Adult hippocampal cell birth and death in relation to stress, aging and the vasculature

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Chapter 1

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General Introduction

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In recent years it has become evident that neurogenesis is not only restricted to early development but also occurs during adulthood in restricted brain regions (section 1.1). Particularly the hippocampus, an area involved e.g. in memory formation, has been studied in this respect (1.2). Neurogenesis is determined by many factors, including intrinsic cell cycle factors (1.3), the cellular microenvironment (1.4) and environmental factors such as stress and aging (1.5).

The aim of this thesis (1.6) was to examine the effect of stress and aging on neurogenesis in the hippocampus (part I) and to survey the role of cell cycle markers and microenvironmental factors such as the vasculature (part II).

1.1 Neurogenesis: A Historical Perspective

Ever since early neuroscience, it was assumed that the mammalian Central Neural System (CNS) becomes structurally stable soon after birth and remains that way throughout life. It was common believe that no new neurons are added to or replaced in the brain in adulthood (Gross, 2000). Therefore, neuronal birth was mainly studied for the duration of the CNS development.

In the sixties, a new technique was introduced to label proliferating cells: 3H-thymidine autoradiography. 3H-thymidine can be taken up by dividing cells undergoing DNA synthesis, so that these cells, and their progeny, can later be identified using autoradiography. This method has led to the first description of cytogenesis occurring in the adult rat olfactory bulb, hippocampus and cerebral cortex (Altman & Das, 1965). Years later, ultrastructure studies of these 3H-thymidine-labeled cells indeed confirmed their neuronal identity (Kaplan & Hinds, 1977; Kaplan, 1981; Gross, 2000). However, although the occurrence of adult neurogenesis was generally accepted in nonmammalian vertebrates such as fish, reptiles and birds (Anderson & Waxman, 1985; Lopez-Garcia et al., 1988; Nottebohm, 1989), the pioneering studies involving neuronal birth in adult mammals were still welcomed with skepticism.

It was not until the early 1990s, when the Bromodeoxyuridine (BrdU) labeling technique became available, that many studies convincingly demonstrated that new neurons are indeed born in restricted regions of the adult mammalian CNS (Kempermann & Gage, 2000; Alvarez-Buylla & Garcia-Verdugo, 2002; Gage, 2002). Only in the subventricular zone (SVZ) of the lateral ventricle (Alvarez-Buylla & Garcia-Verdugo, 2002) and in the hippocampal subgranular zone (SGZ) (Gage, 2002), adult neurogenesis has consistently
been found. It is worth noting that birth of new neurons in these regions has not only been demonstrated in rodents, but in humans as well (Eriksson et al., 1998).

Some studies suggested that neurogenesis also occurs in other regions of the intact adult mammalian CNS, such as the cortex (Gould et al., 1999a) and the substantia nigra (Zhao et al., 2003). However, these results contradict other publications and are still subject to debate (Kornack & Rakic, 2001; Rakic, 2002; Lie et al., 2004).

The notion that neurogenesis still occurs in the adult brain was the start of a whole new field of research. More insight in the regulation of neurogenesis not only yields a better understanding of its functional role and molecular control, but may also open up new ways towards the development of new strategies to treat neurodegenerative and hippocampal volume-related diseases, such as Alzheimer’s, Parkinson disease and depression.

1.2 The Hippocampus

The hippocampus is one of the few regions in the adult brain where neurogenesis has consistently been found. It plays a pivotal role in learning and memory, contextual fear conditioning and neuroendocrine regulation.

Cytoarchitecture / Connections

The rodent hippocampal formation comprises six cytoarchitectonically distinct regions: the dentate gyrus, the hippocampus proper, subdivided into area CA3, CA2, and CA1, the subicular complex (subiculum, presubiculum, parasubiculum), and the enthorinal cortex. These hippocampal regions are linked by largely unidirectional projections. For clarification, our usage of the term hippocampus will further constitute the dentate gyrus and the hippocampus proper mainly (Figure 1). Nomenclature of the hippocampus follows the description of Amaral and Witter (1995).

