998) Up-regulation of a serine protease inhibitor in astrocytes mediates the neuroprotective activity of transforming growth factor beta1. Faseb J 12:1683-1691.

Bullwinkel J, Baron-Luhr B, Ludemann A, Wohlenberg C, Gerdes J, Scholzen T (2006) Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. J Cell Physiol 206:624-635.

Buresova O, Bures J, Oitzl MS, Zahalka A (1985) Radial maze in the water tank: an aversively motivated spatial working memory task. Physiol Behav 34:1003-1005.

Burt DW, Law AS (1994) Evolution of the transforming growth factor-beta superfamily. Prog Growth Factor Res 5:99-118.

Bushong EA, Martone ME, Ellisman MH (2004) Maturation of astrocyte morphology and the establishment of astrocyte domains during postnatal hippocampal development. Int J Dev Neurosci 22:73-86.

Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci 22:183-192.

Butt AM, Kiff J, Hubbard P, Berry M (2002) Synantocytes: new functions for novel NG2 expressing glia. J Neurocytol 31:551-565.

Buzsaki G (1984) Feed-forward inhibition in the hippocampal formation. Prog Neurobiol 22:131-153.

Cajal R (1894a) The croonian lecture "la fine estructure des centre nerveux". Proc R Soc Lond B Biol Sci 88:444.

Cajal SRy (1894b) Estructura intima de los centros nerviosos. Rev Ciencias Med 20:145-160.

Caspi M, Atlas R, Kantor A, Sapir T, Reiner O (2000) Interaction between LIS1 and doublecortin, two lissencephaly gene products. Hum Mol Genet 9:2205-2213.

Cauller LJ, Connors BW (1994) Synaptic physiology of horizontal afferents to layer I in slices of rat SI neocortex. J Neurosci 14:751-762.

Cavazos JE, Golarai G, Sutula TP (1992) Septotemporal variation of the supragranular projection of the mossy fiber pathway in the dentate gyrus of normal and kindled rats. Hippocampus 2:363-372.

Cerasti E, Treves A (2010) How informative are spatial CA3 representations established by the dentate gyrus? PLoS Comput Biol 6:e1000759.

Chawla MK, Guzowski JF, Ramirez-Amaya V, Lipa P, Hoffman KL, Marriott LK, Worley PF, McNaughton BL, Barnes CA (2005) Sparse, environmentally selective expression of Arc RNA in the upper blade of the rodent fascia dentata by brief spatial experience. Hippocampus 15:579-586.

Cheifetz S, Weatherbee JA, Tsang ML, Anderson JK, Mole JE, Lucas R, Massague J (1987) The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. Cell 48:409-415.

Chin BY, Petrache I, Choi AM, Choi ME (1999a) Transforming growth factor beta1 rescues serum deprivation-induced apoptosis via the mitogen-activated protein kinase (MAPK) pathway in macrophages. The Journal of biological chemistry 274:11362-11368.

Chin J, Angers A, Cleary LJ, Eskin A, Byrne JH (1999b) TGF-beta1 in Aplysia: role in long-term changes in the excitability of sensory neurons and distribution of TbetaR-II-like immunoreactivity. Learn Mem 6:317-330.

Chin J, Angers A, Cleary LJ, Eskin A, Byrne JH (2002) Transforming growth factor beta1 alters synapsin distribution and modulates synaptic depression in Aplysia. J Neurosci 22:RC220.

Choi KD, Lillehoj HS, Zalenga DS (1999) Changes in local IFN-gamma and TGF-beta4 mRNA expression and intraepithelial lymphocytes following Eimeria acervulina infection. Vet Immunol Immunopathol 71:263-275.

Christie BR, Cameron HA (2006) Neurogenesis in the adult hippocampus. Hippocampus 16:199-207.

Claiborne BJ, Amaral DG, Cowan WM (1986) A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus. J Comp Neurol 246:435-458.

Clelland CD, Choi M, Romberg C, Clemenson GD, Jr., Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ (2009) A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325:210-213.

Cole AJ, Saffen DW, Baraban JM, Worley PF (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. Nature 340:474-476.

Colombo JA, Sherwood CC, Hof PR (2004) Interlaminar astroglial processes in the cerebral cortex of great apes. Anat Embryol (Berl) 208:215-218.

Cook RH (1979) Memory loss in Alzheimer disease. Ann Neurol 5:105-106.

Corkin S, Amaral DG, Gonzalez RG, Johnson KA, Hyman BT (1997) H. M.'s medial temporal lobe lesion: findings from magnetic resonance imaging. J Neurosci 17:3964-3979.

Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ (2010) Running enhances spatial pattern separation in mice. Proc Natl Acad Sci U S A 107:2367-2372.

D'Hooge R, De Deyn PP (2001) Applications of the Morris water maze in the study of learning and memory. Brain Res Brain Res Rev 36:60-90.

da Cunha A, Vitkovic L (1992) Transforming growth factor-beta 1 (TGF-beta 1) expression and regulation in rat cortical astrocytes. J Neuroimmunol 36:157-169.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1-105.

Daopin S, Piez KA, Ogawa Y, Davies DR (1992) Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. Science 257:369-373.

de Larco JE, Todaro GJ (1978) Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci U S A 75:4001-4005.

de Sampaio e Spohr TC, Martinez R, da Silva EF, Neto VM, Gomes FC (2002) Neuro-glia interaction effects on GFAP gene: a novel role for transforming growth factor-beta1. Eur J Neurosci 16:2059-2069.

Demmer J, Dragunow M, Lawlor PA, Mason SE, Leah JD, Abraham WC, Tate WP (1993) Differential expression of immediate early genes after hippocampal long-term potentiation in awake rats. Brain Res Mol Brain Res 17:279-286.

Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 11:339-350.

Deniselle MC, Lavista-Llanos S, Ferrini MG, Lima AE, Roldan AG, De Nicola AF (1999) In vitro differences between astrocytes of control and wobbler mice spinal cord. Neurochem Res 24:1535-1541.

Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV (1985) Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature 316:701-705.

deToledo-Morrell L, Geinisman Y, Morrell F (1988) Age-dependent alterations in hippocampal synaptic plasticity: relation to memory disorders. Neurobiol Aging 9:581-590.

Dhandapani KM, Hadman M, De Sevilla L, Wade MF, Mahesh VB, Brann DW (2003) Astrocyte protection of neurons: role of transforming growth factor-beta signaling via a c-Jun-AP-1 protective pathway. J Biol Chem 278:43329-43339.

Dheen ST, Kaur C, Ling EA (2007) Microglial activation and its implications in the brain diseases. Curr Med Chem 14:1189-1197.

Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ (1995) Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development 121:1845-1854.

Diemel LT, Jackson SJ, Cuzner ML (2003) Role for TGF-beta1, FGF-2 and PDGF-AA in a myelination of CNS aggregate cultures enriched with macrophages. J Neurosci Res 74:858-867.

Docagne F, Nicole O, Marti HH, MacKenzie ET, Buisson A, Vivien D (1999) Transforming growth factor-beta1 as a regulator of the serpins/t-PA axis in cerebral ischemia. Faseb J 13:1315-1324.

Dong H, Goico B, Martin M, Csernansky CA, Bertchume A, Csernansky JG (2004) Modulation of hippocampal cell proliferation, memory, and amyloid plaque deposition in APPsw (Tg2576) mutant mice by isolation stress. Neuroscience 127:601-609.

Dorr AE, Lerch JP, Spring S, Kabani N, Henkelman RM (2008) High resolution three-dimensional brain atlas using an average magnetic resonance image of 40 adult C57Bl/6J mice. Neuroimage 42:60-69.

Douglas (1972) Pavlovian conditioning and the brain. London, New York,: Academic Press.

Dragunow M, Robertson HA (1987a) Generalized seizures induce c-fos protein(s) in mammalian neurons. Neurosci Lett 82:157-161.

Dragunow M, Robertson HA (1987b) Kindling stimulation induces c-fos protein(s) in granule cells of the rat dentate gyrus. Nature 329:441-442.

Dranovsky A, Hen R (2006) Hippocampal neurogenesis: regulation by stress and antidepressants. Biol Psychiatry 59:1136-1143.

Drapeau E, Mayo W, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2003) Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. Proc Natl Acad Sci U S A 100:14385-14390.

Driscoll I, Howard SR, Stone JC, Monfils MH, Tomanek B, Brooks WM, Sutherland RJ (2006) The aging hippocampus: a multi-level analysis in the rat. Neuroscience 139:1173-1185.

Duan X, Kang E, Liu CY, Ming GL, Song H (2008) Development of neural stem cell in the adult brain. Curr Opin Neurobiol 18:108-115.

Dumas TC (2005) Late postnatal maturation of excitatory synaptic transmission permits adult-like expression of hippocampal-dependent behaviors. Hippocampus 15:562-578.

Dupret D, Fabre A, Dobrossy MD, Panatier A, Rodriguez JJ, Lamarque S, Lemaire V, Oliet SH, Piazza PV, Abrous DN (2007) Spatial learning depends on both the addition and removal of new hippocampal neurons. PLoS Biol 5:e214.

Dupret D, Montaron MF, Drapeau E, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2005) Methylazoxymethanol acetate does not fully block cell genesis in the young and aged dentate gyrus. Eur J Neurosci 22:778-783.

Eichenbaum H (2002) The cognitive neuroscience of memory : an introduction. Oxford ; New York: Oxford University Press.

Eichenbaum H (2004a) Hippocampus: cognitive processes and neural representations that underlie declarative memory. Neuron 44:109-120.

Eichenbaum H (2004b) Hippocampus: cognitive processes and neural representations that underlie declarative memory. Neuron 44:109-120.

Eidinoff ML, Cheong L, Rich MA (1959) Incorporation of unnatural pyrimidine bases into deoxyribonucleic acid of mammalian cells. Science 129:1550-1551.

ElBeltagy M, Mustafa S, Umka J, Lyons L, Salman A, Chur-yoe GT, Bhalla N, Bennett G, Wigmore PM (2010) Fluoxetine improves the memory deficits caused by the chemotherapy agent 5-fluorouracil. Behav Brain Res 208:112-117.

Encinas JM, Vaahtokari A, Enikolopov G (2006) Fluoxetine targets early progenitor cells in the adult brain. Proc Natl Acad Sci U S A 103:8233-8238.

Esposito MS, Piatti VC, Laplagne DA, Morgenstern NA, Ferrari CC, Pitossi FJ, Schinder AF (2005) Neuronal differentiation in the adult hippocampus recapitulates embryonic development. J Neurosci 25:10074-10086.

Fanselow MS (1982) The post-shock activity burst. Anim Learn Behav 10:448-454.

Fanselow MS (2000) Contextual fear, gestalt memories, and the hippocampus. Behav Brain Res 110:73-81.

Farioli-Vecchioli S, Saraulli D, Costanzi M, Pacioni S, Cina I, Aceti M, Micheli L, Bacci A, Cestari V, Tirone F (2008) The timing of differentiation of adult hippocampal neurons is crucial for spatial memory. PLoS Biology 6:e246.

Farmer J, Zhao X, van Praag H, Wodtke K, Gage FH, Christie BR (2004) Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. Neuroscience 124:71-79.

Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G (2004) Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron 43:729-743.

Feng R, Rampon C, Tang YP, Shrom D, Jin J, Kyin M, Sopher B, Miller MW, Ware CB, Martin GM, Kim SH, Langdon RB, Sisodia SS, Tsien JZ (2001) Deficient neurogenesis in forebrain-specific presenilin-1 knockout mice is associated with reduced clearance of hippocampal memory traces. Neuron 32:911-926.

Forni PE, Scuoppo C, Imayoshi I, Taulli R, Dastru W, Sala V, Betz UA, Muzzi P, Martinuzzi D, Vercelli AE, Kageyama R, Ponzetto C (2006) High levels of Cre expression in neuronal progenitors cause defects in brain development leading to microencephaly and hydrocephaly. J Neurosci 26:9593-9602.

Freyschlag CF, Smolczyk DR, Janzen E, Schmieder K, Thome C, Lohr F, Wenz F, Weiss C, Tuettenberg J, Seiz M (2011) Prolonged administration of temozolomide in adult patients with anaplastic glioma. Anticancer Res 31:3873-3877.

Frotscher M (1989) Mossy fiber synapses on glutamate decarboxylase-immunoreactive neurons: evidence for feed-forward inhibition in the CA3 region of the hippocampus. Exp Brain Res 75:441-445.

Frotscher M, Drakew A, Heimrich B (2000) Role of afferent innervation and neuronal activity in dendritic development and spine maturation of fascia dentata granule cells. Cereb Cortex 10:946-951.

Fukushima T, Liu RY, Byrne JH (2007) Transforming growth factor-beta2 modulates synaptic efficacy and plasticity and induces phosphorylation of CREB in hippocampal neurons. Hippocampus 17:5-9.

Gaertner RF, Wyss-Coray T, Von Euw D, Lesne S, Vivien D, Lacombe P (2005) Reduced brain tissue perfusion in TGF-beta 1 transgenic mice showing Alzheimer's disease-like cerebrovascular abnormalities. Neurobiology of disease 19:38-46.

Gallagher M, Burwell R, Burchinal M (1993) Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. Behav Neurosci 107:618-626.

Gamble JR, Vadas MA (1988) Endothelial adhesiveness for blood neutrophils is inhibited by transforming growth factor-beta. Science 242:97-99.

Garcia AD, Doan NB, Imura T, Bush TG, Sofroniew MV (2004) GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci 7:1233-1241.

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

Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589-593.

Ge S, Yang CH, Hsu KS, Ming GL, Song H (2007) A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron 54:559-566.

Genovese CR, Lazar NA, Nichols T (2002) Thresholding of statistical maps in functional neuroimaging using the false discovery rate. Neuroimage 15:870-878.

Georgi LL, Albert PS, Riddle DL (1990) daf-1, a C. elegans gene controlling dauer larva development, encodes a novel receptor protein kinase. Cell 61:635-645.

Gerdes J, Schwab U, Lemke H, Stein H (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13-20.

Gilbert PE, Kesner RP (2003) Localization of function within the dorsal hippocampus: the role of the CA3 subregion in paired-associate learning. Behav Neurosci 117:1385-1394.

Gillies D (2000) Philosophical theories of probability. London ; New York: Routledge.

Ginsberg MD, Dietrich WD, Busto R (1987) Coupled forebrain increases of local cerebral glucose utilization and blood flow during physiologic stimulation of a somatosensory pathway in the rat: demonstration by double-label autoradiography. Neurology 37:11-19.

Goldman SA, Nottebohm F (1983) Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A 80:2390-2394.

Goodrich-Hunsaker NJ, Hunsaker MR, Kesner RP (2005) Dissociating the role of the parietal cortex and dorsal hippocampus for spatial information processing. Behav Neurosci 119:1307-1315.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260-265.

Graff JM, Bansal A, Melton DA (1996) Xenopus Mad proteins transduce distinct subsets of signals for the TGF beta superfamily. Cell 85:479-487.

Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER (1992) Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. Science 258:1903-1910.

Gratzner HG, Pollack A, Ingram DJ, Leif RC (1976) Deoxyribonucleic acid replication in single cells and chromosomes by immunologic techniques. J Histochem Cytochem 24:34-39.

Gu Y, Arruda-Carvalho M, Wang J, Janoschka SR, Josselyn SA, Frankland PW, Ge S (2012) Optical controlling reveals time-dependent roles for adult-born dentate granule cells. Nat Neurosci.

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

Guzowski JF, Setlow B, Wagner EK, McGaugh JL (2001) Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes Arc, c-fos, and zif268. J Neurosci 21:5089-5098.

Hafting T, Fyhn M, Molden S, Moser MB, Moser EI (2005) Microstructure of a spatial map in the entorhinal cortex. Nature 436:801-806.

Hailer NP, Heppner FL, Haas D, Nitsch R (1998) Astrocytic factors deactivate antigen presenting cells that invade the central nervous system. Brain Pathol 8:459-474.

Hamaguchi T, Okino S, Sodeyama N, Itoh Y, Takahashi A, Otomo E, Matsushita M, Mizusawa H, Yamada M (2005) Association of a polymorphism of the transforming growth factor-beta1 gene with cerebral amyloid angiopathy. J Neurol Neurosurg Psychiatry 76:696-699.

Hamilton DA, Akers KG, Weisend MP, Sutherland RJ (2007) How do room and apparatus cues control navigation in the Morris water task? Evidence for distinct contributions to a movement vector. J Exp Psychol Anim Behav Process 33:100-114.

Hardie LJ, Laing KJ, Daniels GD, Grabowski PS, Cunningham C, Secombes CJ (1998) Isolation of the first piscine transforming growth factor beta gene: analysis reveals tissue specific

expression and a potential regulatory sequence in rainbow trout (Oncorhynchus mykiss). Cytokine 10:555-563.

Harman A, Meyer P, Ahmat A (2003) Neurogenesis in the hippocampus of an adult marsupial. Brain Behav Evol 62:1-12.

Hastings NB, Gould E (1999) Rapid extension of axons into the CA3 region by adult-generated granule cells. J Comp Neurol 413:146-154.

Hayashi S, McMahon AP (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol 244:305-318.

Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2:185-193.

Hayes NL, Nowakowski RS (2002) Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. Brain Res Dev Brain Res 134:77-85.

Heine VM, Maslam S, Joels M, Lucassen PJ (2004) Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation. Neurobiol Aging 25:361-375.

Henrich-Noack P, Prehn JH, Krieglstein J (1996) TGF-beta 1 protects hippocampal neurons against degeneration caused by transient global ischemia. Dose-response relationship and potential neuroprotective mechanisms. Stroke 27:1609-1614; discussion 1615.

Hensley K, Fedynyshyn J, Ferrell S, Floyd RA, Gordon B, Grammas P, Hamdheydari L, Mhatre M, Mou S, Pye QN, Stewart C, West M, West S, Williamson KS (2003) Message and protein-level elevation of tumor necrosis factor alpha (TNF alpha) and TNF alpha-modulating cytokines in spinal cords of the G93A-SOD1 mouse model for amyotrophic lateral sclerosis. Neurobiol Dis 14:74-80.

Hernandez-Rabaza V, Llorens-Martin M, Velazquez-Sanchez C, Ferragud A, Arcusa A, Gumus HG, Gomez-Pindeo U, Perez-Villalba A, Rosello J, Trejo JL, Barcia JA, Canales JJ (2009) Inhibition of adult hippocampal neurogenesis disrupts contextual learning but spares spatial working memory, long-term conditional rule retention and spatial reversal. Neuroscience 159:59-68.

Herpin A, Lelong C, Favrel P (2004) Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. Dev Comp Immunol 28:461-485.

Herrera-Molina R, von Bernhardi R (2005) Transforming growth factor-beta 1 produced by hippocampal cells modulates microglial reactivity in culture. Neurobiol Dis 19:229-236.

Hinck AP, Archer SJ, Qian SW, Roberts AB, Sporn MB, Weatherbee JA, Tsang ML, Lucas R, Zhang BL, Wenker J, Torchia DA (1996) Transforming growth factor beta 1: three-dimensional structure in solution and comparison with the X-ray structure of transforming growth factor beta 2. Biochemistry 35:8517-8534.

Hirase H, Creso J, Singleton M, Bartho P, Buzsaki G (2004) Two-photon imaging of brain pericytes in vivo using dextran-conjugated dyes. Glia 46:95-100.

Hirrlinger J, Hulsmann S, Kirchhoff F (2004) Astroglial processes show spontaneous motility at active synaptic terminals in situ. Eur J Neurosci 20:2235-2239.

Hogan BL (1996) Bone morphogenetic proteins in development. Curr Opin Genet Dev 6:432-438.

Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L, Wrana JL (1996) MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85:489-500.

Horesh D, Sapir T, Francis F, Wolf SG, Caspi M, Elbaum M, Chelly J, Reiner O (1999) Doublecortin, a stabilizer of microtubules. Hum Mol Genet 8:1599-1610.

Hosseini-Sharifabad M, Nyengaard JR (2007) Design-based estimation of neuronal number and individual neuronal volume in the rat hippocampus. J Neurosci Methods 162:206-214.

Houi K, Kobayashi T, Kato S, Mochio S, Inoue K (2002) Increased plasma TGF-beta1 in patients with amyotrophic lateral sclerosis. Acta Neurol Scand 106:299-301.

Huang YS, Chang CC, Huang TC, Hsieh YL, Shih HM (2012) Daxx interacts with and modulates the activity of CREB. Cell Cycle 11:99-108.

Hunsaker MR, Mooy GG, Swift JS, Kesner RP (2007) Dissociations of the medial and lateral perforant path projections into dorsal DG, CA3, and CA1 for spatial and nonspatial (visual object) information processing. Behav Neurosci 121:742-750.

Ilzecka J, Stelmasiak Z, Dobosz B (2002) Transforming growth factor-Beta 1 (tgf-Beta 1) in patients with amyotrophic lateral sclerosis. Cytokine 20:239-243.

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11:1153-1161.

Imielski Y, Schwamborn JC, Luningschror P, Heimann P, Holzberg M, Werner H, Leske O, Puschel AW, Memet S, Heumann R, Israel A, Kaltschmidt C, Kaltschmidt B (2012) Regrowing the adult brain: NF-kappaB controls functional circuit formation and tissue homeostasis in the dentate gyrus. PLoS One 7:e30838.

Inokuchi K (2011) Adult neurogenesis and modulation of neural circuit function. Curr Opin Neurobiol 21:360-364.

Inokuchi K, Kato A, Hiraia K, Hishinuma F, Inoue M, Ozawa F (1996) Increase in activin beta A mRNA in rat hippocampus during long-term potentiation. FEBS Lett 382:48-52.

Jaholkowski P, Kiryk A, Jedynak P, Ben Abdallah NM, Knapska E, Kowalczyk A, Piechal A, Blecharz-Klin K, Figiel I, Lioudyno V, Widy-Tyszkiewicz E, Wilczynski GM, Lipp HP,

Kaczmarek L, Filipkowski RK (2009) New hippocampal neurons are not obligatory for memory formation; cyclin D2 knockout mice with no adult brain neurogenesis show learning. Learn Mem 16:439-451.

Jang YS, Kim JH, Seo GY, Kim PH (2011) TGF-beta1 stimulates mouse macrophages to express APRIL through Smad and p38MAPK/CREB pathways. Mol Cells 32:251-255.

Jeffery KJ, Morris RG (1993) Cumulative long-term potentiation in the rat dentate gyrus correlates with, but does not modify, performance in the water maze. Hippocampus 3:133-140.

Jeltsch H, Bertrand F, Lazarus C, Cassel JC (2001) Cognitive performances and locomotor activity following dentate granule cell damage in rats: role of lesion extent and type of memory tested. Neurobiol Learn Mem 76:81-105.

Jerman T, Kesner RP, Hunsaker MR (2006) Disconnection analysis of CA3 and DG in mediating encoding but not retrieval in a spatial maze learning task. Learn Mem 13:458-464.

Jessberger S, Kempermann G (2003) Adult-born hippocampal neurons mature into activity-dependent responsiveness. Eur J Neurosci 18:2707-2712.

