CHARLES UNIVERSITY 2ND MEDICAL FACULTY
INSTITUTE OF EXPERIMENTAL MEDICINE
CZECH ACADEMY OF SCIENCES

DISSERTATION

2007 KATEŘINA NÁMĚSTKOVÁ

IMPAIRMENT OF RAT HIPPOCAMPUS AFTER IRRADIATION WITH LEKSELL GAMMA KNIFE AND EVOKED PROLIFERATION IN GYRUS DENTATUS OF THE ADULT RAT

MUDr. KATEŘINA NÁMĚSTKOVÁ

Supervised by:

Professor MUDr. EVA SYKOVÁ DrSc.

Department of Neuroscience
Second Medical Faculty
Charles University
Prague Czech Republic

Institute of Experimental Medicine
Academy of Sciences of the Czech Republic

ACKNOWLEDGEMENTS

I would like to thank on the first place to my supervisor Professor MUDr. Eva Syková DrSc. for introducing me to the scientific world under strong guidance with patience and support, and for providing the so important means to complete experiments from the beginning to the end. I would like to thank very much to my closest colleagues RNDr. Pavla Jendelová PhD. and RNDr. Zuzana Šimonová PhD. for continuous help and letting me use their experience on setting up new methods publishing our results including the great support during this dissertation writing. My big thanks belong to Lenka Kohoutová, Alena Veselá, Jana Káclová and Hana Kučerová for the exact, reliable and friendly technical support, without which the work would not be finished by today. No results would be published without the "always ready to help" kind support from James Dutt, to whom I am thankful for giving the final correct form to any published English text including this dissertation. I would like to thank to all my colleagues from the Department of Neuroscience on the Second medical faculty and the Institute of Experimental medicine for their help, inspiration, friendship and sharing.

I am grateful to all my colleagues outside the department for including me to the team work and great cooperation on the extensive project with Leksell gamma knife, namely to: MUDr. Roman Liščák Csc., Ing. Dan Jirák PhD., Mgr. Vít Herynek Ph.D., Ing. Milan Hájek DrSc., Prof. MUDr. Gustav Brožek DrSc., Doc. MUDr. Vladimír Mareš DrSc. My thanks belong to Prof. Joseph P. Huston, Dr. Maria Angelica de Souza Silva, Dr. rer. nat. Bianca Topic, Dr. Daniela Schulz for introducing me to the techniques of behavioural research and including me to the international team.

I would like to also thank to my opponents for their constructive comments and suggestions.

Special thanks belong to my closest family for their continuous support and motivation.

ABBREVIATIONS

^{3}H-dT	^{3}H Thymidine
ADCw	apparent diffusion coefficient of water
Ara-c	cytosine-beta-D- arabinifuranoside – antimitotic agent
BDNF	brain derived neurotrophic factor
BMP	bone morphogenic proteins
BrdU	bromodeoxyuridine
C – Fos	one of intermediate early genes
CA 1, 2, 3	cornu ammonis
ChAT	cholinacetyltransferase
Cho	choline
CNTF	ciliary neurotrophic factor
Cr	creatine
CSF	cerebrospinal fluid
Dcx	doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
DWI	diffusion weighted imaging
ECS	electro convulsive shock
EE	enriched environment
EGF	epidermal growth factor
FGF	fibroblasts growth factor
GABA	gamma-aminobutyric acid
GAD-67	glutamic acid decarboxylase 67- GABA synthesizing enzyme
GCL	granular cell layer
GDNF	glial-derived neurotrophic factor
GFP	green fluorescent protein
HF	hippocampal formation
HM	patient H.M.
IGF-I	insulin-like growth factor I

IR	input resistance
Lac/Lip	lactate/lipid ratio
LGK	Leksell gamma knife
LIF	leukemia inhibiting factor
MAM	methylazoxymethanol acetate
MAP2	microtubules associated protein
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MTLE	mesial temporal lobe epilepsy
MTLS	mesial temporal lobe sclerosis
MWM	Morris water maze apparatus
NAA	N-acetyl aspartate
NeuN	neuronal nuclei antibody
NG2	proteoglycan – a marker of common oligodendrocyte precursors
NGF	nerve growth factor
NMDA	N-methyl-D-asparate
NSE	neuron specific enolase
OB	olfactory bulb
PDGF	platelet derived growth factor
PET	positron emission tomography
ppm	parts per million
PSA-NCAM	polysialic acid neural cell adhesion molecule
pSFV	Semliki Forest Virus
RMS	rostral migratory stream
SGZ	subgranular zone
SVZ	subventricular zone
TGF	transforming growth factors
TOAD-64	turned on after division
trk A, B, C	thyrosinkinase receptors
TUJ1	beta Tubulin III
VEGF	vascular endothelial growth factor

CONTENT

1. INTRODUCTION

The hippocampus is a brain structure that has been extensively studied in the context of learning and memory has been considered to be involved in processing of emotion as part of the limbic system.

This organ was initially connected with the sense of smell and around 1900 the role of the hippocampus in memory was noted by Vladimir Bechterev. The significance of this structure for processing of memory was revealed after William Scoville and Brenda Millner released a report in 1957 about their 10 patients who showed impaired short-term memory after bilateral lesions to the medial temporal lobe, including the hippocampus. This form of surgery was introduced in 1954 to treat pharmacoresistant epilepsy, psychosis, and depression with therapeutic success. The severity of the mainly anterograde amnesia was related to the size of the removed medial temporal lobe portion (Scoville and Millner 1957). The most radical surgery was performed on the patient H.M., who has been seen by more than hundred investigators until today (Corkin 2002).

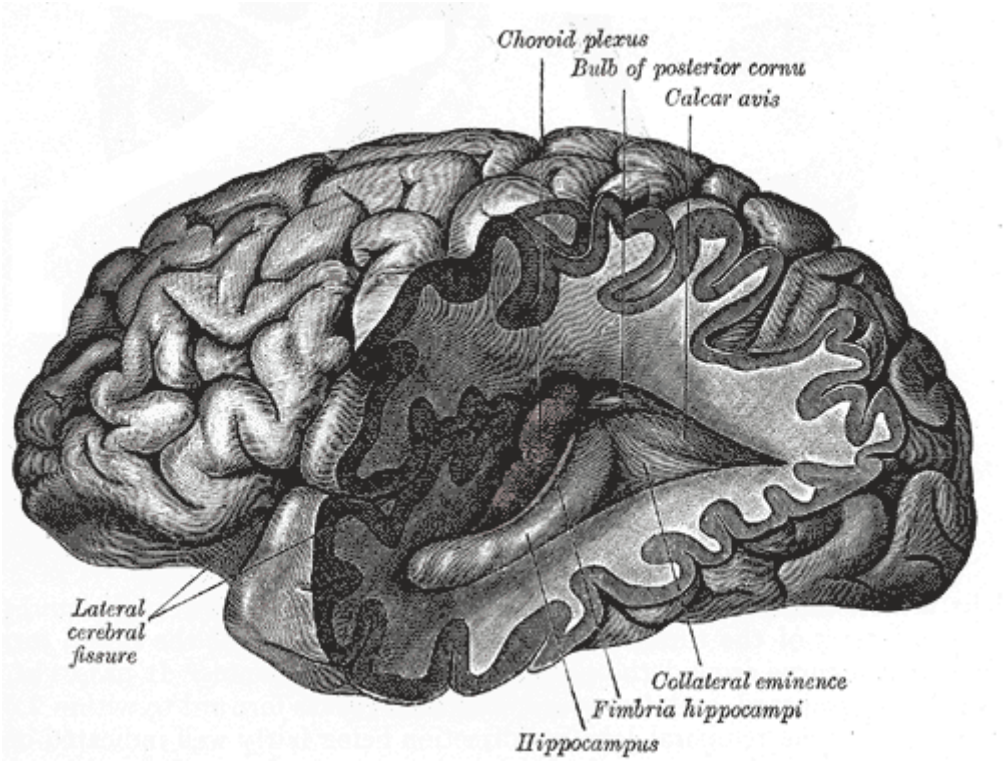

Fig.1 Lithograph plate from Gray's Anatomy showing posterior and inferior cornua of left lateral ventricle exposed from the side including the hippocampus.

The significance of the hippocampus (Fig.1) for processing our memories during every day life has already been partially revealed due to the systematic work of scientists with patient H.M. and other amnesic patients e.g.: D.C., M.B. (Scoville and Millner 1957).

The hippocampus as part of the limbic system is involved in the pathophysiology of mood disorders. Animal and even clinical studies demonstrated that stress and depression lead to reductions of the total volume of this structure due to atrophy and loss of neurons in the adult hippocampus (Frodl et al. 2006). The regulation of adult neurogenesis is one of the cellular mechanisms that could contribute to alterations of hippocampal structure and function. It was demonstrated that neurogenesis is influenced by antidepressants in animal models of depression (Malberg 2004).

Many animal models of hippocampal damage have been developed, and many paradigms to test the impairment of its function have been launched. The significance of adult neurogenesis in hippocampus is extensively being studied.

1.1. ANATOMY OF THE RAT HIPPOCAMPUS

The hippocampus, subiculum and entorhinal cortex together form a functional complex called the hippocampal formation. In the rat brain the hippocampal formation is a prominent structure (Fig.2). Hippocampal formation appears as a “C”-shaped elongated structure, extending from the septal nuclei (located rostrally) over the diencephalon caudoventrally to the temporal lobe. The hippocampus proper (the cornu ammonis and the dentate gyrus) forms the central part of the hippocampal formation. The subiculum lines the dorsal two-thirds of the descending and caudal parts of CA. The entorhinal cortex adheres to the caudo-temporal part of the subiculum.

Fig.2 Drawing of the spatial position of the hippocampus in the rat brain (shaded area) - adapted according to Amaral et al. 1995

The parahippocampal region (perirhinal, parahippocampal and entorhinal cortex) is bi-directionally connected to higher order cortical processing areas (Eichenbaum 2002). For structures within the hippocampal formation, unidirectional neuronal projections are typical (Fig.3). In 1967 Andersen published the idea of trisynaptic circuit. The entorhinal cortex provides the dentate gyrus with its main input, called the **perforant path**. Granule cells of the dentate gyrus project their axons – unmyelinated **mossy fibers** – to the dendrites of large pyramidal cells in the CA3 region (Lawrence et al. 2004). The complex multibranched axonal plexus of pyramidal neurons in the CA3 region projects contralaterally and ipsilaterally as **Schaffer collaterals** to the apical and basal dendrites of the smaller pyramidal cells in the CA1 region. The main efferents from the hippocampus are from the CA1 region projecting to the adjacent subiculum or directly to the entorhinal cortex, where the loop of intrahippocampal unidirectional connections is closed (Amaral et al. 1995). Electrophysiological stimulation of the entorhinal cortex revealed that the neurons within the three subfields of the hippocampus (granule neurons, CA3 and CA1 pyramidal neurons) discharge simultaneously in response to input from a subpopulation of entorhinal cortical neurons. The initial excitation of pyramidal cells is through a monosynaptic connection and is followed by weaker excitatory volleys transmitted through the trisynaptic pathway. The excitation of CA3 and CA1 pyramidal cells can occur in the absence of granule cell excitation, which suggests the significance of monosynaptic afferentation from the entorhinal cortex (Yeckel et al. 1990).

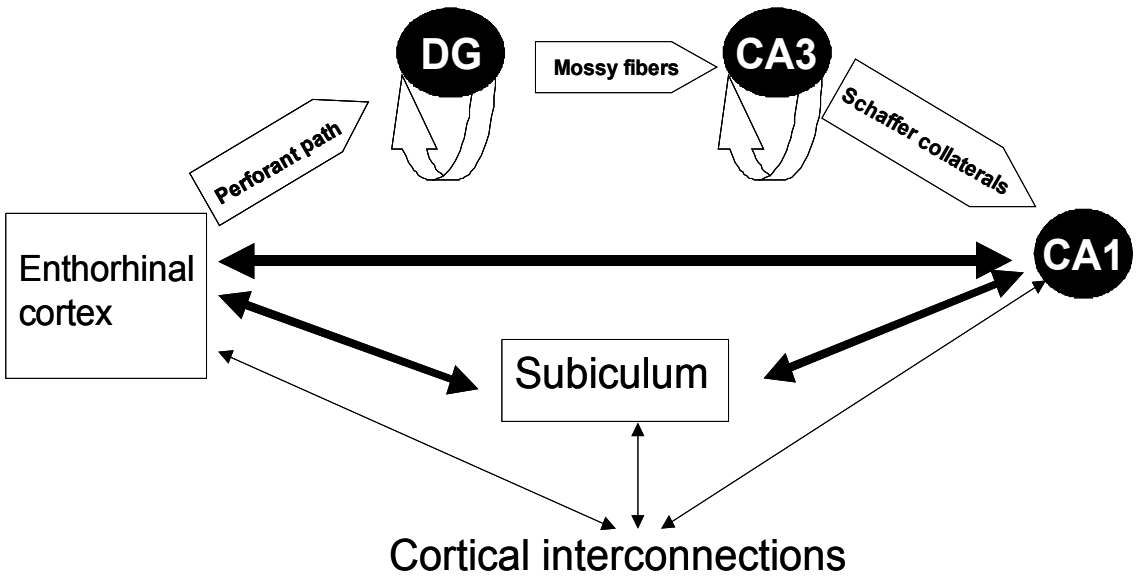

Fig.3 Simplified diagram of connections within the hippocampus, according to Amaral et al. 1995.

A second partly bi-directional modulatory connection is formed between the hippocampus and the subcortical areas via the fornix. There is a *cholinergic* connection originating in the septum, which together with the projection from the hypothalamus terminates primarily in the dentate gyrus (Lawrence et al. 2006). *Noradrenergic* input from the nucleus coeruleus, *dopaminergic* fibers from the ventral tegmentum and *serotoninergic* projections from the raphe nuclei terminate in the dentate gyrus and the hippocampus (CA1, 2 and 3 regions). The serotoninergic axons form synapses with a class of interneurons in the polymorphic and subgranular layers of the dentate gyrus (McMahon et al. 1997).

The hippocampus (CA 1, 2, 3), the dentate gyrus and the subiculum consist of only three layers forming the so-called alocortex. The presubiculum, parasubiculum and entorhinal cortex form the multilaminate retro- or parahippocampal cortex.

1.1.2. CELLS IN THE DENTATE GYRUS

The dentate gyrus consists of a “V”-shaped granular cell layer, which is surrounded by a molecular layer while within its convexity is a polymorphic cell layer called the hilus (or the CA4 due to the extension of the pyramidal cell layer to the hilus). The primary cells are the **granular cells** that form a layer of densely packed nuclei. Spiny dendrites extend to the molecular cell layer receiving afferentation from the perforant path. Seress and Pokorny (1981) observed that granular cells located deeper towards the hilus extend a narrower dendritic tree into the molecular layer than the granule cells located in proximity to the molecular layer. There are prominent presynaptic varicosities on the terminals of the mossy fibers and similarly created spines - “thorny excrescences” - are present as well on the postsynaptic part of the CA3 pyramidal cell dendrites (Seress et al.1995).

The **pyramidal basket cells** are GABAergic interneurons in the granular cell layer; their axons form extensive basket plexuses around the bodies and shafts of the apical dendrites of many granular cells (Seress and Pokorny 1981). A second inhibitory input is mediated by **chandelier cells** that are located in the molecular layer and form axo-axonal contacts with the axons of granule cells. A third inhibitory input was revealed by antibodies to somatostatin (Morrison et al. 1982). There are several somatostatin-positive cells scattered in the polymorphic cell layer, extending their axonal plexus to the molecular layer. The major cell in the polymorphic cell layer is the glutamatergic **mossy cell**, which gives rise to the ipsilateral associational and commissural connections. The dendrites of mossy

cells are covered with very large and complex spines - thorny excrescences - where some of the unmyelinated mossy fibers terminate (Amaral et al. 1995).

1.1.3. CELLS IN THE CORNU AMONIS

The hippocampus proper – the cornu amonis - consists of distinct regions called the **pyramidal cell layers** (CA 1, 2, 3). Pyramidal neurons in CA3 and CA2 are larger than those in CA1. A clear definition of the regions is given by connectivity. **CA3** pyramidal neurons, unlike those in the the CA2, form synapses with the mossy fibers. Mossy fibers travel temporally within the stratum lucidum, which forms an end bulb at the border of the CA3/CA2 region.

The pyramidal neurons in the transitional **CA2 region** are also large but do not receive mossy fiber afferentation. Pyramidal cells in CA2 region show denser labeling for calcium binding protein that provides the cells with greater resistance against excitotoxic cell death (Sloviter et al. 1991). Pyramidal cells have a basal dendritic tree that extends to the stratum oriens and an apical dendritic tree that extends through the stratum radiatum and lacunosum moleculare up to the hippocampal fissure. The total length of all dendrites in the tree can measure up to 16 mm in in the case of **CA1** pyramidal neurons. Several basket cells of various sizes have bodies localized in the pyramidal cell layer and dendritic trees with only a few dendritic spines. The axons form plexus around the pyramidal cell bodies. Several nonpyramidal cells appear in the adjacent lamina (Fig.4). Some of these interneurons are GABAergic and some co-localize with other neuroactive substances (Amaral et al. 1995).

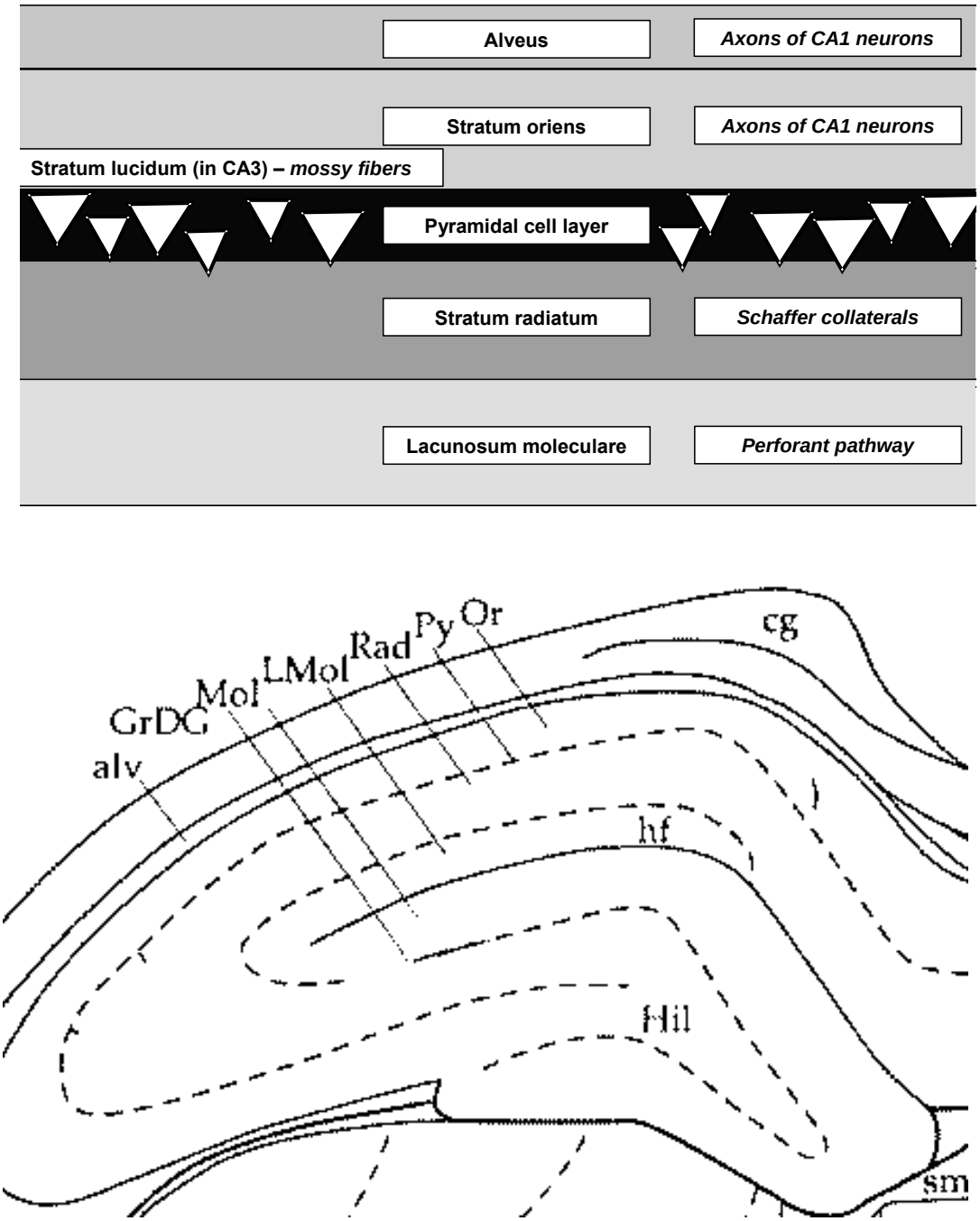

Fig.4 Top: Simplified diagram of the laminar organization of the CA1 region in a coronal section through the rat hippocampus, according to Amaral et al. 1995
Bottom: Drawing (according to Paxinos stereotactic atlas) showing the lamelar organisation of the rat hippocampus on the coronal section. Or - stratum oriens, Py - pyramidal cell layer, Rad - stratum radiatum, LMol - lacunosum moleculare, Mol - molecular cell layer, GrDG - glanular layer of the dentate gyrus, Hil - hilus, alv - alveolus

The mapping of the CA3 pyramidal neurons projections, with help of scanning laser photostimulation of caged glutamate, showed topographic organization. The projections from CA3 to CA1 region are organized so that neighboring cells in CA3 project to neighboring neurons in CA1 (Brivanlou et al. 2004).