The dentate gyrus consists of three main layers: the molecular layer, the granular cell layer (GCL) and the polymorphic layer (hilus) (Amaral & Witter, 1995). The narrow, two to three-cell layer thick region between the hilus and the GCL is called the subgranular zone (SGZ). The GCL is made up of densely packed granule cells, the primary cell type in the dentate gyrus. Their dendrites reach into the molecular layer, a relatively cell-free layer closest to the hippocampal fissure, receives its major input from the enthorinal cortex via the perforant path. The axons of the granule cells (mossy fibers, so called by Ramon y Cajal)
collateralize in the hilus before exiting the dentate gyrus and projecting to the CA3 area of the hippocampus (Claiborne et al., 1986; Amaral & Witter, 1995). Mossy fiber terminals in the hilus also establish contacts with the proximal dendrites of the glutamatergic mossy cells, the basal dendrites of the GABAergic pyramidal basket cells and other unidentified cells (Ribak & Seress, 1983; Ribak et al., 1985; Scharfman et al., 1990).

The principal cell layer in the hippocampus proper (Ammon’s horn) is the pyramidal cell layer which can be divided into three fields: CA3, CA2, and CA1 (Lorente de No). The narrow, cell-free layer beneath the pyramidal layer is called stratum oriens. The stratum lucidum, a narrow layer just above the pyramidal cell layer of the CA3, is occupied by the mossy fibers originating from the dentate gyrus. Superficial to this layer and just above the pyramidal layer of the CA2 and CA1, is the stratum radiatum. In this layer the CA3 to CA3 associative, and the CA3 to CA1 Schaffer collateral connections are located. The perforant path fibers from the entorhinal cortex end in the stratum lacunosum-moleculare, the most superficial layer of the hippocampus (Amaral & Witter, 1995).

Figure 1: Schematic representation of the hippocampal layers and connections  
(adapted from: Ascoli et al., 1998, Computational Neuroanatomy of the Hippocampus – SFN98, Los Angeles)  
EC, entorhinal cortex; PP, perforant path; DG, dentate gyrus; MF, mossy fibers; SC, Schaffer collateral; slm, stratum lacunosum-moleculare; sr, stratum radiatum; sl, stratum lucidum; so, stratum oriens.
**General Introduction**

**Development**

The development of the hippocampal formation has been widely studied. The majority of its neurons are generated in the ventricular zone of the neural tube during embryonic development. Hereafter, daughter cells migrate away to distant sites where they differentiate (Angevine, 1965; Sidman & Rakic, 1973). In rodents, the majority of neurons of the entorhinal cortex, subicular complex and Ammon’s horn are formed before birth (Angevine, 1970; Bayer, 1980, 1982). However, approximately 85% of the granule cells in the hippocampal dentate gyrus are born postnatally (Bayer, 1980; Altman & Bayer, 1990). The cells in the dorsal blade tend to be formed earlier than those in the ventral blade. This also holds for the more caudal cells and those in the more superficial layers versus the rostral and deeper layer of the stratum granulosum. Neurogenesis in the DG peaks shortly after birth, during the first two postnatal weeks, continuing at a slower pace into adulthood (Schlessinger et al., 1975; Bayer, 1980). The progenitor cells, displaced from the subventricular zone during the embryonic phase (Eckenhoff & Rakic, 1988), reside in the subgranular zone at the base of the granular cell layer, and in the hilar region of the dentate gyrus during adulthood. These cells continue to generate new neurons in adulthood.

**Adult Neurogenesis in the Dentate Gyrus**

Altman and colleagues (Altman & Das, 1965) were the first to show cell birth in the adult dentate gyrus. In the dentate gyrus of rodents, monkeys and humans, adult-generated cells arise from progenitor cells located in the hilus or subgranular zone that migrate the short distance to the granule cell layer, where they differentiate into neurons (Figure 2) (Kempermann et al., 2003). Next to morphological characteristics of mature granule neurons (Altman & Das, 1965), later studies have shown that these adult-generated cells indeed receive synaptic input, extend axons into the mossy fiber pathway, and express a number of markers specific for mature neurons (Hastings & Gould, 1999; Markakis & Gage, 1999; Cameron & McKay, 2001; Gould et al., 2001; Seri et al., 2001). Furthermore, by labeling newly generated cells with a retrovirus expressing a reporter gene, Van Praag et al. (2002) showed that after 4 weeks the newborn cells develop electrophysiological characteristics that are very similar to neighboring mature granule cells (van Praag et al., 2002). The complexity and density of their dendritic spines, however, continues to grow for at least several more months. So, these adult-generated neurons are integrated as dentate granule cells in the hippocampal circuit and do become functional *in vivo*. Over the past few years, it became clear that adult neurogenesis is a common
feature in the mammalian hippocampus, as this phenomenon has been shown in adult rats, mice, tree shrews, marmosets, macaques, and even humans (Eriksson et al., 1998; Gould et al., 1999c; Kornack & Rakic, 1999; Gould et al., 2001). This conservation throughout evolution suggests an important physiological role for adult neurogenesis (Kempermann et al., 2004).