Jessberger S, Romer B, Babu H, Kempermann G (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. Exp Neurol 196:342-351.

Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen L, Roberts AB, Smith GH, Merlino G (1993) Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. Embo J 12:1835-1845.

Johns LD, Flanders KC, Ranges GE, Sriram S (1991) Successful treatment of experimental allergic encephalomyelitis with transforming growth factor-beta 1. J Immunol 147:1792-1796.

Jones SP, Rahimi O, O'Boyle MP, Diaz DL, Claiborne BJ (2003) Maturation of granule cell dendrites after mossy fiber arrival in hippocampal field CA3. Hippocampus 13:413-427.

Josselyn SA, Frankland PW (2012) Infantile amnesia: a neurogenic hypothesis. Learn Mem 19:423-433.

Jourdain P, Bergersen LH, Bhaukaurally K, Bezzi P, Santello M, Domercq M, Matute C, Tonello F, Gundersen V, Volterra A (2007) Glutamate exocytosis from astrocytes controls synaptic strength. Nat Neurosci 10:331-339.

Jung B, Kim MO, Yun SJ, Lee EH (2003) Down-regulation of the expression of rat inhibitor-of-apoptosis protein-1 and -3 during transforming growth factor-beta1-mediated apoptosis in rat brain microglia. Neuroreport 14:857-860.

Jung MW, McNaughton BL (1993) Spatial selectivity of unit activity in the hippocampal granular layer. Hippocampus 3:165-182.

Kanaji M, Tada T, Kobayashi S (1997) A murine model of communicating hydrocephalus: Role of TGF-beta1. J Clin Neurosci 4:51-56.

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092-1094.

Katsuno M, Adachi H, Banno H, Suzuki K, Tanaka F, Sobue G (2011) Transforming growth factor-beta signaling in motor neuron diseases. Curr Mol Med 11:48-56.

Kee N, Sivalingam S, Boonstra R, Wojtowicz JM (2002) The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods 115:97-105.

Kee N, Teixeira CM, Wang AH, Frankland PW (2007a) Imaging activation of adult-generated granule cells in spatial memory. Nat Protoc 2:3033-3044.

Kee N, Teixeira CM, Wang AH, Frankland PW (2007b) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat Neurosci 10:355-362.

Kee NJ, Preston E, Wojtowicz JM (2001) Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. Exp Brain Res 136:313-320.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447-452.

Keski-Oja J, Moses HL (1987) Growth inhibitory polypeptides in the regulation of cell proliferation. Med Biol 65:13-20.

Kesner RP (2007) A behavioral analysis of dentate gyrus function. Prog Brain Res 163:567-576.

Kim JJ, Fanselow MS (1992) Modality-specific retrograde amnesia of fear. Science 256:675-677.

Kim WK, Hwang SY, Oh ES, Piao HZ, Kim KW, Han IO (2004) TGF-beta1 represses activation and resultant death of microglia via inhibition of phosphatidylinositol 3-kinase activity. J Immunol 172:7015-7023.

Kim WR, Park OH, Choi S, Choi SY, Park SK, Lee KJ, Rhyu IJ, Kim H, Lee YK, Kim HT, Oppenheim RW, Sun W (2009) The maintenance of specific aspects of neuronal function and behavior is dependent on programmed cell death of adult-generated neurons in the dentate gyrus. Eur J Neurosci 29:1408-1421.

Kim Y, Lee C (2006) The gene encoding transforming growth factor beta 1 confers risk of ischemic stroke and vascular dementia. Stroke 37:2843-2845.

Kimelman D, Kirschner M (1987) Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell 51:869-877.

Kitamura T, Saitoh Y, Takashima N, Murayama A, Niibori Y, Ageta H, Sekiguchi M, Sugiyama H, Inokuchi K (2009) Adult neurogenesis modulates the hippocampus-dependent period of associative fear memory. Cell 139:814-827.

Knowles WD (1992) Normal anatomy and neurophysiology of the hippocampal formation. J Clin Neurophysiol 9:252-263.

Knowles WD, Schwartzkroin PA (1981) Local circuit synaptic interactions in hippocampal brain slices. J Neurosci 1:318-322.

Ko H-G, D-J. J, Son J, Kwak C, Choi J-H, Ji Y-H, Lee Y-S, Son H, Kaang B-K (2009) Effect of ablated hippocampal neurogenesis on the formation and extinction of contextual fear memory. Mol Brain 2:1.

Kohler C (1985) A projection from the deep layers of the entorhinal area to the hippocampal formation in the rat brain. Neurosci Lett 56:13-19.

Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A, Ebina M, Nukiwa T, Miyazawa K, Imamura T, Miyazono K (2003) Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. Embo J 22:6458-6470.

Kondaiah P, Taira M, Vempati UD, Dawid IB (2000) Transforming growth factor-beta5 expression during early development of Xenopus laevis. Mech Dev 95:207-209.

Kowalczyk A, Filipkowski RK, Rylski M, Wilczynski GM, Konopacki FA, Jaworski J, Ciemerych MA, Sicinski P, Kaczmarek L (2004) The critical role of cyclin D2 in adult neurogenesis. J Cell Biol 167:209-213.

Krieglstein K, Zheng F, Unsicker K, Alzheimer C (2011) More than being protective: functional roles for TGF-beta/activin signaling pathways at central synapses. Trends Neurosci 34:421-429.

Kuan CY, Schloemer AJ, Lu A, Burns KA, Weng WL, Williams MT, Strauss KI, Vorhees CV, Flavell RA, Davis RJ, Sharp FR, Rakic P (2004) Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. J Neurosci 24:10763-10772.

Kuhn HG, Biebl M, Wilhelm D, Li M, Friedlander RM, Winkler J (2005) Increased generation of granule cells in adult Bcl-2-overexpressing mice: a role for cell death during continued hippocampal neurogenesis. Eur J Neurosci 22:1907-1915.

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

Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci 17:5820-5829.

Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 94:325-337.

Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S (1993) Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A 90:770-774.

Kutty G, Kutty RK, Samuel W, Duncan T, Jaworski C, Wiggert B (1998) Identification of a new member of transforming growth factor-beta superfamily in Drosophila: the first invertebrate activin gene. Biochem Biophys Res Commun 246:644-649.

Lacaille JC (1991) Postsynaptic potentials mediated by excitatory and inhibitory amino acids in interneurons of stratum pyramidale of the CA1 region of rat hippocampal slices in vitro. J Neurophysiol 66:1441-1454.

Lacaille JC, Schwartzkroin PA (1988) Stratum lacunosum-moleculare interneurons of hippocampal CA1 region. II. Intrasomatic and intradendritic recordings of local circuit synaptic interactions. J Neurosci 8:1411-1424.

Lagace DC, Whitman MC, Noonan MA, Ables JL, DeCarolis NA, Arguello AA, Donovan MH, Fischer SJ, Farnbauch LA, Beech RD, DiLeone RJ, Greer CA, Mandyam CD, Eisch AJ (2007) Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. J Neurosci 27:12623-12629.

Lamberty Y, Gower AJ (1991) Simplifying environmental cues in a Morris-type water maze improves place learning in old NMRI mice. Behav Neural Biol 56:89-100.

Lamouille S, Derynck R (2007) Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. The Journal of cell biology 178:437-451.

Lamouille S, Derynck R (2011) Emergence of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin axis in transforming growth factor-beta-induced epithelial-mesenchymal transition. Cells Tissues Organs 193:8-22.

Lanahan A, Lyford G, Stevenson GS, Worley PF, Barnes CA (1997) Selective alteration of long-term potentiation-induced transcriptional response in hippocampus of aged, memory-impaired rats. J Neurosci 17:2876-2885.

Laping NJ, Grygielko E, Mathur A, Butter S, Bomberger J, Tweed C, Martin W, Fornwald J, Lehr R, Harling J, Gaster L, Callahan JF, Olson BA (2002) Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol Pharmacol 62:58-64.

Laplagne DA, Esposito MS, Piatti VC, Morgenstern NA, Zhao C, van Praag H, Gage FH, Schinder AF (2006) Functional convergence of neurons generated in the developing and adult hippocampus. PLoS Biol 4:e409.

Laplagne DA, Kamienkowski JE, Esposito MS, Piatti VC, Zhao C, Gage FH, Schinder AF (2007) Similar GABAergic inputs in dentate granule cells born during embryonic and adult neurogenesis. Eur J Neurosci 25:2973-2981.

Larkum ME, Senn W, Luscher HR (2004) Top-down dendritic input increases the gain of layer 5 pyramidal neurons. Cereb Cortex 14:1059-1070.

Lassalle JM, Bataille T, Halley H (2000) Reversible inactivation of the hippocampal mossy fiber synapses in mice impairs spatial learning, but neither consolidation nor memory retrieval, in the Morris navigation task. Neurobiol Learn Mem 73:243-257.

Lazarov O, Mattson MP, Peterson DA, Pimplikar SW, van Praag H (2010) When neurogenesis encounters aging and disease. Trends Neurosci 33:569-579.

Lazic SE (2012a) Modeling hippocampal neurogenesis across the lifespan in seven species. Neurobiol Aging 33:1664-1671.

Lazic SE (2012b) Using causal models to distinguish between neurogenesis-dependent and -independent effects on behaviour. J R Soc Interface 9:907-917.

Lee I, Kesner RP (2004a) Differential contributions of dorsal hippocampal subregions to memory acquisition and retrieval in contextual fear-conditioning. Hippocampus 14:301-310.

Lee I, Kesner RP (2004b) Encoding versus retrieval of spatial memory: double dissociation between the dentate gyrus and the perforant path inputs into CA3 in the dorsal hippocampus. Hippocampus 14:66-76.

Lee JW, Kim WR, Sun W, Jung MW (2012) Disruption of Dentate Gyrus Blocks Effect of Visual Input on Spatial Firing of CA1 Neurons. J Neurosci 32:12999-13003.

Lefebvre PP, Martin D, Staecker H, Weber T, Moonen G, Van de Water TR (1992) TGF beta 1 expression is initiated in adult auditory neurons by sectioning of the auditory nerve. Neuroreport 3:295-298.

Lehrmann E, Kiefer R, Christensen T, Toyka KV, Zimmer J, Diemer NH, Hartung HP, Finsen B (1998) Microglia and macrophages are major sources of locally produced transforming growth factor-beta1 after transient middle cerebral artery occlusion in rats. Glia 24:437-448.

Lehrmann E, Kiefer R, Finsen B, Diemer NH, Zimmer J, Hartung HP (1995) Cytokines in cerebral ischemia: expression of transforming growth factor beta-1 (TGF-beta 1) mRNA in the postischemic adult rat hippocampus. Exp Neurol 131:114-123.

Lei ZM, Rao CV (1992) Expression of epidermal growth factor (EGF) receptor and its ligands, EGF and transforming growth factor-alpha, in human fallopian tubes. Endocrinology 131:947-957.

Lerch JP, Sled JG, Henkelman RM (2011a) MRI phenotyping of genetically altered mice. Methods Mol Biol 711:349-361.

Lerch JP, Yiu AP, Martinez-Canabal A, Pekar T, Bohbot VD, Frankland PW, Henkelman RM, Josselyn SA, Sled JG (2011b) Maze training in mice induces MRI-detectable brain shape changes specific to the type of learning. Neuroimage 54:2086-2095.

Letsou A, Arora K, Wrana JL, Simin K, Twombly V, Jamal J, Staehling-Hampton K, Hoffmann FM, Gelbart WM, Massague J, et al. (1995) Drosophila Dpp signaling is mediated by the punt gene product: a dual ligand-binding type II receptor of the TGF beta receptor family. Cell 80:899-908.

Leutgeb S, Leutgeb JK, Treves A, Moser MB, Moser EI (2004) Distinct ensemble codes in hippocampal areas CA3 and CA1. Science 305:1295-1298.

Levy WB, Steward O (1979) Synapses as associative memory elements in the hippocampal formation. Brain Res 175:233-245.

Lew AR (2011) Looking beyond the boundaries: Time to put landmarks back on the cognitive map? Psychol Bull 137:484-507.

Li XG, Somogyi P, Tepper JM, Buzsaki G (1992) Axonal and dendritic arborization of an intracellularly labeled chandelier cell in the CA1 region of rat hippocampus. Exp Brain Res 90:519-525.

Li XG, Somogyi P, Ylinen A, Buzsaki G (1994) The hippocampal CA3 network: an in vivo intracellular labeling study. J Comp Neurol 339:181-208.

Lin AH, Luo J, Mondshein LH, ten Dijke P, Vivien D, Contag CH, Wyss-Coray T (2005) Global analysis of Smad2/3-dependent TGF-beta signaling in living mice reveals prominent tissue-specific responses to injury. J Immunol 175:547-554.

Lin CH, Cheng FC, Lu YZ, Chu LF, Wang CH, Hsueh CM (2006) Protection of ischemic brain cells is dependent on astrocyte-derived growth factors and their receptors. Exp Neurol 201:225-233.

Lin HY, Wang XF (1992) Expression cloning of TGF-beta receptors. Mol Reprod Dev 32:105-110.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130-1132.

Lindholm D, Castren E, Kiefer R, Zafra F, Thoenen H (1992) Transforming growth factor-beta 1 in the rat brain: increase after injury and inhibition of astrocyte proliferation. J Cell Biol 117:395-400.

Link J, Soderstrom M, Olsson T, Hojeberg B, Ljungdahl A, Link H (1994) Increased transforming growth factor-beta, interleukin-4, and interferon-gamma in multiple sclerosis. Ann Neurol 36:379-386.

Liu F, Hata A, Baker JC, Doody J, Carcamo J, Harland RM, Massague J (1996) A human Mad protein acting as a BMP-regulated transcriptional activator. Nature 381:620-623.

Lomo T (1971a) Patterns of activation in a monosynaptic cortical pathway: the perforant path input to the dentate area of the hippocampal formation. Exp Brain Res 12:18-45.

Lomo T (1971b) Potentiation of monosynaptic EPSPs in the perforant path-dentate granule cell synapse. Exp Brain Res 12:46-63.

Luedecking EK, DeKosky ST, Mehdi H, Ganguli M, Kamboh MI (2000) Analysis of genetic polymorphisms in the transforming growth factor-beta1 gene and the risk of Alzheimer's disease. Hum Genet 106:565-569.

Luo J, Ho PP, Buckwalter MS, Hsu T, Lee LY, Zhang H, Kim DK, Kim SJ, Gambhir SS, Steinman L, Wyss-Coray T (2007) Glia-dependent TGF-beta signaling, acting independently of the TH17 pathway, is critical for initiation of murine autoimmune encephalomyelitis. J Clin Invest 117:3306-3315.

Luskin MB (1994) Neuronal cell lineage in the vertebrate central nervous system. FASEB J 8:722-730.

Macklis JD (2001) Neurobiology: New memories from new neurons. Nature 410:314-315, 317.

Madsen TM, Kristjansen PE, Bolwig TG, Wortwein G (2003) Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. Neuroscience 119:635-642.

Madsen TM, Treschow A, Bengzon J, Bolwig TG, Lindvall O, Tingstrom A (2000) Increased neurogenesis in a model of electroconvulsive therapy. Biol Psychiatry 47:1043-1049.

Maei HR, Zaslavsky K, Teixeira CM, Frankland PW (2009) What is the Most Sensitive Measure of Water Maze Probe Test Performance? Front Integr Neurosci 3:4.

Maffei A (2011) The many forms and functions of long term plasticity at GABAergic synapses. Neural Plast 2011:254724.

Maguire EA, Frackowiak RS, Frith CD (1997) Recalling routes around london: activation of the right hippocampus in taxi drivers. J Neurosci 17:7103-7110.

Maguire EA, Gadian DG, Johnsrude IS, Good CD, Ashburner J, Frackowiak RS, Frith CD (2000) Navigation-related structural change in the hippocampi of taxi drivers. Proc Natl Acad Sci U S A 97:4398-4403.

Maguire EA, Mummery CJ (1999) Differential modulation of a common memory retrieval network revealed by positron emission tomography. Hippocampus 9:54-61.

Maguire EA, Spiers HJ, Good CD, Hartley T, Frackowiak RS, Burgess N (2003) Navigation expertise and the human hippocampus: a structural brain imaging analysis. Hippocampus 13:250-259.

Maguire EA, Woollett K, Spiers HJ (2006) London taxi drivers and bus drivers: a structural MRI and neuropsychological analysis. Hippocampus 16:1091-1101.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

Marques G, Bao H, Haerry TE, Shimell MJ, Duchek P, Zhang B, O'Connor MB (2002) The Drosophila BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function. Neuron 33:529-543.

Marr D (1969) A theory of cerebellar cortex. J Physiol 202:437-470.

Marrone DF, Ramirez-Amaya V, Barnes CA (2011) Neurons generated in senescence maintain capacity for functional integration. Hippocampus.

Martin A (1999) Automatic activation of the medial temporal lobe during encoding: lateralized influences of meaning and novelty. Hippocampus 9:62-70.

Martin A, Wiggs CL, Weisberg J (1997) Modulation of human medial temporal lobe activity by form, meaning, and experience. Hippocampus 7:587-593.

Massague J (2012) TGFbeta signalling in context. Nat Rev Mol Cell Biol 13:616-630.

Mathews LS, Vale WW (1991) Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65:973-982.

Matthias K, Kirchhoff F, Seifert G, Huttmann K, Matyash M, Kettenmann H, Steinhauser C (2003) Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. J Neurosci 23:1750-1758.

McBain CJ, Fisahn A (2001) Interneurons unbound. Nat Rev Neurosci 2:11-23.

McDowell N, Gurdon JB (1999) Activin as a morphogen in Xenopus mesoderm induction. Semin Cell Dev Biol 10:311-317.

McNaughton BL, Barnes CA, O'Keefe J (1983) The contributions of position, direction, and velocity to single unit activity in the hippocampus of freely-moving rats. Exp Brain Res 52:41-49.

McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387:83-90.

Meck WH, Church RM, Olton DS (1984) Hippocampus, time, and memory. Behav Neurosci 98:3-22.

Meltzer LA, Yabaluri R, Deisseroth K (2005) A role for circuit homeostasis in adult neurogenesis. Trends Neurosci 28:653-660.

Merrill DA, Karim R, Darraq M, Chiba AA, Tuszynski MH (2003) Hippocampal cell genesis does not correlate with spatial learning ability in aged rats. J Comp Neurol 459:201-207.

Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9:729-731.

Michaelson JS, Bader D, Kuo F, Kozak C, Leder P (1999) Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes Dev 13:1918-1923.

Miller MW, Nowakowski RS (1988) Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res 457:44-52.

Milner B, Corkin, S., Teuber, H.L. (1968) Further analysis of the hippocampal amnesic syndrome: 14- year followup of H.M. . Neuropsychologia 6:215-234.

Milner R, Campbell IL (2003) The extracellular matrix and cytokines regulate microglial integrin expression and activation. J Immunol 170:3850-3858.

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

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

Mirshafiey A, Mohsenzadegan M (2009) TGF-beta as a promising option in the treatment of multiple sclerosis. Neuropharmacology 56:929-936.

Miyashita T, Kubik S, Haghighi N, Steward O, Guzowski JF (2009) Rapid activation of plasticity-associated gene transcription in hippocampal neurons provides a mechanism for encoding of one-trial experience. J Neurosci 29:898-906.

Miyazono K, Koinuma D (2011) Arkadia--beyond the TGF-beta pathway. J Biochem 149:1-3.

Mizumatsu S, Monje ML, Morhardt DR, Rola R, Palmer TD, Fike JR (2003) Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. Cancer Res 63:4021-4027.

Mogi M, Harada M, Kondo T, Narabayashi H, Riederer P, Nagatsu T (1995) Transforming growth factor-beta 1 levels are elevated in the striatum and in ventricular cerebrospinal fluid in Parkinson's disease. Neurosci Lett 193:129-132.

Mogi M, Harada M, Kondo T, Riederer P, Inagaki H, Minami M, Nagatsu T (1994) Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from parkinsonian patients. Neurosci Lett 180:147-150.

Moreno MM, Linster C, Escanilla O, Sacquet J, Didier A, Mandairon N (2009) Olfactory perceptual learning requires adult neurogenesis. Proc Natl Acad Sci U S A 106:17980-17985.

Morgan JI, Cohen DR, Hempstead JL, Curran T (1987) Mapping patterns of c-fos expression in the central nervous system after seizure. Science 237:192-197.

Morris RG, Garrud P, Rawlins JN, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297:681-683.

Morris RG, Moser EI, Riedel G, Martin SJ, Sandin J, Day M, O'Carroll C (2003) Elements of a neurobiological theory of the hippocampus: the role of activity-dependent synaptic plasticity in memory. Philos Trans R Soc Lond B Biol Sci 358:773-786.

Morris RGM (1981) Spatial localization does not require the presence of local cues. . Learning and Motivation 12:239-260.

Mu Y, Gudey SK, Landstrom M (2012) Non-Smad signaling pathways. Cell Tissue Res 347:11-20.

Mulhern RK, Palmer SL, Merchant TE, Wallace D, Kocak M, Brouwers P, Krull K, Chintagumpala M, Stargatt R, Ashley DM, Tyc VL, Kun L, Boyett J, Gajjar A (2005) Neurocognitive consequences of risk-adapted therapy for childhood medulloblastoma. J Clin Oncol 23:5511-5519.

Nagai N, De Mol M, Lijnen HR, Carmeliet P, Collen D (1999) Role of plasminogen system components in focal cerebral ischemic infarction: a gene targeting and gene transfer study in mice. Circulation 99:2440-2444.

Nanry KP, Mundy WR, Tilson HA (1989) Colchicine-induced alterations of reference memory in rats: role of spatial versus non-spatial task components. Behav Brain Res 35:45-53.

Newlands ES, Blackledge GR, Slack JA, Rustin GJ, Smith DB, Stuart NS, Quarterman CP, Hoffman R, Stevens MF, Brampton MH, et al. (1992) Phase I trial of temozolomide (CCRG 81045: M&B 39831: NSC 362856). Br J Cancer 65:287-291.

Nichols NR, Finch CE (1991) Transforming growth factor-beta1 mRNA decreases in brain in response to glucocorticoid treatment of adrenalectomized rats. Mol Cell Neurosci 2:221-227.

Nicolakakis N, Aboulkassim T, Aliaga A, Tong XK, Rosa-Neto P, Hamel E (2011) Intact memory in TGF-beta1 transgenic mice featuring chronic cerebrovascular deficit: recovery with pioglitazone. J Cereb Blood Flow Metab 31:200-211.

Nicole O, Docagne F, Ali C, Margaill I, Carmeliet P, MacKenzie ET, Vivien D, Buisson A (2001) The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. Nat Med 7:59-64.

Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS (1999) Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. J Neurobiol 39:569-578.

Nolte C, Matyash M, Pivneva T, Schipke CG, Ohlemeyer C, Hanisch UK, Kirchhoff F, Kettenmann H (2001) GFAP promoter-controlled EGFP-expressing transgenic mice: a tool to visualize astrocytes and astrogliosis in living brain tissue. Glia 33:72-86.