1.2. THE HIPPOCAMPUS AND LEARNING

After early reports on H.M. and other amnesic patients, there have been attempts to develop animal models of amnesia. The prior experience of these animals, as well as the exact anatomy of the tissue damage, can be better specified than in a heterogeneous group of patients with cranio-cerebral injury.

1.2.1. THE METHODS FOR STUDYING LEARNING IN RODENTS

Different procedures to perform an ablation of the hippocampus in rats were developed, and their effect on hippocampal damage was tested in a variety of ways (Squire et al. 1996, Jarrad 1995, Morris 1984, Stubley-Weatherly et al. 1995, Hollup et al. 2001, Eijkenboom et al. 1999, Bures et al. 1997).

The first years of developing a model of global amnesia in rats were very disappointing. After hippocampal ablation, the animals showed similar results as did controls on the **conditioning tests** of "shuttle box avoidance". In a "passive avoidance" test, they were able to learn to approach a small compartment containing food, but although they showed a deficit in the avoidance phase (when they re-entered the small chamber again after receiving a foot shock), this was not convincing proof of an impaired ability to learn (Eichenbaum 2002).

The **visual discrimination test** in a Y maze, in which a rat is rewarded for consistently entering one of the arms discriminated by a black or white color, also did not show impaired learning (Eichenbaum 2002).

Those very ambiguous results led to the conclusion that for some simple forms of learning, the hippocampus is not an essential structure. The idea that a higher order form of memory is processed by the hippocampus was proposed by John O'Keefe and Lynn Nadel in 1978. They observed that rats with damage to the hippocampus show impaired exploration and spatial orientation rather than impaired conditioning and discrimination. The observation of cellular units that show activity in relation to the environment supported the following idea: a topological map of the surroundings and the ability to navigate in this environment are processed in the hippocampus (O'Keefe et al. 1978). Large pyramidal place cells were observed in the CA1 and CA3 and were defined by John O'Keefe in 1979 as follows: "a place cell is a cell which constructs the notion of a place in the environment by connecting together several multisensory inputs each of which can be perceived when the animal is in particular part of the environment" (Eichenbaum 2002).

The activity of the place cells provided solid stepping-stones to the cognitive mapping hypothesis, which was pioneered by Tolman in 1948. Further study of

place cells provided evidence that those cells encode the spatial relation to sets of environmental cues that may not be necessarily spatial. Such a cue may be, for example, an odor or even a behavioral event (Eichenbaum 2002).

Damage to the hippocampus caused by conventional lesion methods, such as aspiration or electrolytic radio frequency, has always been accompanied by damage to the adjacent structures of the hippocampal formation, such as the subiculum or the fibers passing through the fimbria (Morris et al. 1990). To avoid this indiscriminate damage, the use of the neurotoxin ibotenic acid to create lesions was proposed by Jarrard in 1989. Small amounts of ibotenic acid were delivered by glass micropipettes to multiple sites in the hippocampus. A stereotactic frame was used to deliver the microinjections exactly to 26 sites within the granular and pyramidal cell layers. This method was able to selectively eliminate most of the pyramidal cells in the CA 1, 2 and 3 regions (Fig. 5) while sparing the fibers and remaining structures of the hippocampal formation (Jarrard et al. 1995). Those selectively hippocampectomised animals were then tested on two very instructive “within subject experiments” in an eight arm maze.

In the intramaze cue task, the floors of the arms were marked by different textures (sandpaper, carpeting, etc); these cues were randomly distributed among the arms between the trials. Rats were rewarded for entering those arms that were always associated with a certain cue on the floor. Direct reflector lightning above the maze left the rest of the room dark and minimized room cues.

In the spatial version of this experiment, there were the same four out of eight arms of the maze consistently baited for each rat, with regards to the position in the room. There were obvious extramaze cues in the room (door, window…), which remained in the same positions throughout the experiment. These experiments made it possible to test **the reference or procedural memory** – the ability to learn not to enter the never baited arms – and the working memory – to avoid entering the visited arms twice in one trial. The rats with a damaged hippocampus were not able to learn the spatial task, but on the cued version, they only needed a longer time to avoid the **working memory** errors of entering already visited arms a second time (Jarrard 1995). This clearly demonstrated that selective hippocampal damage impairs the processing of spatial memory.

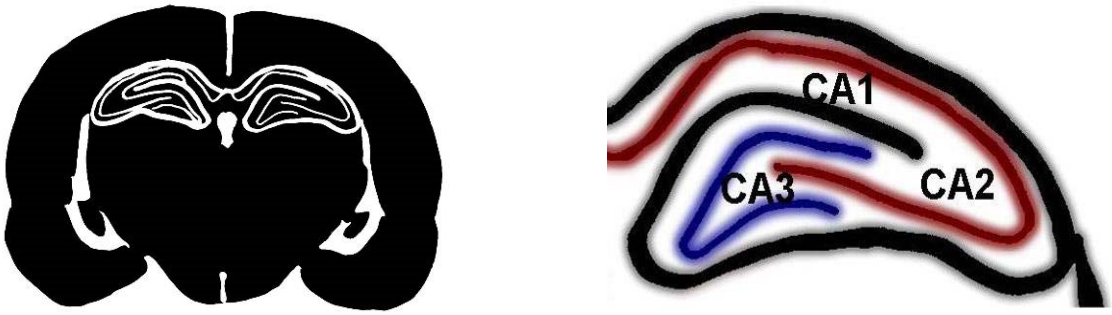

Fig.5 LEFT: Schema of the location of the rat hippocampus in a frontal slice positioned 3 to 4 mm from the rat's bregma.
RIGHT: More detailed drawing of the granular cell layer (blue) that forms the dentate gyrus and the pyramidal cell layer (red) that is then divided into the three parts of the cornu amonis (CA1, 2, 3)

1.2.2. THE MORRIS WATER MAZE

In 1981 Richard G. Morris introduced an apparatus for studying hippocampus-dependent spatial learning. The Morris water maze apparatus (MWM) is a circular pool with a diameter usually around 200cm and walls about 60cm high. The pool contains enough water that a rat cannot touch the bottom and at the same time is not able to climb the walls. The temperature of the water should be around 22±2°C. In this swimming pool (Fig.6), a rat is exposed to a navigational challenge: to swim in the direction of a submerged platform that is beyond the rat's perception. The rat has to use cognition to link external cues in order to locate the platform, which is hidden from the rat's perspective (under opaque water). The diameter of the submerged platform is usually around 10 cm. Rats are very good swimmers, but an environment of cold water motivates them to seek escape from the pool. After an initial phase of following its instinct and swimming around the walls, the animal finds the submerged platform by chance, climbs on to it and tries to locate its position within the experimental room. There is evidence that the animals are using only external (i.e., extra-maze) cues to locate the platform. Removal of the platform after the acquisition phase results in persistent crossing of the target location (annulus crossings).

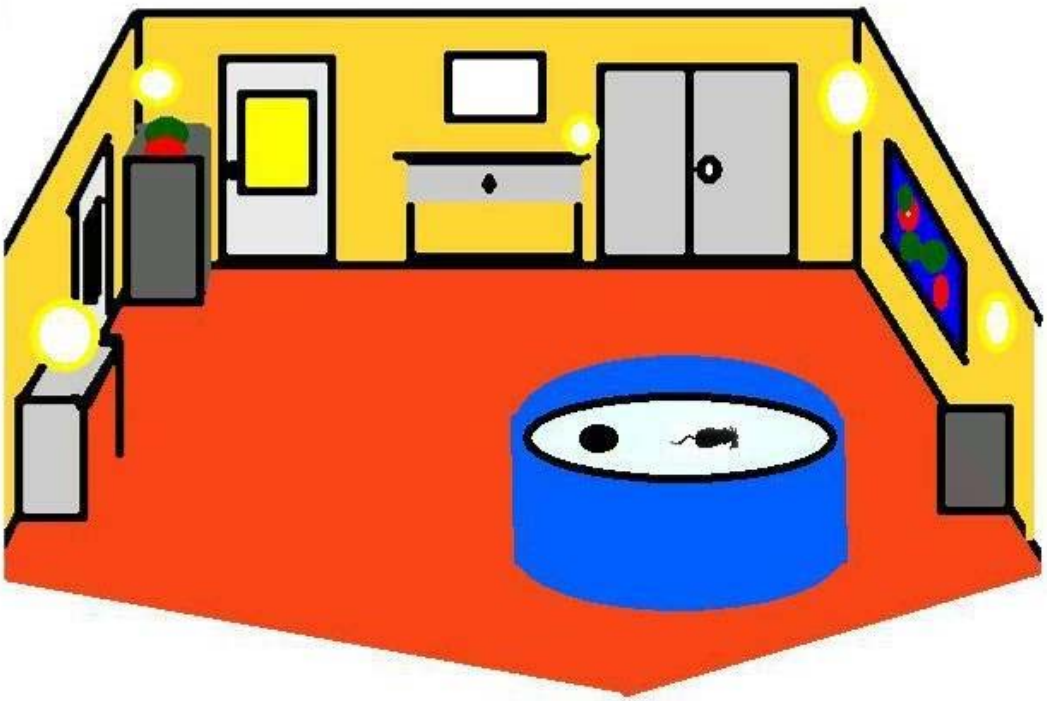

Fig.6 The experimental setting of the room for MWM testing. The circular pool is filled with lukewarm water, and a platform is hidden underneath the water surface. Prominent items in the room serve as spatial cues (particularly windows, doors, lightning, shelves, and artwork).

When the environment surrounding the pool is covered with curtains, a trained rat is able to find the platform only by chance. Interestingly, when part of the curtain is missing, this provides the rat with a sufficient set of cues to localize the platform correctly (Morris et al. 1984).

Several parameters of behavior in the water maze can be observed. The *latency required to reach the platform*, the *length of the swim path* and the *swim speed* can be measured from tracks obtained by a tracking system. There is evidence that the most representative measure of hippocampal impairment is the manual scoring of the latency required to find the platform (Eijkenboom et al. 1999).

The simplest test in the water maze is a **place navigation task** on a single submerged platform that remains in a stable position. Rats acquire long-term memory of the platform location. The rat is placed in the pool facing the wall during 4 to 8 trials a day initiated from 4 different positions (North, East, South and West) in the pool in random order. After approximately 5 days of training, young adult hooded male rats approach the asymptote of the learning curve. The design of the experiment may vary according to the strain, age and sex of the animals (albino rats require more trials; working memory performance varies with the estrous cycle) (Warren et al. 1997). A **transfer test, retention or probe test** can

be conducted on the last day of acquisition. The platform is removed from the pool. In one trial the rat is placed in the water and allowed to swim for 60 seconds. The amount of time spent in the target quadrant or the number of annulus crossings is measured with the use of a tracking system. This test has been widely used and improved by O. Buresova (Buresova et al. 1985), who introduced an “on demand platform” feature in which the platform is connected to a computer tracking system and is made available for the rat to climb onto only when the rat remains for a certain time (1 sec) within a certain distance (10 cm) of the virtual center of the platform. This feature mimics the properties of a probe trial, yet offers a reward for the correct solution of the task (Bures et al. 1997).

1.2.3. LEARNING AND LONG-TERM MEMORY

The process of learning induces a number of neuronal changes, i.e. a so-called engram. These changes form a memory, which is stored in the same neuronal system that was involved in the perception, analysis and processing during the learning. Separate regions of the neocortex simultaneously carry out the computation of the external environment representation (Squire 1986).

Different brain structures are involved in the conscious recollection of facts and events (declarative memory) and non-conscious learning (nondeclarative memory), which is expressed through performance. The evidence for distinguishing between these two kinds of memory was obtained during studies of amnesic patients (e.g. H.M.) who had bilateral damage to the medial temporal lobe or diencephalic brain structures. These patients showed learning on some tasks, while others remained impaired. The following overview expands on the schema presented by LR Squire (Squire et al. 1996).

Nondeclarative memory is implicit and is acquired and recalled unconsciously.

- *Habituation and sensitization* are the simplest forms of learning. This non-associative learning occurs on the level of reflex pathways (habituation is a decrease in the response to a benign stimulus presented repeatedly and sensitization is an enhanced response to a variety of stimuli after a noxious stimulus is presented).
- *Classical conditioning* is associative and involves the amygdala for processing emotional responses. The cerebellum controls learning acquired through the movement of the skeletal musculature (conditioning is the learning of a relationship between two stimuli, e.g. a protective eyeblink reflex can be established when an auditory stimulus is associated with an air puff to the eye, which causes an eyeblink)

- *Procedural* skills and habits require the participation of the striatum.
- *Priming* is nondeclarative learning in which a long-term trace is stored in the cerebral cortex (e.g. the recall of words or objects is improved by prior exposure to these words or objects).

Declarative memory is explicit and is acquired and recalled by deliberate conscious effort. The learning is highly flexible and involves the association of several pieces of information.

- *Episodic memory* is memory of events or personal experiences (Vargha-Khadem et al. 1997).
- *Semantic memory* is a collection of facts (Levy et al. 2004).

The knowledge stored as an explicit memory is first acquired through processing in one or more of the three associational cortices (prefrontal, limbic, parietooccipital) where auditory, visual and somatic information is synthesized. Then, the information is mediated through the parahippocampal and perirhinal cortices to the entorhinal cortex from where it enters the dentate gyrus and cornu amonis in the hippocampus to return back to the entorhinal cortex (Squire et al. 1996). The entorhinal cortex is the main input to the hippocampus but also the main output leading the information back to the polymodal associational cortices. Impairment of the entorhinal cortex, such as during Alzheimer's disease, disconnects the hippocampus from processing the long-term declarative memory (Hyman et al. 1984). Episodic memory depends primarily on the hippocampal component of the system, whereas semantic memory depends primarily on the underlying cortices (Vargha-Khadem et al. 1997).

The hippocampus is the essential structure for processing declarative learning and for the consolidation of a memory before the engrams are fully established (Squire 1986). Explicit knowledge involves the following processes:

- **Encoding** - associating the information systematically with already existing knowledge, motivation strengthens the storage
- **Consolidation** - structural changes involving protein synthesis to stabilize the trace (Bailey et al. 1996)
- **Storage** – the information is retained over time (Martin et al. 2000)
- **Retrieval** - bringing the different pieces of information together from different storage sites during a constructive process that is critically dependent on short-term working memory.

1.2.4. THE HIPPOCAMPUS AND IMPAIRED SPATIAL LEARNING

The ability of a rat to solve the maze task will depend on the processing of visual, olfactory and spatial information by the respective systems (Squire 1986). **Lesions to the hippocampus** and its projection areas cause impaired spatial navigation in the water maze. An ibotenate **lesion to the hippocampus and subiculum** prevented rats from developing a search strategy or showing spatial learning. The rats circled around the walls and found the platform only by chance (Morris et al. 1990).

Extensive overtraining on acquisition trials of rats with lesions to the hippocampus only diminished the impairment of spatial learning. The lesions caused spatial learning to occur at a slower rate. Preoperatively trained rats were as accurate as controls on annulus crossings of the former platform location, suggesting that the hippocampus is unlikely to be the only site of long-term storage of spatial information. In a probe trial (without a platform), rats with lesions to the hippocampus swam in a circle that had a constant distance from the wall equal to the distance of the platform from the wall (platform biased) (Morris et al. 1990).

A kainic acid lesion to the CA3 with 88% cellular damage or a lesion to the CA1 region damaging only 50% of cells caused increased latencies in locating the platform even after 25 acquisition trials in the water maze (Stubley-Weatherly 1996). Rats with ibotenic acid damage to the hippocampus and a minimal adjacent part of the subiculum also failed to recognize the goal location in an annular water maze task. These lesioned rats did not slow down during a probe trial above the previously present platform, showing that they did not recognize the location of the target (Hollup 2001).

Rats with a lesion to the subiculum only showed slower learning than did the controls, but they also reached the asymptote after overtraining. In a probe trial, they displayed a “random search” strategy that led to fewer annulus crossings than the “localized search” performed by controls. The subiculum-lesioned rats showed better relearning of a new platform location than did rats with lesions in the hippocampus only. As the subiculum is the main output from the hippocampus to the neocortex, long-term spatial learning was impaired, but the learning of a new location was impaired less than in animals with a lesion to the hippocampus only. There is little impairment in processing, navigation, or short-term memory when the subiculum is lesioned (Morris et al. 1990).

The hippocampus plays a role in the retrieval and consolidation of information stored in the memory. Demonstrative was a test on working memory task in the

MWM. Rats were pre-trained on a reference memory task, and then the platform was moved to a new position. The new position of the platform was learned within the first trial of the working memory task due to the already acquired procedural part of the learning and the rats' familiarity with the extra-maze cues in the experimental room. A functional bilateral blockade of the hippocampus by lidocaine applied 3 min after the first working memory trial resulted in the impaired retrieval of the new platform location when the second trial was started during the period of full effectiveness of the blockade. The consolidation of the new location was unimpaired when the effect of lidocaine wore off (30 min after injection) (Bohbot et al. 1996).

Electroconvulsive shock (ECS) immediately or up to 30 sec after acquiring a working memory engram in the MWM prevented the consolidation of the spatial location of the target. The consolidation of this spatial information was, however, very quick, as an ECS applied 30 to 45 seconds after the trial did not impair the rats' progress in the next trials (Bohbot et al. 1996).

There are several neurochemical systems, and their functional integrity is required for place learning in the water maze. The importance of the cholinergic system has been revealed by studies of mnemonic dysfunction in patients with Alzheimer's disease. Transection of the fimbria-fornix, the main pathway connecting the subcortical systems to the hippocampus, has been found to impair spatial learning similarly as do lesions to the medial septum and the nucleus basalis magnocelularis. Aged rats that were impaired on place learning, showed a significant reduction of cholinacetyl transferase (ChAT) in the basal forebrain, striatum and sensomotoric cortexes, but not in the hippocampus. The transplantation of cholinergic septal grafts into the hippocampus ameliorated the deficit. Muscarinic antagonists (atropine and scopolamine) impair place learning as do central nicotinic receptor antagonists (mecamylamine). Interestingly, physostigmine - a cholinacetyl esterase inhibitor that slows down the enzymatic breakdown of acetylcholin - can attenuate the learning deficit in rats with lesions to the medial septum, but it impairs place learning when administered at higher doses to nonlesioned rats (Mc Namara et al. 1993).

1.3. ADULT NEUROGENESIS IN THE HIPPOCAMPUS

The last decade has seen many convincing proofs that the process of neurogenesis continues into adulthood in a number of animal species. The initial theory from 1928 by Ramón y Cajal, that neuronal cells in the adult brain are not renewed, was based on the inability to detect mitotic figures and transient forms from simple to more complex neuronal morphologies (Rakic 2002). Recently it has been clearly established that there are at least two neurogenic regions in adult mammals. The **subventricular zone (SVZ**) supplies newborn neurons to the olfactory bulb through the rostral migratory system. In the **subgranular zone (SGZ)** of the dentate gyrus (DG) in the hippocampus, the newborn neurons finally reside within the granular cell layer.

1.3.1. INTRODUCTION TO PRENATAL NEUROGENESIS

There are abundant numbers of neuronal and glial cells produced during the embryonic development of the nervous systems. The numbers of cells that mature are regulated by programmed cell death according to the presence of trophic factors. The molecular mechanisms by which the developing cells acquire a glial or neuronal phenotype have been revealed in invertebrates during the past decade. The studied organisms include Drosophila melanogaster (Lu et al. 1998) and Xenopus levis (Kandel et al. 2000).

Small clusters of cells that are able to give rise to neurons are recruited within the uniform population of ectodermal cells. Neuronal fate is decided by a process of signalling between adjacent cells in the proneural region. The process depends on the level of activation of the transmembrane proteins encoded by the neurogenic genes **Notch** and **Delta** (Lu et al. 1998). Delta functions as a ligand and Notch is the receptor. Both are expressed among adjacent cells at similar levels. The activation of Notch by Delta is rapidly amplified by a cascade activating the transcription factor suppressor of hairless, which leads to a decrease in the expression of delta on the cell surface. Cells with highly activated Notch are inhibited from acquiring a neuronal fate (Lai et al. 2004). A cytoplasmic protein called Numb binds the intracellular domain of Notch and leads to an inhibition of Notch activation. **Cells with Numb become neurons**. This system regulates the ability of daughter cells to give rise to both cell types (i.e. the anterior cell will follow a neuronal fate and the posterior will continue in the glial lineage; Kandel et al. 2000)

One of the best-understood systems for studying the distinction between neural and glial cells is the *neural crest tube*. Nicole Le Douarin used the different

condensation of chromatin in chick and quail cells as a marker for permanent cell lineage. Grafting quail neural crest cells into different levels of the rostro-caudal axis in the neural system of the chick led to the generation of the cell types that are typical for the level into which the graft was placed rather than the level from which it was taken. This led to the idea that the environments of migrating or grafted cells produce distinct signals that have a critical role in controlling their fate (Le Douarin 2005).