**Figure 2: Neurogenesis in the adult hippocampal dentate gyrus**
(adapted from Lie et al., 2004)

Fibers from Entorhinal cortex →

1) proliferation 2) fate determination 3) migration 4) integration → to CA3

**Functional Role of the Hippocampus in Health and Disease**

**Learning and Memory**

In rodents, the hippocampus is generally considered to be involved in ‘spatial’ types of memory. So called ‘place cells’, discovered by O’Keefe and Dostrovsky (1971) (O’Keefe & Dostrovsky, 1971), have the ability to fire when a rat is in a specific spatial location in an environment. Later, more studies reported on various hippocampal manipulations (lesions, drugs, environmental stimulations, stress and gene mutations), which could alter performance in spatial-memory tasks, such as the Morris water maze,
radial-arm maze, circular maze and contextual conditioning (Morris et al., 1982; Morris et al., 1986; Tsien et al., 1996; Kim & Diamond, 2002). Furthermore, the hippocampus is thought to have access to cortically processed sensory information and to the memory storage sites in the neocortex through various afferent and efferent connections (Mishkin, 1982; Lavenex & Amaral, 2000). In addition, this circuit has been regarded as one of the sites in which association of multimodal sensory input and formation of associative memory occurs (Black et al., 1977; McNaughton & Morris, 1987). In humans, the hippocampus is thought to be crucial for ‘declarative’ memory, but not ‘non-declarative’ memory (Squire & Cave, 1991; Eichenbaum, 2000).

**Neuroendocrine Regulation**

In addition to its role in memory formation, the hippocampus is also involved in feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Box 1) (De Kloet et al., 1998). Following activation of the HPA-axis, as occurs e.g. during stressful experiences, corticosteroid levels in the blood increase. Corticosteroids can mobilize energy, increase cardiovascular tone, and suppress aspects of the immune system. In the brain these lipophilic hormones bind to two types of corticosteroid receptors: mineralocorticoid receptors (MR, or Type I), which bind corticosterone and cortisol with high affinity; and glucocorticoid receptors (GR, or Type II), which have approximately one tenth the affinity of MRs (Reul & de Kloet, 1985), but are more widely distributed. Although cells in most brain regions mainly contain GR, neurons in the hippocampus contain high amounts of both receptor types except for the CA3 area that generally has low GR expression. As a consequence of the difference in affinity, MRs are already occupied to a large extent when corticosteroid levels are low e.g. during rest, while GRs become only fully activated when corticosteroid levels rise, e.g. during stress.

Unbound GRs are predominantly located in the cytoplasm where they are bound to large heterocomplexes with heat shock protein 90 and other heat shock proteins. Upon hormone binding, the hormone-receptor complex is activated and causes the heterocomplex to dissociate to an activated hormone-receptor complex monomer, that becomes highly phosphorylated and translocates to the nucleus (Beato & Sanchez-Pacheco, 1996). Conventionally, homodimers of MRs or GRs were thought to bind to hormone-responsive elements in the DNA, thereby affecting the transcription of specific genes. Meanwhile it has become clear that various steroid actions rely on protein-protein interactions between corticosteroid-receptor monomers and other transcription factors (Reichardt & Schutz, 1998), such as cAMP-response-element-binding protein or immediate-early genes.
Box 1: Hypothalamic-pituitary-adrenal (HPA) axis