Nunez G, del Peso L (1998) Linking extracellular survival signals and the apoptotic machinery. Curr Opin Neurobiol 8:613-618.

Nurse S, Lacaille JC (1999) Late maturation of GABAB synaptic transmission in area CA1 of the rat hippocampus. Neuropharmacology 38:1733-1742.

O'Keefe GM, Nguyen VT, Benveniste EN (1999) Class II transactivator and class II MHC gene expression in microglia: modulation by the cytokines TGF-beta, IL-4, IL-13 and IL-10. Eur J Immunol 29:1275-1285.

O'Keefe J (1976) Place units in the hippocampus of the freely moving rat. Exp Neurol 51:78-109.

O'Keefe J, Dostrovsky J (1971) The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain Res 34:171-175.

O'Keefe J, Nadel L (1978) The hippocampus as a cognitive map. Oxford

New York:

Oxford University Press.

O'Reilly RC, McClelland JL (1994) Hippocampal conjunctive encoding, storage, and recall: avoiding a trade-off. Hippocampus 4:661-682.

Ohta M, Greenberger JS, Anklesaria P, Bassols A, Massague J (1987) Two forms of transforming growth factor-beta distinguished by multipotential haematopoietic progenitor cells. Nature 329:539-541.

Olton DS, RJ. (1976) Remembrance of places passed: Spatial memory in rats. Journal of Experimental Psychology: Animal Behavior Processes 2:97-116.

Olton DSB, James T.; Handelmann, Gail E. (1979) Hippocampus, space, and memory. Behavioral and Brain Sciences 2:313-365.

Padgett RW, St Johnston RD, Gelbart WM (1987) A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. Nature 325:81-84.

Palmer TD, Ray J, Gage FH (1995) FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci 6:474-486.

Pan YW, Chan GC, Kuo CT, Storm DR, Xia Z (2012) Inhibition of adult neurogenesis by inducible and targeted deletion of ERK5 mitogen-activated protein kinase specifically in adult neurogenic regions impairs contextual fear extinction and remote fear memory. J Neurosci 32:6444-6455.

Parri HR, Gould TM, Crunelli V (2001) Spontaneous astrocytic Ca2+ oscillations in situ drive NMDAR-mediated neuronal excitation. Nat Neurosci 4:803-812.

Patil SS, Boddul SV, Schlick K, Kang SU, Zehetmayer S, Hoger H, Lubec G (2011) Differences in hippocampal protein levels between C57Bl/6J, PWD/PhJ, and Apodemus sylvaticus are paralleled by differences in spatial memory. Hippocampus 21:714-723.

Patton PE, McNaughton B (1995) Connection matrix of the hippocampal formation: I. The dentate gyrus. Hippocampus 5:245-286.

Paul CM, Magda G, Abel S (2009) Spatial memory: Theoretical basis and comparative review on experimental methods in rodents. Behav Brain Res 203:151-164.

Peissner W, Kocher M, Treuer H, Gillardon F (1999) Ionizing radiation-induced apoptosis of proliferating stem cells in the dentate gyrus of the adult rat hippocampus. Brain Res Mol Brain Res 71:61-68.

Perea G, Araque A (2005) Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. J Neurosci 25:2192-2203.

Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA (2001) TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat Cell Biol 3:708-714.

Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 106:274-285.

Pierce DF, Jr., Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, Daniel CW, Hogan BL, Moses HL (1993) Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. Genes Dev 7:2308-2317.

Pierreux CE, Nicolas FJ, Hill CS (2000) Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. Mol Cell Biol 20:9041-9054.

Poucet B (1989) Object exploration, habituation, and response to a spatial change in rats following septal or medial frontal cortical damage. Behav Neurosci 103:1009-1016.

Pratt BM, McPherson JM (1997) TGF-beta in the central nervous system: potential roles in ischemic injury and neurodegenerative diseases. Cytokine Growth Factor Rev 8:267-292.

Raber J, Rola R, LeFevour A, Morhardt D, Curley J, Mizumatsu S, VandenBerg SR, Fike JR (2004) Radiation-induced cognitive impairments are associated with changes in indicators of hippocampal neurogenesis. Radiat Res 162:39-47.

Racke MK, Dhib-Jalbut S, Cannella B, Albert PS, Raine CS, McFarlin DE (1991) Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-beta 1. J Immunol 146:3012-3017.

Raftery LA, Twombly V, Wharton K, Gelbart WM (1995) Genetic screens to identify elements of the decapentaplegic signaling pathway in Drosophila. Genetics 139:241-254.

Rahimi O, Claiborne BJ (2007) Morphological development and maturation of granule neuron dendrites in the rat dentate gyrus. Prog Brain Res 163:167-181.

Ramirez-Amaya V, Marrone DF, Gage FH, Worley PF, Barnes CA (2006) Integration of new neurons into functional neural networks. J Neurosci 26:12237-12241.

Ratey JJ, Loehr JE (2011) The positive impact of physical activity on cognition during adulthood: a review of underlying mechanisms, evidence and recommendations. Rev Neurosci 22:171-185.

Reilly JF, Maher PA, Kumari VG (1998) Regulation of astrocyte GFAP expression by TGF-beta1 and FGF-2. Glia 22:202-210.

Reynisdottir I, Polyak K, Iavarone A, Massague J (1995) Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev 9:1831-1845.

Richter-Levin G, Canevari L, Bliss TV (1995) Long-term potentiation and glutamate release in the dentate gyrus: links to spatial learning. Behav Brain Res 66:37-40.

Roberts AB, Anzano MA, Lamb LC, Smith JM, Frolik CA, Marquardt H, Todaro GJ, Sporn MB (1982) Isolation from murine sarcoma cells of novel transforming growth factors potentiated by EGF. Nature 295:417-419.

Roberts AB, Lamb LC, Newton DL, Sporn MB, De Larco JE, Todaro GJ (1980) Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc Natl Acad Sci U S A 77:3494-3498.

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, et al. (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A 83:4167-4171.

Rogers LR (2012) Neurologic complications of radiation. Continuum (Minneap Minn) 18:343-354.

Rolls ET (1996) A theory of hippocampal function in memory. Hippocampus 6:601-620.

Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, Konnerth A (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature 426:74-78.

Rubin H (2011) The early history of tumor virology: Rous, RIF, and RAV. Proc Natl Acad Sci U S A 108:14389-14396.

Ruggiero A, Cefalo G, Garre ML, Massimino M, Colosimo C, Attina G, Lazzareschi I, Maurizi P, Ridola V, Mazzarella G, Caldarelli M, Di Rocco C, Madon E, Abate ME, Clerico A, Sandri A, Riccardi R (2006) Phase II trial of temozolomide in children with recurrent high-grade glioma. J Neurooncol 77:89-94.

Ruocco A, Nicole O, Docagne F, Ali C, Chazalviel L, Komesli S, Yablonsky F, Roussel S, MacKenzie ET, Vivien D, Buisson A (1999) A transforming growth factor-beta antagonist unmasks the neuroprotective role of this endogenous cytokine in excitotoxic and ischemic brain injury. J Cereb Blood Flow Metab 19:1345-1353.

Ruth RE, Collier TJ, Routtenberg A (1982) Topography between the entorhinal cortex and the dentate septotemporal axis in rats: I. Medial and intermediate entorhinal projecting cells. J Comp Neurol 209:69-78.

Ruth RE, Collier TJ, Routtenberg A (1988) Topographical relationship between the entorhinal cortex and the septotemporal axis of the dentate gyrus in rats: II. Cells projecting from lateral entorhinal subdivisions. J Comp Neurol 270:506-516.

Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 472:466-470.

Sanchez-Capelo A, Colin P, Guibert B, Biguet NF, Mallet J (2003) Transforming growth factor beta1 overexpression in the nigrostriatal system increases the dopaminergic deficit of MPTP mice. Mol Cell Neurosci 23:614-625.

Sandoval CJ, Martinez-Claros M, Bello-Medina PC, Perez O, Ramirez-Amaya V (2011) When are new hippocampal neurons, born in the adult brain, integrated into the network that processes spatial information? PLoS One 6:e17689.

Sanes JR (1989) Analysing cell lineage with a recombinant retrovirus. Trends Neurosci 12:21-28.

Santello M, Bezzi P, Volterra A (2011) TNFalpha Controls Glutamatergic Gliotransmission in the Hippocampal Dentate Gyrus. Neuron 69:988-1001.

Saunders RC, Rosene DL, Van Hoesen GW (1988) Comparison of the efferents of the amygdala and the hippocampal formation in the rhesus monkey: II. Reciprocal and non-reciprocal connections. J Comp Neurol 271:185-207.

Savage C, Das P, Finelli AL, Townsend SR, Sun CY, Baird SE, Padgett RW (1996) Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. Proc Natl Acad Sci U S A 93:790-794.

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proc Natl Acad Sci U S A 103:17501-17506.

Schlunegger MP, Cerletti N, Cox DA, McMaster GK, Schmitz A, Grutter MG (1992) Crystallization and preliminary X-ray analysis of recombinant human transforming growth factor beta 2. FEBS Lett 303:91-93.

Schlunegger MP, Grutter MG (1992) An unusual feature revealed by the crystal structure at 2.2 A resolution of human transforming growth factor-beta 2. Nature 358:430-434.

Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429:184-187.

Schnell SA, Staines WA, Wessendorf MW (1999) Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. J Histochem Cytochem 47:719-730.

Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. J Cell Physiol 182:311-322.

Schreiber SS, Maren S, Tocco G, Shors TJ, Thompson RF (1991) A negative correlation between the induction of long-term potentiation and activation of immediate early genes. Brain Res Mol Brain Res 11:89-91.

Schwartz SP, Coleman PD (1981) Neurons of origin of the perforant path. Exp Neurol 74:305-312.

Schwarz S, Froelich L, Burns A (2012) Pharmacological treatment of dementia. Curr Opin Psychiatry 25:542-550.

Scott JW, Wellis DP, Riggott MJ, Buonviso N (1993) Functional organization of the main olfactory bulb. Microsc Res Tech 24:142-156.

Scoville WB, Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. J Neurol Neurosurg Psychiatry 20:11-21.

Seki T, Arai Y (1995) Age-related production of new granule cells in the adult dentate gyrus. Neuroreport 6:2479-2482.

Seki T, Namba T, Mochizuki H, Onodera M (2007) Clustering, migration, and neurite formation of neural precursor cells in the adult rat hippocampus. J Comp Neurol 502:275-290.

Selden JR, Dolbeare F, Clair JH, Nichols WW, Miller JE, Kleemeyer KM, Hyland RJ, DeLuca JG (1993) Statistical confirmation that immunofluorescent detection of DNA repair in human fibroblasts by measurement of bromodeoxyuridine incorporation is stoichiometric and sensitive. Cytometry 14:154-167.

Sellheyer K, Bickenbach JR, Rothnagel JA, Bundman D, Longley MA, Krieg T, Roche NS, Roberts AB, Roop DR (1993) Inhibition of skin development by overexpression of transforming growth factor beta 1 in the epidermis of transgenic mice. Proc Natl Acad Sci U S A 90:5237-5241.

Seoane J, Le HV, Shen L, Anderson SA, Massague J (2004) Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell 117:211-223.

Seress L (1978) Pyramid-like basket cells in the granular layer of the dentate gyrus in the rat. J Anat 127:163-168.

Sherry DF, Hampson E (1997) Evolution and the hormonal control of sexually-dimorphic spatial abilities in humans. Trends Cogn Sci 1:50-56.

Shi Y, Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685-700.

Shoji-Kasai Y, Ageta H, Hasegawa Y, Tsuchida K, Sugino H, Inokuchi K (2007) Activin increases the number of synaptic contacts and the length of dendritic spine necks by modulating spinal actin dynamics. J Cell Sci 120:3830-3837.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372-376.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578-584.

Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al. (1992) Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 359:693-699.

Singer BH, Jutkiewicz EM, Fuller CL, Lichtenwalner RJ, Zhang H, Velander AJ, Li X, Gnegy ME, Burant CF, Parent JM (2009) Conditional ablation and recovery of forebrain neurogenesis in the mouse. J Comp Neurol 514:567-582.

Skelton RW, Miller JJ, Phillips AG (1985) Long-term potentiation facilitates behavioral responding to single-pulse stimulation of the perforant path. Behav Neurosci 99:603-620.

Sloviter RS (1983) "Epileptic" brain damage in rats induced by sustained electrical stimulation of the perforant path. I. Acute electrophysiological and light microscopic studies. Brain Res Bull 10:675-697.

Snyder JS, Hong NS, McDonald RJ, Wojtowicz JM (2005) A role for adult neurogenesis in spatial long-term memory. Neuroscience 130:843-852.

Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 32:638-647.

Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. Acta Neuropathol 119:7-35.

Somogyi P, Freund TF, Hodgson AJ, Somogyi J, Beroukas D, Chubb IW (1985) Identified axo-axonic cells are immunoreactive for GABA in the hippocampus and visual cortex of the cat. Brain Res 332:143-149.

Sousa Vde O, Romao L, Neto VM, Gomes FC (2004) Glial fibrillary acidic protein gene promoter is differently modulated by transforming growth factor-beta 1 in astrocytes from distinct brain regions. Eur J Neurosci 19:1721-1730.

Stahnisch FW, Nitsch R (2002) Santiago Ramon y Cajal's concept of neuronal plasticity: the ambiguity lives on. Trends Neurosci 25:589-591.

Steinhauser C, Berger T, Frotscher M, Kettenmann H (1992) Heterogeneity in the Membrane Current Pattern of Identified Glial Cells in the Hippocampal Slice. Eur J Neurosci 4:472-484.

Steward O, Huang F, Guzowski JF (2007) A form of perforant path LTP can occur without ERK1/2 phosphorylation or immediate early gene induction. Learn Mem 14:433-445.

Stoeck K, Bodemer M, Zerr I (2006) Pro- and anti-inflammatory cytokines in the CSF of patients with Creutzfeldt-Jakob disease. J Neuroimmunol 172:175-181.

Stone SS, Teixeira CM, Zaslavsky K, Wheeler AL, Martinez-Canabal A, Wang AH, Sakaguchi M, Lozano AM, Frankland PW (2010) Functional convergence of developmentally and adult-generated granule cells in dentate gyrus circuits supporting hippocampus-dependent memory. Hippocampus.

Stone SS, Teixeira CM, Zaslavsky K, Wheeler AL, Martinez-Canabal A, Wang AH, Sakaguchi M, Lozano AM, Frankland PW (2011) Functional convergence of developmentally and adult-generated granule cells in dentate gyrus circuits supporting hippocampus-dependent memory. Hippocampus 21:1348-1362.

Stringer SM, Rolls ET, Trappenberg TP (2004) Self-organising continuous attractor networks with multiple activity packets, and the representation of space. Neural Netw 17:5-27.

Suga H, Ono K, Miyata T (1999) Multiple TGF-beta receptor related genes in sponge and ancient gene duplications before the parazoan-eumetazoan split. FEBS Lett 453:346-350.

Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH (2007) In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. Cell Stem Cell 1:515-528.

Sultan S, Mandairon N, Kermen F, Garcia S, Sacquet J, Didier A (2010) Learning-dependent neurogenesis in the olfactory bulb determines long-term olfactory memory. Faseb J 24:2355-2363.

Sun W, Winseck A, Vinsant S, Park OH, Kim H, Oppenheim RW (2004) Programmed cell death of adult-generated hippocampal neurons is mediated by the proapoptotic gene Bax. J Neurosci 24:11205-11213.

Sutherland R. J, Chew G.L, Baker J.C, R.C. L (1987) Some limitations in the use of distal cues in place navigation by rats. Psychobiology 15:48-57.

Sutherland R.J, J.W. R (1989) Configural association theory: the role

of the hippocampal formation in learning, memory, and amnesia. Psychobiology 17:129-144.

Sutherland RJ, Whishaw IQ, Kolb B (1983) A behavioural analysis of spatial localization following electrolytic, kainate- or colchicine-induced damage to the hippocampal formation in the rat. Behav Brain Res 7:133-153.

Swanson LW, Wyss JM, Cowan WM (1978) An autoradiographic study of the organization of intrahippocampal association pathways in the rat. J Comp Neurol 181:681-715.

Tada T, Kanaji M, Kobayashi S (1994) Induction of communicating hydrocephalus in mice by intrathecal injection of human recombinant transforming growth factor-beta 1. J Neuroimmunol 50:153-158.

Tanila H (1999) Hippocampal place cells can develop distinct representations of two visually identical environments. Hippocampus 9:235-246.

Tashiro A, Makino H, Gage FH (2007) Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage. J Neurosci 27:3252-3259.

Teixeira CM, Pomedli SR, Maei HR, Kee N, Frankland PW (2006) Involvement of the anterior cingulate cortex in the expression of remote spatial memory. J Neurosci 26:7555-7564.

ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K (1993) Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 8:2879-2887.

Tesseur I, Wyss-Coray T (2006) A role for TGF-beta signaling in neurodegeneration: evidence from genetically engineered models. Curr Alzheimer Res 3:505-513.

Tilson HA, Rogers BC, Grimes L, Harry GJ, Peterson NJ, Hong JS, Dyer RS (1987) Time-dependent neurobiological effects of colchicine administered directly into the hippocampus of rats. Brain Res 408:163-172.

Todaro GJ, De Larco JE, Fryling C, Johnson PA, Sporn MB (1981) Transforming growth factors (TGFs): properties and possible mechanisms of action. J Supramol Struct Cell Biochem 15:287-301.

Todaro GJ, Fryling C, De Larco JE (1980) Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. Proc Natl Acad Sci U S A 77:5258-5262.

Tomac A, Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, Olson L (1995) Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. Nature 373:335-339.

Toni N, Laplagne DA, Zhao C, Lombardi G, Ribak CE, Gage FH, Schinder AF (2008) Neurons born in the adult dentate gyrus form functional synapses with target cells. Nat Neurosci 11:901-907.

Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T (2005) GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 47:803-815.

Traub RD, Miles R (1991) Neuronal networks of the hippocampus. Cambridge ; New York: Cambridge University Press.

Treves A, Rolls ET (1994) Computational analysis of the role of the hippocampus in memory. Hippocampus 4:374-391.

Treves A, Tashiro A, Witter MP, Moser EI (2008) What is the mammalian dentate gyrus good for? Neuroscience 154:1155-1172.

Trouche S, Bontempi B, Roullet P, Rampon C (2009) Recruitment of adult-generated neurons into functional hippocampal networks contributes to updating and strengthening of spatial memory. Proc Natl Acad Sci U S A 106:5919-5924.

Tsirka SE (1997) Clinical implications of the involvement of tPA in neuronal cell death. J Mol Med 75:341-347.

Tulving E, Donaldson W, Bower GH, United States. Office of Naval Research. (1972) Organization of memory. New York,: Academic Press.

Ueda S, Sakakibara S, Yoshimoto K (2005) Effect of long-lasting serotonin depletion on environmental enrichment-induced neurogenesis in adult rat hippocampus and spatial learning. Neuroscience 135:395-402.

Valley MT, Mullen TR, Schultz LC, Sagdullaev BT, Firestein S (2009) Ablation of mouse adult neurogenesis alters olfactory bulb structure and olfactory fear conditioning. Front Neurosci 3:51.

Van Dam D, Lenders G, De Deyn PP (2006) Effect of Morris water maze diameter on visual-spatial learning in different mouse strains. Neurobiol Learn Mem 85:164-172.

Van der Borght K, Havekes R, Bos T, Eggen BJ, Van der Zee EA (2007) Exercise improves memory acquisition and retrieval in the Y-maze task: relationship with hippocampal neurogenesis. Behav Neurosci 121:324-334.

van der Staay FJ (2000) Effects of the size of the morris water tank on spatial discrimination learning in the CFW1 mouse. Physiol Behav 68:599-602.

van der Worp HB, Claus SP, Bar PR, Ramos LM, Algra A, van Gijn J, Kappelle LJ (2001) Reproducibility of measurements of cerebral infarct volume on CT scans. Stroke 32:424-430.

van Groen T, Wyss JM (1990) Extrinsic projections from area CA1 of the rat hippocampus: olfactory, cortical, subcortical, and bilateral hippocampal formation projections. J Comp Neurol 302:515-528.

van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999a) Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A 96:13427-13431.

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

van Praag H, Shubert T, Zhao C, Gage FH (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci 25:8680-8685.

Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 6:626-640.

von Zahn J, Moller T, Kettenmann H, Nolte C (1997) Microglial phagocytosis is modulated by pro- and anti-inflammatory cytokines. Neuroreport 8:3851-3856.

Wainwright ML, Zhang H, Byrne JH, Cleary LJ (2002) Localized neuronal outgrowth induced by long-term sensitization training in aplysia. J Neurosci 22:4132-4141.

Walker JA, Olton DS (1979) Spatial memory deficit following fimbria-fornix lesions: independent of time for stimulus processing. Physiol Behav 23:11-15.

Wallraff A, Odermatt B, Willecke K, Steinhauser C (2004) Distinct types of astroglial cells in the hippocampus differ in gap junction coupling. Glia 48:36-43.

Walsh TJ, Schulz DW, Tilson HA, Schmechel DE (1986) Colchicine-induced granule cell loss in rat hippocampus: selective behavioral and histological alterations. Brain Res 398:23-36.

Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM (1988) Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci U S A 85:9484-9488.

Wang SH, Morris RG (2010a) Hippocampal-neocortical interactions in memory formation, consolidation, and reconsolidation. Annu Rev Psychol 61:49-79, C41-44.

Wang SH, Morris RG (2010b) Hippocampal-neocortical interactions in memory formation, consolidation, and reconsolidation. Annu Rev Psychol 61:49-79.

Wang SH, Teixeira CM, Wheeler AL, Frankland PW (2009) The precision of remote context memories does not require the hippocampus. Nat Neurosci 12:253-255.

Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA (1998) Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. Nat Med 4:228-231.

Watanabe M, Masuyama N, Fukuda M, Nishida E (2000) Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. EMBO Rep 1:176-182.

Wati H, Kudo K, Qiao C, Kuroki T, Kanba S (2006) A decreased survival of proliferated cells in the hippocampus is associated with a decline in spatial memory in aged rats. Neurosci Lett 399:171-174.

Wei L, Meaney MJ, Duman RS, Kaffman A (2011) Affiliative behavior requires juvenile, but not adult neurogenesis. J Neurosci 31:14355-14345.

Westerhausen DR, Jr., Hopkins WE, Billadello JJ (1991) Multiple transforming growth factor-beta-inducible elements regulate expression of the plasminogen activator inhibitor type-1 gene in Hep G2 cells. J Biol Chem 266:1092-1100.

Whishaw IQ, Tomie J (1996) Of mice and mazes: similarities between mice and rats on dry land but not water mazes. Physiol Behav 60:1191-1197.

Wilcox JN, Derynck R (1988) Localization of cells synthesizing transforming growth factor-alpha mRNA in the mouse brain. J Neurosci 8:1901-1904.

Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S (2006) Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 16:296-304.

Witter MP (1993) Organization of the entorhinal-hippocampal system: a review of current anatomical data. Hippocampus 3 Spec No:33-44.