The **bone morphogenic proteins (BMP)** are responsible not only for the induction of premigratory neural crest cells in early developmental stages, but also for the differentiation of autonomic neurons. Glial growth factor (GGF) expressed on the surface of autonomic neurons directs the nearby cells along the pathway to glial differentiation. There is therefore a feedback system that ensures the appropriate balance between Schwann cells and neurons within each ganglion (Shah et al. 1996).The generation of glia cells is controlled by diffusible factors (Fig.7). Oligodendrocyte progenitors are maintained in a self-renewing progenitor state by **platelet derived growth factor (PDGF)**. In the absence of PDGF the progenitors stop dividing and differentiate. **Ciliary neurotrophic factor (CNTF)** promotes the differentiation of progenitor cells into astrocytes. PDGF and CNTF are produced by astrocytes and ensure that the proper ratio of glial subtypes is created (Lillien et al. 1990)

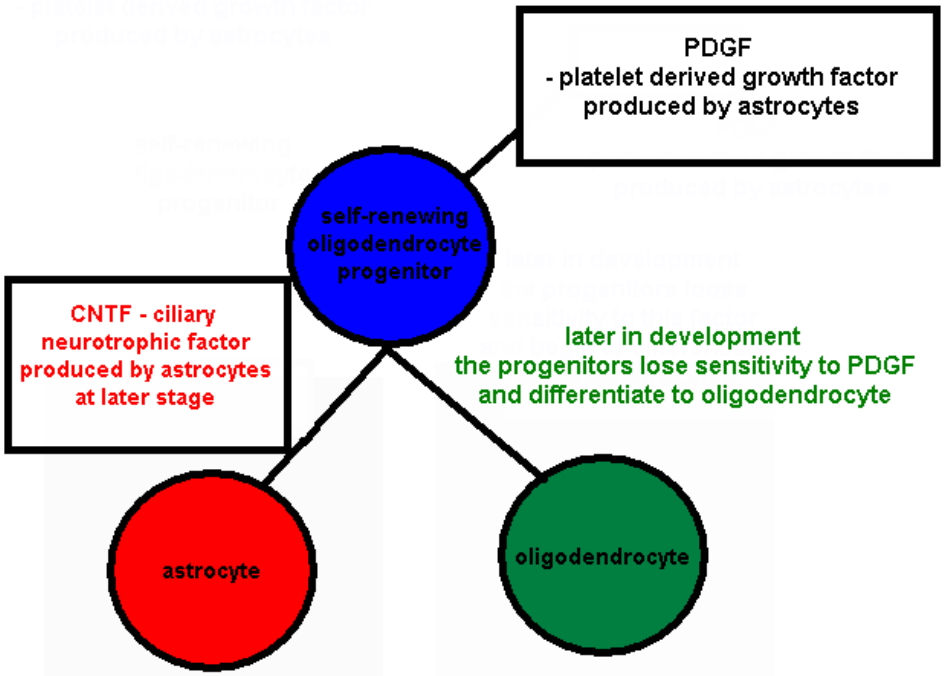

Fig.7 Schematic drawing showing the influence of trophic factor on the differentiation of an oligodentrocyte progenitor. The presence of platelet derived growth factor (PDGF) maintains the progenitor in its proliferative state, while the absence of this factor leads to the differentiation of the progenitor into a mature oligodentrocyte. Cilliary neurotrophic factor (CNTF) leads the common glial progenitor to differentiate into the astrocytic lineage. Adapted according to Kandel et al. 2000.

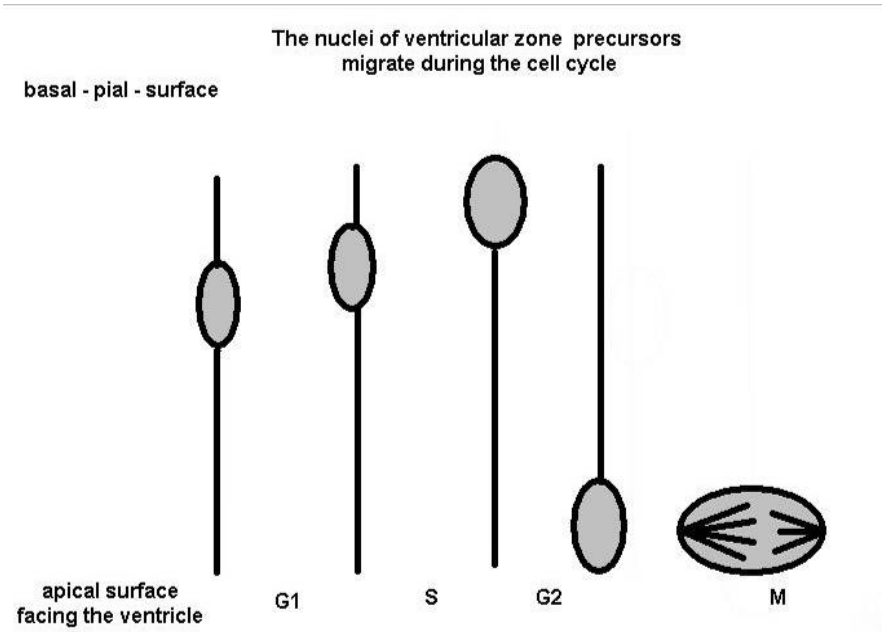

Fig.8 Drawing showing the movement of neuronal progenitors within the subventricular zone during the cell cycle. Adapted according to Kandel et al. 2000

Cortical progenitor cells are attached to the apical surface facing the ventricle, and the distance from the ventricle varies during the cell cycle (Fig.8). Early in cortical development the neuronal progenitors divide symmetrically (Fig.9) and give rise to two daughter progenitor cells (Nadarajah and Parnavelas 2002)

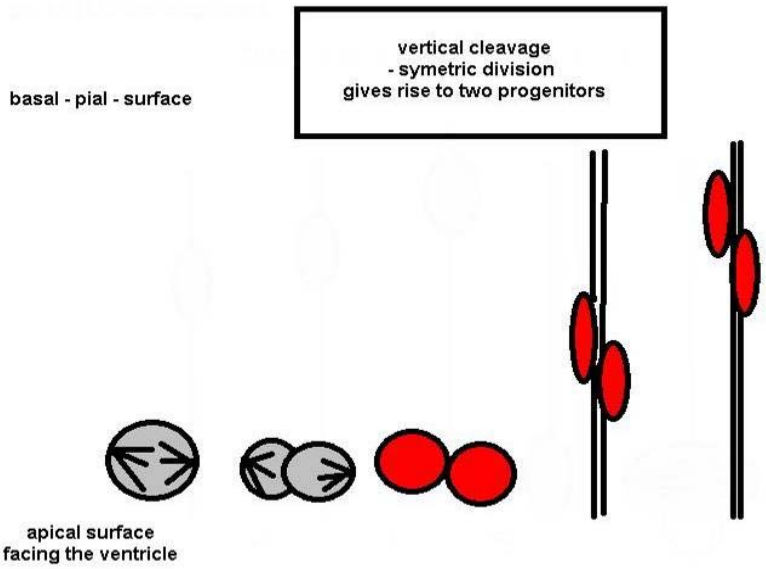

Fig.9 Drawing showing the symmetric horizontal cleavage of progenitors within the ventricular zone. Two progenitors are generated that reside within the subventricular zone. Adapted according to Kandel et al. 2000

At later stages the progenitor cells alter their program of cell division and produce neurons and progenitors (Fig.10). At a still later stage only neurons are produced.

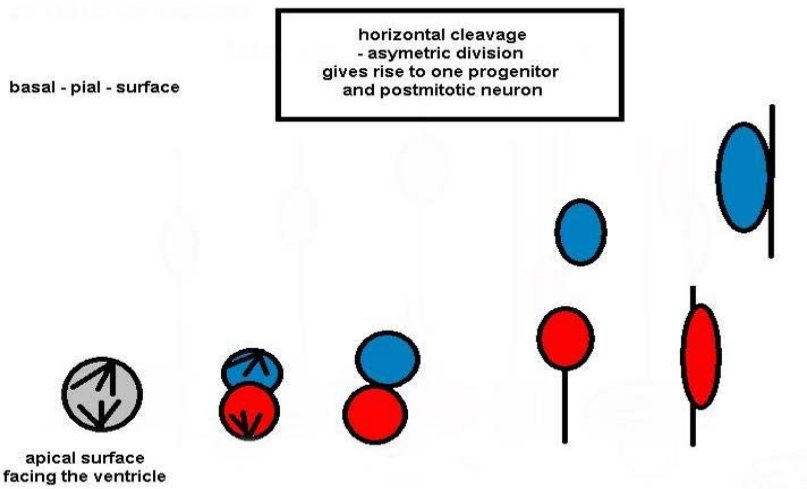

Fig.10 Drawing showing the asymmetric vertical cleavage of progenitors within the ventricular zone. Adapted according to Kandel et al. 2000

Different neuronal subtypes of the cerebral cortex adopt different modes of migration. Neuronal migration in the cerebral cortex begins when the first group of post-mitotic neurons leaves the germinal ventricular zone and the cortical plate is

initiated. The cortical layers II to VI are then formed in an inside-out sequence so that the cells that are born early reside in the deepest layers. The neurons in the cortical plate are separated by an intermediate zone that contains the axons of the white matter. During late gestation and early postnatal life, the ventricular zone disappears and the subventricular zone then becomes the main germinal zone. The cortical neurons initially migrate via the process of **somal translocation** – continuous advancement. When the cortical plate is formed, **radial glia guided** saltatory movement becomes the prevalent migration pattern (Nadarajah and Parnavelas 2002). GABA-positive cortical interneurons originate in the ganglionic eminence and migrate **tangentially** along the corticofugal axons (O'Rourke et al. 1995).

The number of surviving neurons is reduced during and after embryonic development. Rita Levi-Montalcini and Victor Hamburger proved that target cells produce factors that prevent the cell death of particular neurons. The removal of a limb from a chick embryo leads to the excessive death of sensory neurons and motor neurons in the lateral spinal column (Levi-Montalcini 2000). Limb muscle activity also influences the number of neurons that survive. Blocking muscle activity with curare will spare a number of motor neurons. Conversely, direct stimulation of muscle activity will increase the death of motor neurons. These experiments led to the isolation of **nerve growth factor (NGF)**. Until now, several classes of neurotrophic factors and several families of their receptors have been identified. The best described is the interaction of neurotrophins with the thyrosinkinase receptors (trk A, B, C). Activation by the ligand leads to the phosphorylation of the intracellular domain and induces several cascades that lead to the transcription of cell death inhibitors.

1.3.2. THE PHENOMENON OF POSTNATAL NEUROGENESIS

Joseph Altman published in 1962 a report describing his observations in young adult rats (Altman 1962). He made an electrolytic lesion in the lateral geniculate nucleus and injected ^{3}H thymidine into the lesioned area. ^{3}H thymidine is incorporated during the S - phase of the cell cycle into the DNA of the dividing cell. The substance is available in the tissue of the animal for less than 10 minutes after injection and marks, therefore, the exact “birth date” of the dividing cell (Rakic 2002a). **Autoradiographic treatment** revealed new neurons (micro-neurons) in the thalamus and pyramidal cells in the cortex of the lesioned animals. Intraperitoneal injections of ^{3}H thymidine resulted in the labeling of a small number of neurons in the neocortex and in the granular cell layer of the hippocampus. By

1967, Altman was able to conclude that **postnatal neurogenesis occurs in specific regions**. In the cerebellar cortex, neurogenesis continues only shortly after birth. In the dentate gyrus, new micro-neurons are added in a process of delayed growth until late adulthood, while in the subventricular zone, adjacent to the lateral ventricles, new micro neurons replace dyeing cells in the olfactory bulb. Professor Altman was very cautious in his interpretation of his results, pointing out that labeling may occur in the adjacent glial wedge and that DNA repair may also contribute to the number of labeled cells that he saw (Nottebohm 2002). The precise electron microscopy of Altman and other pioneers (Kaplan et al. 1977) has been challenged by very high standards of proof that the observed labeled cells are really neurons. A number of studies have provided supporting arguments:

- The newly born cells were identified as granule neurons (30 days after labeling) (Kaplan et al. 1977)
- Bayer observed an increase in the number of granule cells on Nissl - stained sections throughout the maturation of young adult rats (Bayer et al. 1982)
- The newly born granule cells form synapses (Kaplan et al. 1977)

During the 1990s, a number of **neuronal molecular markers** have been discovered, such as **NSE -** neuron specific enolase (Cameron et al. 1993), **NeuN, MAP2 -** microtubules associated protein**, TOAD-64 -** turned on after cell division**, and TUJ1 - beta Tubulin III** (Rakic 2002, Nowakowski 2000). The labeling of dividing cells was achieved using the incorporation of the thymidine analog BrdU (bromodeoxyuridine) into the DNA during the cells' S-phase. BrdU can be detected immunohistochemicaly and thus enables confocal microscopy analysis using multiple cell-specific markers (Nowakowski 2000, Rakic 2002). The last decade has witnessed a much greater understanding of the cellular processes of neurogenesis, the conditions that influence it and even evidence of functional integration.

1.3.2.1. NEURAL PROGENITOR CELLS IN THE ADULT BRAIN

The term **stem cell** has been used in the literature to refer to the phenomenon of adult neurogenesis. A stem cell is defined as an **undifferentiated cell that exhibits the ability to proliferate, self-renew, and differentiate into multiple lineages**. In the adult brain, stem-like cells are in a quiescent stage, except in the neurogenic zones of the SVZ and SGZ. The multi potentiality of these cells is still a matter of debate as is the question of whether these cells are really stem cells. It has been established that these stem-like cells are more committed **neuronal progenitors that have ability to proliferate and give rise to terminally differentiated cells, but which are not capable of indefinite self-renewal.** In in vivo studies, it is not possible to define which cell type is being studied (Mc Kay et al. 1999).

The term **precursor cell refers** to the group of cells that includes stem cells as well as progenitors (Seaberg et al. 2003). Specific markers have been identified that enable researchers to study the origin, development and fate of precursor cells through particular stages of neurogenesis (Fig.11).

<table>
<tr><td></td><td colspan="2">Mitotic cells</td><td colspan="2">Post-mitotic cells</td></tr>
<tr><td></td><td>Self renewing precursor</td><td>Transit amplifying progenitor cell</td><td>Immature neuron</td><td>Mature neuron</td></tr>
<tr><td rowspan="7">Markers that define precursors from differentiated cells</td><td colspan="2">Musashi 1 (neural RNA binding protein)</td><td colspan="2">Beta III tubulin/TuJ1 (cytoskeletal protein)</td></tr>
<tr><td colspan="2">Nestin (intermediate filament)</td><td colspan="2">NeuN (neuronal specific nuclear protein)</td></tr>
<tr><td rowspan="5"></td><td colspan="2">DCX (double cortin- microtubule associated protein in migrating neurons)</td><td>NSE (neuron specific enolase)</td></tr>
<tr><td rowspan="2"></td><td>TOAD 64 – turned on after division (transient cytoplasmic protein)</td><td>MAP – 2 (microtubule associated protein)</td></tr>
<tr><td>PSA-NCAM (cell adhesion molecule)</td><td rowspan="3">absence of glial antigens (GFAP, CNP, S100)</td></tr>
<tr><td>Neuro D (basic helix-loop-helix transcription factor)</td></tr>
<tr><td>Hu and elav (neuron specific RNA binding protein)</td></tr>
<tr><td>Antigens present in cycling cells</td><td colspan="2">BrdU (incorporated into DNA during replication)

Ki – 67 (nuclear protein expressed during the whole course of mitotic activity)

PCNA (auxiliary protein of DNA polymerase expressed during G1-S phase of the cell cycle)

HH 3 (phosphorylated histone present in cells in G2 and M phase of the cell cycle)</td><td colspan="2"></td></tr>
</table>

Fig.11 An overview of the markers used to study adult neurogenesis. Adapted from Abrous et al. 2005.

1.3.2.2. ADULT NEUROGENSIS IN THE SUBVENTRICULAR ZONE

The origin of newborn neurons has been investigated in several vertebrate species. The germinal zones have been studied, and some conditions underlying neurogenic potential have been identified.

Reptiles

Neurons born in adult lizards have been reported in all major subdivisions of the telencephalon; the entire adjacent subventricular zone (SVZ) is neurogenic. The capacity of the reparative system is enormous. Destroyed parts of the brain can be regenerated, possibly due to the maintenance of radial glia throughout adulthood (Doetsch 2003). The proliferating cells in the SVZ have the ultrastructure of radial glial cells (type B with a single cilium) and/or multiciliated ependymal cells (type E) soon after labeling. The labeled cells show the properties of neurons (Type A migrating cells) days after labeling. There are no free glial cells formed during this process. Most of the glial cells in the reptilian brain are radial glia and provide precursors and scaffolding for the short migration of newborn neurons in the reptilian brain (Garcia-Verdugo 2002).

Birds

In canaries neurogenesis in the hyperstriatum ventrale is particularly important and has been studied in association with the function of newborn neurons in long term memory (Nottebohm 2002), but newborn neurons have been seen in most of the major divisions of the telencephalon. Unlike in reptiles, only the SVZ of the lateral ventricles is neurogenic in birds. One day after labeling there are many proliferating cells in the SVZ, while neurons can be identified 2-3 weeks later throughout the telencephalon. The proliferating, tangentially dividing cells (type A cells) are found clustering in so-called hot spots, which are in the proximity of single ciliated radial glial cells (type B cells; Fig.12). Mitosis takes places in proximity to the ventricular wall. Ependymal multiciliated cells (type E cells), as well as type B cells, are in contact with the cerebrospinal fluid, but do not give rise to neuroblasts. Cells in the SVZ in birds use radial migration when guided by radial glial cells or tangential migration in chains or along blood vessels (Garcia-Verdugo 2002).

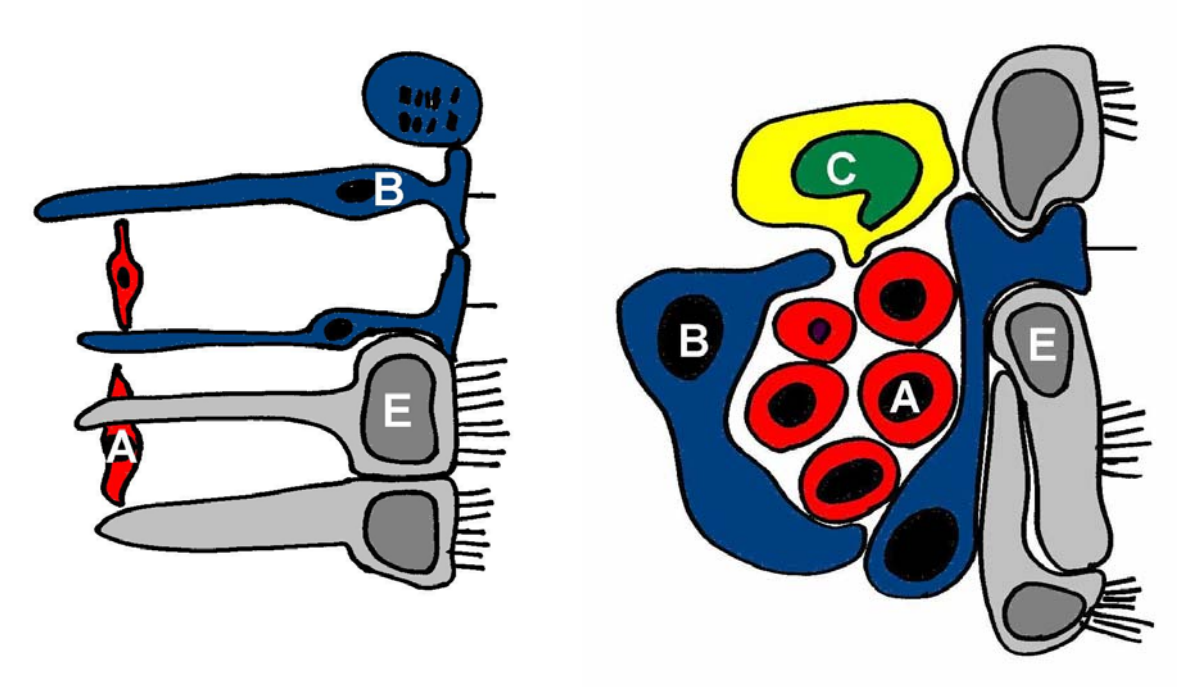

*Fig.12 Schematic drawings of neurogenesis in the **subventricular zone** in birds (left) and mammals (right). Mult-ciliated ependymal - E type cells (gray) - form epithelium. Single-ciliated multipotent stem cells - B cells (blue) - divide and give rise to neuroblasts. In birds have B cells properties of radial glia; proliferation occurs also in cells that are in contact with cerebrospinal fluid. Migrating neuroblasts - type A cells (red) - divide and migrate radially and tangentially. B cells in mammals are slowly dividing astrocytes that give rise to rapidly dividing precursors – transient amplifying C cell (green). Neuroblasts – A cells (red) – then migrate in astrocytic tubes through the rostral migratory stream to olfactory bulb (according to A. Alvares-Buylla 2002).*

Mammals

In contrast to the widespread neurogenesis seen in non-mammalian vertebrates, in mammals, including humans, neurogenesis continues only in restricted regions (Alvarez–Buylla et al. 2002, Brown et al. 2003, Cameron et al. 1993, Doetsch 2003, Duman et al. 2001, Eriksson et al. 1998). Neurogenesis in adult mammals has been proven to continue in the dentate gyrus and the subventricular zone, providing neuroblasts for the olfactory bulb.

The germinal layer of the SVZ in mice consists of four types of cells (A, B, C, E) (Fig.12). Most abundant are the cuboid, multiciliated, GFAP-positive ependymal cells (type E cells), which form an epithelial monolayer. Occasionally, a singleciliated process of a glial type B cell is in contact with the CSF. Type B cells contain bundles of intermediate filaments in the cytoplasm and stain with antibodies against GFAP, nestin and vimentin. Type B cells in the subventricular zone of immature and adult mice are multipotent astrocytic stem-like cells that give rise to proliferating type C cells. The astrocytes divide asymmetrically, giving rise

to one fast proliferating type C cell and one astrocyte. Type C cells are larger and more spherical cells with smooth contours and do not contain any bundles of intermediate filaments. These “transit amplifying progenitor” type C cells are often in contact with type B astrocytes and type A migrating cells. Type A migrating precursor cells have an elongated cell body with either one or two processes. The cells have a smooth outline and are often joined to other type A cells by junctional complexes. Migrating neuroblasts can be detected with antibodies against PSA-NCAM and TUJ1 (Garcia-Verdugo et al., Alvares–Buylla et al. 2002, Hu et al. 1996).