Stressful experiences lead to an activation of the HPA-axis (Figure 3). Following a stressor, the hypothalamus releases corticotrophin releasing factor (CRF) and vasopressin (VP), which activate the pituitary. The anterior lobe responds by secreting adrenocorticotrophin hormone (ACTH), which is released into the blood, detected by the adrenal cortex. This subsequently leads to the release of corticosteroids (corticosterone in rodents and cortisol in humans). Corticosteroids will enter the blood circulation and are the final effectors of the HPA axis. They act by exerting homeostatic regulatory effects including energy mobilization and inhibition of anabolic processes. HPA axis activity is controlled through feedback inhibition, since the release of glucocorticoids into the blood suppresses the release of CRF from the hypothalamus and ACTH secretion from the pituitary, and ultimately results in termination of the stress response. Both the prefrontal cortex and the hippocampus contain high densities of corticosteroid receptors, although the hippocampus contains both MRs (mineralocorticoid receptors) and GRs (glucocorticoid receptors), while the frontal lobes contain mostly GRs. These cortical structures a.o. indirectly inhibit the HPA axis.

Figure 3: Schematic representation of the HPA-axis
General Introduction

Stress and Age-Related Impairments of Hippocampal Structure and Function

Since the hippocampus is crucial in aspects of memory formation, it has a prominent place in many studies involving age- and related changes in cognition. In humans, the hippocampus plays a pivotal role in declarative memory and is e.g. one of the first brain regions to display pathology in Alzheimer’s disease, where memory dysfunction is a prominent and early feature. Furthermore, in different species, processes of learning and memory that involve the hippocampus, often show deficits with aging; aged rodents display impairments in their performances in spatial memory tasks, while old primates perform poorly in delayed response memory tasks, as do old humans in their performances on a delayed recall task as well and in their memory to locate objects (Eberling et al., 1997; Bohbot et al., 1998). Also, a relationship has been shown between larger hippocampal volume and better cognitive performances, both in verbal and spatial memory, as well as in learning of complex spatial tasks (Maguire et al., 1997; Hackert et al., 2002). In addition, MRI studies have demonstrated hippocampal volume reductions in diseases such as dementia, recurrent major depression, post-traumatic stress disorder, and Cushing’s disease (Bremner et al., 1995; Sheline et al., 1999; Starkman et al., 1999; Villarreal et al., 2002), as well as in aging individuals (Pruessner et al., 2001). However, other groups failed to find reduced hippocampal cell numbers in aged rats (Rapp & Gallagher, 1996; Rasmussen et al., 1996; West, 1999).

High levels of corticosteroids, which are found in disorders like Cushing’s disease or major depression and during stressful experiences, cause cognitive impairments and decrease hippocampal volume in humans (Lupien et al., 1997; Lupien et al., 1998; Lupien et al., 1999; Belanoff et al., 2001; Wolf et al., 2002b; Wolf et al., 2002a). However, other studies have yielded conflicting findings (Axelson et al., 1993; Coffey et al., 1993; Vakili et al., 2000). The hippocampus is further involved in terminating the stress-induced release in glucocorticoids (Box 1).

Stress is believed to be involved in precipitating depressive disorders in genetically predisposed individuals (Kendler et al., 1999). Several clinical studies indicate that a large percentage of the patients with depression, suffer from glucocorticoid hypersecretion (Sapolsky, 2000), show dexamethason non-suppression or exhibit a hyperactive HPA-axis (Arborelius et al., 1999). Functional imaging studies of depressed patients showed volume changes in limbic (hippocampus, basal ganglia and amygdala) and cortical brain regions (Sheline et al., 1999; Bremner et al., 2000; Mervaala et al., 2000; Hastings et al., 2004), which are also implicated in the affective and cognitive impairments observed in depressive disorders (Manji et al., 2001). Although this indicates that volume loss is
associated with depressive disorders, histologically, no indications for damage, massive neuronal loss or neuropathology could be found (Lucassen et al., 2001b; Muller et al., 2001; Schweitzer et al., 2001).

Depression research in animal models is therefore in part focused on understanding the changes induced in limbic and cortical brain structures by different stressors and corticosteroids. Most of this research is centered around the hippocampus, as this brain region is particularly susceptible to functional and structural impairments induced by stress (McEwen, 2000; Sapolsky, 2000). Although the stress-induced changes in the hippocampus may not explain all affective symptoms of depression, they may provide a cellular basis for understanding the changes in this brain region as well as in other regions associated with depression.