Witter MP, Groenewegen HJ, Lopes da Silva FH, Lohman AH (1989) Functional organization of the extrinsic and intrinsic circuitry of the parahippocampal region. Prog Neurobiol 33:161-253.

Wojtowicz JM (2006) Irradiation as an experimental tool in studies of adult neurogenesis. Hippocampus 16:261-266.

Wojtowicz JM, Askew ML, Winocur G (2008) The effects of running and of inhibiting adult neurogenesis on learning and memory in rats. Eur J Neurosci 27:1494-1502.

Worley PF, Bhat RV, Baraban JM, Erickson CA, McNaughton BL, Barnes CA (1993) Thresholds for synaptic activation of transcription factors in hippocampus: correlation with long-term enhancement. J Neurosci 13:4776-4786.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. Science 242:1528-1534.

Wyss-Coray T, Feng L, Masliah E, Ruppe MD, Lee HS, Toggas SM, Rockenstein EM, Mucke L (1995) Increased central nervous system production of extracellular matrix components and development of hydrocephalus in transgenic mice overexpressing transforming growth factor-beta 1. Am J Pathol 147:53-67.

Wyss-Coray T, Lin C, Sanan DA, Mucke L, Masliah E (2000) Chronic overproduction of transforming growth factor-beta1 by astrocytes promotes Alzheimer's disease-like microvascular degeneration in transgenic mice. Am J Pathol 156:139-150.

Wyss-Coray T, Lin C, Yan F, Yu GQ, Rohde M, McConlogue L, Masliah E, Mucke L (2001) TGF-beta1 promotes microglial amyloid-beta clearance and reduces plaque burden in transgenic mice. Nat Med 7:612-618.

Wyss-Coray T, Masliah E, Mallory M, McConlogue L, Johnson-Wood K, Lin C, Mucke L (1997) Amyloidogenic role of cytokine TGF-beta1 in transgenic mice and in Alzheimer's disease. Nature 389:603-606.

Xavier GF, Oliveira-Filho FJ, Santos AM (1999) Dentate gyrus-selective colchicine lesion and disruption of performance in spatial tasks: difficulties in "place strategy" because of a lack of flexibility in the use of environmental cues? Hippocampus 9:668-681.

Xiao BG, Bai XF, Zhang GX, Link H (1997) Transforming growth factor-beta1 induces apoptosis of rat microglia without relation to bcl-2 oncoprotein expression. Neurosci Lett 226:71-74.

Xiao BG, Zhu WH, Lu CZ (2007) The presence of GM-CSF and IL-4 interferes with effect of TGF-beta1 on antigen presenting cells in patients with multiple sclerosis and in rats with experimental autoimmune encephalomyelitis. Cell Immunol 249:30-36.

Yamaguchi M, Saito H, Suzuki M, Mori K (2000) Visualization of neurogenesis in the central nervous system using nestin promoter-GFP transgenic mice. Neuroreport 11:1991-1996.

Yang X, Khosravi-Far R, Chang HY, Baltimore D (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell 89:1067-1076.

Ying SY (1987) Inhibins and activins: chemical properties and biological activity. Proc Soc Exp Biol Med 186:253-264.

Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 5:448-453.

Yun HJ, Yoon JH, Lee JK, Noh KT, Yoon KW, Oh SP, Oh HJ, Chae JS, Hwang SG, Kim EH, Maul GG, Lim DS, Choi EJ (2011) Daxx mediates activation-induced cell death in microglia by triggering MST1 signalling. The EMBO journal 30:2465-2476.

Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci U S A 94:3789-3794.

Zar JH (2010) Biostatistical analysis. Upper Saddle River, N.J.: Prentice-Hall/Pearson.

Zetterberg H, Andreasen N, Blennow K (2004) Increased cerebrospinal fluid levels of transforming growth factor-beta1 in Alzheimer's disease. Neurosci Lett 367:194-196.

Zhang CL, Zou Y, He W, Gage FH, Evans RM (2008) A role for adult TLX-positive neural stem cells in learning and behaviour. Nature 451:1004-1007.

Zhang F, Endo S, Cleary LJ, Eskin A, Byrne JH (1997) Role of transforming growth factor-beta in long-term synaptic facilitation in Aplysia. Science 275:1318-1320.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971-982.

Zhang RL, Zhang ZG, Wang Y, LeTourneau Y, Liu XS, Zhang X, Gregg SR, Wang L, Chopp M (2007) Stroke induces ependymal cell transformation into radial glia in the subventricular zone of the adult rodent brain. J Cereb Blood Flow Metab 27:1201-1212.

Zhang S, Ekman M, Thakur N, Bu S, Davoodpour P, Grimsby S, Tagami S, Heldin CH, Landstrom M (2006) TGFbeta1-induced activation of ATM and p53 mediates apoptosis in a Smad7-dependent manner. Cell Cycle 5:2787-2795.

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

Zhao C, Teng EM, Summers RG, Jr., Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26:3-11.

Zhao M, Choi YS, Obrietan K, Dudek SM (2007) Synaptic plasticity (and the lack thereof) in hippocampal CA2 neurons. J Neurosci 27:12025-12032.

Zhao RY, Elder RT (2005) Viral infections and cell cycle G2/M regulation. Cell Res 15:143-149.

Zhou W, Park I, Pins M, Kozlowski JM, Jovanovic B, Zhang J, Lee C, Ilio K (2003) Dual regulation of proliferation and growth arrest in prostatic stromal cells by transforming growth factor-beta1. Endocrinology 144:4280-4284.

Zhu Y, Ahlemeyer B, Bauerbach E, Krieglstein J (2001) TGF-beta1 inhibits caspase-3 activation and neuronal apoptosis in rat hippocampal cultures. Neurochem Int 38:227-235.

Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, Culmsee C, Klumpp S, Krieglstein J (2002) Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. J Neurosci 22:3898-3909.

Appendix I

Hippocampal neurogenesis regulates forgetting during adulthood and infancy

Katherine G. Akers[1*], Alonso Martinez-Canabal[1,2*], Anne L. Wheeler[1,2], Leonardo Restivo[1], Hirotaka Shoji[3], Axel Guskjolen[1,4], Blake A. Richards[1], Hideo Hagihara[3], Tsuyoshi Miyakawa[3], Sheena A. Josselyn[1,2,4†] & Paul W. Frankland[1,2,4†]

* These authors contributed equally

† Corresponding authors

[1] Program in Neurosciences and Mental Health, The Hospital for Sick Children, Toronto, M5G 1X8, Canada.

[2] Institute of Medical Science, University of Toronto, Toronto, M5S 1A8, Canada.

[3] Division of Systems Medical Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan.

[4] Department of Physiology, University of Toronto, Toronto, M5S 1A8, Canada.

ABSTRACT

New neurons are continuously added to the dentate gyrus of the hippocampus throughout life. Although the hippocampus is known to be important for memory, the functional consequences of hippocampal neurogenesis remain unclear. The majority of previous studies have examined the impact of manipulating hippocampal neurogenesis on subsequent memory formation, but few have examined the effects of these manipulations on established, hippocampus-dependent memories. Since the addition of new neurons leads to extensive remodeling of hippocampal circuits, computational models predict this should lead to degradation or forgetting of established memories. Consistent with this, developmental changes in hippocampal neurogenesis are inversely correlated with memory persistence across species: During infancy, when hippocampal neurogenesis levels are high, freshly-generated memories tend to be rapidly forgotten. In contrast, during adulthood, when neurogenesis levels are lower, memories are typically much more persistent. We have conducted two types of experiments that suggest that neurogenesis is causally related to forgetting. First, in adult mice (P60), we find that increasing neurogenesis after memory formation is sufficient to induce forgetting. Second, in infant mice (P17), we find that decreasing neurogenesis after memory formation mitigates normal forgetting observed at this age. These data identify a novel role for hippocampal neurogenesis in the regulation of forgetting, and provide a neurobiological basis for infantile amnesia.

INTRODUCTION

In both artificial systems and brain networks there is a trade-off between plasticity—the ability to incorporate new information—and stability—ensuring that the process of incorporating new information does not degrade information already stored in that network (*1*). In the hippocampus new neurons continue to be generated in the subgranular zone of the dentate gyrus (DG) beyond development and into adulthood (*2*, *3*). These new neurons gradually synaptically integrate into hippocampal circuits (*4-8*), and therefore provide potential substrates for new learning. Consistent with this, promoting the production of new neurons in adult mice facilitates the formation of new hippocampal memories (*9*, *10*). However, at the same time, the continuous integration of new neurons may impact existing memory storage. Indeed, as new neurons integrate into the hippocampus they compete with existing granule cells for inputs and outputs, establishing new synaptic connections that either co-exist with, or even replace, existing synaptic connections in both the DG and CA3 (*5*, *6*, *11*). As such remodeling necessarily alters the configuration of DG-CA3 circuits and likely rescales synaptic weights of pre-existing connections, computational models predict that high levels of hippocampal neurogenesis will lead to forgetting of information already stored in those circuits (*12-14*).

Although hippocampal neurogenesis persists across the lifespan, rates decline dramatically with age (*15*, *16*). Therefore, this predicted remodeling-induced forgetting should be more pronounced during infancy when hippocampal neurogenesis levels are high. Consistent with this, infantile forgetting (or infantile amnesia (*17*)) is observed in a wide variety of species (*18*), including humans (*19*). Neurobiological accounts of infantile amnesia previously emphasized that continued brain maturation might interfere with consolidation and/or storage of infant memories, rendering them inaccessible at later time points (*20*). Here we test whether one specific feature of ongoing hippocampal development—postnatal hippocampal neurogenesis— modulates ontogenetic changes in memory persistence.

Post-natal neurogenesis and memory stability are inversely correlated. We first characterized hippocampal neurogenesis in infant and adult mice using retrovirus expressing GFP (to label neural progenitors and their progeny (*10*)) and immunohistochemical approaches (**Fig. 1**). We observed a pronounced age-dependent reduction in neurogenesis (**Fig. 1A**), reflected by a decrease in the number of proliferating cells ($Ki67^+$) and immature neurons (doublecortin$^+$ or DCX^+) from infancy (postnatal day 17; P17) to early adulthood (P60) (**Fig.**

1B-D; **fig. S1**). Postnatally-generated DG granule cells project a mossy fiber that reaches the CA3 region within about 2 weeks (e.g., **Fig. 1A**), contacting 11-15 pyramidal cells (*5, 7, 21*). Consistent with the age-dependent decrease in neurogenesis we observed a reduction in DCX^+ large mossy fiber terminals (LMTs) in the CA3 region (**Fig. 1E**). Given that there are ~250,000 pyramidal cells in the CA3 region (*22*), we next modeled how this addition of new neurons impacts DG-CA3 circuits during infancy and adulthood. During infancy, new neuron addition altered contacts on virtually all CA3 pyramidal cells after one month. In contrast, during adulthood, new neuron addition led to more modest synaptic rearrangements (**Fig. 1F**). These analyses suggest that the addition of new neurons profoundly impacts DG-CA3 circuits, and that this remodeling is most pronounced during infancy.

To evaluate how age-dependent changes in neurogenesis relate to changes in the ability to form enduring hippocampus-dependent memories, we used an incidental context learning paradigm. When mice receive a footshock immediately upon placement in a novel context, they fail to condition to that context. However, previous exposure to this context alleviates this immediate shock deficit (*23*). Accordingly, the ability of the pre-exposure to rescue the immediate shock deficit may be used to evaluate the integrity of this context-only memory. Infant (P17) or adult (P60) mice were pre-exposed to the context and then trained with an immediate shock 1, 7, 14 or 28 days later (**Fig. 1G**). Importantly, this pre-exposure engaged the hippocampus, consistent with previous studies in adult rodents (**fig. S2**). Conditioned freezing was assessed following shock delivery and in a separate test 24 h later. In adult mice, context pre-exposure rescued the immediate shock deficit at all pre-exposure-training delays, suggesting this context-only memory endured for at least a month. In contrast, in infant mice, context pre-exposure only rescued the immediate shock deficit when pre-exposure occurred one day before training. Progressively weaker conditioning at longer pre-exposure-training delays therefore suggests that infant mice forget this context-only memory with time (**Fig. 1H**). Next we trained additional age-matched infant and adult mice identically, but without pre-exposure to the context. The absence of freezing in all infant and adult groups (**Fig. 1I**) confirmed that pre-exposure is necessary for conditioning, and, furthermore, indicated that differing freezing levels in infant mice that were pre-exposed to the context cannot be accounted for by age-dependent changes in propensity to freeze following an immediate shock. This pattern of accelerated forgetting in infant mice was replicated using a standard contextual fear conditioning task (**fig.**

S3). Therefore, these data indicate that memory persistence and neurogenesis are inversely correlated. To test whether they are causally related, we next conducted two types of experiments. First, we tested whether increasing hippocampal neurogenesis after learning destabilizes memories in adult mice. Second, we tested whether decreasing hippocampal neurogenesis after learning counteracts forgetting in infant mice (*24*) (**Fig. 1J**).

Increasing neurogenesis destabilizes established memories in adult mice. To test whether transiently increasing hippocampal neurogenesis after memory formation in adult mice promotes forgetting of newly-acquired information we used voluntary running, a naturalistic intervention that robustly increases neurogenesis (*25*). Confirming previous reports, running increased neurogenesis in the DG of adult mice (**Fig. 2A-D**). Relative to sedentary controls, running induced an approximate two-fold elevation in the number of $Ki67^+$ cells (**Fig. 2B**), DCX^+ cell bodies in DG (**Fig. 2C**) and DCX^+ LMTs in CA3 (**Fig. 2D**). Increases were evident within 7 days of commencing running, were sustained for the duration of exercise, and were not associated with increased death of developmentally-generated granule cells (**fig. S4**). To examine the impact of running-induced increases in neurogenesis on DG-CA3 circuit remodeling, we injected retrovirus expressing GFP into the DG. Four weeks later we observed many GFP^+ neurons in the innermost layers of the DG, and GFP^+ LMTs in stratum lucidum of CA3. Following counterstaining for a marker of mature presynaptic terminals, zinc transporter-3 (ZnT3) (*26*), we identified many new LMTs ($GFP^+/ZnT3^+$) in close apposition to pre-existing LMTs ($GFP^-/ZnT3^+$) (**Fig. 2E**), suggesting that new contacts co-exist with established connections (*5*). Furthermore, running increased the ratio of new-to-established LMTs, indicating that running induces greater levels of remodeling of DG-CA3 circuits (**fig. S5**).

To ask whether running-induced increases in hippocampal remodeling weaken an established hippocampus-dependent memory, we used the incidental context learning paradigm. Following context pre-exposure, adult mice were allowed continuous access to a running wheel in their home cage. Four weeks later mice were replaced in the same context and shocked immediately. Freezing was assessed following shock delivery and in a separate test 24 h later. A control group was treated identically, but did not have access to a running wheel in their home cage (**Fig. 2F**). As hypothesized, running both increased proliferation in the DG (**fig. S6**) and reduced the ability of the context pre-exposure to rescue the immediate shock deficit (**Fig. 2G-**

H). Importantly, there was a strong, inverse correlation between memory strength and proliferation levels in both the running and sedentary groups (**Fig. 2I**)

Voluntary running induces a number of neural and physiological changes, other than increasing hippocampal neurogenesis (*27*). Therefore, to control for the possibility that running might nonspecifically decrease freezing, we reversed the order of context pre-exposure and running (**Fig. 2J**). As before, running robustly increased proliferation in the DG (**fig. S7**). However, running before (rather than after) context pre-exposure did not alter the effectiveness of context pre-exposure (**Fig. 2K-L**). Importantly, in this experiment we found no relationship between proliferation and freezing levels (**Fig. 2M**), indicating that running does not nonspecifically reduce freezing levels and that levels of neurogenesis do not regulate the ability to form a new context-only memory and use that memory for up to 48 h. We found similar dissociable effects of pre- vs. post-learning running on forgetting using another hippocampus-dependent task (contextual fear conditioning; **fig. S8**). This indicates that running-induced changes in neurogenesis have more profound retrograde than anterograde effects on memory.

These data strongly suggest that neurogenesis levels regulate the stability of newly-acquired memories. However, to evaluate whether our data are consistent with a causal relationship between neurogenesis and forgetting we used Bayesian causal model analysis (*28*). In this analysis we constructed a model in which running-induced forgetting could be mediated by neurogenesis-*dependent* and/or neurogenesis-*independent* mechanisms (**Fig. 2N; fig. S9**). Using Markov Chain Monte Carlo sampling, we found that our data provide strong support for the neurogenesis-dependent model ($P = 0.99$ for neurogenesis-dependent versus $P = 0.13$ for the neurogenesis-independent model). Therefore, while voluntary running induces a number of neural and physiological changes other than increasing hippocampal neurogenesis, these Bayesian causal model analyses indicate that the running-induced effects on forgetting are most likely mediated via an increase in neurogenesis.

Specificity of running-induced forgetting. We next asked whether the amount and/or timing of running influenced the degree of running-induced forgetting. Using the incidental context learning paradigm, we found that home cage access to an operational running wheel for 2 or more weeks was sufficient to induce forgetting (**Fig. 3A-B**). However, two weeks of running did not induce forgetting when initiated 4 weeks after training, indicating that as context memories age they become increasingly invulnerable to the effects of running-induced increases

in neurogenesis (**Fig. 3C-D**). This temporally-graded pattern is similar to that observed following hippocampal lesions (*29*), and suggests that remote memories are invulnerable, in part, because they become dependent on extra-hippocampal structures (*30*).

New granule cells require ~16 days to establish functional output connections onto CA3 pyramidal cells (*5*, *7*). Therefore, if forgetting critically depends on the integration of newborn granule cells into established hippocampal circuits, then the emergence of running-induced forgetting should follow a similar time-course. Consistent with this, when mice were allowed to run for 2 weeks following context pre-exposure, and then tested immediately, rather than after a 2 week delay, there was no forgetting (**Fig. 3E-F**).

Finally, we evaluated whether hippocampus-independent memories are affected by post-training running using a conditioned taste aversion paradigm (**Fig. 3G**). When a novel taste (saccharin) is paired with a malaise-inducing agent (lithium chloride), mice later avoid the saccharin in a choice test. Neither acquisition nor expression of this aversion depends on the hippocampus (*31*). Consistent with this, we found that 4 weeks of post-training running did not alter aversion compared to sedentary controls (**Fig. 3H**), suggesting that running-induced remodeling of hippocampal circuits does not impact memories that depend on extra-hippocampal structures.

Running induced forgetting is adaptive. What might be the adaptive value of neurogenesis-mediated forgetting? One possibility is that forgetting enhances memory function by improving the efficiency of new encoding (*32*, *33*). To examine this, we trained mice to locate a submerged platform in a fixed location in the water maze. Mice were either allowed access to a running wheel or were sedentary (for 4 weeks). Subsequently, mice were retrained in a reversal phase where the platform was placed in a position opposite to its initial location (**Fig. 4A**). In this reversal phase, therefore, memory of the original location impedes learning of the new location. Mice that ran after the initial learning phase (but before the reversal phase), showed weaker memory for original platform location (**Fig. 4B**), consistent with running-induced forgetting of context-only memories. However, these running mice also learned the new platform location more efficiently in the reversal phase (**Fig. 4C**), exhibiting a stronger spatial bias for the new platform location in a probe test at the end of training (**Fig. 4D**). Running did not alter swim speed during probe tests (**fig. S10**). These results suggest that post-training running led to loss of specific (e.g., exact platform location) but not general (e.g., how to perform task, overall spatial

layout) features of learning, a form of selective forgetting that might allow mice to more rapidly adapt to changing contingencies in an otherwise stable environment (*32*).

Chemical and genetic elevation of neurogenesis induces forgetting in young adult mice. We next asked whether increasing neurogenesis using either chemical or genetic approaches would similarly induce forgetting of hippocampus-dependent memories in adult mice. The non-competitive N-methyl-d-aspartate (NMDA) receptor antagonist, memantine, promotes adult neurogenesis (*34*). Similar to post-training running for 4 weeks, we found that memantine treatment (25 mg/kg once per week for 4 weeks) induced forgetting of a context-only memory (**Fig. 5A-B**). We found a similar pattern of results using contextual fear conditioning. Prolonged post-training memantine treatment for 4 weeks induced forgetting and this forgetting was observed even after memantine treatment was stopped. Importantly, a single post-training injection did not impair context fear memory tested the next day, suggesting that memantine did not affect initial memory consolidation (**fig. S11**). SSRIs such as fluoxetine also increase adult neurogenesis (*35*), and similarly we found that post-training fluoxetine treatment induced forgetting of a spatial memory (**Fig. 5C-E**). In addition to these chemical interventions that increase adult neurogenesis, we also found that two genetic mutations (deletion of Schnurri-2 (*36*) and calcineurin (*37*)) that increase adult, hippocampal neurogenesis also induce forgetting of spatial memories (**fig. S12**). Therefore, convergent behavioral results using multiple, mechanistically distinct interventions (that differ in their off-target effects but have similar effects on neurogenesis) provide compelling evidence that the observed forgetting is mediated by a neurogenic mechanism.

Reducing neurogenesis increases memory stability in infant mice. In infancy, there are both high levels of hippocampal neurogenesis and forgetting. We next examined whether reducing neurogenesis after learning counteracts normal forgetting in infant mice using the incidental context learning paradigm (**Fig. 1H**). Following context pre-exposure at P17, infant mice received daily injections of temozolomide (TMZ), a DNA alkylating agent that reduces hippocampal neurogenesis (*38*), or vehicle for 4 weeks (**Fig. 6A**). While TMZ treatment suppressed neurogenesis (reduced number of $Ki67^+$ cells, DCX^+ cell bodies in DG and DCX^+ LMTs in CA3) (**Fig. 6B-E**), strikingly, it improved retention of the context-only memory. TMZ-treated mice froze more during training (following shock delivery; **Fig. 6F**) and testing (**Fig. 6G**) compared to vehicle-treated mice, suggesting that reducing neurogenesis after learning mitigated

forgetting in infant mice. Consistent with this interpretation, there was a strong inverse relationship between freezing and proliferation levels (**Fig. 6H**).

It is also possible that TMZ nonspecifically increases the propensity to freeze. To evaluate this possibility, we conducted an additional experiment in which the order of pre-exposure and drug treatment was reversed (**Fig. 6I**). As before, TMZ treatment reliably decreased neurogenesis (**Figs. 6J-M**). However, decreasing neurogenesis before learning did not alter freezing levels during training (following shock delivery; **Fig. 6N**) or testing (**Fig. 6O-P**), indicating that TMZ treatment does not decrease the threshold for freezing by, for example, reducing activity or increasing anxiety.

Discussion

To our knowledge, in examining the relationship between hippocampal neurogenesis and memory, all previous studies have used essentially the same design: They have manipulated hippocampal neurogenesis before training and subsequently examined memory formation (i.e., they have studied the anterograde effects of manipulating neurogenesis) (*39*). The view that has emerged from these numerous studies is that, once sufficiently mature, new neurons contribute to encoding of new hippocampus-dependent memories (*39*). In considering how similar manipulations of neurogenesis retrogradely impact established hippocampus-dependent memories, our studies reveal a novel role for neurogenesis in forgetting or memory clearance, in line with theoretical predictions (*12-14*).