Cells born in the adult mouse SVZ migrate (Fig. 13) in chains along glial tubes, which are formed by astrocytes or their processes. This restricted pathway is called the rostral migratory stream (RMS) and guides neuroblasts to the olfactory bulb where they differentiate into GABA-ergic granule and periglomerular neurons. The migration path in rodents is about 5mm long, and migration takes up to six days (Winner et al. 2002). Transsection of the RMS or removal of the rostral OB impedes neuroblast migration. It has been proposed that there is a diffusible chemoattractant produced in a specific layer of the olfactory bulb (Liu et al. 2003). The number of the neurons in the OB is, however, maintained by continuous turnover. Massive cell death occurs within 15 to 45 days after cell birth after marked by a BrdU pulse. Cells that survived the first 3 months after BrdU injection persisted for up to 19 months. The majority of the BrdU-positive cells that reach the olfactory bulb differentiate into granule cells, but a small fraction migrate further into the glomerular layer (Winner et al. 2002). The newly born granule and periglomerular cells are integrated into existing ciruits, as proven by the injection of the GFP- (green fluorescent protein) expressing pseudorabies virus into the piriform cortex. The adult-born neurons are capable of early c-Fos gene induction, demonstrating their ability to respond to odor stimuli (Carlen et al. 2002).

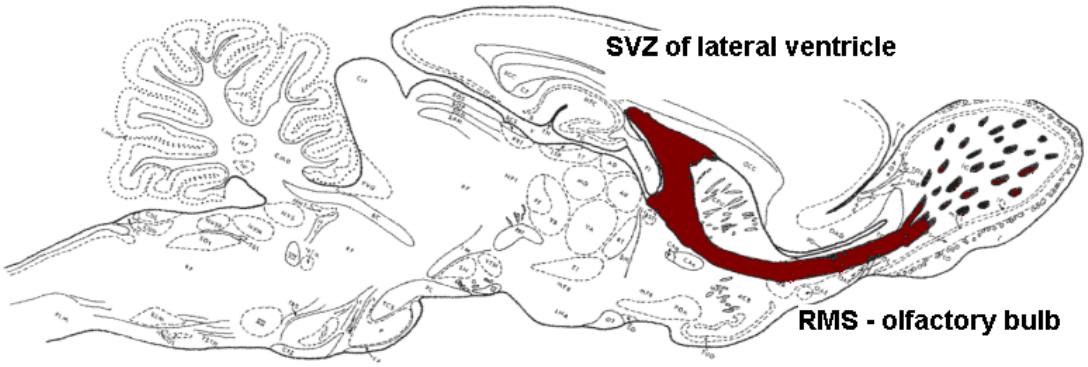

*Fig.13 Schematic drawing of neurogenic region the **sub ventricular zone (red)** in rodent. The neuroblasts migrate in the rostral migratory stream (RMS) to the **olfactory bulb** where they mature and some of them functionally integrate. Modified drawing adapted on drawing from Pellegrino et al. 1967.*

From the SVZ cells can be isolated that are believed to have the properties of stem cells – renewal and multipotency. These cells can be isolated in culture using epidermal growth factor (EGF), fibroblast growth factor (FGF) or both (Alvares-Buylla et al. 2002). Stem cells can also be isolated from other brain regions using several growth factors, but only cells from the SVZ are able to generate glia and neurons under the influence of EFG (Alvares-Buylla et al. 2002). Neurospheres can be grown on an astrocyte monolayer isolated from the SVZ in cultures free of serum and other exogenous factors. The intimate presence of the astrocytic microenvironment suggests the importance of cell-cell contact between SVZ astrocytes and the precursors (Lim et al. 1999). There are several arguments supporting the statement that GFAP-positive astrocytes are neuronal precursors. Treatment with an antimitotic agent (Ara-C) eliminates all type A and C cells (neuroblasts and rapidly dividing cells), sparing only some astrocytes and ependymal cells. After twelve hours, the astrocytes begin dividing and after ten days the whole SVZ regenerates. The ependymal cells do not incorporate the mitotic markers (Alvares–Buylla et al. 2002, Doetsch 1999). Astrocytes can also be visualized in transgenic mice by detecting the alkaline phosphatase (AP) produced by the avian retrovirus. The receptor for the avian leucosis virus is expressed under the control of the GFAP promotor in these transgenic mice. The injection of the retrovirus allows the virus to infect the dividing GFAP-expressing astrocytes. The progeny of these infected cells keep producing alkaline

phosphatase and can be easily followed (Doetsch 1999). There are no ependymal cells labeled after 12 days of continuous intraventricular infusion of 3H Thymidine (Doetsch et al. 1999). Ependymal cells, however, play an important role in creating a neurogenic niche. Bone morphogenic proteins (BMP) are produced by astrocytes and suppress the neurogenic potential of these cells. Ependymal cells produce noggin, which neutralizes the inhibitory effect of BMP (Alvares–Buylla et al. 2002, Doetsch 2003).

1.3.2.3. ADULT NEUROGENESIS IN THE SUBGRANULAR ZONE

In the adult dentate gyrus neurons are formed in situ and become excitatory granule neurons (Garcia-Verdugo 2002). Astrocytes are believed to be the primary precursors that give rise to new granule neurons. Astrocytes in the SGZ have a very similar ultrastructure as astrocytes in the SVZ. In the SGZ (Fig. 14), astrocytes extend radial processes that reach through the granular cell layer and the shorter tangential processes that line the blades of the granular cell layer (Alvares–Buylla et al. 2002). It has been proposed that Type I/**B cells** divide asymmetrically, generating neuronal progenitors (type II/**D cells**), and glial lineage restricted progenitor cells (Abrous et al. 2005).

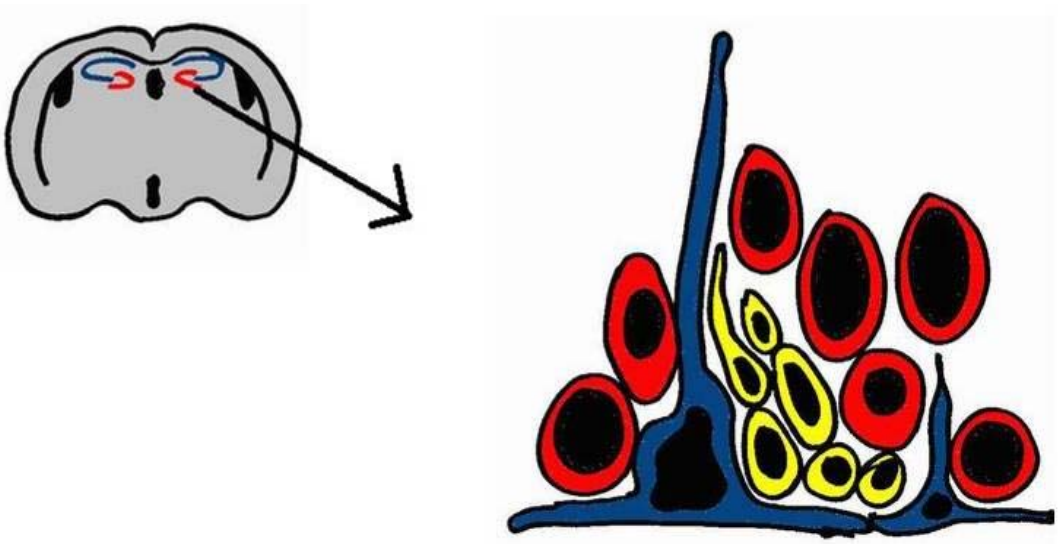

*Fig.14 Schematic drawings showing neurogenesis in the **subgranular zone** in the rodent hippocampus. At the schematic drawing of a cross section at the level of the hippocampus, the germinal granular cell layer is shown in red while blue indicates the pyramidal cell layer of the cornu amonis (CA1-3). Schema of the cellular evolution in the subgranular zone: B cells (blue; astrocytes) give rise to D cells (yellow), which extend processes (dendrites) through the granular cell layer (red cells). After maturation, some of the D cells show properties of granular neurons (adapted from A. Alvares-Buylla 2002).*

Electrophysiological studies of transgenic mice in which GFP (green fluorescent protein) is produced under the control of the *nestin promoter* have revealed two precursor cell subpopulations. **Type I cells** are GFAP+, S100-, DCX-, PSA-NCAM- and show lower input resistance (IR) and complex branching with processes stretching towards the molecular layer. These cells are referred to as the **B cells** (Seri et al. 2001). **Type II** cells are positive for *PSA-NCAM* (GFAP–, S100–, and DCX+)**,** show high IR and sodium-dependent voltage currents, and their shape is rather spherical. These cells correspond to **D cells** (Abrous et al. 2005). Interestingly, the IR of Type II cells is much higher than in mature granule neurons and lower than the IR measured in the developing rat dentate gyrus. These finding describe the transient stages of granule cell development (Fukuda et al. 2003). Clusters of dividing D cells give rise to granular cells and often can be seen in the proximity of growing capillaries (Alvares-Buylla et al. 2002).

Experiments with the antimitotic agents Ara-C and Procarbazol resulted in the elimination of D cells and some astrocytes. The surviving astrocytes began dividing after the termination of this treatment, and D cells reappeared 4 days later. After five months, the D cells matured to granule neurons, astrocytes, and oligodentrocytes (Alvares-Buylla et al. 2002).

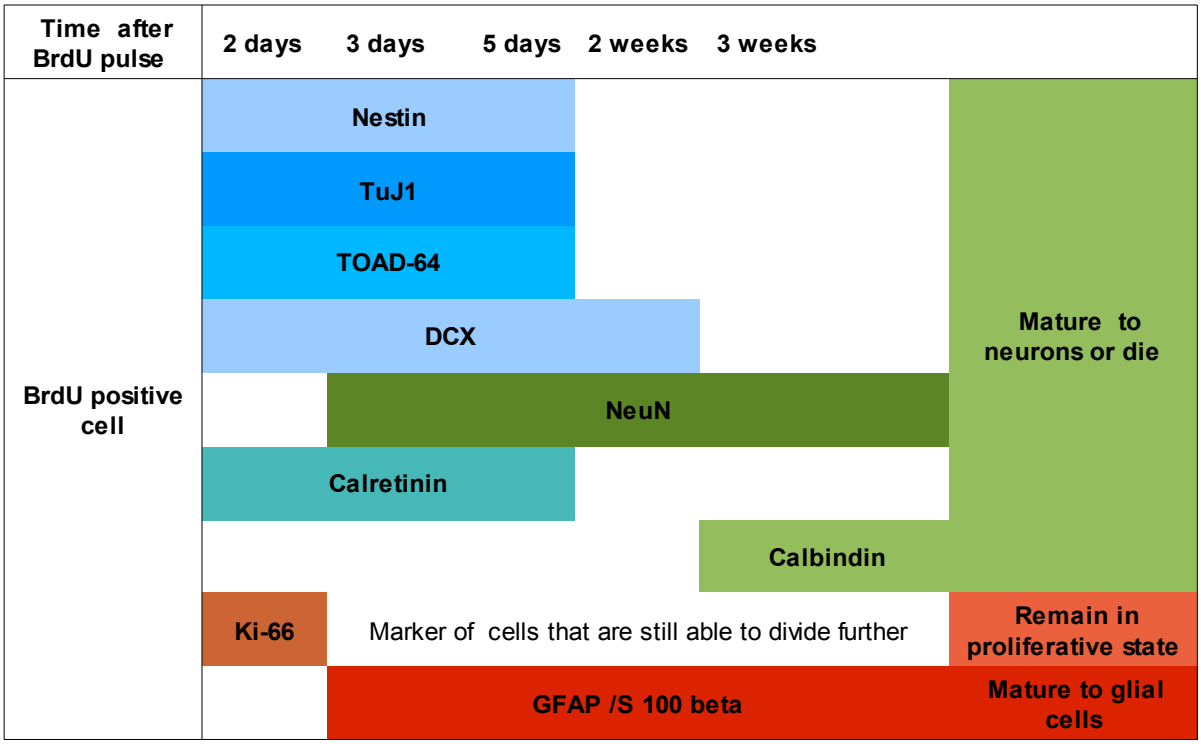

Fig.15 An overview of immunoreactivity through the maturation of a proliferating precursor during the first weeks after a BrdU pulse. Adapted from Abrous et al. 2005.

Alvares – Buylla and his colleagues performed experiments on transgenic mice, in which developing cells in the dentate gyrus produced the avian retrovirus receptor

under the control of the GFAP promotor. The cells that produced GFAP during their development were, at the time of GFAP production, prone to infection by the avian retrovirus. The virus was responsible for the production of detectable alkaline phosphatase (AP) in these cells. These avian retrovirus-infected cells then matured into neurons and extended some of their processes (dendrites) through the granular cell layer and axons into the CA3 region; they also showed the potential for functional integration (Alvares–Buylla et al. 2002). More intensive GFP staining was achieved by infection with a mutant Semliki Forest Virus (pSFV), which revealed the morphologies of inhibitory neurons in the DG, **the basket cells**. Newborn basket cells are also positive for **glutamic decarboxylase,** a marker of immature inhibitory neurons (Liu et al. 2003).

It has been observed that the cell cycle of the proliferating cells is about 24 hours. The number of proliferating cells doubles during the first 24 hours of observation and then doubles again. Figure 15 shows the presence of detectable antigens during the maturation of the proliferating cells. Fifty percent of the cells die until 22 days after birth (Dayer et al. 2003). Newborn cells were proven to survive as long as eleven months after labeling in mice (Kempermann et al. 2003).

1.3.3. INTRINSIC FACTORS REGULATING ADULT NEUROGENESIS

Studies of adult neurogenesis have provided evidence that this process can be influenced in at least two phases. There are factors that influence the processes of **proliferation** and **survival**. Early studies in rodents contributed to an understanding of how alterations in physiological signalling systems affect the rate of cellular proliferation, and some insight has been gained into the fate of newborn cells. In some cases of induced proliferation, the proliferating cells are not able to survive. The table below shows an overview of the studied systems that affect neurogenesis in its initial phase. The table (Fig.16) provides overview of various hormonal systems that participate in regulation of neurogenesis.

Proliferation is mainly regulated by the levels of hormones. The number of cells proliferating in the DG of animals that underwent **adrenalectomy** was higher than in controls (Gould 2002). Proliferation in the SVZ seemed to be unaffected by adrenalectomy. The maximal proliferation rate of postnatal granule cells occurs during the early postnatal period, when there is the stress hyporesponsive period. Young pups do not react to stress with a rise in **circulating corticosteroid** levels. In aged animals, the levels of corticosteroids naturally increase, and granular cell proliferation declines in these animals (Gould 2002). The survival of cells generated during proliferation increases 1 to 6 days after adrenalectomy is

corticosterone independent; the cells survive at least 4 weeks (Rodrigues et al. 1998).

Corticosterone seems to act on the neighboring glial cells, which express corticoid receptors and could control the cell cycle by releasing growth factors (Sousa et al. 2002). The down regulation (Fig.18) of insulin-like growth factor (IGF-I) has also been proposed as a possible negative influence on the rate of neurogenesis. Corticosteroids also increase **glutamate release**. The negative effect of high corticosterone levels can be blocked by NMDA receptor inactivation (Cameron et al. 1997).

Ovarian steroids have stimulatory effects (Fig.16). Additional new cells in female rats are produced during proestrus (when estrogen levels are the highest), but under standard laboratory housing conditions, these cells are lost. In wild rats single estrous cycles are usually followed by pregnancy (Gould 2002). During estrus and diestrus in female rats, there is a similar proliferation rate as in males, but many more pyknotic cells are detectable (Tanapat 1999). Interestingly, low levels of estradiol are correlated with lower synapse density in the CA1 stratum oriens of the hippocampus (Woolley et al. 1992).

Growth factors are produced by cells adjacent to the neurogenic zones. Some extrinsic factors, such as pharmacological manipulation and environmental effects, are believed to enhance their production. An overview is presented on figures 17 and 18. Epidermal growth factor (**EGF**), fibroblast growth factor (**FGF**) or both can stimulate the in vitro cultivation of stem-like cells from the SVZ (Alvares - Buylla et al 2002).

Hormones that enhance proliferation	Effect on proliferation	Effect on differentiation and survival	Reference
Depletion of **corticoids** – **adrenalectomy**	Adrenalectomy increases proliferation	Manipulations with corticosteroids do not alter cell differentiation and survival.	Cameron et al. 1994
Gonadal hormons – **estradiol**	Higher proliferation is observed in proestrus of cycling rats. Administration of estradiol in ovarectomised rats increases proliferation.	Newborn cells produced in proestrus in females do not survive- therefore there is no sex difference in amount of produced cells	Tanapat et al. 1999
Neurosteroids: **DHEA** (dehydroxyepiandrosteron), **prenenolon –** is produced in glial cells in hippocampal formation	Antagonists of GABA A receptor stimulates cell proliferation after 24 hrs (the proliferation is blocked by muscimol – GABA A agonist). Acts as posible antistress hormon - blocs effect of corticosterone	Newborn cells stimulated by DHEA pelets in male rats - survive and express NeuN	Karishma et al. 2002

Fig.16 An overview of the influence, that some of the studied, hormonal systems have on proliferating precursors. Adapted from Abrous et al. 2005.

Growth factors that enhance proliferation I	Effect on proliferation	Effect on differentiation and survival	Reference
FGF-2 or bFGF **–** basic fibroblast growth factor	Increases the proliferation in SVZ		Kuhn et al. 1997
EGF – epidermal growth factor and **HB-EGF** - heparin binding EGF	HB-EGF stimulates birth in the SVZ and DG	HB-EGF increases the number of newborn cells expressing the NeuroD, Dcx and reaching the OB. EGF decreases the number of newly born cell reaching the OB.	Jin et al. 2002, a
TGF- alpha – transforming growth factor	icv administration induces dramatic increase in proliferation of the cells in SVZ, TGF-alfa null mice show decreased proliferation and number of the cells in the OB	No data have been revealed on the effect to the hipocampal precursors	Tropepe et al. 1997

Fig. 17 First part of an overview showing the influence that studied growth factors have on proliferating precursors. Adapted from Abrous et al. 2005

Growth factors that enhance proliferation II	Effect on proliferation	Effect on differentiation and survival	Reference
IGF-I – growth promoting peptide hormon	increases the proliferation in DG and hilus in hypophysectomised rats, the increased proliferation in normal rats is not associated with enhancement of differentiation to the neuronal fate	In hypophys-ectomised rats is the increased proliferation followed by neuronal differentiation to BrdU/calbindin + neurons (unlike in nonhypophys-ectomised rats).	Aberg et al. 2000
BDNF – brain derived neurotrophic factor	Icv infusion increased number of f BrdU labeled cell in OB and DG. RILUZOL- stimulator of endogenous BDNF- increased only cell birth and not survival, this effect was blocked by antibodies against BDNF	BDNF is preventing neurons from cell death and seems to be positive regulator o both proliferation and survival.	Zigova et al. 1998 and Katoh-Semba et al. 2002
VEGF – vascular endothelial factor (its neurotrophic properties are shown by the fact that clustes of dividing cell are in close proximity to blood vessels)	Icv administration for 7 days of VEGF stimulates proliferation in rodent SVZ and SGZ.	The proliferating cells then express s NeuN and Dcx neuronal markers.	Jin et al. 2002, b

Fig. 18 Second part of an overview showing the influence, that studied growth factors have on proliferating precursors. Adapted from Abrous et al. 2005

1.3.4. EXTRINSIC FACTORS REGULATING ADULT NEUROGENESIS

Environmental and physiological stimulations have been examined in several rodent studies, revealing some aspects of the functional significance of neuronal turnover in the hippocampus. Some pathological states have been found to be associated with alterations of adult neurogenesis in the hippocampal formation.

An enriched environment (EE) has been defined as a more physiological counterpart to normal laboratory conditions (Rosenzweig 1996). The availability of physical and cognitive exercise, together with social interaction, has been found to modify brain functioning in many aspects such as learning, neurochemistry, and structural organization (Van Praag et al. 2000). The exposure of 3-month-old female C57BL/6 mice to 12 days of an enriched environment did not affect cell proliferation in the DG, but it **increased the number of cells that survived** and differentiated into a neuronal phenotype (van Praag et al. 1999, Nilsson et al. 1998). A comparison of different mouse strains revealed that their genetic background plays an important role. C57BL/6 mice have high baseline neurogenesis unlike 129/Svj mice, which respond to EE exposure with a higher rate of proliferation (Kempermann et al. 1997). The effects of a social environment have also been studied. Isolation caused a decrease in cell proliferation. The decreased rate was reversed by subsequent group housing for 4 weeks (Lu et al. 2003).

The key factor inducing higher rates of proliferation has been found to be motor exercise. Mice housed with a running wheel, who thus could engage in **voluntary wheel running, displayed higher proliferation rates** than mice housed in an EE without a running wheel. Those newborn cells also survived and differentiated into neurons (van Praag et al. 1999, Kroneberg et al. 2003). Running increased the number of cells corresponding to Type II nestin - positive progenitors, some of which expressed early neuronal markers such as Doublecortin or PSA-NCAM, suggesting this type of stimulation positively affects the neuronal lineage of asymmetrically dividing cells (Kroneberg et al. 2003). Voluntary wheel running has been associated with the increased production of several growth factors, e.g. BDNF, IGF-I and VEGF. It has been proposed that the stimulatory effect of wheel running on proliferation during neurogenesis is mediated by these factors (Neeper et al. 1996, Russo-Neustadt et al. 2000).