While reduction in hippocampal volume in relation to aging and stress has thus been observed, little was known at the start of this project about neurogenesis and cell death under these conditions. This was one of the subjects of the present investigation (see further section 1.5).

1.3 The Role of Cell Cycle Factors in Neurogenesis

During development of the mammalian CNS, neurons and glia, traditionally viewed, arise from multipotent stem cells in a stereotyped sequence in which generally neurons are generated first, primarily during embryonic period, followed by glia, the majority of which differentiate after most neurons are born (McKay, 2000; Temple, 2001). Generation of new glia in the adult CNS continues to occur throughout life in many different regions (Kuhn et al., 1996; Horner et al., 2000; Lie et al., 2004). Radial glia are an exception in that radial glia are generated before neurogenesis and guide neuronal migration. Presently, several studies even show that radial cells are the neuronal progenitor cells, giving rise to both neurons and astrocytes (Noctor et al., 2001; Alvarez-Buylla & Garcia-Verdugo, 2002; Goldman, 2003). The general view, though, is that those cells in the nervous system, that remain undifferentiated, are the neural progenitor or stem cells.

The process of neurogenesis involves proliferation and differentiation of new cells. For proliferating cells to differentiate, they need to exit the cell cycle to take up a particular neuronal or glia fate. In most systems, there is a clear correlation between birth date and fate. One of the examples in the brain is the generation of neurons before glial cells. Also in vitro experiments show that glial cells are formed after neurons, suggesting
that this may be an intrinsic property of mammalian neuroblasts (Qian et al., 2000). Important determinants to influence cell fate are transcription factors. For example the proneural genes, especially the basic helix-loop-helix (bHLH) type, have a major role in cell fate choice (Kanekar et al., 1997; Brown et al., 1998). Further, components of the cell cycle are shown to be involved in coordinating this aspect of histogenesis. The cell cycle inhibitor p27Kip e.g. gradually increases in cultures of glial progenitors, and when it accumulates to sufficient levels, it causes oligodendrocyte precursors to exit the cell cycle and differentiate (Durand & Raff, 2000).

**Cell Cycle Control**

The eukaryotic cell cycle (Sherr, 1994) (see Box 2, Figure 4) is controlled by two core intrinsic components: 1) the cyclin-dependent kinases (Cdks), which phosphorylate target proteins on critical serine and threonine sites, and 2) the cyclins, which are produced in a cyclic fashion and are able to guide the Cdks to appropriate substrates and activate their catalytic activity. A variety of cyclin / Cdk complexes are formed during distinct phases of the cell cycle, each dedicated to phosphorylate a specific set of proteins which will trigger different phases of the cell cycle at the right time and in the right sequence. In general, the levels of Cdks are relatively constant throughout the cell cycle, while the levels of the cyclins vary substantially and in a cyclic manner (Box 2, Figure 5). Cdk inhibitors are able to block the assembly or activity of the cyclin / Cdk complexes. Two families of Cdk inhibitors have been identified in mammalian cells, the Cip/Kip family and the Ink4 family.

Regulation of mammalian cell proliferation by extracellular signals is thought to occur primarily during the G₁ phase of the cell cycle (Pardee, 1989) (Box 2, Figure 4). During this interval, stimulatory and inhibitory signals derived from the extracellular environment converge onto the cell cycle control machinery. The activation and regulation of G₁ phase specific regulatory proteins like cyclin D₁ and E (Box 2, Figure 5; reviewed in Sherr, 1993), bind to Cdk-4 and Cdk-2, respectively, to form complexes, which, upon activation by cyclin-activating kinase (Cak), phosphorylate and subsequently inactivate the retinoblastoma protein (pRb) (Kato et al., 1993; Weinberg, 1995). This results in the release of transcription factor E2F, which then stimulates other factors necessary for the initiation of the S phase.
Box 2: The Cell Cycle Machinery

Cell division is a complex and carefully controlled process, as a cell progresses through the cell cycle. The cell cycle consists of the following phases: $G_0$, $G_1$ (Gap 1), S (Synthesis), $G_2$ (Gap 2), and M (Mitosis) (Figure 4). The $G_1$ phase is characterized by cell growth, gene expression and protein synthesis necessary for DNA synthesis. In this phase, the cell cycle can be regulated by extracellular stimuli (like growth factors). Cdk4 / cyclin D complexes are formed and are able to phosphorylate pRb in mid $G_1$. Cdk2 / cyclin A and Cdk2 / cyclin E phosphorylate pRb at the $G_1$ to S transition.