The hippocampus is thought to rapidly and automatically encode experiences (*40*). As not all experiences are ultimately remembered, however, there likely exist forgetting processes that continuously degrade or clear stored information from the hippocampus. Our experiments identify neurogenesis as one such process that promotes degradation of hippocampal memories. In adult mice, we found that transiently increasing neurogenesis after learning induced forgetting of hippocampus-dependent (but not hippocampus-independent) memories. These effects of increasing neurogenesis were dose-dependent and temporally-graded, with more pronounced impact on recently-formed memories. Neurogenesis likely induces forgetting by reconfiguring DG-CA3 circuits. As memory fidelity depends on the precise spatio-temporal activation of hippocampal neurons, reconfiguration of hippocampal circuits would reduce the ability of a

given set of cues (or inputs) to re-invoke the same pattern of activity (akin to a failure to pattern complete) (*41*).

In addition to neurogenesis, there may be other forgetting mechanisms, each acting on different timescales (*40*, HYPERLINK \l "page15" 42, 43). The clearance of memories from the hippocampus might facilitate the formation of new memories by increasing capacity for new learning and/or reducing the likelihood of old, irrelevant memories interfering with encoding of new information (*33*). Not all memories are destined to be forgotten. Neuromodulatory influences selectively strengthen the initial encoding of more biologically-salient experiences in hippocampal and cortical circuits (*44*). Post-encoding reactivation of these circuits is thought to incrementally strengthen inter-cortical connections and allow the memory to be expressed independently of the hippocampus (*30*). While previous studies suggest that neurogenesis may regulate the transformation of memories from hippocampus-dependent to hippocampus-independent forms (*45*), our findings suggest that neurogenesis represents a form of structural plasticity that continuously clears old memories from the hippocampus.

As neurogenesis levels decline during development, the ability to form persistent memories emerges. Strikingly, in infant mice, we found that reduction of hippocampal neurogenesis after learning promoted memory persistence, suggesting that elevated levels of hippocampal neurogenesis during infancy are responsible, in part, for infantile amnesia. However, our rescue of infantile amnesia was incomplete. While it is possible that more comprehensive suppression of neurogenesis might have led to more complete rescue, perhaps more plausibly, the continued maturation of prefrontal cortical regions that are necessary for permanent consolidation of memories additionally contributes to infantile forgetting (*46*).

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research to P.W.F. (MOP86762) and S.A.J. (MOP74650) and CREST/Japan Science and Technology Agency to T.M. We thank S. Ishii for the Schnurri-2 knockout mice and I. Graeff and G. Crabtree for the calcineurin knockout mice. K.G.A. and A.M.-C. were supported by fellowships from the Canadian Institutes of Health Research and El Consejo Nacional de Ciencia y Tecnología (Mexico), respectively. A.L.W. received support from the Ontario Mental Health Foundation,

and A.G. and B.A.R. were supported by fellowships from the Natural Sciences and Engineering Research Council of Canada.

Figure Captions

Fig. 1. **Age-dependent levels of hippocampal neurogenesis and memory stability are inversely related.** (**A**) Higher levels of neurogenesis in infancy versus adulthood. Dentate gyrus (DG) granule cells in DG and CA3 regions one month after retrovirus labeling with GFP (green) in infant (P17, left) and adult (P60, right) mice. *Left.* Low magnification of DG and CA3 regions. Scale bar = 200 μm. *Right*. High magnification of CA3 region showing GFP^+ mossy fibers and large mossy fiber terminals (LMTs). Gray = DAPI. Scale bar = 50 μm. (**B**) DCX^+ cells (green) in infant (P17) and adult (P60) mice in DG (left) and CA3 (right). Scale bar = 100 μm. Left, inset. High magnification of DG. Right, inset. High magnification of CA3. Scale bar = 50 μm. (**C-E**) Infant mice show increased (**C**) proliferating ($Ki67^+$) cells ($t(7) = 6.55$, $P < 0.001$), (**D**) immature neurons (DCX^+) in DG ($t(6) = 13.59$, $P < 0.001$) and (**E**) LMTs in CA3 ($t(6) = 15.03$, $P < .001$) compared to adult mice. (**F**) Cumulative predicted synaptic remodeling (proportion of CA3 cells with altered synaptic inputs as a consequence of new neuron addition) in DG-CA3 circuits in infants (high neurogenesis) is higher than in adults (low neurogenesis). A CA3 cell is defined as being remodeled if 2-4% (dashed lines) of its synaptic inputs are altered by new neuron addition. (**G**) Experimental design. (**H**) Adults retained a context-only memory for at least 28 days, but infants showed forgetting (age × delay, $F(3,102) = 3.04$, $P < 0.05$; post hoc t-test, 7 d, $P < 0.05$; 14 d, $P < 0.05$; 28 d, $P < 0.001$). (**I**) Infant and adult mice not pre-exposed to the context showed no context-only memory ($P > 0.05$). (**J**) Inverse relationship between neurogenesis and memory persistence predicts increasing neurogenesis in adults will induce forgetting, whereas decreasing neurogenesis in infants will counteract forgetting. * indicates $P < 0.05$.

Fig. 2. **Voluntary running both increases neurogenesis and promotes forgetting in adult mice.** (**A**) Running increases immature (DCX^+, green) neurons in DG and mossy fibers in CA3 in mice given access to running wheel for 28 d (lower panel) versus sedentary mice (upper panel). Scale bar = 50 μm. (**B-D**) Running increased number of (**B**) proliferating ($Ki67^+$) cells (running × day, $F(4,70) = 4.22$, $P < 0.005$; post hoc t-test, 7 d, $P < 0.001$; 14 d, $P < 0.001$; 28 d,

$P < 0.01$), (**C**) immature neurons (DCX^+) in DG (running × day, $F(4,30) = 7.73$, $P < 0.001$; 7 d, $P < 0.05$; 14 d, $P < 0.001$; 28 d, $P < 0.05$) and (**D**) LMTs in CA3 (running × day, $F(4,30) = 3.45$, $P < 0.05$; 7 d, $P < 0.001$; 14 d, $P < 0.05$; 28 d, $P < 0.05$) compared to sedentary mice. (**E**) DG-CA3 circuit remodeling induced by neurogenesis. Retrovirally labeled pre-synaptic LMTs from new neurons (GFP^+, green) in close apposition to existing pre-synaptic LMTs from mature neurons ($ZnT3^+$, red) (top). Scale bar = 50 μm. Confocal stacks (middle) used to reconstruct representative 3-D images of new LMTs (green) in close contact with existing LMTs (red) in CA3 (lower). (**F**) Experimental design. (**G-I**) Adult mice allowed to run after context pre-exposure showed forgetting of the context-only memory both (**G**) after immediate shock ($t(29) = 2.58$, $P < 0.05$) and (**H**) during test ($t(29) = 2.20$, $P < 0.05$). (**I**) An inverse relationship between neurogenesis ($Ki67^+$ cells) and memory (freezing during test) ($r = -0.69$). (**J**) In contrast, mice that ran before context pre-exposure showed no changes in freezing (**K**) after immediate shock ($P > 0.05$) or (L) during test ($P > 0.05$), and (**M**) there was no relationship between neurogenesis and memory ($r = -0.09$). (**N**) A Bayesian causal model analysis was used to estimate the probability that running-induced forgetting was mediated by a neurogenesis-dependent and/or neurogenesis-independent mechanism. The probability that forgetting was mediated by a neurogenesis-dependent mechanism was high ($P(\beta 1 \times \beta 3 < 0) = 0.99$), while the probability that these effects were neurogenesis-independent was low ($P(\beta 2 < 0) = 0.13$). * indicates $P < .05$.

Fig. 3. **Duration, timing and specificity of running-induced forgetting.** (**A**,**B**) Access to operational (but not locked) running wheel for at least 2 weeks induced forgetting (14d: $t(42) = 2.23$, $P < 0.05$, 28d: $t(39) = 2.15$, $P < 0.05$). (**C**, **D**) Two weeks of running immediately after context pre-exposure, but not later, induced forgetting ($t(22) = 4.10$, $P < .001$). (**E**, **F**) Two weeks of running did not induce forgetting in mice tested immediately after running ($P > 0.05$). (**G**, **H**) Running did not induce forgetting of a non-hippocampus-dependent conditioned taste aversion memory (choice, $F(1,22) = 309.84$, $P < .001$; sedentary: $t(11) = 13.92$, $P < .001$, running: $t(11) = 11.71$, $P < .001$). * indicates $P < .05$.

Fig. 4. **Running-induced forgetting is adaptive**. (**A**) Adult mice that ran after learning a hidden platform location (Target) in the water maze subsequently showed weaker memory for the original platform location (**B**, $t(29) = 2.38$, $P < 0.05$), but faster (**C**, group × day, $F(1,29) = 6.31$,

$P < 0.05$; day 2, $t(29) = 3.34$, $P < 0.005$) and more efficient (**D**, $t(28) = 3.75$, $P < .001$) learning of a new platform location compared to sedentary controls. Density plots show where mice concentrated their search for the platform during probe tests (color legend: average number of visits per mouse per 5 × 5 cm area). Original platform location (black dot) is upper left, subsequent location is bottom right. * indicates $P < .05$.

Fig. 5. **Chemical treatments that increase neurogenesis promote forgetting in adult mice.** (**A, B**) Adult mice treated with the neurogenesis-enhancing agent memantine (MEM) after context pre-exposure showed forgetting of the context-only memory ($t(18) = 2.73$, $P < 0.05$). (**C**) Adult mice were treated with the neurogenesis-enhancing agent fluoxetine (FLX) after learning an escape location in the Barnes maze. Following training, mice showed a spatial bias for the escape location (Target) compared to other locations (**D**, left (vehicle): $t(9) = 3.53$, $P < 0.01$; right (FLX): $t(7) = 2.71$, $P < 0.05$), but prolonged FLX treatment induced forgetting of the escape location (**E**, left (vehicle): $t(9) = 2.96$, $P < 0.05$; right (FLX), $P > 0.05$). * indicates $P < .05$.

Figure 6. **Decreasing neurogenesis mitigates forgetting in infant mice**. (**A**) Experimental design. (**B**) Temozolomide (TMZ) reduced DCX^+ (green) cells and DCX^+ LMTs in CA3 in P17 mice. Scale bar = 50 µm. There were fewer (**C**) proliferating ($Ki67^+$) cells ($t(57) = 3.98$, $P < 0.001$), (**D**) immature neurons (DCX^+) in DG ($t(10) = 3.37$, $P < 0.01$), and (**E**) LMTs in CA3 ($t(10) = 3.23$, $P < 0.01$) compared to mice treated with vehicle. (**F-G**) Infant mice treated with TMZ after context pre-exposure showed stronger context-only memory both (**F**) after immediate shock ($t(57) = 3.69$, $P < 0.001$) and (**G**) during test ($t(57) = 2.47$, $P < 0.05$). (**H**) An inverse relationship between neurogenesis ($Ki67^+$ cells) and memory (freezing during test) ($r = -0.47$). (**I**) Experimental design. (**J**) Similarly, TMZ reduced DCX^+ (green) cells and DCX^+ LMTs in CA3 in P17 mice. Scale bar = 50 µm. There were fewer (**K**) proliferating ($Ki67^+$) cells ($t(20) = 3.21$, $P < 0.005$), (**L**) immature neurons (DCX^+) in DG ($t(12) = 4.23$, $P < 0.001$) and (**M**) LMTs in CA3 ($t(12) = 2.20$, $P < 0.05$) compared to mice treated with vehicle. (**N-P**) In contrast, infant mice that were treated with TMZ before context pre-exposure showed no changes in freezing (**N**) after immediate shock ($P > 0.05$) or (**L**) during test ($P > 0.05$), and (**M**) there was no relationship between neurogenesis and memory ($r = 0.01$). * indicates $P < .05$

References

1. W. C. Abraham, A. Robins, *Trends Neurosci* **28**, 73 (Feb, 2005).
2. C. Zhao, W. Deng, F. H. Gage, *Cell* **132**, 645 (Feb 22, 2008).
3. G. L. Ming, H. Song, *Annu Rev Neurosci* **28**, 223 (2005).
4. D. A. Laplagne *et al.*, *PLoS Biol* **4**, e409 (Nov, 2006).
5. N. Toni *et al.*, *Nat Neurosci* **11**, 901 (Aug, 2008).
6. N. Toni *et al.*, *Nat Neurosci* **10**, 727 (Jun, 2007).
7. C. Zhao, E. M. Teng, R. G. Summers, Jr., G. L. Ming, F. H. Gage, *J Neurosci* **26**, 3 (Jan 4, 2006).
8. S. Ge *et al.*, *Nature* **439**, 589 (Feb 2, 2006).
9. A. Sahay *et al.*, *Nature* **472**, 466 (Apr 28, 2011).
10. S. S. Stone *et al.*, *J Neurosci* **31**, 13469 (Sep 21, 2011).
11. M. Yasuda *et al.*, *Neuron* **70**, 1128 (Jun 23, 2011).
12. K. Deisseroth *et al.*, *Neuron* **42**, 535 (May 27, 2004).
13. L. A. Meltzer, R. Yabaluri, K. Deisseroth, *Trends Neurosci* **28**, 653 (Dec, 2005).
14. V. I. Weisz, P. F. Argibay, *Cognition* **125**, 13 (Oct, 2012).
15. H. G. Kuhn, H. Dickinson-Anson, F. H. Gage, *J Neurosci* **16**, 2027 (Mar 15, 1996).
16. T. Seki, Y. Arai, *Neuroreport* **6**, 2479 (Dec 15, 1995).
17. S. Freud, *Childhood and concealing memories*. A. A. Brill, Ed., The basic writings of Sigmund Freud. (Norton, New York, 1905).
18. B. A. Campbell, in *Comparative perspectives on the development of memory*, R. V. Kail, N. E. Spear, Eds. (Routledge, 1984), pp. 23-35.
19. D. C. Rubin, *Memory* **8**, 265 (Jul, 2000).
20. B. A. Campbell, N. E. Spear, *Psychol Rev* **79**, 215 (May, 1972).
21. L. Acsady, A. Kamondi, A. Sik, T. Freund, G. Buzsaki, *J Neurosci* **18**, 3386 (May 1, 1998).
22. M. Hosseini-Sharifabad, J. R. Nyengaard, *J Neurosci Methods* **162**, 206 (May 15, 2007).
23. M. S. Fanselow, *Pavlov J Biol Sci* **15**, 177 (Oct-Dec, 1980).
24. S. A. Josselyn, P. W. Frankland, *Learn Mem* **19**, 423 (2012).
25. H. van Praag, G. Kempermann, F. H. Gage, *Nat Neurosci* **2**, 266 (Mar, 1999).
26. R. McGonigal, N. Tabatadze, A. Routtenberg, *Hippocampus* **22**, 1242 (Jun, 2012).
27. H. van Praag, *Trends Neurosci* **32**, 283 (May, 2009).
28. S. E. Lazic, *J R Soc Interface* **9**, 907 (May 7, 2012).
29. J. J. Kim, M. S. Fanselow, *Science* **256**, 675 (May 1, 1992).
30. P. W. Frankland, B. Bontempi, *Nat Rev Neurosci* **6**, 119 (Feb, 2005).
31. S. A. Josselyn, S. Kida, A. J. Silva, *Neurobiol Learn Mem* **82**, 159 (Sep, 2004).
32. P. J. Kraemer, J. M. Golding, *Psychonomic Bulletin & Review* **4**, 480 (1997).
33. E. S. Rosenzweig, C. A. Barnes, B. L. McNaughton, *Nat Neurosci* **5**, 6 (Jan, 2002).
34. M. Maekawa *et al.*, *Neurosci Res* **63**, 259 (Apr, 2009).
35. J. E. Malberg, A. J. Eisch, E. J. Nestler, R. S. Duman, *J Neurosci* **20**, 9104 (Dec 15, 2000).
36. T. Takagi, J. Harada, S. Ishii, *Nat Immunol* **2**, 1048 (Nov, 2001).
37. J. R. Neilson, M. M. Winslow, E. M. Hur, G. R. Crabtree, *Immunity* **20**, 255 (Mar, 2004).
38. A. Garthe, J. Behr, G. Kempermann, *PLoS One* **4**, e5464 (2009).
39. W. Deng, J. B. Aimone, F. H. Gage, *Nat Rev Neurosci* **11**, 339 (May, 2010).
40. R. L. Redondo, R. G. Morris, *Nat Rev Neurosci* **12**, 17 (Jan, 2011).

41. A. Treves, E. T. Rolls, *Hippocampus* **4**, 374 (Jun, 1994).
42. D. M. Villarreal, V. Do, E. Haddad, B. E. Derrick, *Nat Neurosci* **5**, 48 (Jan, 2002).
43. Y. Shuai *et al.*, *Cell* **140**, 579 (Feb 19, 2010).
44. J. L. McGaugh, *Science* **287**, 248 (Jan 14, 2000).
45. T. Kitamura *et al.*, *Cell* **139**, 814 (Nov 13, 2009).
46. P. J. Bauer, *Trends Cogn Sci* **10**, 175 (Apr, 2006).

Figure 1

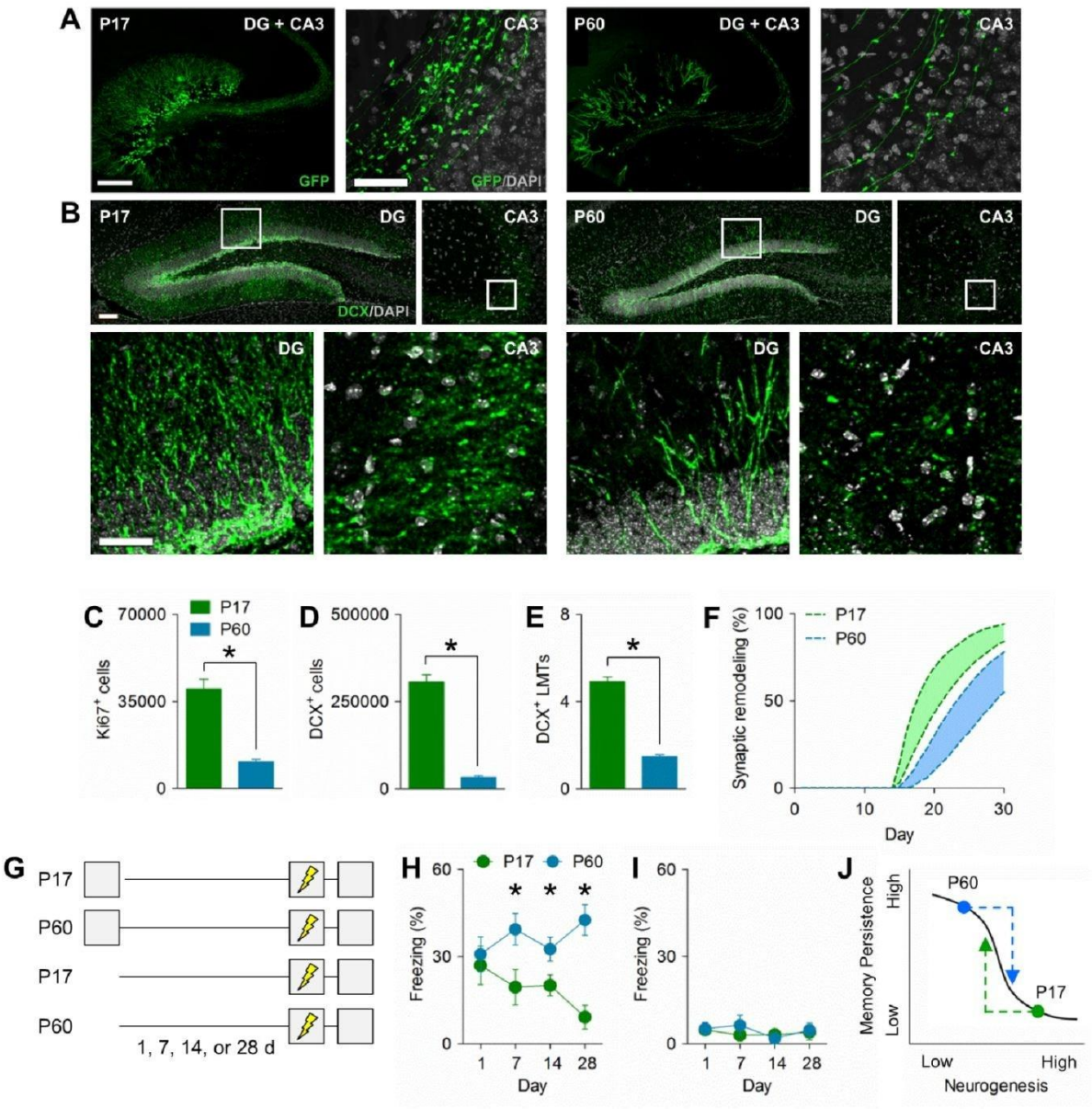

A
P17
DG + CA3
GFP
CA3
GFP/DAPI
P60
DG + CA3
CA3
B
P17
DG
CA3
DCX/DAPI
P60
DG
CA3
DG
CA3
DG
CA3
C
P17
P60
Ki67+ cells
70000
35000
0
*
D
DCX+ cells
500000
250000
0
*
E
DCX+ LMTs
8
4
0
*
F
Synaptic remodeling (%)
100
50
0
P17
P60
0
10
20
30
Day
G
P17
P60
P17
P60
1, 7, 14, or 28 d
H
P17
P60
Freezing (%)
60
30
0
* * *
1
7
14
28
Day
I
Freezing (%)
60
30
0
1
7
14
28
Day
J
Memory Persistence
High
Low
P60
P17
Low
High
Neurogenesis