It was suggested that the major positive effect of environmental enrichment (without a running wheel) is the exposure to novel conditions, which in turn leads to exploration and **learning**. Exposure to enriched conditions for an extended period does not further benefit hippocampal neurogenesis (Kempermann et al.

1999). The effect of learning on hippocampal neurogenesis has been investigated in several hippocampus-dependent tests. **Spatial navigation tests in the Morris water maze** have provided a number of controversial results, even leading to the suggestion that the challenge of the water maze does not create demands for the generation of new cells (Shors et al. 2002). Three-month-old mice exposed to 12 days of an acquisition task did not show a greater rate of either proliferation or cell differentiation (van Praag et al. 1999). Four days of acquisition learning in the MWM increased the number of surviving cells in 3-month-old rats (Gould et al. 1999). Increased proliferation was shown after five days exposure to the MWM in 3-month-old rats (Lemaire et al. 2000). These different results were caused by variations in experimental design. **The trace protocol in eye blink conditioning** presents a challenge to the hippocampal formation. The animals have to associate an auditory tone - the conditioned stimulus - with an electric foot shock - the unconditioned stimulus - that are separated by a trace time interval. Blocking cell proliferation with the toxin methylazoxymethanol acetate (MAM) for two weeks resulted in impaired trace conditioning. When MAM was administered for only 6 days, the acquisition learning of the task was comparable to that of controls. This suggests that cells born 1 to 2 weeks before the learning challenge contribute significantly to better performance on this learning task.

Several hypotheses can be put forward to explain the function of the cellular turnover process in the hippocampus for learning:

- The newborn cells have a greater chance to integrate and survive when an occasion for learning is presented by a hippocampus-dependent task or simple novelty exploration in an EE. This could be termed the **“Use it or lose it“ hypothesis**. The contribution of the newborn cells can be studied by examining hippocampus-demanding tasks.
- The cellular turnover is associated with the cell death of possibly unnecessary or non-integrated neurons. The learning-related synaptic connections **may be consolidating the memory trace**.
- The ability to induce cell proliferation may be involved in memory flexibility. Presenilin-1 knockout mice, with normal basal proliferation but reduced enrichment-induced cell proliferation, show better retrieval of contextual fear memory traces than do wild-type mice. Deficient up-regulation of cell proliferation may prevent **memory clearance** and impair **forgetting processes** (Mc Guire et al. 2001, Abrous et al. 2005).

1.4. THE HIPPOCAMPUS AND STRESS

The hippocampus has been observed to be a brain structure altered by stressful experiences. Atrophy and cell death in the hippocampus, together with changes in the prefrontal cortex, have been observed in a number of clinical studies (Potter et al. 1998).

A histopathological study was performed on captive primates that were exposed to psychosocial stress from other animals in the cage. These animals had bite wounds, gastric ulcers and hyperplastic adrenal glands. Brain autopsies revealed **neuronal degeneration in the CA3, CA1 and hilus of the hippocampus** and in parts of the cortex (frontal prefrontal cingulated and postcentral gyres), whereas neurons in other brain regions as well as in **the granular cell layer appeared intact**. The damaged CA3 neurons had reduced and irregularly shaped pericarya associated with dispersed Nissl bodies and atrophic dendritic branches. The numbers of mossy fibre terminals and synaptic vesicles were reduced and the dendritic branches wereedematous with only a few neurotubules. There were also changes in oligodentrocytes, which displayed anedematous cytoplasm, dispersed ribosomes and irregular vesicles (Uno et al. 1989).

Hippocampal neurons are extremely vulnerable to the effect of cortisol, therefore damage to the hippocampus might also prevent the inhibition that the hippocampus usually exerts on the HPA axis (Potter et al. 1998). This leads to a vicious circle resulting in an inability to adapt to new situations.

- **Stress in its acute phase** is known to induce a cascade of beneficial endocrine and behavioural responses. A rising level of cortisol provides **additional energy to neurons, modulates excitability, influences the magnitude of long-term potentiation and produces long- term depression** (Mc Ewen 2001).
- **Chronic or repeated stress** leads to adverse effects on neuronal function and even to damage resulting in cell death. Repeated stress induces a **shortening and debranching of apical dendrites in the CA3 region,** reversible if the stressful situation is terminated before the 21st day of exposure. This phenomenon is modulated by excitatory amino acids, since the pharmacological blockade of the NMDA receptors by phenytoin prevented the remodelling (Mc Ewen 2001). Stress presented by restraining for 21 days caused a decrease in synaptic density on the mossy fibre terminals in the CA3 region of young rats. The density returned to normal levels when the animals were exposed to four sessions of learning in the Morris water maze after the termination of the restrained stress (Sandi et al. 2003). In this experiment, the novel stressful experience in the water maze was not inescapable; on the contrary, the animals learned that finding

escape on the platform would interrupt the activity of the stressor. This implies that when animals are able to adapt to a stressful condition through a learning process, the effect of the stress might not cause damage.

Stress has been found to be the major regulator of the proliferation phase of adult neurogenesis (Pham et al. 2003). One week after adrenalectomy, the turnover of proliferating cells in the GCL was increased in young and aged (20 months) rats. The age-related decline in progenitor cell turnover (the number of proliferating and apoptotic cells) was 'reversed in this experiment (Cameron et al. 1999). A similar result was observed when the effect of glutamate was blocked by an antagonist and deafferentation – the destruction of the entorhinal cortex (Cameron et al. 1995). The administration of NMDA, in contrast, decreased the number of proliferating cells but did not completely terminate DNA synthesis.

1.5. THE HIPPOCAMPUS AND AGING

There are large individual differences in the cognitive abilities of aging rats. Not all aged rats exhibit cognitive disorders; some rats perform as well as young controls, whereas others present spatial memory impairments (Rapp et al. 1991). The extent of memory dysfunction is related to the severity of the age-related changes occurring in the hippocampus. Neurogenesis (i.e. cell proliferation and cell survival) has been shown to be higher in rats that were less impaired on spatial learning in a water maze (Drapeau et al. 2003).

The absolute number of granule cells does not differ between age-impaired and -unimpaired rats (Rapp et al. 1996). The preservation of cognitive functions is associated with the maintenance of a neurogenesis level that contributes to the rejuvenation of the granule cell pool. Exhaustion of the potential for neurogenesis at an advanced age could lead to cognitive deficits. The differences in hippocampal neurogenesis in aging animals may be due to the lack or presence of stimulating or inhibiting factors that accelerate aging (Abrous et al. 2005). The hypothesis that hippocampal cognitive aging is the result of long-term exposure to glucocorticoids is difficult to challenge. The detrimental effect of HPA axis deregulation in old age supports the theory of a steep cognitive decline towards the end of mammalian life. The following hypotheses (Fig. 19) have been proposed (Potter et al. 1998).

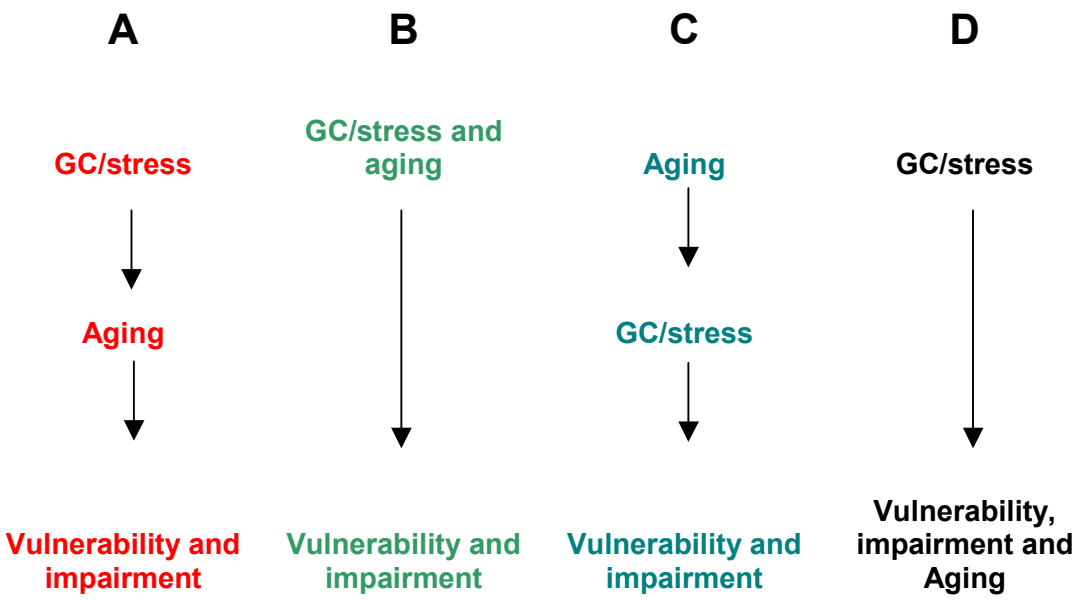

Fig.19 Diagrams of four hypotheses proposed by Potter et al. – capturing the link between aging and glucocorticoid (GC) exposure. Adapted from Potter et al. 1998.

Gene microarray analysis from the hippocampal tissue of behaviourally phenotyped rats in three age groups revealed alterations in gene expression associated with aging and cognitive decline. **Aging- and cognition-related genes** were compared between the age groups and the figure 20 presents the most significant geen groups to be down- or up- regulated in aging rats (Blalock et al. 2003).

The transcription of genes involved in the following processes was:	
Down-regulated	**Up-regulated**
Signalling	Inflammation, immunity, oxidative stress
Energy metabolism, mitochondrial transporters	Negative transcriptional regulation
Transcription factors (early re-sponse)	Cholesterol and lipid metabolism
Biosynthesis	Myelin related proteins
Extracelullar matrix	Iron utilisation
Activity regulated synaptic plasti-city	Amino acid metabolism
Protein trafficking	

Fig. 20 An overview of the Aging- and cognition-related genes that were compared between the age groups in aging rats (Blalock et al. 2003)

In contrast to these modern high-tech research methods, the most potent therapeutic approach to postponing age-related cognitive decline is very simple and affordable. Mark Mattson postulated this effective idea in simple words: “TAKE AWAY MY FOOD AND LET ME RUN”.

Physical activity and caloric restriction are the manipulations that have been proven to prolong the life span of laboratory animals, and numerous observations have documented the relationship between lifestyle and successful aging in humans (Mattson 2000).

Physical activity has been found to increase neurogenesis and enhance performance on spatial learning tasks in rodents. The proposed mechanism of action was an increase in the levels of the mediators serotonin and BDNF in response to exercise. This is consistent with the fact that the administration of antidepressants, as well as of BDNF, increases the proliferation of neuronal precursors (Fig. 21). A great deal of evidence has been presented demonstrating enhanced synaptic plasticity as a result of housing an animal in a complex environment. Also important are the effects on non-neuronal elements in the brain. The process surface density of astrocytes in the visual cortex and the volume of astrocytic processes in the cerebellum were increased in animals reared in an enriched environment (Churchill et al. 2002).

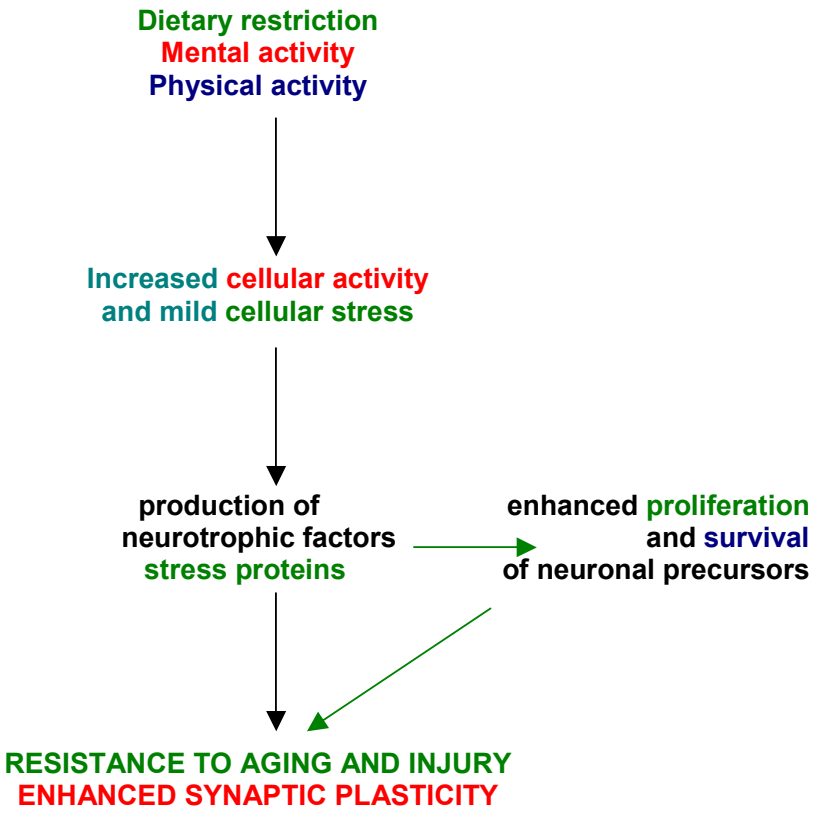

Fig. 21 Schematic diagram of the proposed action of the manipulations postponing hippocampal aging. Adapted from Mattson et al. 2000.

The mechanisms of action underlying the beneficial effects of **caloric restriction** have been extensively studied. The mechanisms are complex and act on many levels. Frequently discussed mechanisms involve *reduced oxidative damage, lowered blood glucose, and the decreased secretion of growth hormone, insulin and insulin-like growth hormone*. The reduction in the levels of these hormones leads to a decreased rate of cellular proliferation. Caloric restriction, however, paradoxically elevates blood corticosterone levels**.** Reduced food intake results in a homeostatic response and the need for constant flexibility to increase gluconeogenesis.

How can elevated corticosterone in this case be beneficial for sparing cognitive abilities and increasing longevity? The secretion of cortisol during caloric restriction is diurnal, and the level of free cortisol is elevated due to a reduction in the concentration of corticoid binding plasma protein. Caloric restriction increases the expression of **heat shock proteins**, which may improve calcium homeostasis and increase neuronal protection against future stress and toxins. Short-term caloric restriction also increases the production of **BDNF** and **glia-derived neurotrophic factor** – trophic factors that increase the survival of newborn neurons. Caloric restriction attenuates age-related **GFAP expression** and the hypertrophy of astrocytes that prevent neurite outgrowth in the rat hippocampus.

Advanced glycation end-products are reduced during caloric restriction when the level of blood glucose is decreased by 15%. These glycated proteins might initiate an **inflammatory process** and activate microglia.

Occupation of the high affinity mineralocorticoid receptor in the hippocampus is necessary for **optimal long-term potentiation**. The activation of lower affinity glucocorticoid receptors in the hippocampus during higher blood cortisol levels leads to higher extracellular glutamate concentrations and shifts the balance away from LTP. The number of the glucocorticoid receptors in the CA1 of the hippocampus is, reduced after 3 months of caloric restriction (alternate day feeding), which might enhance learning and reduce the vulnerability of hippocampal neurons to insults (Patel et al. 2002). Generally, dietary restriction and physical activity might induce a mild stress response in neurons, presumably due to the reduced availability of glucose. Such a preconditioning mechanism might increase the resistance of neurons to injury (Mattson 2000).

1.6. THE HIPPOCAMPUS AND EPILEPSY

Epilepsy is a common neurological disorder that affects approximately 0.7% of the worldwide population. Epilepsy is characterised by recurrent spontaneous seizures that originate in particular parts of the brain (Bluemcke et al. 1999). The overview of the current seizure types classification is presented on figure 22.

Generalized seizures
* Tonic-clonic seizures
  * Clonic seizures
    - o Without tonic features
    - o With tonic features
  * Typical absence seizures
  * Atypical absence seizures
  * Myoclonic absence seizures
  * Tonic seizures
  * Spasms
  * Myoclonic seizures
  * Massive bilateral myoclonus
  * Eyelid myoclonia
    - o Without absences
    - o With absences
  * Myoclonic atonic seizures
  * Negative myoclonus
* Atonic seizures
  * Reflex seizures in generalized epilepsy syndromes
  * Seizures of the posterior neocortex
  * Neocortical temporal lobe seizures

Focal seizures
  * Focal sensory seizures
    - o With elementary sensory symptoms (e.g., occipital and parietal lobe seizures)
    - o With experiential sensory symptoms (e.g., temporo parieto occipital junction seizures)
  * Focal motor seizures
    - o With elementary clonic motor signs
    - o With asymmetrical tonic motor seizures (e.g., supplementary motor seizures)
    - o With typical (temporal lobe) automatisms (e.g., mesial temporal lobe seizures)
    - o With hyperkinetic automatisms
    - o With focal negative myoclonus
    - o With inhibitory motor seizures
  * Gelastic seizures
  * Hemiclonic seizures
  * Secondarily generalized seizures
  * Reflex seizures in focal epilepsy syndromes

Continuous seizure types
Generalized status epilepticus
  * Generalized tonic-clonic status epilepticus
  * Clonic status epilepticus
  * Absence status epilepticus
  * Tonic status epilepticus
  * Myoclonic status epilepticus

Focal status epilepticus
  * Epilepsia partialis continua of Kojevnikov
  * Aura continua
  * Limbic status epilepticus (psychomotor status)
  * Hemiconvulsive status with hemiparesisGeneralized seizures

Fig. 22 The International League Against Epilepsy (ILAE classification of the seizure types) (http://www.ilae-epilepsy.org/Visitors/Centre/ctf/seizure_types.cfm):

In a fraction of these patients, the seizures originate in the mesial temporal lobe and involve the hippocampal formation and amygdala.

Mesial temporal lobe epilepsy can be classified as a type of focal seizure. Simple focal seizures do not involve a loss of consciousness. Such seizures usually present with symptoms such as a sensation of epigastric rising, emotional changes – fear, and occasionally olfactory or gustatory hallucinations. A **complex partial seizure** starts as a stare *w*ith arrest of motion, followed by altered responsiveness, oroallimentary and gestural automatisms and later reactive automatisms. There is usually amnesia for the ictal event. These complex partial seizures involve the temporal lobe (Engel 1996).

Mesial temporal lobe epilepsy (MTLE) does not have a significant genetic component, but neuropathological damage has been described in many patients (Blumcke et al. 1999). The focal lesions observed within the hippocampus of patients with MTLE are mainly **mesial temporal lobe sclerosis** and lesions that belong to the group of glioneuronal neoplasms and malformations.

About 60% of patients with MTLE show unilateral atrophy of the hippocampal formation – **Amon's horn sclerosis**. Histopathologicaly, the hippocampus of these patients undergoes segmental neuronal loss in the CA1 and hilus, whereas granule cells in the dentate gyrus and the pyramidal neurons in the CA2 region are more resistant. The loss of neurons is accompanied by dense fibrillar astrogliosis, which leads to the shrinkage and hardening of the tissue in the proximity of the neuronal damage. The selective loss of hillar interneurons, the excitatory mossy cells and inhibitory basket cells has also been described (Blumcke et al. 1999). There is **aberrant axonal reorganisation of the mossy fibres** and other axonal pathways. The recurrent connectivity has been proposed as the major epileptogenic mechanism compromising the inhibitory functions in the dentate gyrus. Timm's staining is used to detect the sprouting fibers (Sutula 2002). Aberrant sprouting is enhanced by molecules of the extracelullar matrix produced by astrocytes that regulate the axonal guidance (Blumcke et al. 1999). Signals for sprouting neurites might be also mediated through the activation of the NMDA receptors.

The development of **animal models of MTLE** allowed for the comparative study of the cellular and molecular mechanisms involved in the pathogenesis of mesial temporal lobe epilepsy. Some aspects of human MTLE can be reproduced in these animal models. The most commonly used animal models for focal limbic epilepsies are chronic seizures induced **by kainic acid, pilocarpine or kindling**. The induction of status epilepticus by an intraperitoneal or intracerebral infusion of

neuroactive drugs such as pilocarpin or kainic acid induces segmental neuronal loss in the hippocampal formation, which corresponds to findings in human Amon's horn sclerosis. The subconvulsive electrical kindling of the amygdala results in sustained hippocampal seizure activity, which usually does not lead to gross histological alterations.

The ethiopathogenesis of MTLE in humans is still not completely understood. Animal studies suggest that an initial event involving synchronised neuronal activity, such as status epilepticus, induces hippocampal pathology - Amon's horn sclerosis. In humans it is often impossible to identify the initial event. However, a number of patients with MTLE experienced febrile seizures in childhood (Blumcke et al 1999). The question of whether the febrile seizures are the cause or the first manifestation of a congenital predisposition is still not answered. The increased incidence of MTLE in some families suggests a genetic predisposition (Engel, 1996). There is also evidence that patients with Amon's horn sclerosis have dysplastic abnormalities - heterotopia in addition to hippocampal sclerosis – a dual pathology (Engel 1996).

Glioneuronal neoplasms and malformations are the cause of MTLE in approximately 30% of cases. Among these lesions are predominating gangliogliomas, dysembryoplastic neuroepithelial tumors, low-grade astrocytomas, as well as hamartomas (Blumcke et al. 1999). Vascular malformations located in the temporal lobe can induce mesial temporal lobe epilepsy.

Temporal lobe epilepsy is often refractory to the available antiepileptic drugs. The only therapeutic possibility that then remains is often the surgical removal of the affected brain tissue

In the clinical environment mesial temporal lobe sclerosis can be revelaed by combination of **several diagnostic methods**. There is atrophy and increased signals on T2 weighted MRI in the medial parts of temporal lobe. Positron emission tomography (PET) and ^{1}H magnetic resonance spectroscopy allow metabolism studies. Electronecephalography (EEG) with use of deep brain electrodes can confirm the epileptogenic focus (Hájek et al. 2003).

Adult neurogenesis, which continues in the subgranular zone of the GCL, is enhanced during recurrent seizures. Granule cells that are born after seizures can migrate into the hilus, and some newly born granule cells migrate as far as the CA3 cell layer, where they become integrated abnormally into the CA3 network although they retain granule cell intrinsic properties (Scharfmann et al. 2000). Gamma irradiation of the rat brain abolishes adult neurogenesis, and no new cells are born even after status epilepticus. Blocking neurogenesis, however, does not

prevent the sprouting of the mossy fibres, suggesting that the sprouting is independent of neurogenesis (Parent et al. 1999). Recent reports suggest that the newborn neurons might contribute to maladaptive consequences supporting epileptogenesis as well as memory dysfunction in patients with MTLE (Parent et al. 2005, Sutula et al. 2003).

1.6.1. RADIOSURGERY TREATMENT OF THE TEMPORAL EPILEPSY

Amygdalohippocampectomy has become the accepted standard of treatment in patients with refractory mesial temporal lobe epilepsy. The rates of seizure-free patients are about 70 to 80% (Bazil et al. 2001). Gamma knife radiosurgery can be appealing as a bloodless alternative (Khalil 2004). The first clinical studies have been performed including patients with mesial temporal sclerosis, and there have been numbers of experimental studies on animals with focal epilepsy. The results of animal experiments have shown that a decrease in the frequency of epileptic seizures can be achieved with subnecrotic doses of radiation. The application of 20 Gy to the ventral part of the hippocampus led to a decreased frequency of epileptic seizures in a rat model of epilepsy within 2 months after gamma knife radiosurgery, and this effect was even more pronounced 4-6 months after the radiosurgery. A dose of 40 Gy led to a decrease in epileptic seizures within 1 month with the maximum effect seen during the second month after radiosurgery (Chen et al. 2001). A positive antiepileptic effect on animals could be achieved with a low, subnecrotic dose of 20 Gy. An antiepileptic effect of a dose - 20 Gy to patients with temporal epilepsy has been observed in some studies (Barcia-Salorio et al. 1998), but failed in others, as was recently reported (Srikijvilaikul et al 2004). Experience with the radiosurgical treatment of brain tumors and arterio-venous malformations with secondary epilepsy also showed, that an effect on secondary epilepsy can already be achieved with relatively low doses of radiation.

Patients with bilateral epileptic foci have been disqualified for epileptosurgery or radiosurgery as well as some patients, whose results on amobarbital and neuropsychological tests do not allow sacrificing the hippocampus. At the time, when our experiments were planned, radiosurgery using subnecrotic doses to the amygdalo-hippocampal complex was supposed that it might bring relief to the patients without anatomical ablation of this structure. These patients could have been indicated to subnecrotic radiosurgery on the focus in hippocampus to reduce the epileptic activity without impairing the function of hippocampus in memory.

The clinical observation of patients, that were treated by stereotactic radiosurgery with dose of 50 Gy on hippocampus during 1996-1997 revealed, within last years, a necesity to remove the epileptogenic focus during open operation for several reasons. Today is the treatment of mesiotemporal lobe sclerosis and epilepsy not included among the typical indications for stereotactic radiosurgery.

This dissertation is based on two different approaches to study the relationship between function and structure of hippocampus in laboratory rats.

In the first set of experiments hippocampus was damaged by gamma irradiation applied in several doses. Development of functional impairment in dependency on the structural changes was followed. Radiation was exactly delivered by the Leksell gamma knife. The function was examined in regular time intervals with the Morris water maze test (MWM). This test is revealing rat's ability to use spatial navigation and show tendency of hippocampus dependent learning. Structural impairment was observed on regular MRI scans and post mortem on the histological evaluation.

The second project used the apparatus of Morris water maze to study the outcome of exposure to this challenge (requiring the process of lhippocampus dependent earning) on the number of newly proliferating cells in neurogenic region - the dentate gyrus of the hippocampus. The aim was to compare the effect of learning in environment of this benchmark test alone to the effect of combination with pharmacological stimulator of proliferation - the antidepressant fluoxetine. The proliferating cells incorporated the fake nucleotide BrdU before the sacrifice and were then detected with help of antibodies against BrdU. Compared were the responses of three age groups.

2. EXPERIMENTAL AIMS

1. What is the dose of gamma irradiation to the rat hippocampus that causes functional impairment of spatial navigation in rats?

We created and tested an animal model of radiation-caused functional ablation of the hippocampus. In pilot study we created the irradiation plan with help of high irradiation dose (150 Gy) to position the isocenters to impair the functionaly relevant parts of hippocampus. Consequently we studied what would be the maximal radiation dose that would spare the ability of a rat to perform spatial learning.

2. Can a subnecrotic dose of gamma radiation to the rat hippocampus cause a functional impairment of learning?

The highest subnecrotic dose was determined from the previous experiment. Animals exposed to doses equal to or lower than 50 Gy did not show impaired learning or the development of necrosis 1, 3, 6 or 12 months after irradiation. However, long-term survival and regular observation of our animal model was necessary to rule out any minor transient changes that might show an adverse effect of this therapy.

3. Does combining a spatial navigation task in the MWM with antidepressant Fluoxetine affect the proliferation of neuronal precursors in the rat hippocampus?

Neurogenesis can be influenced by the animal's experience and environment. The effect of complex stimulation in the Morris water maze, where a rat is exposed to physical activity and a spatial learning challenge, on proliferation has not been fully established. We studied the effect hippocampus-dependent learning, the administration of the antidepressant fluoxetine, and their combination on cell proliferation in the granular cell layer of the dentate gyrus of adult rats.

4. Does the proliferation of neuronal precursors in the rat hippocampus respond to spatial learning and fluoxetine treatment differently during aging? We exposed three age groups of animals (3 months, 9 to10 months and 28 to 31 months) to the challenge in the water maze. The different groups of animals received fluoxetine treatment alone or concomitantly with water maze training. We studied the animals' response to these manipulations in the three age groups of adult rats.

3. METHODS

3.1 METHODS: RADIOSURGERY OF THE RAT HIPPOCAMPUS

In the first project hippocampus was damaged by gamma irradiation applied in several doses. Development of functional impairment in dependency on the structural changes was followed. Radiation was exactly delivered by the Leksell gamma knife. The function was examined in regular time intervals with the Morris water maze test (MWM). This test is revealing rat's ability to use spatial navigation and show tendency of hippocampus dependent learning. Structural impairment was observed noninvasively on regular MRI scans and post-mortem on the histological evaluation.

Animals: We used 3-month-old male Long- Evans hooded rats whose weight ranged between 320 and 380g.

The gamma knife surgery: The gamma knife surgery was performed using a Leksell gamma knife (ELEKTA Instrument AB, Stockholm, Sweden) (Fig.23 A). The animals were under deep Thiopental anesthesia (40 mg/kg) and fixed in a specially developed MRI-compatible stereotactic frame (Fig. 23B). The exact position of the hippocampus was established using a 1.5 T Siemens Expert MRI, and the MR images were exported to a GammaPlan treatment planning workstation.

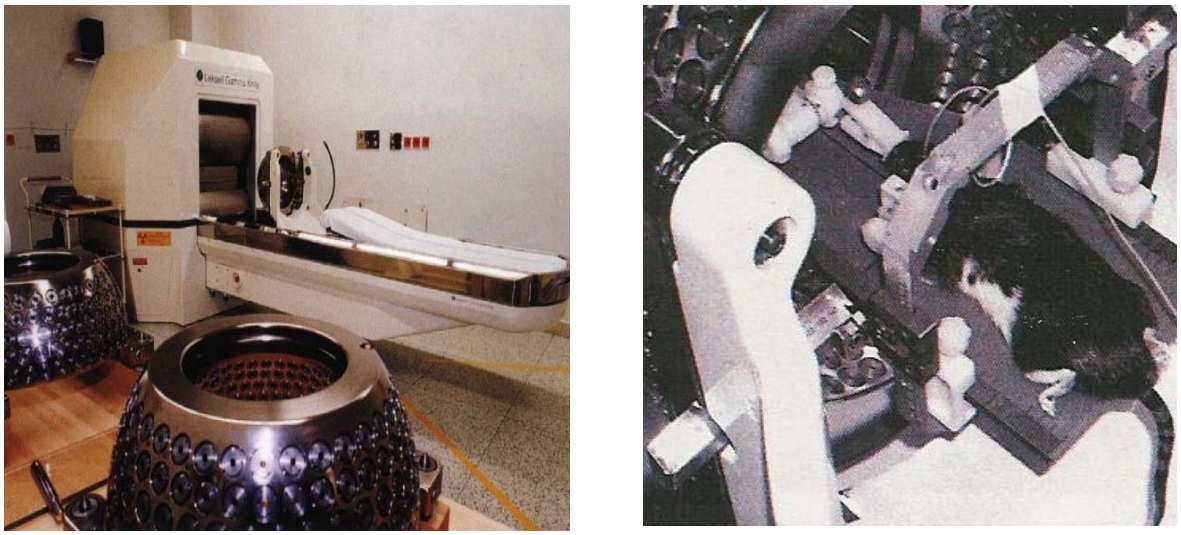

Fig. 23 A. (left) The apparatus for stereotactic radiosurgery in the Department of Stereotactic and Radiation Neurosurgery, Na Homolce Hospital in Prague. The collimator is seen in the front of the picture. B. (right). The experimental animal is placed in a nonparamagnetic homemade stereotactic apparatus and prepared for radiation treatment.

In the pilot study the dose was delivered with the use of a 4 mm collimator through two isocenters (Fig. 23 A) on each dorsal hippocampus, while four isocenters were used (Fig. 25) to cover the complete dorsal hippocampus with a 70% isodose in the long-term experiments (in detail described in our publication Liscak et al. 2002).

The **Morris water maze testing:** Behavioral testing of learning was performed in a Morris water maze, which is revealing impairment of spatial learning that is known to depend on intact hippocapus.

The Morris water maze used for these experiments was a circular pool, 196 cm in diameter, filled with 20-23°C water. Escape was possible on a hidden platform with a diameter of 10 cm, which was submerged 2 cm below the water surface so that the rat could not see it. The platform remained at the same position during the five consecutive days of acquisition testing. Multiple extra-maze landmarks (on the walls and ceiling of the room) provided cues to the spatial location of the hidden platform. The testing was performed 8 times each day. During the first few trials, the naive rats swam around the shores (natural behavior); the greatest chance for escape from water in nature is usually via the shore. Then, cruising through the pool in different directions led to finding the platform by chance. If this did not happen within 60 seconds, the rat was lead to the platform by hand. The rats were allowed to rest on the platform for 15 seconds; during this time the animals tried to determine the spatial coordinates and to remember the position of the platform. On the fifth day an unimpaired rat was usually able to find the platform in about 7 seconds when released from any location. A rat with impaired hippocampal function looked for the platform for significantly longer or even failed to find it at all. From the 8 measurements performed during a single day, the result of the first trial was omitted and the sum of the 7 remaining latencies was calculated. The theoretical maximal value of the sum of latencies was 420 seconds for each individual rat. When the group of rats treated with the same radiation dose was evaluated, the group average of these sums of latencies was calculated and plotted on the presented graphs (Fig. 3 on page 668 of our publication Liscak et al. 2002).

MRI follow up observations using a 4.7 T experimental MR spectrometer: All the groups, together with 10 controls, were scanned (Fig. 24) using a Bruker Biospec 47/20 spectrometer equipped with a homemade surface coil. The imaging was always performed following the functional behavioral testing. The animals were placed on a warming pad to maintain a constant temperature during measurements. During the examination, the animals were anesthetized by the

spontaneous inhalation of isoflurane (Forane, Abbott, CR). Anesthesia was induced by a 3% concentration of isoflurane in air and maintained at a 1.5% concentration during the entire measurement. A set of T2-weighted images was acquired using a standard turbo spin echo sequence, repetition time TR=2000 ms, echo time TE = 35.1 ms, field of view FOV = 4 X 4 cm, matrix 256 x 256, slice thickness/distance =1/1mm. The size of the lesion was evaluated by manual segmentation using the MaZda program (Hajek et al. 2006). T2 relaxation times were evaluated from a set of T2-weighted images obtained using a CPMG sequence with 30 echoes, TE = 8.63 ms, TR = 2500 ms. Sets of diffusion-weighted images were obtained using STEAM sequences, TR = 2000 ms, TE = 38 ms, mixing time TM = 11.1 ms, Δ = 30 ms, b-values were b=19, 58, 135, 404, 826, 1400 s/mm2, matrix 128x128, FOV = 3.5 x 3.5 cm^2, slice thickness 1 mm. The diffusion gradient was applied in the anterior-posterior direction. The apparent diffusion coefficient of water (ADCw) was evaluated using 2-parameter fitting. Spectra were obtained using a single voxel STEAM sequence, with an echo time TE = 3 ms and a repetition time TR = 5000 ms. The volume of interest was approximately 30 mm^3 for each hemisphere. The spectra were evaluated using the LCModel (Provencher 1993) to obtain absolute metabolite concentrations.

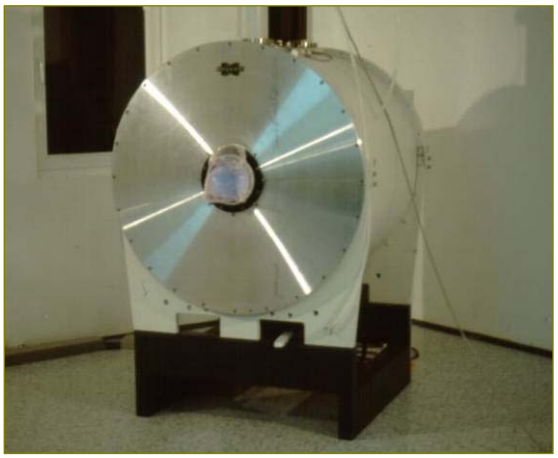

Fig.24 The 4.7 T experimental MR spectrometer in the Institute for Clinical and Experimental Medicine in Prague

For the **histological and imunohistochemical examinations** the animals were sacrificed by exsanguination under deep, whole body anesthesia (5% Narkamon Spofa, CR, 0.2 ml/100 g of the animal weight, i.p.). The brains were transcardially perfused by 0.9% NaCl followed by 4% buffered formaldehyde. For basic histology, the whole brains, or only their right hemispheres (see below), were post-fixed in either 4% buffered formaldehyde or Carnoy's solution (6:3:1) and embedded in Paraplast-Plus (Polysciences Inc, Warrington, PA). Seven micron -

thick coronary sections were cut using a sliding microtom and stained with toluidine blue. Immunohistochemical analyses were carried out on coronary cryostat sections prepared from unfixed or prefixed left hemispheres. They included the immunocytochemical detection of GFAP (Mabs GF-01, 1:25, Exbio, Prague, CR), Synaptohysin and Syntaxin (Mabs Clone SPV-38 and HPC-1, Sigma, St. Louis, Mo), followed by incubation with biotinylated goat anti-mouse IgG (Fab fragment, 1:200) and Extravidin-Peroxidase conjugate (1:100, both Sigma, St. Louis, Mo); diaminobenzidine was used as the substrate. Morphometric evaluations, including the determination of the width of the neocortex and hippocampus, the density of pyramidal hippocampal neurons and the intensity of the immunocytochemical stainings, were carried using an eye-piece micrometer and an ocular grid (20x20) or by using the Advanced Image Data Analyzer Program (AIDA 2.11, Raytest Isotopengeraete GmbH, Germany) applied to digitalized photomicrographs (Olympus Provis).

An additional **histological examination** was employed in the long-term survival experiment: The animals were sacrificed with a barbiturate overdose (200 mg/kg) and transcardially perfused with buffered saline followed by 4% buffered formaldehyde, 17 months after irradiation. Every fourth brain section (40 microns-thick) was stained with cresyl violet to visualize the extent of the changes induced by irradiation. Additional slices were stained with luxol fast blue. The slices were observed using an Axioskop 2; photographs were taken by an Axiovision set (Carl Zeiss, Germany).

Three pilot experiments were performed to develop the rat model for Gamma knife surgery on the hippocampus:

1. A pilot experiment performed with the aim of finding the ideal focus for irradiation on the structure essential for spatial learning.

Six animals were irradiated bilaterally using 1 isocentre aimed at each dorsal hippocampus. A second group of six animals was irradiated using two isocenters on each hippocampus. Three animals in each group received a dose of 100 and three of 150 Gy. A dose of 120 Gy is known to cause necrosis within 4 weeks (Kamiryo et al. 1996). The animals were pretrained on the acquisition spatial task for five days and were then tested every third or fourth day to evaluate their retention of the platform position in the water maze. When a worsening of their performance in the water maze occurred due to developing necrotic damage in the

hippocampus, the animal was sacrificed and the area of the lesion was examined (results in our publication Liscak et al. 2002)

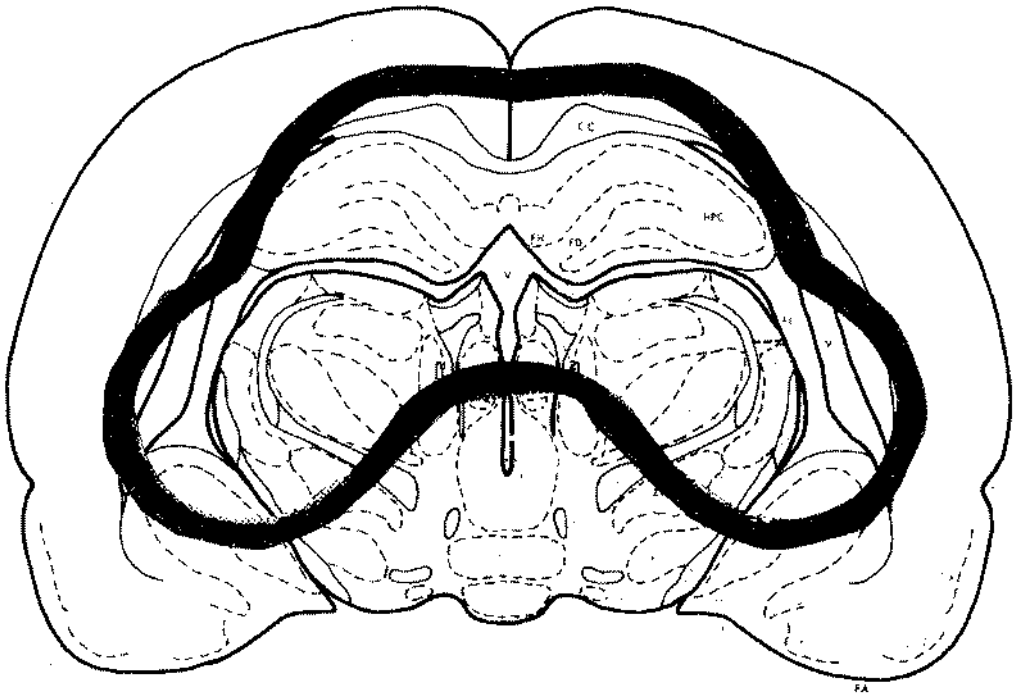

Fig. 25 The approximate target of irradiation (70 to 50 % isodose) and its location within the rat brain is shown on modified drawing according to Pellegrino et al. 1967. The schema of isodoses was created using GammaPlan software on the MRI reconstructed slice aquired on Siemens Expert 1.5 T imager. During the experiment was each animal individually positioned to the MRI compatible stereotactic frame and planning was done on basis of the individual MR image.

2. An experiment to determine the relationship of the dosage and time course of irradiation that would result in impaired spatial learning and structural changes in the hippocampus

All together fifty-two rats were irradiated according to an improved irradiation plan (after the first pilot study) using two isocenters (Fig. 25) for each hemisphere and covering the whole hippocampus with a 70% isodose.

The maximal doses applied to the centers of irradiation were as follows:

150 Gy 3 animals

100 Gy 9 animals

75 Gy 12 animals

50 Gy 16 animals

25 Gy 12 animals

The groups of animals were irradiated in four sets so that three animals irradiated with the same dose were present in one set (i.e. 3 animals irradiated with 25Gy, 3 with 50Gy, 3 with 75Gy…) together with 3 control animals (underwent only anesthesia). Each set of irradiated animals was tested at a given time point after irradiation; the time points were 1, 3, 6 or 12 to 14 months after irradiation. Histological analysis was performed after the rats were tested in the water maze 1, 3, 6, or 12 to 14 months after irradiation (results are published in our publication Liscak et al. 2002)

3. A third experiment studied the minimal and transient changes during 16 months after irradiation using the highest subnecrotic dose from the previous experiment (50 Gy)

Six animals were irradiated bilaterally with two isocenters on the dorsal hippocampus, utilizing the same irradiation plan as was used in the previous experiment. These were matched with six control animals to eliminate the effects of natural aging and the possible confounding effect of repeated long anesthesia during MRI measurements. Four irradiated animals and four controls underwent MRI analysis. MRI volumetry and magnetic resonance spectroscopy (MRS) were performed on two animals, while two other animals underwent MRI, using diffusion weighted imaging (DWI), approximately once a month. The remaining two animals were examined by MRI, MRS and DWI only at the end of the observation period, i.e. 16 months after irradiation. The animals were tested on acquisition in the MWM 6, 10 and 12 months after irradiation in order to correlate functional impairment with the changes that could be observed by MRI (results are published in our publications Herynek et al. 2004 and Jirak et al. 2007).