Figure 2

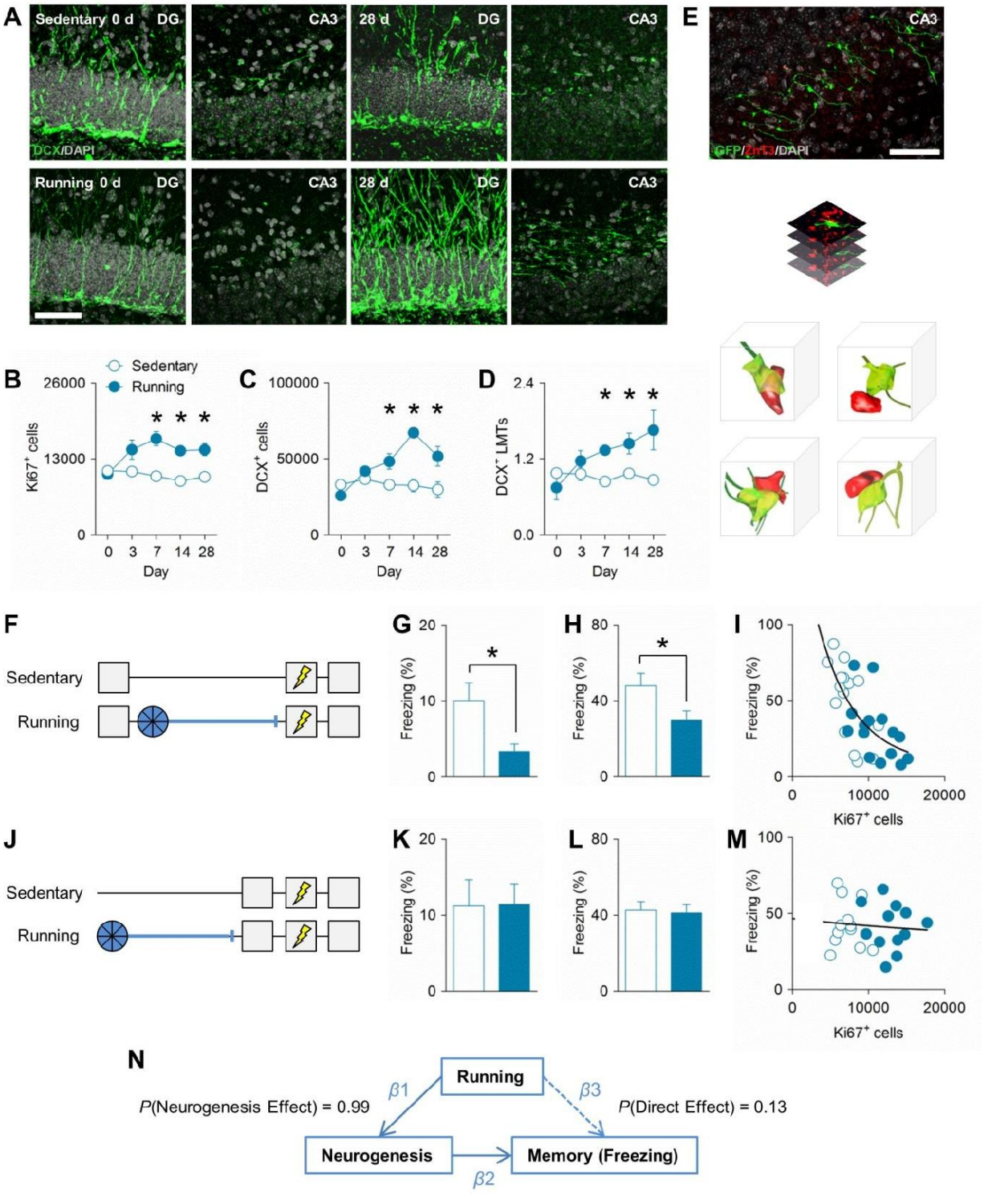

A
Sedentary 0 d
DG
CA3
28 d
DCX/DAPI
Running 0 d
E
GFP/Zn13/DAPI
B
Sedentary
Running
Ki67+ cells
Day
C
DCX+ cells
D
DCX- LMTs
F
Sedentary
Running
G
Freezing (%)
H
I
Ki67+ cells
J
K
L
M
N
P(Neurogenesis Effect) = 0.99
β1
Running
β3
P(Direct Effect) = 0.13
Neurogenesis
Memory (Freezing)
β2

Figure 3

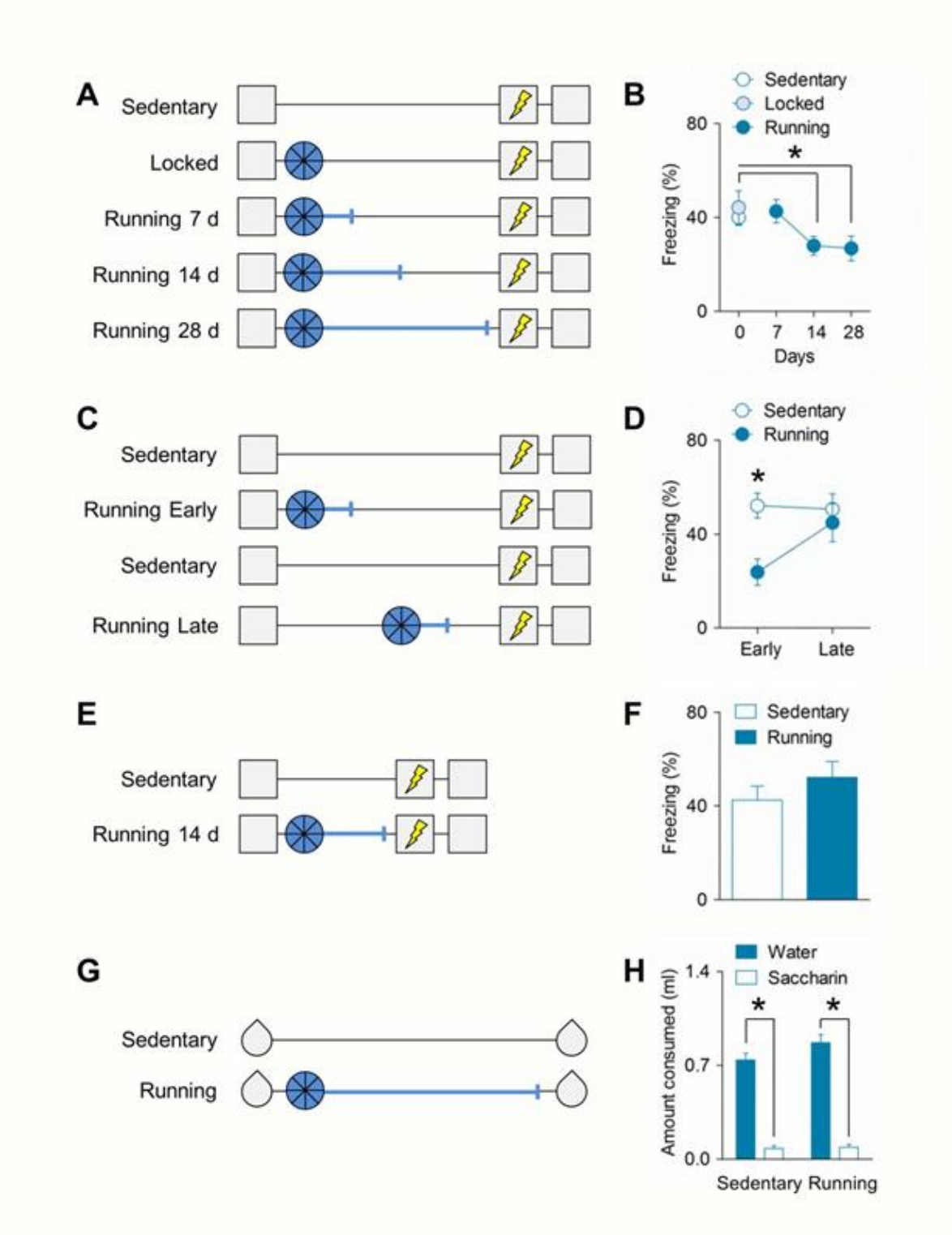

A
Sedentary
Locked
Running 7 d
Running 14 d
Running 28 d
B
Sedentary
Locked
Running
*
Freezing (%)
80
40
0
0
7
14
28
Days
C
Sedentary
Running Early
Sedentary
Running Late
D
Sedentary
Running
*
Freezing (%)
80
40
0
Early
Late
E
Sedentary
Running 14 d
F
Sedentary
Running
Freezing (%)
80
40
0
G
Sedentary
Running
H
Water
Saccharin
*
*
Amount consumed (ml)
1.4
0.7
0.0
Sedentary Running

Figure 4

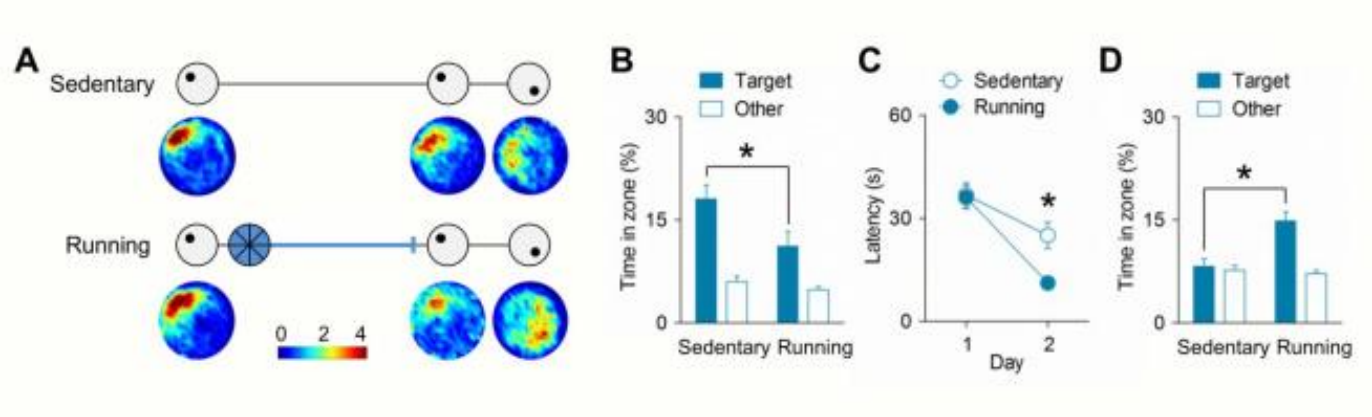


Figure 5

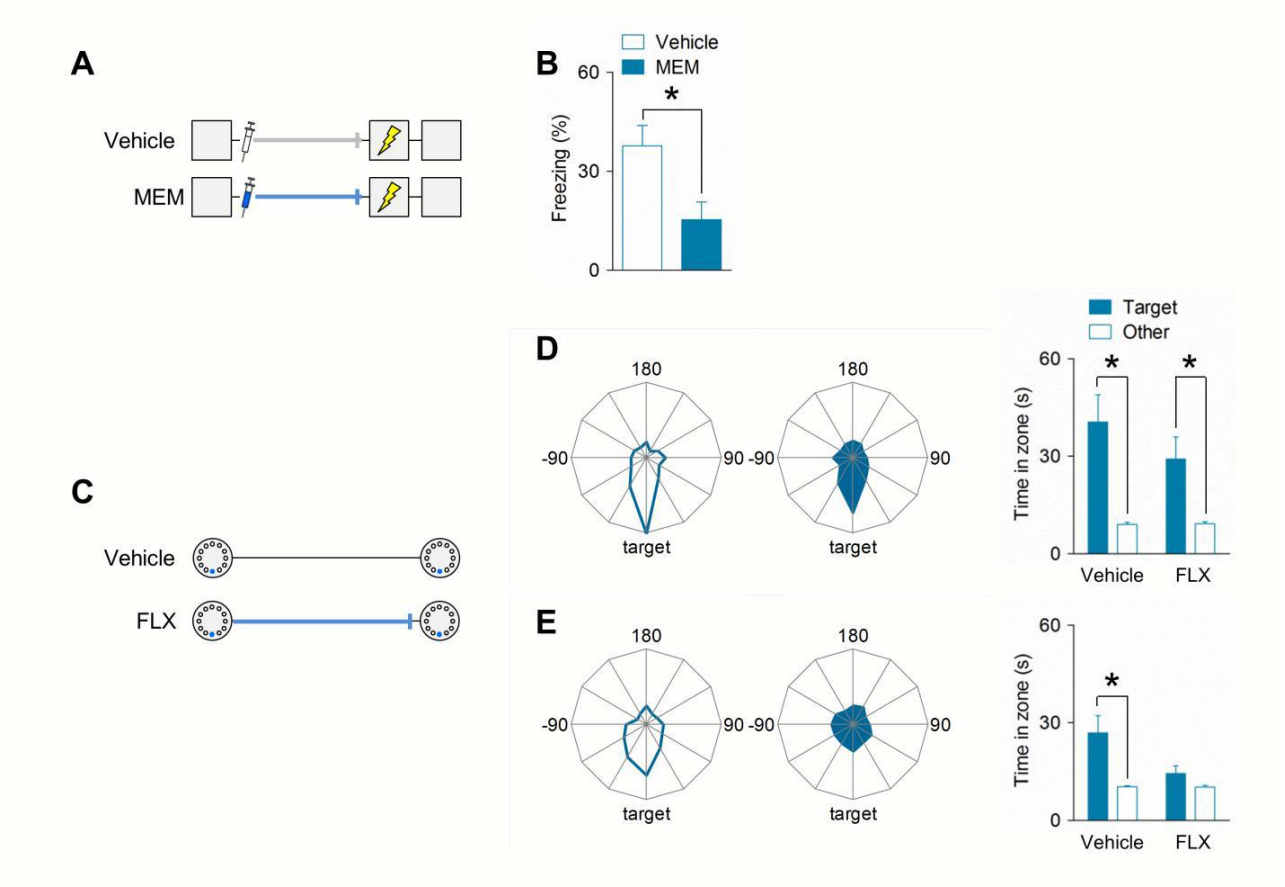
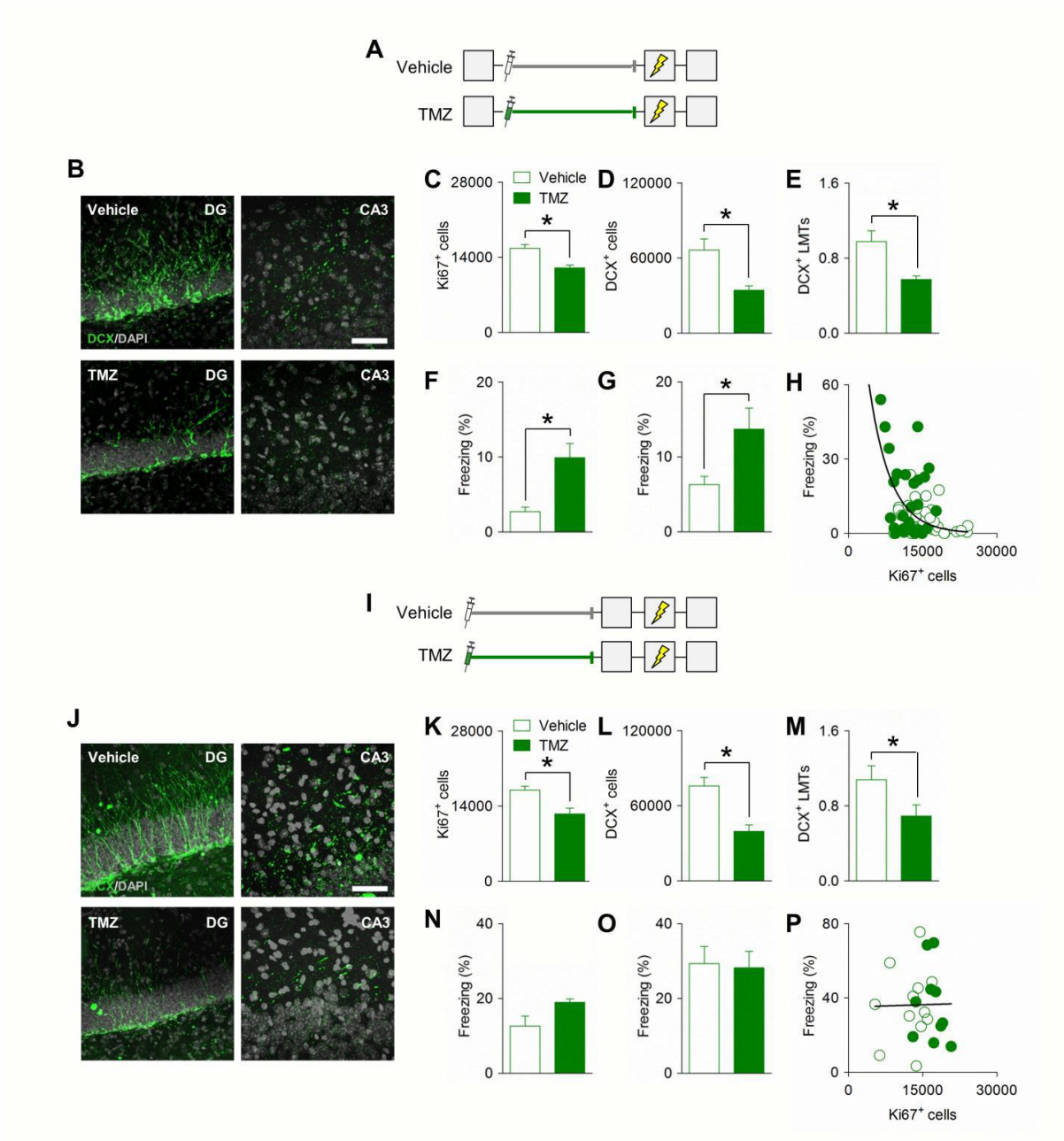


Figure 6


A
Vehicle
TMZ
B
Vehicle
DG
CA3
DCX/DAPI
TMZ
DG
CA3
C
Vehicle
TMZ
Ki67+ cells
28000
14000
0
D
DCX+ cells
120000
60000
0
E
DCX+ LMTs
1.6
0.8
0.0
F
Freezing (%)
20
10
0
G
Freezing (%)
20
10
0
H
Freezing (%)
60
30
0
0
15000
30000
Ki67+ cells
I
Vehicle
TMZ
J
Vehicle
DG
CA3
DCX/DAPI
TMZ
DG
CA3
K
Vehicle
TMZ
Ki67+ cells
28000
14000
0
L
DCX+ cells
120000
60000
0
M
DCX+ LMTs
1.6
0.8
0.0
N
Freezing (%)
40
20
0
O
Freezing (%)
40
20
0
P
Freezing (%)
80
40
0
0
15000
30000
Ki67+ cells

Appendix II

SUPPORTING ONLINE MATERIAL (SOM)

Hippocampal neurogenesis regulates forgetting during adulthood and infancy

Katherine G. Akers[1*], Alonso Martinez-Canabal[1,2*], Anne L. Wheeler[1,2], Leonardo Restivo[1], Hirotaka Shoji[3], Axel Guskjolen[1,4], Blake A. Richards[1], Hideo Hagihara[3], Tsuyoshi Miyakawa[3], Sheena A. Josselyn[1,2,4†] & Paul W. Frankland[1,2,4†]

MATERIALS AND METHODS

Mice

All procedures were approved by the Animal Care and Use Committees at the Hospital for Sick Children and Fujita Health University. Mice were bred in the animal facilities at the Hospital for Sick Children or Fujita Health University and maintained on a 12 hr light/dark cycle (lights on at 0700 hrs). Day of birth was designated P0. After weaning on P21, mice were group-housed (2-5 per cage) in transparent plastic cages (31 × 17 × 14 cm) with free access to food and water unless otherwise specified.

For a subset of experiments, hippocampal neurogenesis was tracked using reporter mice, which were generated from a cross between nestin-Cre^{ERT2} line and Rosa-LacZ or Rosa-49YFP lines maintained in a C57Bl/6 background (*1*). In these mice, injection with tamoxifen (TAM) leads to expression of the LacZ or YFP transgene only in nestin^{+} cells and their progeny. Genotypes were determined using PCR analysis of tail DNA samples.

For the Barnes maze experiments, three different types of mice were used. First, Calcineurin knockout mice (*2, 3*) were generated from a cross between floxed CNB1 (fCNB) and forebrain-specific, CaMKII promoter-driven Cre mice (CaMKII-Cre) maintained in a C57Bl/6 background. Comparisons were made between Calcineurin$^{-/-}$ mice (fCNB1/fCNB1, Cre/+) and a pooling of control littermate mice [(+/+, CaMKII-Cre/+), (+/+, +/+), and (fCNB1/fCNB1, +/+)]. Second, Schnurri-2 knockout mice (*4*) were generated from a first generation cross between Schnurri-2 heterozygous mice in a C57Bl/6 background and Schnurri-2 heterozygous mice in a BALB/cA background. Comparisons were made between Schnurri-2$^{-/-}$ mice and WT control littermates. Third, C57Bl/6 mice were implanted with fluoxetine (FLX) or

placebo pellets.

For all other experiments, mice were a first generation cross between C57Bl/6 and 129Svev strains.

Treatments

Running. Mice in running groups were given voluntary access to a running wheel (Med Associates ENV-044) placed in their home cage. Mice in sedentary groups were not given a running wheel.

Tamoxifen. TAM (Sigma) was dissolved in 10% ethanol/90% sunflower seed oil and injected (180 mg/kg, i.p.) once per day for 5 consecutive days.

BrdU. BrdU (Sigma) was dissolved in phosphate-buffered saline (PBS). For C57Bl/6 × 129Svev mice, BrdU was injected (100 mg/kg, i.p.) twice per day (12 h apart) for 5 consecutive days. For Calcineurin mice, a single BrdU injection (50 mg/kg) was given. For Schnurri-2 mice, BrdU was injected (50 mg/kg, i.p.) once per day for 4 consecutive days.

Memantine. Memantine (MEM; Sigma) was dissolved in 0.9% saline and injected (25 mg/kg, i.p.) once per week.

Fluoxetine. FLX (15 mg/kg/day, 21-day release, Innovative Research of America) or placebo pellets were implanted subcutaneously in the dorsal interscapular region under chloral hydrate anesthesia (400 mg/kg).

Temozolomide. Temozolomide (TMZ, Sigma) was dissolved in 10% DMSO/90% saline (0.9% NaCl). Four rounds of treatment at one week intervals were given. Each round consisted of one injection (25 mg/kg, i.p.) per day for 4 consecutive days.

Retrovirus. New neurons were labeled by CAG promoter-driven green fluorescent protein (GFP) expression following infection with a replication-deficient retroviral vector (based on the Moloney murine leukemia virus) (*5, 6*). Viral vector was prepared by transfecting

Plat-gp cells with two plasmids containing an amphotropic envelope (vsvg) and the transgene (pCAG-GFP), followed by collection through ultra-speed centrifugation. Plat-E cells were then infected to generate a stable virus-producing cell line and concentrated virus solution was obtained by ultra-speed centrifugation (average 3.5 x 109 iu/ml). Mice were treated with atropine (0.1 mg/kg) and anesthetized with chloral hydrate (400 mg/kg). Using stereotaxic procedures, 0.5 µl of retrovirus was infused into the dentate gyrus (DG) bilaterally (-2.2 mm AP, ±1.8 mm ML, and 2.2 mm DV relative to bregma) with a glass micropipette. A pump maintained the infusion rate at 0.15 µl/min. The pipette was left in place for 5 min after each infusion. Mice were postoperatively treated with ketoprofen (5 mg/kg) and perfused 4 weeks later.

Behavioral Testing

Incidental Context Learning Paradigm. Incidental context learning occurred in test chambers (31 cm × 24 cm × 21 cm; Med Associates) with shock-grid floors (bars 3.2 mm in diameter spaced 7.9 mm apart). The front, top and back of the chamber were clear acrylic and the two sides were modular aluminum. During the context pre-exposure session, mice were placed in the chambers for 10 min. During the immediate shock session, mice were returned to the chambers, and a single foot shock was immediately delivered (≤ 1 s after entry, 1 mA, 2 s duration). Mice were removed from the chambers after 1 min. During the test session, mice were placed in the chambers for 3 min. Behavior was recorded by overhead cameras. Freezing (i.e. absence of movement except for breathing) was measured using an automated scoring system (Actimetrics).