3.1. METHODS: PROLIFERATION IN THE ADULT RAT HIPPOCAMPUS

The second project used the apparatus of Morris water maze to study the outcome of exposure to this challenge requiring the process of hippocampus dependent learning on the number of newly proliferating cells in neurogenic region the dentate gyrus of hippocampus. The aim was to compare the effect of learning in environment of this benchmark test alone to the effect of combination with pharmacological stimulator of proliferation - the antidepressant fluoxetine. The proliferating cells incorporated the fake nucleotide BrdU before the sacrifice and were then detected with help of antibodies against BrdU. Compared were the responses of three age groups to the manipulations.

Animals: Male Wistar rats were aged 3 months in the young group (Y), 9 to 10 months in the middle-aged group (MA) or 27 to 31 months in the group of aged rats (A). Prof. J.P. Huston (Institute of Physiological Psychology, University of Düsseldorf) kindly provided the older two groups of animals.

The Morris water maze (MWM) acquisition training and drug administration:

After initial handling, the rats were randomly assigned to one of four groups (within each age group) and underwent 15 days of one of four different treatments. The first (control - C) group received a daily i.p. injection of 1.5 ml saline for 15 days. The second group (WM) received a daily i.p. injection of saline and underwent four trials per day of acquisition training in the MWM for 15 days. The third group (F) received for 15 days a daily i.p. injection of fluoxetine (5 mg/kg) diluted in saline. The fourth group (FWM) received an i.p. injection of fluoxetine one hour prior to learning and underwent the same acquisition training in the water maze as did the second group. The acquisition training took place during four consecutive trials per day. The Morris water maze apparatus was a circular tank 196 cm in diameter filled with lukewarm water (22±2°C). A submerged invisible platform 10 cm in diameter was kept in one quadrant for ten days and then moved to another quadrant on days eleven and thirteen. This modification of the acquisition learning, together with a change in light conditions on days 11 and 13, was introduced in order to keep the animals learning and motivated to solve the task under new conditions. After finding the submerged platform, the rats were allowed to rest on the platform for 10 seconds and then the next trial started. The cut off time was 60 seconds.

BrdU administration: On day 14, all animals in all groups received an i.p. injection of BrdU solution in distilled water (75mg/kg of body weight). The BrdU injection was repeated after 8 and 16 hours (three injections in 24 hours). The animals were sacrificed 24 hours after the first BrdU injection by barbiturate overdose (200 mg/kg) and transcardially perfused by phosphate buffered saline pH 7.4 (PBS) followed by freshly made 4% paraformaldehyde in PBS.

Tissue processing and immunohistochemistry: The brains were removed from the skull and stored in 4% paraformaldehyde for at least 3 days, and then transferred to solutions with ascending concentrations of sucrose to a maximum of 30%. After saturation, 40 µm - thick coronal sections were cut on a freezing sliding microtome. The antibodies used were mouse monoclonals directed against 5-bromo-2-deoxyuridine and against doublecortin C-18 (DCX).

For **peroxidase immunohistochemistry to detect BrdU**, peroxidase-conjugated rabbit anti-mouse IgG and peroxidase-conjugated goat anti-mouse were used as secondary antibodies with diaminobenzidine (DAB substrate kit). For DCX immunostaining, the C-18 primary antibody was detected using a Vectastain Elite ABC kit. For BrdU immunostaining, the following procedure was used: The sections were incubated in 3N HCl for 20 minutes, washed in dH_2O, then neutralized in 0.1 M borate buffer for 10 minutes. Endogenous peroxidase was then quenched in 0.3% H_2O_2 in MeOH for 30 minutes. Subsequently, the sections were incubated in 5% fetal bovine serum in PBS for 20 minutes, then overnight with anti-BrdU at 4°C. After rinsing in PBS, the slices were incubated for 30 minutes in peroxidase-conjugated goat anti-mouse IgG diluted in PBS with 5% fetal bovine serum, followed by rinses and 30-minute incubation in peroxidase-conjugated rabbit anti-mouse IgG. The peroxidase reaction was detected by a solution of DAB in PBS and 0.06% H_2O_2. All steps were performed at room temperature.

Images and cell counting: Coronal brain sections were cut through the entire hippocampal formation containing the U shaped granular cell layer. An Axioskop 2 plus microscope (Carl Zeiss, Germany), equiped with a 63x Plan Neofluar objective, was used to count BrdU-positive cells as well as DCX-positive cells. Every sixth section was used in the group of young (Y) animals and every third section was used for counting the BrdU-positive cells in middle-aged (MA) and aged (A) rats, which allowed us to evaluate 11 sections from each animal. Cells were counted separately in the ventral and dorsal blades of the granular cell layer and in the area of the hilus; cells that were located more than 2 cells away from the subgranular zone were considered to be in the hilus. Figure 28 shows the

estimated number of all cells based on the number in every 3rd respective 6th slice of an average animal in the group. The quantification of DCX-positive cells was done in four hemisections from each animal. Only cells that showed the typical appearance of an unstained nucleus surrounded by staining in the cell matrix and membrane were counted. Differences among the treatment groups and the age groups were then evaluated.

4. PUBLISHED PAPERS ON WHICH IS BASED THIS DISSERTATION

4.1. PUBLICATIONS: GAMMA KNIFE SURGERY OF THE RAT HIPPOCAMPUS

Liscak R, Vladyka V, Novotny J Jr, Brozek G, Namestkova K, Mares V, Herynek V, Jirak D, Hajek M, Sykova E, **Leksell gamma knife lesioning of the rat hippocampus: the relationship between radiation dose and functional and structural damage**, J Neurosurg 2002; 97 (5 Suppl): 666-73

Herynek V, Burian M, Jirak D, Liscak R, Namestkova K, Hajek M, Sykova E, **Metabolite and diffusion changes in the rat brain after Leksell Gamma Knife irradiation**, Magn Reson Med. 2004;52(2):397-402

Jirak D, Namestkova K, Herynek V, Liscak R, Vymazal J, Mares V, Sykova E, Hajek M, **Lesion evolution after gamma knife irradiation observed by magnetic resonance imaging,** Int. J. Radiat. Biol. 2007 ,83 (4): 2007 237 - 244

4.2. PUBLICATIONS: PROLIFERATION IN THE ADULT RAT HIPPOCAMPUS

Namestkova K, Simonova Z, Sykova E.**, Decreased proliferation in the adult rat hippocampus after exposure to the Morris water maze and its reversal by fluoxetine,** Behav Brain Res. 2005;163(1):26-32

Namestkova K, Simonova Z, Sykova E**, Proliferation of granule neurons in the rat hippocampus after stimulation in the Morris water maze and fluoxetine treatment is different across the life span** (Manuscript)

5. GENERAL DISCUSSION

The dose of gamma irradiation to the rat hippocampus that causes functional impairment of spatial navigation in rats (Liscak et al. 2002).

There has been limited previous knowledge about creating a complex lesion in the rat brain. Usually, a single isocenter has been used (collimator 4 mm) to investigate changes in the rat brain (Kamiryo et al. 1996 and Kondziolka et al. 1992). The effect of radiosurgery depends on the irradiated volume, therefore, the tolerance of the rat brain to the use of more isocenters (and consequently a larger volume of irradiated brain tissue) was unknown. A group in Charlottesville at the University of Virginia used two isocenters to irradiate the rats hippocampus (Chen et al. 2001). We used four isocenters with a 4 mm collimator to irradiate the whole hippocampus bilaterally; we discovered that higher doses could be used in a rat model, creating a complex brain lesion. Irradiated animals were tested over a period of more than one year.

Based on previously published results (Hock et al. 1998, Moser et al. 1995), we believed that irradiation of the dorsal part of the hippocampus would be sufficient. Therefore, in the first pilot group of rats, the radiosurgical lesion was performed using only two isocenters and only in the dorsal part of the hippocampus. From previously published radiobiological studies, we knew that 150 Gy would produce a necrotic lesion in the parietal cortex within one month in all animals and 100 Gy in about half of the rats within 3 months after radiosurgery, when only one isocenter with a 4 mm collimator is used (Kamiryo et al. 1996 and Kondziolka et al. 1992). Rats irradiated with 150 Gy only in the dorsal part of the hippocampus (one isocenter on each hippocampus bilaterally) showed no sign of memory impairment within one month, when tested twice a week (two animals were impaired 35 days after the radiosurgery and one 50 days after the surgery). Therefore, in the second pilot group the whole hippocampus was irradiated using 4 isocenters (collimator 4 mm) with doses of 150 or 100 Gy bilaterally (two isocenters on each hippocampus). A dose of 100 Gy was used because we did not know if the irradiated volume could cause diffuse postirradiation edema of the rat brain. Although radionecrosis after a dose of 150 Gy should develop within 30 days, the rats showed no impairment within 36 days after the radiosurgery.

In these pilot groups, memory retention was tested after the rats were trained to find the hidden platform, and the radiosurgery was performed subsequently.

Memory impairment was observed in rats irradiated with 150 Gy not earlier than the 35th day after radiosurgery and in rats irradiated with 100 Gy not earlier than

70 days after the radiosurgery, when extensive necrotic lesions involving also the neocortex developed.

The late onset of impairment can be explained by the fact that repeated testing of memory track retention in the Morris water maze served to stimulate plasticity processes and that the memory track was probably restored in residual preserved regions in the situation in which the lesion developed gradually. Therefore, this test could not be used as it was not sensitive enough. We introduced the acquisition learning test in naive rats instead of the retention test.

When naive rats were irradiated with a dose of 150 Gy to maximum and tested one month after radiosurgery, their results were 2-3 times worse compared to the control group, and histology revealed necrotic changes. Naive rats irradiated with 25-100 Gy showed no impairment of acquisition one month after the radiosurgery, MRI revealed no changes and histology did not show any necrosis.

Three months after the irradiation of naive rats with doses ranging from 25-100 Gy, there were no changes in the behavioral acquisition test. Similarly, MRI did not reveal any changes, and histology did not show any necrosis, although an astrocytic reaction and a lack of PCNA-positive cells were observed in all rats. These changes were more pronounced after higher doses.

Naive rats irradiated with a dose of 25 or 50 Gy showed no changes in the behavioral acquisition test, except one rat irradiated with 50 Gy **six months** after radiosurgery. MRI revealed no changes and histology showed no necrosis, except in the one impaired rat from the 50 Gy group. A different reaction was observed in the group of 4 rats irradiated with 50 Gy to maximum, which were examined on MRI repeatedly, while the rest of the animals were examined on MRI only once. In this group of 4 rats, the changes observed on MRI were more prominent. These animals, unlike the rest, underwent repeated anesthesia during the MRI examination, which lasted for several hours. We believe that the effect of protracted anesthesia with eventual hypoxia can potentiate the development of structural and functional changes in the irradiated tissue; this hypothesis should be tested in further studies. All animals irradiated with 75 Gy developed significant impairment in the behavioral acquisition test; MRI revealed brain edema and histology showed necrotic cavities. None of the rats irradiated with 100 Gy survived six months after radiosurgery to be tested. The groups of animals tested twelve months after irradiation showed similar results to results at 6 month.

Taken together, these results demonstrate that a rat can be used for animal experiments that create complex lesions in the brain using 4 isocenters with a 4 mm collimator. When the doses to maximum do not exceed 75 Gy, the rats

survive and can be tested at least 12 months after radiosurgery. A retention behavioral test after bilateral radiosurgery of the whole hippocampus is not sensitive enough, and acquisition learning should be tested to track the functional changes in the hippocampus. A significantly higher frequency of edema and necrosis, together with behavioral changes, was observed for doses greater than 50 Gy. There was no edema, necrosis or behavioral changes observed in animals irradiated with 25 Gy. There seems to be a therapeutic window, when the dose that could be affecting the epilepsy does not impair function of hippocampus in the rat. This therapeutic window should be further tested in epileptic rats, to verify the sensitivity to memory impairment of a hippocampus harboring an epileptic focus.

The subnecrotic dose (50Gy) of gamma radiation to the rat hippocampus causes impairment of learning and necrotic changes during long-term observation (Herynek et al. 2004 and Jirak et al. 2007)

The first signs of necrotic damage occurred on diffusion maps as late as 6 months after irradiation with 50 Gy to 70% isodose covering the dorsal hippocampus. The changes become visible on T2 weighted images 8 months after irradiation together with metabolite changes observed by MRS. The observed hyperintense area on the T2 weighted images was a sign of an edema, which led to severe necrotic damage and, in some animals, to the development of a postnecrotic cavity. The edema was reflected by an increased apparent diffusion coeficient and by metabolic changes observed by MRS.

We observed an increase of lipids/lactate signals and a decrease of Cr concentration in the edematous tissue, where blood circulation and metabolism was impaired. The subsequent partial decrease of lactate/lipid signals (approximately 12 months after the irradiation) can be explained by the release of pressure afteredema regression and lower lactate production. A decrease in NAA concentration was observed 3 months after the first signs of the lesion. Therefore, we suggest that during the first stage, edema occurs and the neurons are still present and alive. The decrease in NAA concentration three months later was a sign of neuronal and myelin loss in the lesion. This observation corresponds well with the results obtained from a human study (published by our group in Hájek et al. 2003).

Histological examination confirmed the severe damage visible on the MRI images. In four animals radiation-induced damage was observed in the corpus calosum, and there was a loss of cells in layers of the somatosensory cortex and the stratum oriens hippocampi. In another two animals necrotic damage led to the

development of hydrocephalus and to the creation of a postnecrotic cavity filled with cerebrospinal fluid (CSF). The presence of a post necrotic cavity with residual metabolites in the area of the edema correlates with the NAA decrease found by MRS and the high ADCw values in the lesion 12 months and later after irradiation.

Hypointense areas observed inside the edematous necrotic tissue, in the thalamus and in the medial part of the corpus calosum were confirmed by histology as posthaemorrhagic changes.

The long-term response to irradiation reveals that a dose of 50 Gy leads, besides damage of the hippocampus, to damage of the corpus calosum and the somatosensory cortex. The volume of irradiated tissue was larger than in previous studies (Kamiryo et al. 1996 and Kondziolka et al. 1992), and the delayed effect of radiation on compromising the cerebral microcirculation exceeded a tolerable volume. Thus, a small variability in isodose volume caused by the individual treatment plan for each animal could be responsible for the observed inconsistency. The focal point for irradiation was focused using a whole body 1,5 T imager that does not have sufficient resolution for small animals, so we could not avoid possible partial irradiation of neighbouring structures in the rat brain.

In summary, we observed and quantified the evolution of diffusion and metabolic changes in the rat brain after irradiation by the Leksell gamma knife. The first signs of edema accompanying necrotic changes occurred 6 months after irradiation with a dose of 50 Gy using four isocenters with a 4 mm collimator. Three months later, an edema was observed and MRS revealed the start of further cell death. Twelve months after irradiation, a regression of the edema in the tissue was observed, but in some cases an extensive fluid-filled cavity remained in the location of the previously edematous tissue. In all animals severe damage to the corpus calosum was revealed by histology 17 months after irradiation, while in 50% of the rats hydrocephalus developed with severe damage to the cortex and hippocampus.

The spatial navigation task in the MWM, together with antidepressant treatment affects the proliferation of neuronal precursors in the adult rat hippocampus (Namestkova et al. 2005)

Our study showed that spatial learning acquisition trials in the Morris water maze and fluoxetine treatment affect the rate of cell proliferation in the neurogenic region – the granular cell layer – and in the hilus of the dentate gyrus in the

hippocampus. Complex stimulation and learning in the water maze for 15 days resulted in a massive down-regulation of granular cell proliferation. This was a very surprising finding since learning has generally been considered as a factor that positively influences the generation of new neurons in adulthood. It has been clearly demonstrated that hippocampus-dependent learning of temporal or spatial events enhances the survival of newborn cells. Proliferation, on the other hand, remained at control levels after training with a trace paired eyeblink protocol (Gould et al. 1999) and after 12 days of MWM acquisition training (van Praag et al. 1999). In an experiment done by Lemaire et al., five days of spatial task learning in the Morris water maze did increase the rate of proliferation (Lemaire et al. 2000). This finding seems to be contradictory to our results; however, there are important differences between the studies. The length of exposure to the MWM task was only five days in the Lemaire et al. study, and the BrdU injections were given just before the training session for five days, thus also labelling cells that had been proliferating before the exposure to the MWM. Five days of exposure led to an increase in the rate of proliferation, but in our study 15 days of MWM training caused a decrease in the proliferation rate in animals of a similar age. The MWM challenge not only involves spatial learning and physical activity, but it also has a stressful component. The motivation for finding the location of the hidden platform is to escape from a life-threatening situation in a novel, unpleasant environment of cold water. The different results of the two studies suggest that the benefit from proliferation enhancing factors during the test, such as physical activity, may be diminished by prolonged exposure to a stressful experience.

Our results can be compared to a study (Fig. 28) in which mice were exposed to either 12 days of water maze acquisition or only to swimming (van Praag et al.1999); in that study, the number of labeled cells was not different between the test groups and controls when BrdU was administered every day during all 12 training days. The down-regulating effect of stress was probably masked by a normal rate of neurogenesis prior to the decline in the proliferative rate during the water maze training. A supporting argument for our results can be also found in the work of Shors et al. (2002), who suggested that the acquisition of spatial memory in the water maze may be a task that requires only a small percentage of new neurons; trace eye blink and fear conditioning were impaired by blocking neurogenesis with cytostatics (Shors et al. 2001, 2002), but spatial learning performance in the water maze was unaffected by such a blockade (Shors et al. 2002).

The administration of fluoxetine to animals that were not exposed to the WM increased the rate of proliferation as expected in the examined regions. Proliferation was very high in the hilus of the DG. The work of Cameron et al. in 1993 suggested that the neurogenic region in the hilus, observed in the early postnatal period, may persist to adulthood (Cameron et al. 1993). One to 24 hours after labelling newborn cells with [^{3}H] thymidine, the majority of positive cells were observed in the area of the hilus. Three weeks after labelling a majority of positive cells was observed in the granular cell layer, suggesting that the cells migrate to the granular cell layer (Cameron et al. 1993). This suggestion cannot, however, be supported by our observations since there were no DCX-positive cells present in the area of the hilus. Animals treated with fluoxetine for 15 days did not show a greater number of DCX-positive newborn neurons. The higher proliferation rate caused by the chronic application of fluoxetine was not reflected in the number of the DCX-positive cells. These results suggest that fluoxetine treatment increases proliferation but not the differentiation and survival of newborn neurons.

Evidence for a stress-related decrease in proliferation after water maze learning is provided by the reversal of the effect of stress by the application of fluoxetine. The animals in the WM trained group that received fluoxetine showed similar numbers of BrdU-positive cells in the GCL as did the control rats. The significant decrease in proliferation seen in the hilus remains unexplained.

Immunohistochemical staining for DCX allowed us to determine the number of cells differentiating into new neurons born during approximately the last 14 days preceding sacrifice, the time period when newborn cells show this early neuronal marker (Brown et al. 2003, Kempermann et al. 2003). It is likely that proliferation contributes to the number of DCX-positive cells, as well as the survival and differentiation of cells generated earlier. The number of DCX-positive cells in the GCL of animals exposed to a combination of fluoxetine treatment and stimulation in the water maze was increased when compared to animals exposed to WM acquisition alone or treatment with fluoxetine alone, but was similar to control animals. Gould et al. showed (Fig. 28) that 4 days of acquisition in the water maze increased the survival of already labeled newborn cells (Gould et al. 1999). Our results are not in discrepancy with this experiment in view of the fact that the cells were labeled before exposure to the potentially stressful experience in the water maze. Stress has been shown to suppress the proliferation of granule cells (Gould et al. 1999, Lemaire et al. 2000, Malberg et al. 2003). The water maze training in our study did not result in decreased proliferation in the granular cell layer when the stress was reduced by the administration of fluoxetine.

Subgranular zone astrocytes are considered to be the primary precursors of the D cells that differentiate into neurons (Alvarez-Buylla et al. 2002). Astrocytes that extend their basal processes under the blades of the granular cell layer and their apical processes through the layer, together with endothelial cells from apposed blood vessels, form a niche for developing newborn neurons (Doetsch et al. 2003). The survival of newborn cells is likely to be successful when stimulating substances diffusing from the blood stream as well as impulses from the functional neuronal network are delivered through the astrocytic synticyum. We have shown previously (Sykova et al. 2002) that learning deficits in aged rats are related to the disorganization of astrocytic processes and the loss of extracellular matrix in the hippocampus. Similar changes were observed by our group after postnatal hypobaric hypoxia and were also related to learning deficits (Simonova et al. 2003). The reduction in the number of surviving BrdU-positive cells takes place particularly in the first month after labelling, when more than half of the cells die, but then the number remains stable for several months (Kempermann et al. 2003). It has been shown in young female rats that BrdU-positive cells that express DCX survive to maturation and express a marker of mature neurons, NeuN (Kempermann et al. 2003, Brown et al. 2003).

In our study we have not examined the survival of newborn cells for an extended period of time. It would be of interest to see if the combination of fluoxetine, which increases the proliferation rate, and hippocampus-dependent learning in the water maze, which increases survival, would dramatically change the number of surviving cells four weeks after labelling. Moreover, determining the survival of the cells stimulated by fluoxetine would also be clinically relevant. A further question to be addressed is the mechanism of action in stressful learning combined with fluoxetine on the rate of neurogenesis. Our study revealed the negative effect of long-term exposure to the conditions of the Morris water maze on the proliferation of new neurons that are believed to be a potential substrate for new learning capacity. This should be considered for future behavioural studies using the Morris water maze test.

Proliferation of neuronal precursors in the rat hippocampus respond to spatial learning and fluoxetine treatment differently during aging (Namestkova et al. manuscript)

Our study revealed that proliferation in the GCL responds differently to the presented challenges during the lifespan of Wistar male rats. The proliferation of neuronal precursors declines with aging, in parallel with the worsening of performance in spatial learning tasks (Bizon et al. 2004, Heine et al. 2004).