Standard Contextual Fear Conditioning. Standard contextual fear conditioning occurred in the same chambers as described above. During training, mice were placed in the chambers,

and 3 foot shocks (0.5 mA, 2 s duration, 1 min apart) were delivered after 2 min. Mice were removed 1 min after the last shock. During testing, mice were placed in the chambers for 5 min.

Water Maze. A circular plastic pool (120 cm diameter, 50 cm height) was filled to a depth of 40 cm with water (~26º C) made opaque by the addition of nontoxic paint. A circular escape platform (10 cm diameter) was submerged 0.5 cm below the surface of the water in the center of one of the pool quadrants. The pool was surrounded by curtains that were located at least 1 m from the pool wall and painted with distinct geometric cues. During training, mice were given 3 trials per day for 4 days. Trials started when mice were released into the pool, facing the wall, from one of 4 possible points. A different release point was used for each trial on each day, and the order of release points varied pseudorandomly across days. Trials ended when mice reached the hidden platform or 60 s elapsed. If a mouse failed to find the platform, it was guided by the experimenter. During reversal training, the hidden platform was moved to the center of the opposite quadrant, and mice were given 3 trials per day for 2 days. During the probe tests, the platform was removed from the pool, and mice were allowed to swim for 60 s. Swim paths were recorded by an overhead video camera and tracked using automated software (Actimetrics). Latency to reach the platform was recorded during training, and time spent in a circular zone (15 cm radius) centered on the platform location was measured during the probe tests. Density plots depicting areas of the pool visited more frequently during the probe tests were generated using a custom program (Matlab) developed in our laboratory.

Barnes Maze. A white circular platform (100 cm diameter, 70 cm above the floor) contained 12 holes equally spaced around its perimeter. Under one of the holes, there was a blackPlexiglas escape box (17 × 13 × 7 cm) filled with paper bedding. The location of this

escape hole was consistent for a given mouse but randomized across mice. To prevent navigation based on olfactory or proximal cues within the maze, the platform was rotated before each trial, and the spatial location of the escape hole remained in a fixed location with respect to the distal room cues. During habituation, mice were allowed 5 min to freely explore the maze, with no escape box present. During training, mice were given 3 trials per day for 6 days. On each trial, mice were released in the center of the maze and allowed 5 min to enter the escape box, where it remained for 30 s. If a mouse failed to find the escape box, it was guided by the experimenter. During probe tests, the escape box was removed from the maze, and mice were allowed to search for 3 min. Time spent around each hole was recorded. Search paths were recorded by an overhead video camera and tracked using automated software (Image BM) based on the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/) and custom modifications of ImageJ software (http://rsbweb.nih.gov/ij/).

Conditioned Taste Aversion. During habituation, water-restricted mice were placed in individual cages and given access to two bottles containing tap water for gradually decreasing amounts of time across days: 4 hours on day 1, 2 hours on day 2, 1 hour on day 3, and 30 min on days 4 and 5. During conditioning, the water bottles were replaced by a single bottle containing a saccharin solution (0.1% saccharin in tap water). Mice were allowed to drink for 30 min and then injected with LiCl (0.30M in PBS, 2% body weight, i.p.). During testing, mice were given access to one bottle containing tap water and one bottle containing saccharin solution. During the delay between conditioning and testing, mice were kept on water restriction for 4 days each week, during which they were placed in individual cages and given access to two water bottles for 1 hour a day. Throughout the experiment, a limited amount of wet food was provided daily in the home cage to maintain ~90% of baseline body weight.

Histology

Tissue Preparation. Mice were perfused transcardially with PBS followed by 4% paraformaldehyde (PFA). Brains were post-fixed in PFA and transferred to 30% sucrose. Coronal sections (50 µm) were cut along the entire anterior-posterior extent of the DG using a cryostat. For Calcineurin and Schnurri-2 mice, 8 µm sections were cut. Sections were kept in sequential order and stored free-floating in 50% glycerol and 10% ethylene glycol in PBS. A 1/4 section sampling fraction was used to create four sets (each containing sections at 200 µm intervals) for immunohistochemistry.

Immunohistochemistry. Unless otherwise stated, all incubations occurred at room temperature.

For Ki67 labeling, sections were pre-treated with 0.01M citrate buffer (pH 6.0) in a 97º C steamer for 1 h followed by 3% H_20_2 and 20% methanol in PBS for 10 min. Sections were then incubated with primary (rabbit anti-Ki67, 1:1000, Abcam) and secondary (biotinylated goat anti-rabbit, 1:1000, Jackson ImmunoResearch) antibodies. $Ki67^+$ cells were visualized using avidin-biotin-peroxidase complex (ABC, Vector Laboratories) followed by diaminobenzidine (DAB, Sigma).

For DCX labeling, sections were pre-treated with 0.01M citrate buffer (pH 6.0) in a 97º C steamer for 1 h. Sections were then incubated with primary (rabbit anti-DCX, 1:1000, Cell Signalling) and secondary (biotinylated goat anti-rabbit, 1:2000, Jackson ImmunoResearch) antibodies. DCX^+ cells were visualized using ABC, then signal amplification using a TSA system (Invitrogen), followed by SAV Alexa-488 (Invitrogen). DAPI (1:10000, Sigma) was used as a counterstain.

For c-Fos labeling, sections were pre-treated with 0.01M citrate buffer (pH 6.0) in a 97º C steamer for 1 h followed by 3% H_20_2 and 20% methanol in PBS for 10 min. Sections were then incubated with primary (rabbit anti-c-Fos (Ab-5), 1:5000, Abcam) and secondary (biotinylated goat anti-rabbit, 1:1000, Jackson ImmunoResearch) antibodies. c-Fos$^+$ cells were visualized using avidin-biotin-peroxidase complex (ABC, Vector Laboratories) followed by diaminobenzidine (DAB, Sigma).

For YFP/GFP and ZnT3 double-labeling, sections were incubated with primary (rabbit anti-GFP, 1:1000, Invitrogen; goat anti-ZnT3, 1:1000, Sigma) and secondary (donkey anti-rabbit Alexa-488, 1:1000; donkey anti-goat Alexa-568, 1:1000, Invitrogen) antibodies. DAPI was used as a counterstain.

For BrdU labeling, sections were pre-treated with 1N HCl at 45°C for 30 min. Sections were then incubated with primary (rat anti-BrdU monoclonal, 1:1000, Accurate Chemicals) and secondary (Alexa-488 goat anti-rat, 1:1000, Invitrogen) antibodies.

For TUNEL labeling, sections were pre-treated with 0.01M citrate buffer (pH 6.0) in a 97º C steamer for 1 h. Sections were then processed using the DeadEnd Colorimetric TUNEL System (Promega). Methyl green was used as a counterstain.

For LacZ labeling, sections were incubated with primary (rabbit anti-Laz, 1:1000, Invitrogen) and secondary (donkey anti-rabbit Alexa-488, 1:1000, Invitrogen) antibodies.

Imaging and Quantification. Ki67$^+$, DCX$^+$ and LacZ$^+$ cell quantification was performed using Olympus BX61 microscope and StereoInvestigator 9.0 software (MBF Bioscience). The total number of labeled cells in the DG was estimated using the optical fractionator technique. With a 60× oil-immersion objective (Olympus, N.A. 1.43), labeled cells were counted inside 90 µm × 90 µm counting frames equally spaced across a 250 µm × 250 µm grid with a 20

μm optical dissector height. The counting parameters were chosen to achieve a Gundersen coefficient of error less than 0.1 (*7*).

For quantification of YFP^+ LMTs, DCX^+ LMTs, and $ZnT3^+$ puncta, a LSM-710 confocal microscope with a 20× objective (Zeiss, N.A. 0.8) was used to acquire serial Z-stack images (step: 0.8 μm) of the CA3 region at a resolution of 1024 × 1024 pixels (212 × 212 μm, digital zoom: 1×). Using imageJ software (http://rsbweb.nih.gov/ij/), labeled structures were counted in a 70 × 70 μm square within the stratum lucidum of the CA3b region. Counts were expressed as number of LMTs per 5000 μm^3.

Three-dimensional reconstructions of GFP^+ LMTs were created using an LSM-710 confocal microscope with Zen 2009 software (Zeiss). With a 63× oil-immersion objective (Zeiss, N.A. 1.40), serial Z-stack images were acquired (step: 0.25 μm). The contours of the GFP^+ LMT and $ZnT3^+$ zone were traced in 3 dimensions using Neurolucida 9.1 software (MBF Bioscience), and surface reconstruction was performed using the 3D visualization module.

$BrdU^+$, $TUNEL^+$, and c-Fos^+ cell quantification was performed manually using a Nikon microscope. With a 40× objective, $BrdU^+$ cells were counted in the granule cell layer, $TUNEL^+$ cells were counted from the entire DG, and c-Fos^+ cells were counted separately from the CA3 and the DG. An estimate of the total number of labeled cells per DG was obtained by multiplying the number of cells counted by 4. For Calcineurin and Schnurri-2 mice, counts were expressed as number of labeled cells per mm^2 or per section, respectively.

Specific experimental procedures

Age-dependent changes in hippocampal neurogenesis. To track age-dependent changes in neurogenesis, GFP-expressing retrovirus was infused into the DG of mice at P17

or P60. Mice were perfused 28 days later, and sections were stained for GFP. In a second experiment, mice were perfused at P17 (n = 5), P28 (n = 5), P60 (n = 4), P120 (n = 4), or P180 (n = 4), and sections were stained for Ki67 and DCX.

Age differences in synaptic remodeling. We developed a computational model to estimate differences between P17 and P60 mice in the amount of DG-CA3 synaptic remodeling occurring across time. First, the number of $Ki67^+$ cells in the DG was estimated by fitting a double exponential curve ($Y = (a \times \exp(-b \times X)) + (c \times \exp(-d \times X))$, $r^2 = 0.91$) to the data shown in Fig. 1C. Thirty percent of $Ki67^+$ cells were assumed to survive and mature into granule cells that project mossy fibers to the CA3 and grow large mossy terminals (LMTs) onto CA3 pyramidal cells within 14 days (*8, 9*). Next, the number of LMTs making contact with CA3 cells was calculated by multiplying the number of LMTs per DG cell (n = ~14 (*10, 11*), generated by a Gaussian function) by the number of DG cells added each day. Finally, the number of new LMTs making contacts with CA3 cells was divided by the total number of LMTs per CA3 cell (n = 50 (*10, 11*)). A CA3 cell was defined as being remodeled if 2-4% of its synaptic inputs were altered by new neuron addition, and cumulative predicted synaptic remodeling was then expressed as the proportion of CA3 cells (from a total of 250,000 *(12)*) with altered synaptic inputs as a consequence of new neuron addition..

Age differences in memory stability. To track age-dependent changes in memory persistence, mice were trained in the incidental context learning paradigm on P17 or P60. Shock and test sessions then occurred either 1 (P17 n = 13, P60 n = 13), 7 (P17 n = 13, P60 n = 15), 14

(P17 n = 13, P60 n = 14), or 28 (P17 n = 15, P60 n = 14) days later. Age-matched groups of mice (1 day: P17 n = 10, P60 n = 8; 7 days: P17 n = 9, P60 n = 10; 14 days: P17 n = 9, P60 n

= 8; 28 days: P17 *n* = 9, P60 *n* = 9) were not exposed to the context prior to shock and test sessions. To determine whether context exposure activates the hippocampus in infant mice, additional groups of P17 mice were perfused 90 min after context exposure (*n* = 5) or immediately after removal from the home cage (*n* = 5), and sections were stained for c-Fos. To assess the generality of observed infantile forgetting, we trained other groups of infant and adult mice in standard contextual fear conditioning on P17 or P60. Mice were subsequently tested 1 (P17 *n* = 9, P60 *n* = 9), 7 (P17 *n* = 9, P60 *n* = 8), 14 (P17 *n* = 9, P60 *n* = 8), or 28 (P17 *n* = 9, P60 *n* = 9) days later.

Effect of running on adult hippocampal neurogenesis. To assess whether voluntary running increases neurogenesis, mice remained sedentary or were allowed access to a running wheel for 0 (sedentary *n* = 8, running *n* = 8), 3 (sedentary *n* = 8, running *n* = 8), 7 (sedentary *n* = 8, running *n* = 8), 14 (sedentary *n* = 8, running *n* = 8), or 28 (sedentary *n* = 8, running *n* = 8) days starting at P60. Mice were then perfused, and sections were stained for Ki67 and DCX. In another experiment, GFP-expressing retrovirus was infused in the DG on P60. Mice then ran for 28 days before being perfused, and sections were stained for GFP and ZnT3 and used for 3-dimensional reconstruction of pre-synaptic terminals in CA3. Finally, nestin-cre^{ERT2} × YFP reporter mice were injected with TAM starting at P55, and then remained sedentary (*n* = 6) or ran (*n* = 6) for 28 days starting at P60. Mice were then perfused, and sections were stained for GFP and ZnT3.

Effect of running on hippocampal cell death. At P30, mice were injected with BrdU for 5 days. Starting at P60, mice remained sedentary (*n* = 8) or ran (*n* = 8) for 7 days. Mice were then perfused, and sections stained for BrdU and TUNEL.

Effect of running-induced increase in neurogenesis on memory. Mice were trained in the incidental context learning paradigm on P60, then remained sedentary (*n* = 16) or ran (*n*

= 16) for 28 days, and then given shock and test sessions. Separate groups of mice remained sedentary (n = 12) or ran (n = 12) for 28 days before training, shock, and test sessions. Mice were perfused after testing, and sections were stained for Ki67. In another experiment, nestin-cre^{ERT2} × Rosa-LacZ reporter mice were injected with TAM starting on P55. Half of the mice underwent standard contextual fear conditioning on P60, then remained sedentary (n = 9) or ran (n = 8) for 42 days, and then were tested. The other half remained sedentary (n = 9) or ran (n = 8) for 42 days before conditioning and testing. Mice were perfused after testing, and sections were stained for LacZ.

Neurogenesis-dependent versus -independent effects of running on memory. To determine whether data from the incidental context learning experiment support a neurogenesis-dependent or neurogenesis-independent effect of running on memory, a Bayesian causal model based on the work of Lazic (*13*) was used. The model was implemented in the R software environment (version 2.14.1). Model parameters were estimated via Markov-Chain Monte-Carlo Gibbs sampling using the OpenBUGS and R2OpenBUGS software packages (*14*). The equations describing the model were

$$G \sim N(\mu_G, \sigma_G^2)$$
$$F \sim N(\mu_F, \sigma_F^2)$$
$$\mu_G = \alpha_1 + \beta_1 R$$
$$\mu_F = \alpha_2 + \beta_2 RT + \beta_3 GT$$

where G was the level of neurogenesis (as measured by Ki67 staining); F was the amount of freezing during testing; R was assignment to running or sedentary groups; T was assignment to the running before training or running after training conditions; μ_G and μ_F were the means for neurogenesis levels and freezing, respectively; and α_i, β_i, σ_G, and σ_F were parameters estimated by Gibbs sampling. Non-informative priors were used; α_i and β_i were given

Normal priors with means of zero and variances of 0.00001, whereas σ_G and σ_F were given Gamma priors with shape and scale parameters of 0.001. Three chains were run for 50,000 iterations with a burn-in of 25,000 iterations. Chain convergence and mixing were verified by inspecting the sampled values for the parameters across iterations (Fig. S9a). The resulting estimates of the posteriors for $\beta_1 \times \beta_3$ and β_2 were used to assess the probability of neurogenesis-dependent forgetting, $P(\beta_1 \times \beta_3 < 0)$, and neurogenesis-independent forgetting, $P(\beta_2 < 0)$, respectively (Fig. S9b).

Specificity of running-induced forgetting. Mice were trained in the incidental learning paradigm on P60. Separate groups either remained sedentary for 28 days (n = 28), were given a locked running wheel for 28 days (i.e., no running, n = 10), or ran for 7 (n = 13), 14 (n = 16), or 28 (n = 13) days; shock and test sessions occurred 28 days after context pre-exposure. In a second experiment, mice were trained in the incidental context learning paradigm on P60. Separate groups either remained sedentary for 8 weeks (n = 10, n = 11), ran for 14 days immediately after training (n = 14), or ran for 14 days late after training (i.e., running started 28 days after training, n = 10); shock and test sessions occurred 56 days after context pre-exposure. In a third experiment, mice were trained in the incidental learning paradigm on P60 and then remained sedentary (n = 16) or ran (n = 16) for 14 days; shock and test sessions occurred 14 days after context pre-exposure. In a fourth experiment, mice underwent taste aversion conditioning on P60 and then remained sedentary (n = 12) or ran (n = 12) for 28 days; a test session occurred 28 days after conditioning.

Adaptiveness of running-induced forgetting. Starting at P60, mice were trained to locate a hidden platform in the water maze. An initial probe test occurred immediately after training.

Mice then remained sedentary (n = 16) or ran (n = 15) for 28 days, followed by a second

probe test. Finally, the hidden platform was moved to the opposite quadrant, and mice underwent re-training followed by a third probe test.

Effect of chemical-induced elevation of neurogenesis on memory. Mice were trained in the incidental context learning paradigm on P60, then received weekly injections of MEM (n = 10) or vehicle (n = 10) for 4 weeks, and then given shock and test sessions. In a second experiment, mice underwent standard contextual fear conditioning on P60. Separate groups were then either given a single injection of MEM (n = 8) or vehicle (n = 8) 24 hours after conditioning with a test session 48 hours after conditioning, weekly injections of MEM (n = 8) or vehicle (n = 8) for 6 weeks with a test session 1 week after the final injection, or weekly injections of MEM (n = 8) or vehicle (n = 8) for 6 weeks with a test session 3 weeks after the final injection. Mice were perfused after testing, and sections were stained for DCX. In a third experiment, mice were subcutaneously implanted with FLX (n = 8) or placebo (n = 10) pellets on P70 and then trained to locate an escape box in the Barnes maze. Probe tests occurred 2 and 30 days after training.

Effect of genetic-induced elevation of neurogenesis on memory. Calcineurin$^{-/-}$ (n = 8) or control (n = 53) mice were trained to locate an escape box in the Barnes maze on P60. Probe tests occurred 2 and 30 days after training. In a second experiment, Schnurri-2$^{-/-}$ (n = 6) or control (n = 6) mice were trained to locate an escape box in the Barnes maze on P60. Probe tests occurred 2 and 30 days after training. Separate groups of mice were injected with BrdU starting on P60 and perfused the day after the final injection.

Effect of chemical-induced reduction in neurogenesis on memory in infants. Mice were trained in the incidental context learning paradigm on P17, then given 4 rounds of injection with vehicle (n = 30) or TMZ (n = 29), and then given shock and test sessions. Separate groups of

mice given 4 rounds of injection with vehicle (n = 22) or TMZ (n = 22) before training, shock, and test sessions. Mice were perfused after testing, and sections were stained for Ki67 and DCX.

Statistical Analysis

Data were analyzed using ANOVA and two-tailed t-tests, with alpha set at 0.05. To examine individual-level relationships between measures of neurogenesis and memory, Pearson's r was reported after using nonlinear regression to calculate exponential rate of decay ($y = A \times e^{(-k \times x)}$).

SUPPLEMENTAL FIGURES

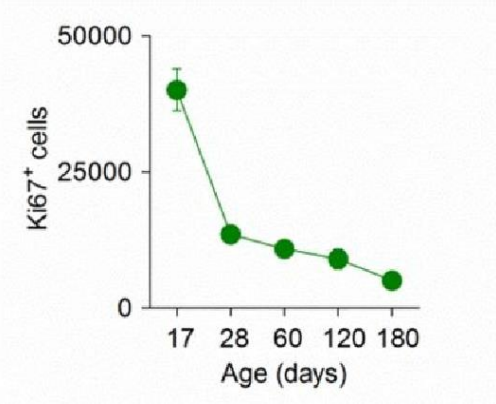


Figure S1. The number of Ki67$^+$ cells in the DG decreased with age (*age* main effect, $F_{4,17} = 43.64$, $P < 0.001$).

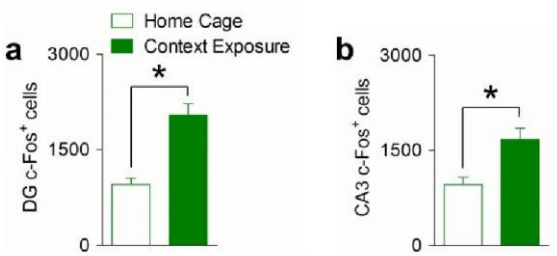


Figure S2. Infant mice that were exposed to a context showed greater activation of the DG (**a**, $t_8 = 5.43$, $P < 0.001$) and CA3 (**b**, $t_8 = 3.32$, $P < 0.05$) compared to home cage control

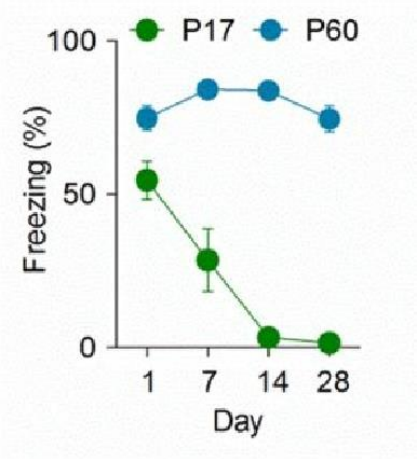


Figure S3. Adult mice retained a context fear memory for at least 28 days, but infant mice showed forgetting of the context fear memory (*age* × *day* interaction, $F_{1,62} = 13.08$, $P < 0.001$).

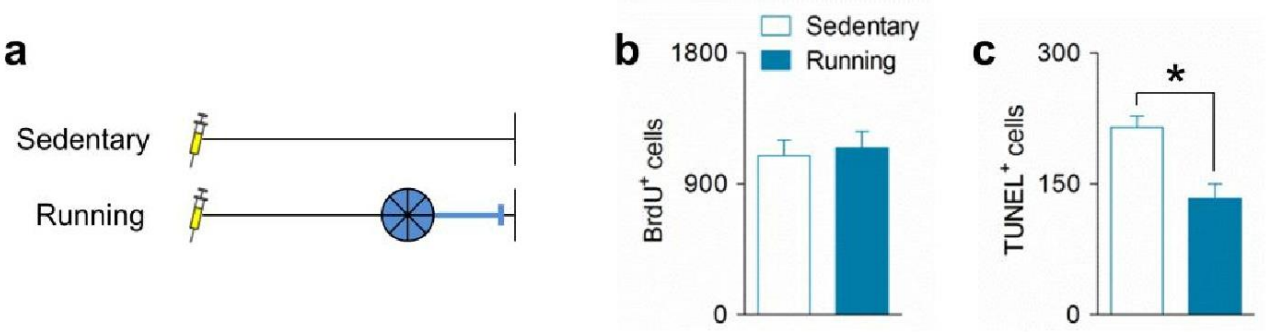


Figure S4. Adult mice remained sedentary or ran after BrdU injection at P30 (**a**). Compared to sedentary mice, running mice showed equivalent numbers of surviving $BrdU^+$ cells (**b**) and fewer numbers of cells undergoing apoptosis (i.e., $TUNEL^+$; **c**, $t_{14} = 3.83$, $P < 0.01$).

These results indicate that running-induced increases in neurogenesis are not associated with increased death of granule cells generated earlier during development.

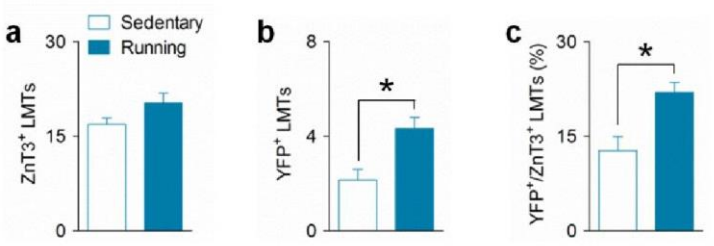


Figure S5. Sedentary and running adult mice had equivalent numbers of LMTs in the CA3 (**a**, per 5000 µm^3), but running mice showed an increase in number (**b**, per 5000 µm^3, t_{10} = 2.67, $P < 0.05$) and proportion (**c**, t_{10} = 2.47, $P < 0.05$) of newly-generated LMTs.

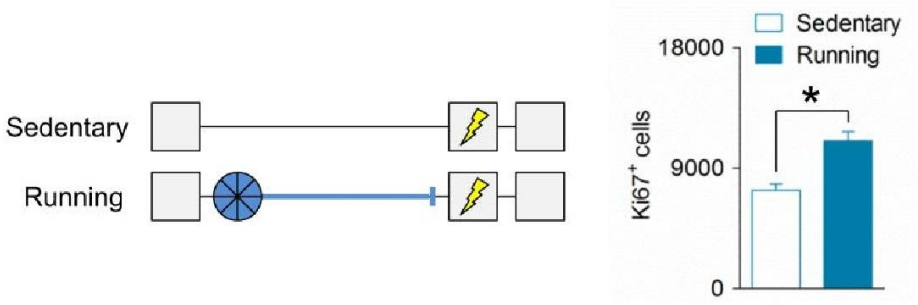


Figure S6. Adult mice that ran after context pre-exposure showed more Ki67$^+$ cells in the DG compared to sedentary mice (t_{28} = 4.65, $P < 0.001$).

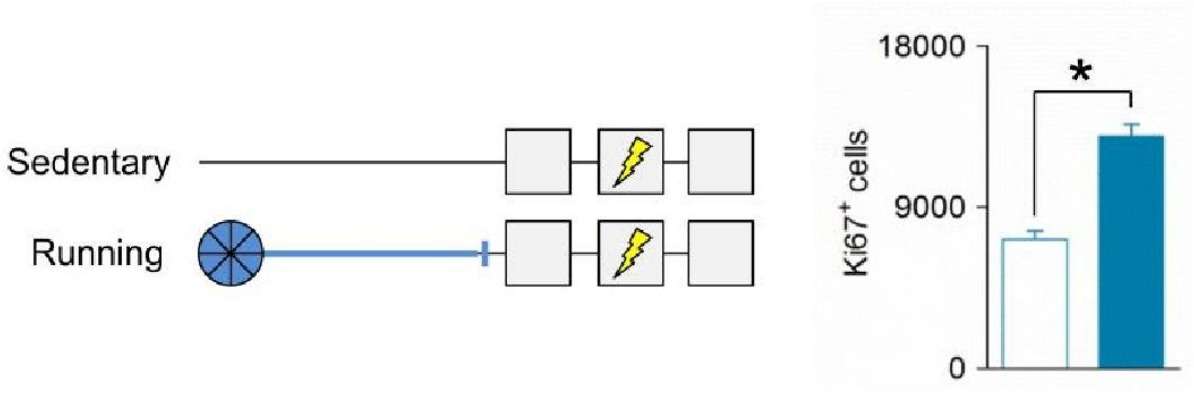


Figure S7. Adult mice that ran before context pre-exposure showed more Ki67^{+} cells in the DG compared to sedentary mice (t_{22} = 6.90, P < 0.001).

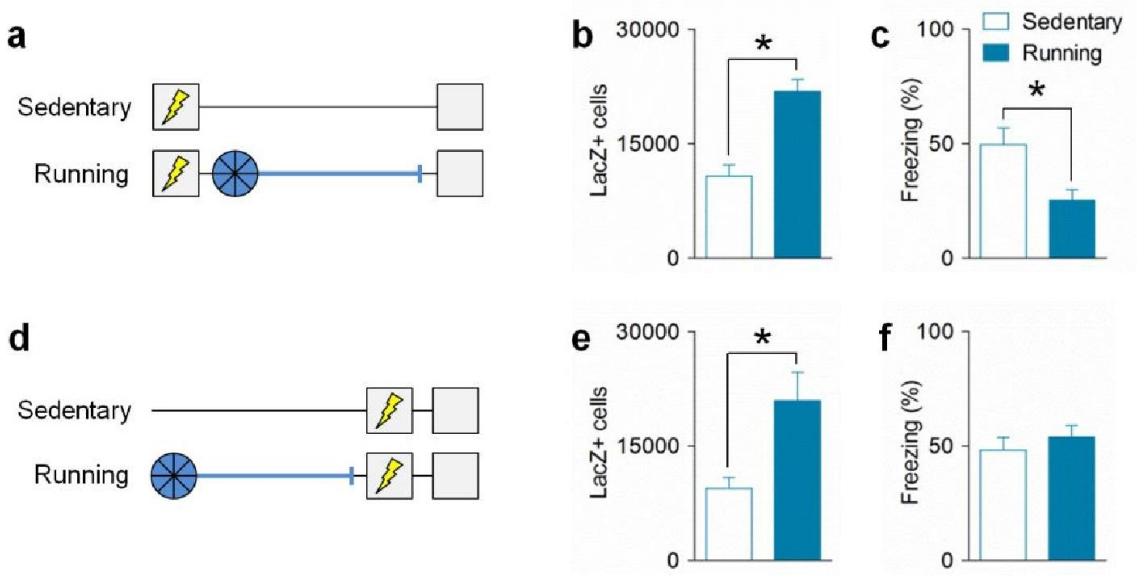


Figure S8. Adult mice that ran after standard contextual fear conditioning (**a**) had more new LacZ^{+} neurons in the DG (**b**, t_{15} = 2.94, P < 0.01) and showed forgetting of the context fear memory (**c**, t_{15} = 2.75, P < 0.05). In contrast, adult mice that ran before contextual fear conditioning (**d**) had more new LacZ^{+} neurons in the DG (**e**, t_{13} = 2.91, P < 0.05) but showed no change in freezing (**f**)

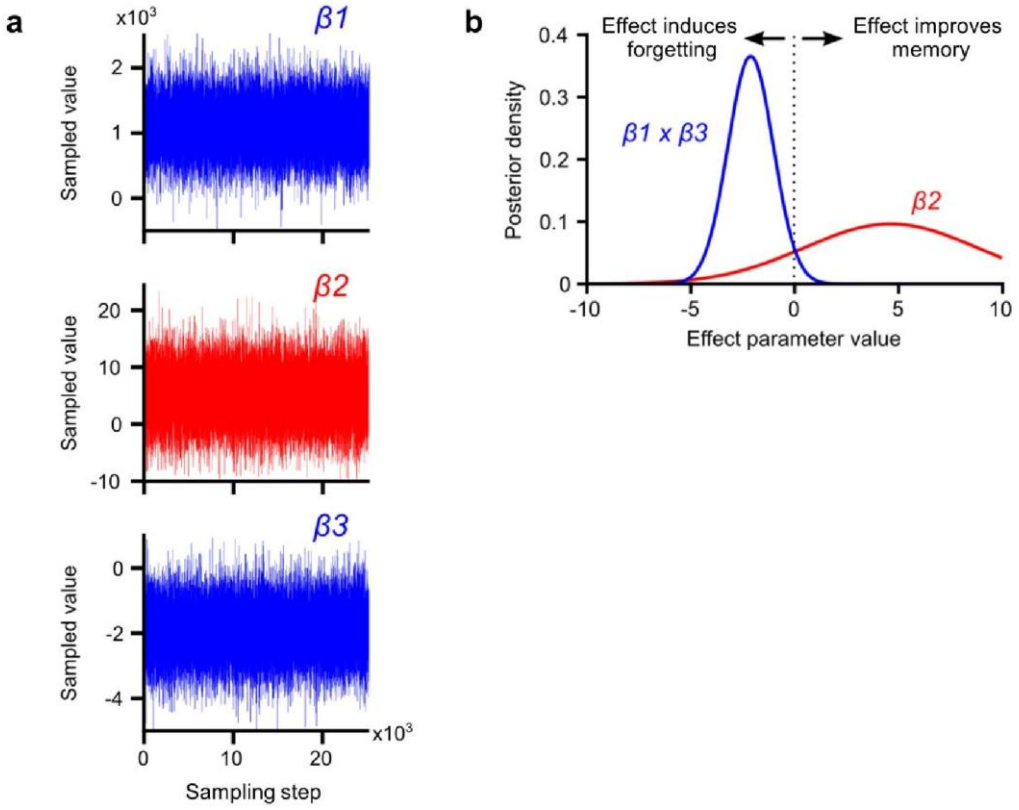


Figure S9. Bayesian causal model analysis was used to estimate the probability that running-induced forgetting was mediated by a neurogenesis-dependent and/or neurogenesis-independent mechanism. (**a**) Sample values for the three parameters (β1, β2, β3) from the

25000 steps of Markov Chain Monte Carlo simulation. The stable distribution of values indicates that the model converged. (**b**) Posterior distributions for the neurogenesis-dependent and neurogenesis-independent effects. These distributions suggest that forgetting is a result of a neurogenesis-dependent mechanism.

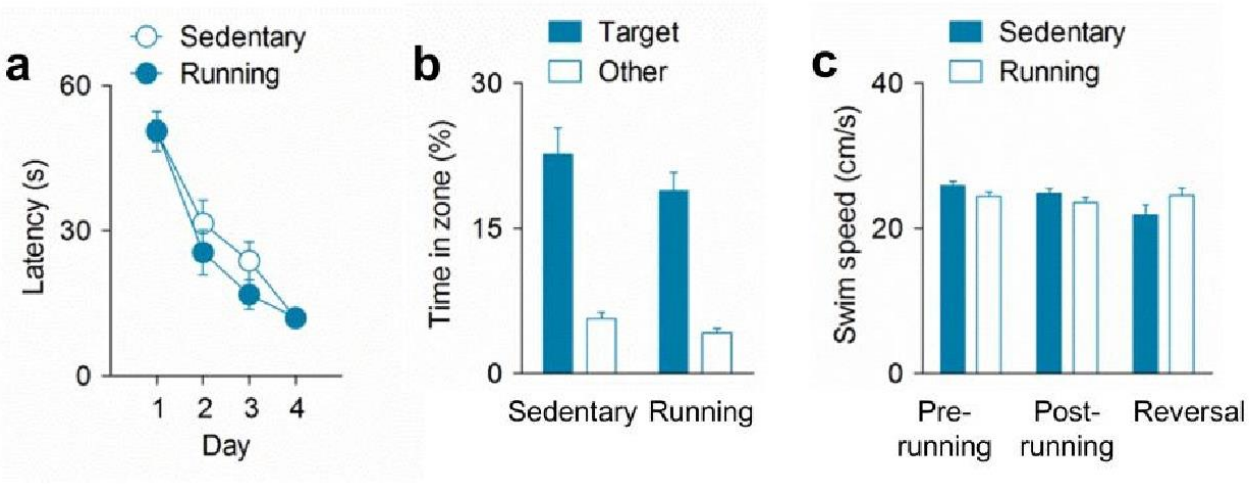


Figure S10. Running and sedentary adult mice showed equivalent learning of the hidden platform location during water maze training (**a**), equivalent spatial bias toward the platform location (Target) during an initial probe test (**b**), and equivalent swim speed during the initial, post-running, and reversal probe tests (**c**).

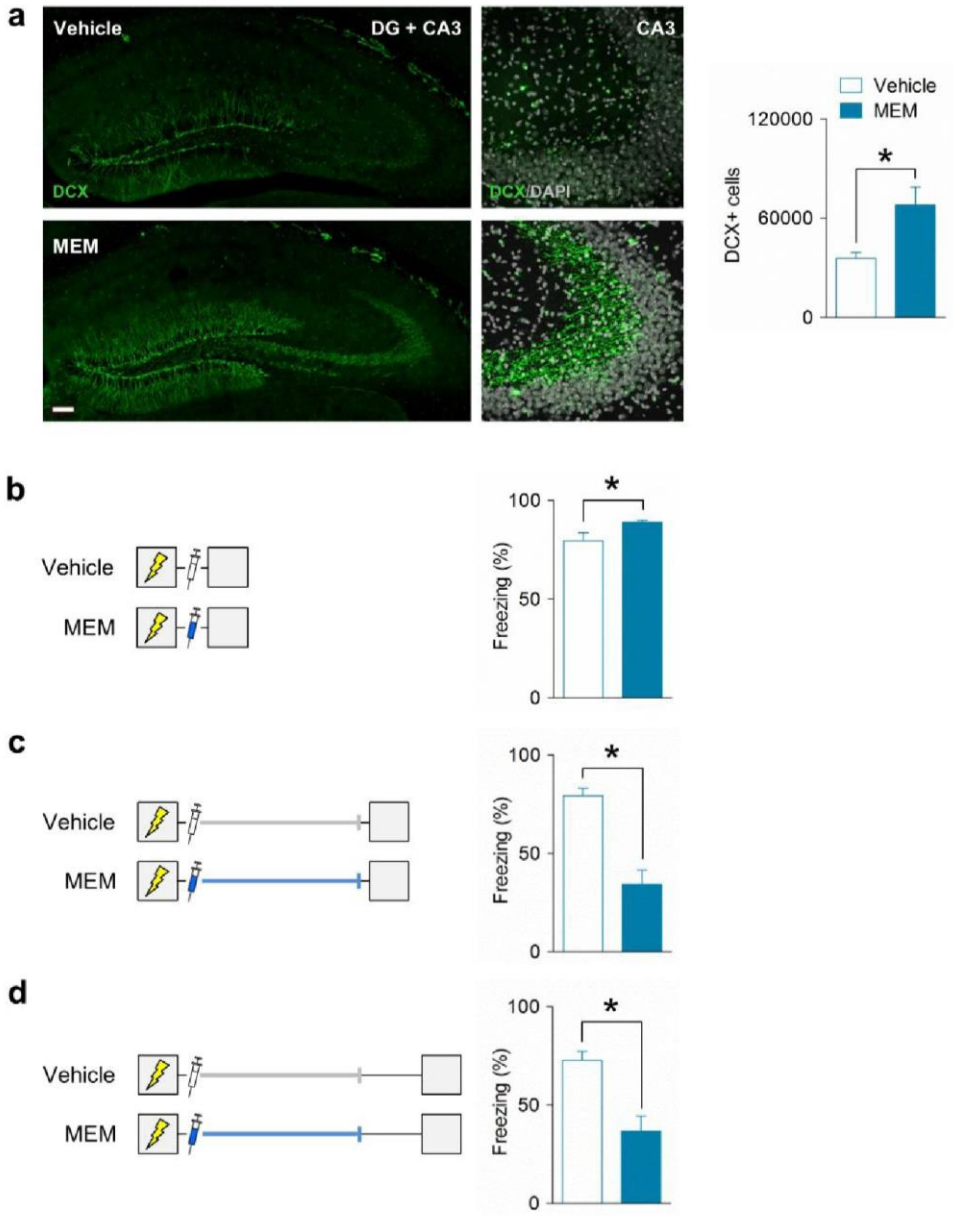


Figure S11. Prolonged MEM treatment increased the number of DCX$^+$ cells in the DG (a, left, scale bar = 100 µm; $t_{11} = 3.03$, $P < 0.05$) and LMTs in the CA3 (a, right, scale bar = 50 µm) of adult mice. A single MEM injection did not disrupt initial consolidation of a context fear memory (b; $t_{14} = 2.26$, $P < 0.05$), but prolonged MEM treatment resulted in forgetting of a context fear memory 6 weeks (c; $t_{14} = 5.47$, $P < 0.001$) or 8 weeks (d; $t_{12} = 4.11$, $P < 0.001$) later.

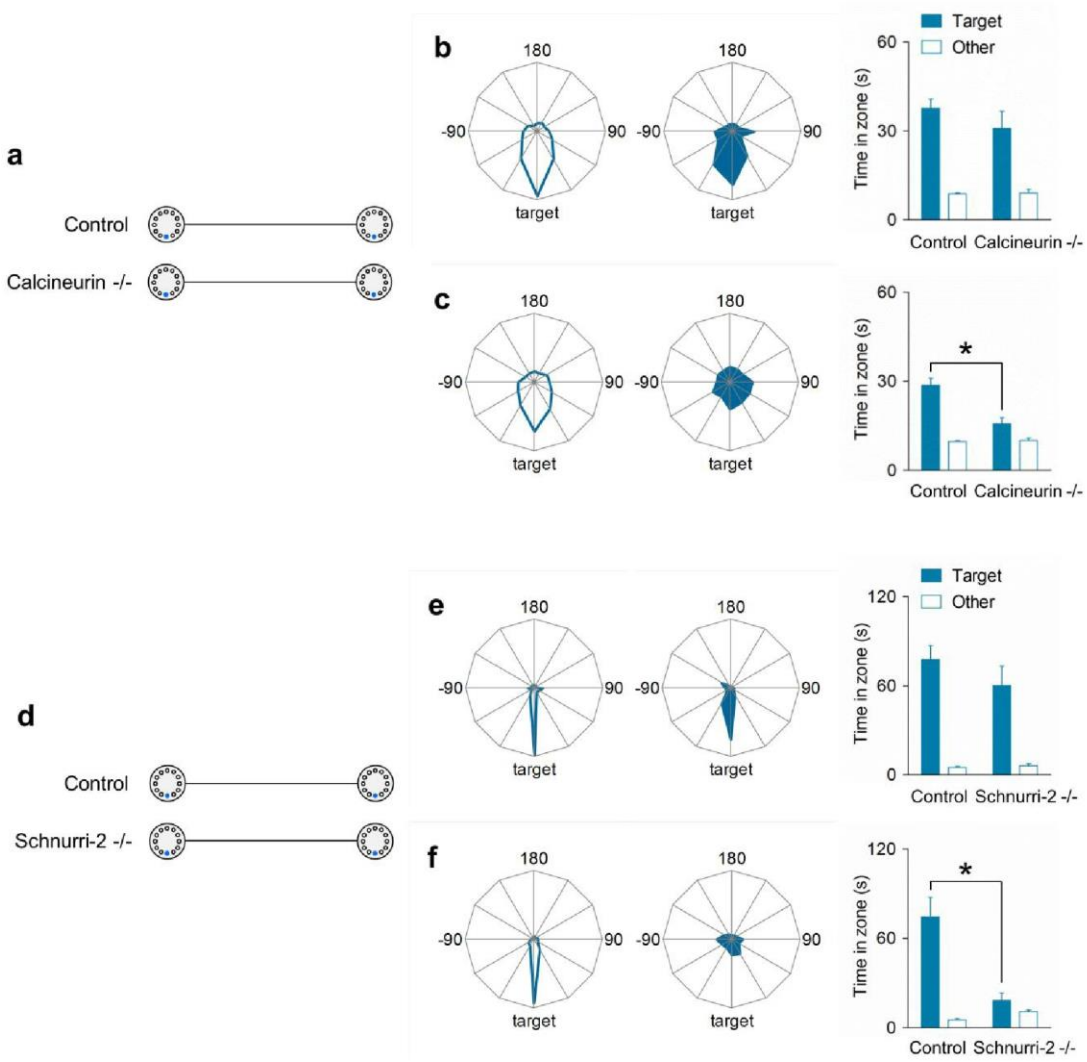


Figure S12. Adult control or Calcineurin$^{-/-}$ mice with a 69% elevation of DG neurogenesis (BrdU^{+} cells/mm^2, control: 22.99 ± 2.27, Calcineurin$^{-/-}$: 38.84 ± 5.94, $t_{10} = 2.94$, $P < 0.05$) were trained to locate an escape hole in the Barnes maze (**a**). Immediately after training, control (left) and Calcinceurin$^{-/-}$ (right) mice showed equivalent spatial bias toward the escape location (Target) (**b**), but 30 d after training, Calcinceurin$^{-/-}$ mice (right) showed poorer memory for the escape location compared to control mice (left) (**c**, $t_{55} = 2.24$, $P < 0.05$). Adult control or Schnurri-2$^{-/-}$ mice with a 78% elevation of DG neurogenesis (BrdU^{+} cells/section, control: 5.28 ± 0.53, Schnurri-2$^{-/-}$: 9.39 ± 1.09, $t_4 = 3.38$, $P < 0.05$) were trained to locate an escape hole in the Barnes maze (**d**). Immediately after training, control (left) and Schnurri-2$^{-/-}$ (right) mice showed equivalent spatial bias toward the escape location (**e**), but 30 d after training, Schnurri-2$^{-/-}$ mice (right) showed poorer memory for the escape location compared to control mice (left) (**f**, $t_{10} = 3.96$, $P < 0.01$).

SUPPLEMENTAL REFERENCES

41. M. Arruda-Carvalho, M. Sakaguchi, K. G. Akers, S. A. Josselyn, P. W. Frankland, *J Neurosci* 31, 15113 (Oct 19, 2011).
42. J. Neilson, M. Winslow, E. Hur, G. Crabtree, *Immunity* 20, 255 (2004).
43. T. Takagi *et al.*, *Brain Res* 1108, 88 (2006).
44. T. Takagi, J. Harada, S. Ishii, *Nat Immunol* 2, 1048 (2001).
45. A. Leslie *et al.*, *Transl Psychiatry* 1, doi: 10.1038/tp.2011.38 (2011).
46. S. Stone *et al.*, *J Neurosci* 31, 13469 (2011).
47. H. Gundersen, E. Jensen, K. Kieu, J. Nielsen, *J Microsc* 193, 199 (1989).
48. G. Kempermann, F. H. Gage, *Brain research. Developmental brain research* 134, 1 (Mar 31, 2002).
49. A. G. Dayer, A. A. Ford, K. M. Cleaver, M. Yassaee, H. A. Cameron, *The Journal of comparative neurology* 460, 563 (Jun 9, 2003).
50. D. A. Henze, N. N. Urban, G. Barrionuevo, *Neuroscience* 98, 407 (2000).
51. D. G. Amaral, J. A. Dent, *The Journal of comparative neurology* 195, 51 (Jan 1, 1981).
52. M. Hosseini-Sharifabad, J. Nyengaard, *J Neurosci Methods* 162, 206 (2007).
53. S. E. Lazic, *J R Soc Interface* 9, 907 (2012).
54. D. Lunn, D. Spiegelhalter, A. Thomas, N. Best, *Stat Med* 28, 3049 (2009).