Interestingly, the number of neurons in the dentate gyrus of aged animals remains similar to that in adult rats (Heine et al. 2004), and aged rats impaired on spatial learning also have a preserved number of granule cells (Rapp et al. 1996). The fact that the number of neurons in the DG does not decrease with age or impairment implies that the proliferation of neuronal precursors in the hippocampus provides a reserve for situations that place a greater demand on plasticity, such as exposure to a novel environment. This reserve might also be available to replenish the loss of neurons due to cellular damage (Abrous et al. 2005).

The experimental conditions of the water maze challenge used in this study did not allow the aged animals to show learning in all cases, although some male Wistar rats at 24 months of age are capable of learning (showed by our group in Sykova et al. 2002). Every individual animal in the younger age groups, up to 3 and 10 months, showed learning curves under the same conditions (presented by our previous publication in Namestkova et al. 2005). However, the aged animals used in this study were four to six month older than the animals used in any of the previous studies (Sykova et al. 2002, Heine et al. 2004, Bizon et al. 2004), so age-related cognitive impairment might be apparent due to the greater age of the animals. Three out of four aged animals receiving fluoxetine during learning showed a very slowly descending learning curve. Although there is great variability in the performance of aged animals in the water maze (showed by our group in Sykova et al. 2002), our experiment suggests that the stressful component of the water maze challenge can impair learning and that this can be prevented by fluoxetine administration. Our water maze testing procedure was standard, but for the aged animals that did not learn and showed floating, might have presented a situation of inescapable stress that ultimately leads to depressive-like behavior (Shors 2004). Aging animals are possibly more susceptible to the stress presented by the water maze due to the overall reduced adaptability of their cardiovascular systems and age-related alterations in the HPA axis (Gallagher et al. 2003, Stoelzel et al. 2002).

Proliferation in the neurogenic subgranular zone was only twice as great in the middle-aged control group when compared to the control group of aged animals (Fig. 26). However, the proliferation in this area decreased almost ten times between the third and tenth months of the animals' lives according to our earlier study (Namestkova et al. 2005), which is consistent with the results reported by other authors (Heine et al. 2004).

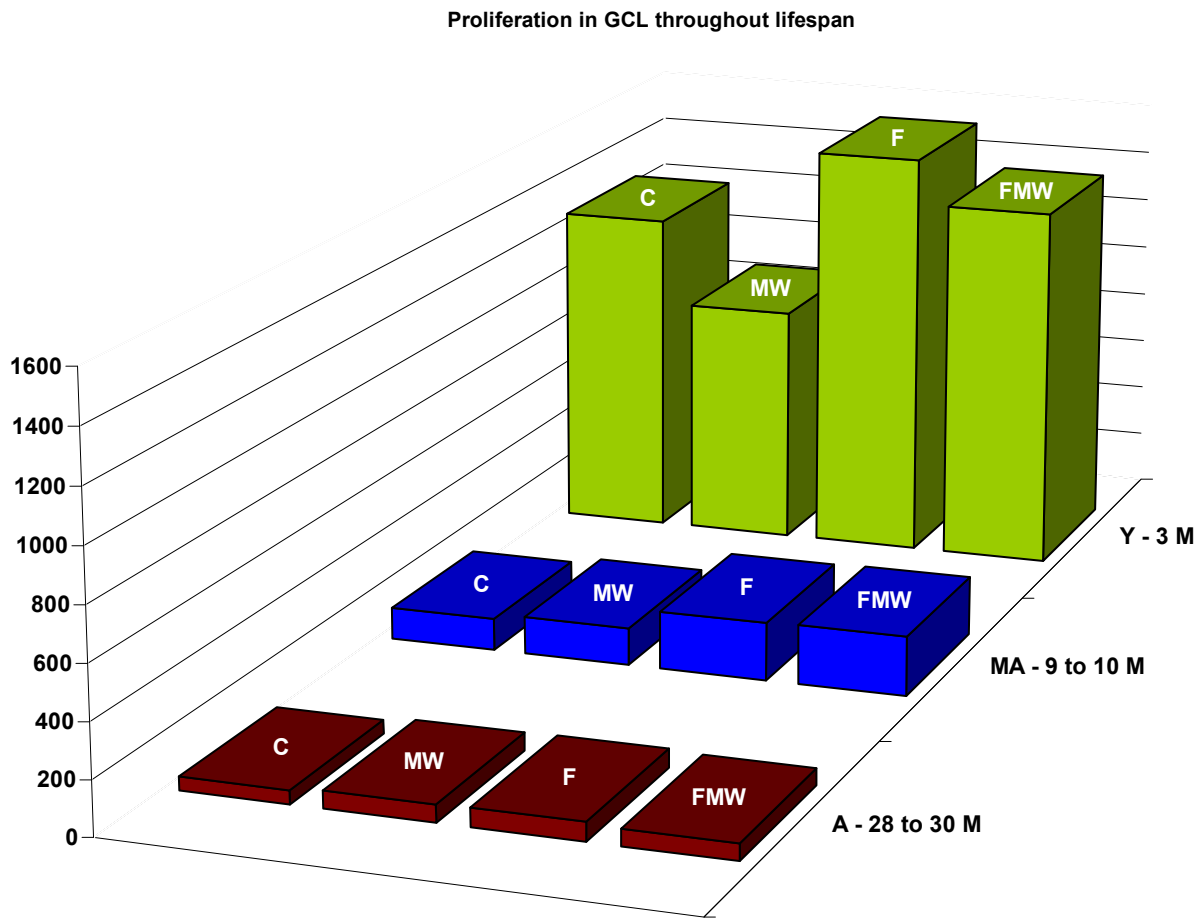

*Fig. 26 Graph based on estimates of the total number of proliferating cells within the **granular cell layer (GCL)** of rat the hippocampus. The cells were calculated in every sixth (Y) or third (MA and A) slice throughout the granular cell layer; the final estimate was calculated by the appropriate multiplication. The three age groups are shown in columns of the same color, and the treatments are indicated by abbreviations C- control, MW- exposed to water maze alone, F- treated by fluoxetine and FWM exposed to water maze and fluoxetine.*

The effects of the treatments on proliferation in the GCL of the middle-aged group showed interesting results when compared to the group of young animals (Fig. 26 and 28) that underwent the same procedure (by our group in Namestkova et al. 2005). In young animals, the water maze challenge might have represented a stressful experience that led to decreased proliferation in the GCL. This negative effect of stress was, in young animals, blocked by the administration of fluoxetine during water maze learning, resulting in the same rate of proliferation as in the control group, whereas fluoxetine treatment alone dramatically increased the number of proliferating cells. Animals aged 9 to 10 months responded to the treatment in a very different way. The availability of a learning challenge stimulated proliferation, and the addition of fluoxetine even increased the effect of cognitive stimulation on proliferation. The stressful component did not evoke negative effects on proliferation in this age group, suggesting that the animals respond differently to the water maze challenge over the course of their life span.

The effects of treatment on proliferation were not observed in the group of aged rats in the GCL, and the variability of the results was high. Great variability was also observed in other parameters examined in aging animals (Gallagher et al. 2003). Our results are consistent with the results of a study observing alterations in HPA axis reactivity during the lifespan (Heine et al. 2004). A mild stressor such as a tail knick increased the peak corticosterone levels in young 6-week-old animals more dramatically than in middle-aged 12-month-old animals. Aged animals at 24 months of age did not show elevated basal levels or an increased stress response in the Heine study, nor did the levels of corticosterone correlate with the extent of proliferation (Heine et al. 2004). This would explain why the water maze experience that is stressful to young animals does not cause a decrease in proliferation in middle-aged animals. The effect of general physical activity and stimulation by novelty in middle-aged animals has a beneficial effect on proliferation in the GCL. Very interesting is the finding of spared proliferation potential in aged animals in the area of hilus (Fig. 27). Proliferation in the hilus was almost twice as great in aged animals as in the middle-aged. This phenomenon can be explained by increased astrogliosis during aging (showed by our group in Sykova et al. 1998).

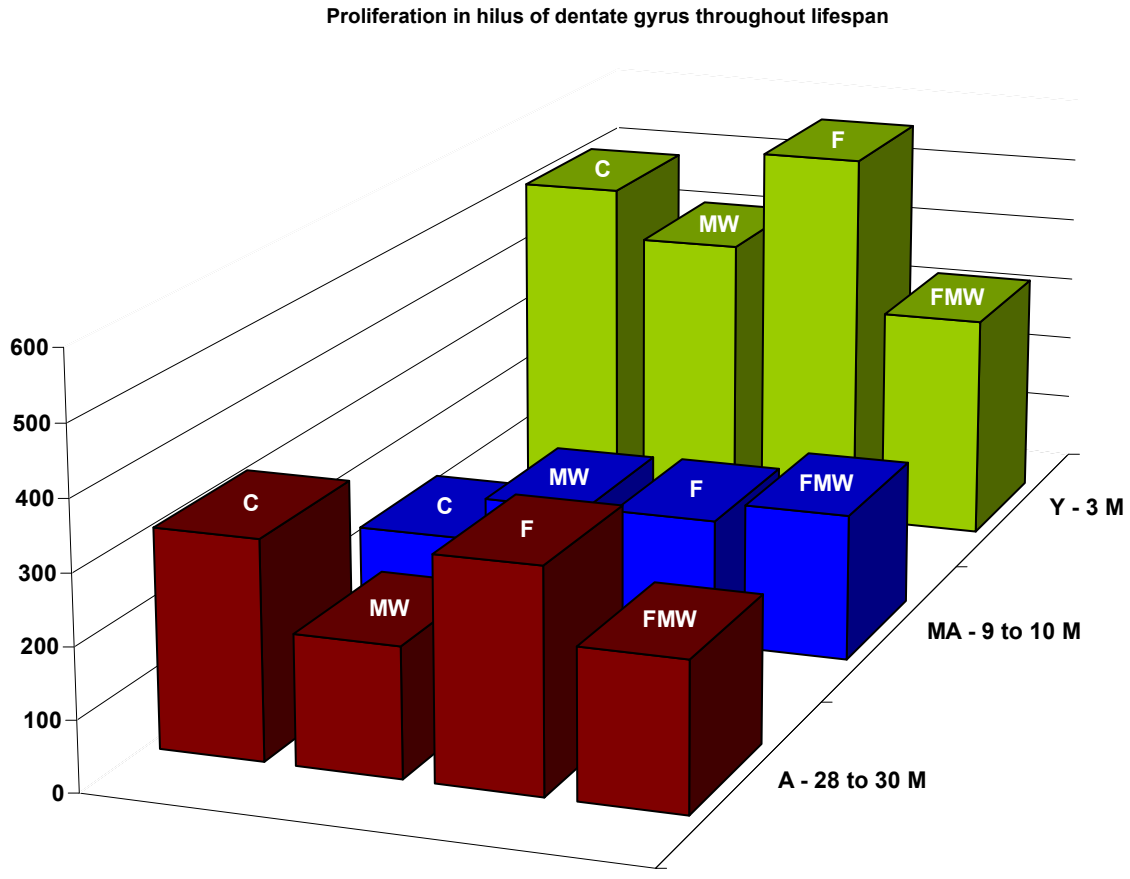

*Fig. 27 Graph based on estimates of the total number of proliferating cells within the **hilus** of the rat hippocampus. The cells were calculated in every sixth (Y) or third (MA and A) slice throughout the granular cell layer. The three age groups are*

shown in columns of the same color, and the treatments are indicated by abbreviations C- control, MW- exposed to water maze alone, F- treated by fluoxetine and FWM exposed to water maze and fluoxetine. Please note the high proliferation rate in the hilus of aged animals.

Proliferation in the hilus was affected by the treatments in both age groups. The water maze challenge led to increased proliferation in the middle-aged group when the animals were concomitantly treated with fluoxetine. This increase was parallel to the increase in the GCL. A similar effect of fluoxetine was observed when the astrocytic protein S100β was found to be up regulated in the hippocampus after 21 days of treatment (Manev et al. 2003). The group of aged animals responded to water maze exposure by a decreased number of BrdU-positive cells, which might be in this case a beneficial effect of physical activity and learning preventing the need to compensate by increased gliogenesis. An activity-dependent decrease in age-related astrogliosis was also observed when rats were housed for 2 months in an enriched environment or trained on a hippocampus-dependent task at the age of 23 months (Soffie et al. 1999).

The significance of the newborn cells proliferating in the hippocampus during adulthood and aging is still an unanswered question. Aged Long Evans rats, 25 months old, showed that higher numbers of surviving BrdU-positive cells are associated with poorer performance on a spatial learning task (Bizon et al. 2004).

This implies that the process of neurogenesis can compensate for cognitive impairments on tasks that are solved easily at a younger age, but which are a great challenge when the animal is older. The exhaustion of this compensatory mechanism during aging might hinder an animal's ability to adapt to novel environments. This limited adaptation might progress to a further stressful experience, which closes a negative feedback loop that additionally suppresses the proliferation of neuronal precursors in the hippocampus. We can speculate that interrupting this negative feedback through the use of antidepressants might postpone age-related cognitive decline in humans, in whom high cortisol exposure is associated with poor performance on spatial tasks (Porter et al. 1998). However, there are numerous differences between human and rodent aging.

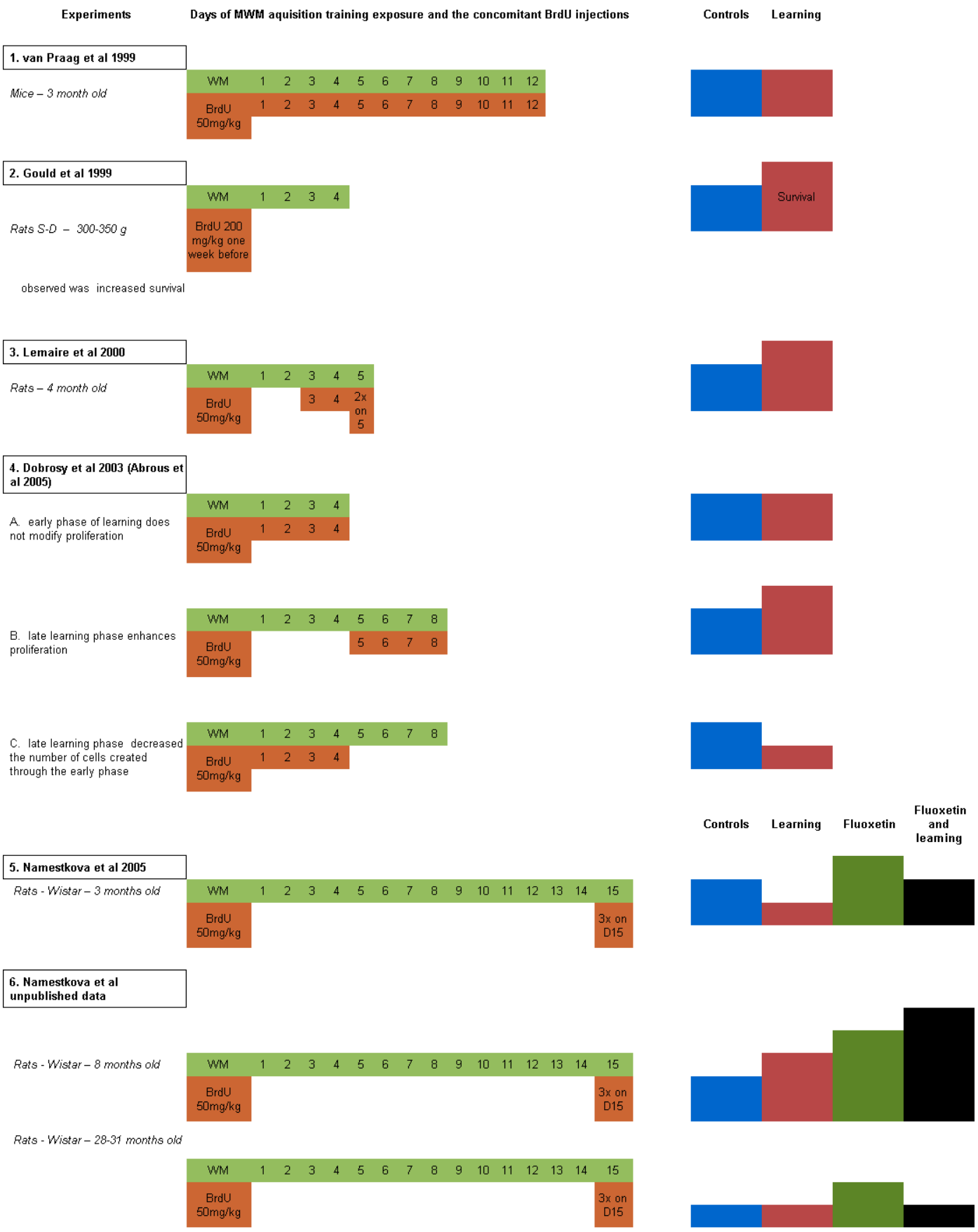

Fig. 28 An overview of experiments performed by several investigators studying the effect of learning on the proliferation and survival of adult-born granular cells in the hippocampus of rodents. The experimental designs are schematised noting the length of the water maze training and the dose and time schedule of administration.

The neurodegenerative diseases that lead to cognitive impairment in humans such as Alzheimer's and Parkinson's diseases, do not occur in rodents. With regards to adult neurogenesis, the hippocampiof rodents impaired in spatial tasks do not show atrophy (Heine et al. 2004), which has been observed, however, in aged humans with spatial cognitive deficit (Driscoll et al. 2003).

Our study showed that male Wistar rats might react to water maze exposure differently during different stages of their lifespan (Fig. 28). It might be a stressful experience at a young age, an occasion for stimulation during adulthood and a stressful challenge in old age. We have shown that spatial learning and the concomitant administration of fluoxetine have an additive effect on proliferation in the hilus in middle-aged animals, which is a phenomenon that should deserve further attention with respect to aging. We detected proliferation and neurogenesis in the GCL in rats of such advanced age as 31 months, but the proliferation was very low and no effect of the manipulations could be observed. However, an effect of water maze exposure on suppressing proliferation in the hilus was observed – a phenomenon that is difficult to interpret. We can only speculate that water maze stimulation prevents the proliferation of astrocytes that play a role in cognitive decline during aging (see Sykova et al. 2002).

Generally, neurogenesis during aging offers a pool for the turnover of cells used in the process of adapting to a new situation. The newborn cells can possibly compensate through modulatory action for the functional deficit caused by cellular loss, when a pool of proliferating cells would be still available in older age.

6. CONCLUSIONS

The dose of gamma irradiation to the rat hippocampus that causes functional impairment of spatial navigation in rats

We established that a rat model could be used in animal experiments studying the effect of irradiation of the hippocampus on spatial learning. After doses higher than 50 Gy, edema and necrotic damage developed, accompanied by behavioral impairment. A dose of 50 Gy can be described as marginal because necrotic damage occurred only in some animals; the majority of animals irradiated with this dose did not show behavioral impairment or necrotic damage. There was no edema, necrosis or behavioral change after a dose of 25 Gy. The dose that was found to suppress epileptic activity (25 Gy) did not impair the function of the hippocampus.

Gamma radiation with 50Gy to the rat hippocampus causes a functional impairment of learning

The long-term survival and regular observations of our animals revealed the evolution of diffusion and metabolic changes in the rat brain after LGK irradiation of 50 Gy.

The first signs of edema occurred 6 months after irradiation. MRS revealed the beginning of further cell death in theedematous tissue nine months after irradiation. Twelve months after irradiation a regression of theedema was observed, but in some cases an extensive water-filled cavity remained in the location of the previously edematous tissue. There was severe damage to the corpus calosum in all animals, as revealed by histology, 17 months after irradiation. Half of the examined animals developed hydrocephalus accompanied by severe damage to the cortex and hippocampus.

The spatial navigation task in the MWM, together with antidepressant treatment, affects the proliferation of neuronal precursors in the rat hippocampus

Our experiments revealed a negative effect of long-term exposure (15 days) to the conditions of the Morris water maze on the proliferation of neuronal precursors in 3-month-old Wistar male rats. The addition of fluoxetine treatment to the water maze training suppressed the negative effects, with the result that proliferation in the GCL was similar to control levels. The administration of fluoxetine alone caused increased proliferation in the GCL by 26% when compared to control

levels. The spatial navigation task in the Morris water maze is a stressful experience that reduced the proliferative rate of granule cells in the hippocampus of 3-month-old Wistar male rats.

The proliferation of neuronal precursors in the rat hippocampus responds to spatial learning and fluoxetine treatment differently during aging

Our experiments revealed that proliferation in the GCL responds differently to the presented challenges during the lifespan of Wistar male rats (3 months, 9 to10 months and 28 to 31 months). Proliferation in the neurogenic SGZ decreased almost ten-fold between the third and tenth months of the animals' lives but decreased only two-fold between the tenth and thirty-first months in the control groups.

The Morris water maze task was a stressful experience that reduced the proliferative rate of granule cells in the hippocampus of 3-month-old Wistar male rats. The addition of fluoxetine to the MWM exposure returned the proliferative rate to control levels.

Middle-aged adults responded to the challenge in the MWM by increasing proliferation in the GCL; the addition of fluoxetine even potentiated the effect of the MWM stimulation.

The aged animals generally showed very little proliferation in the SGZ, and no effect of treatment could be observed.

The proliferation in the hilus of control, aged rats was almost twice as high as in control middle-aged animals. The number of proliferating cells in the hilus of aged animals was decreased by the MWM challenge, implying that the process of astrogliosis during aging can be influenced by exposure to activity.

7. LIST OF PUBLISHED ABSTRACTS AND ARTICLES

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