At the end of $G_1$ phase, a key cell cycle restriction point (R-point) is located. If cells pass this point, they will almost invariably complete the cell cycle. In order to differentiate, cells need to leave the cell cycle in $G_1$ without passing the cell cycle restriction point. During the S phase, the cell replicates its DNA and the Cdk2 / cyclin A complex is active. The cell again undergoes growth and protein synthesis during the $G_2$ phase. In $G_2$, Cdc2 / cyclin A and Cdc2 / cyclin B complexes have catalytic activity. Once this phase is complete, the cell enters the final stage of the cell cycle: the M phase. During the M phase, the cell splits apart (called cytokinesis) into two daughter cells. As in both $G_1$ and $G_2$, there is a checkpoint in the middle of mitosis (metaphase checkpoint) that ensures the cell is ready to complete cell division. When division is complete, cells can either start the cycle again by entering $G_1$, or become quiescent by entering $G_0$.

There are two distinct families of mammalian Cdk inhibitors. The first class, so-called inhibitors of Cdk4 (Ink4 proteins), is composed of four distinct members (p16Ink4a, p15Ink4b, p18Ink4c, and p19Ink4d), each capable of specifically inactivating the mitogen-dependent cyclin D-dependent kinases Cdk4 and Cdk6 (Ruas & Peters, 1998). Ink4 proteins interact only with the Cdk subunit of cyclin D-dependent kinases, ultimately displacing D-type cyclins from their Cdk partners and leading to the formation of inactive CDK-Ink4 dimers. By contrast, the Cip/Kip family of Cdk inhibitors is composed of three members (p21Cip1, p27Kip1, and p57Kip2), each of which contains independent binding sites for both cyclin and Cdk subunits (Sherr & Roberts, 1999).
Figure 4: The cell cycle.

Extracellular signals (e.g., growth factor pathways, stress response)

- p21
- p27
- p57

Cdk4, cyclin D
Cdk6, cyclin D

G1 arrest / Apoptosis
Differentiation

Cdk2, cyclin A
Cdk2, cyclin E

G1 phase (cell growth)

G0

Interphase

Cytokinesis

M phase

G2 phase (growth and preparation for cell division)

S phase (DNA replication)

R-point

Cell division

Figure 5: Fluctuation of the cyclin protein levels during the cell cycle phases

p27

G0 G1 S G2-M G0
Chapter I

**Proliferation Markers**

In the 1990s the introduction of cell type-specific markers allowed for the first time immunohistochemical identification of the phenotype of newly generated cells (Cameron *et al.*, 1993; Okano *et al.*, 1993; Seki & Arai, 1993), and thereby helped to establish the occurrence of neurogenesis in the adult brain. The development of thymidine analog Bromodeoxyuridine (BrdU) immunohistochemistry to identify S phase cells in the brain (Miller & Nowakowski, 1988; Seki & Arai, 1995; Kuhn *et al.*, 1996) was an important improvement over 3H-thymidine autoradiography. Since BrdU can be double labeled with cell type-specific markers, the phenotype of the new cells can be determined using confocal microscopy. So α-BrdU not only identifies the labeled proliferating cells in S phase as well as their progeny, but can also be used to study the phenotypic development of the new surviving cells over time.

Also, with antibodies against the endogenous protein Ki-67, it is possible to detect cell proliferation in fixed tissue (Kee *et al.*, 2002). Ki-67 antigen is a 345 to 395 kDa non-histone protein complex present only in proliferating cells during G1, S, G2 and M, but not the G0 phase of the cell cycle (Gerdes *et al.*, 1984; Endl & Gerdes, 2000). α-Ki-67 is an often used and well-accepted proliferation marker in tumor biology (Gerdes *et al.*, 1991). By using combinations of these markers, several laboratories confirmed that new neurons are added to the adult brain (Kaplan & Hinds, 1977; Altman & Bayer, 1990; Cameron *et al.*, 1993; Kuhn *et al.*, 1996; Kempermann *et al.*, 1997; Eriksson *et al.*, 1998).

**Cell Death**

To maintain tissue homeostasis, there must be a balance between the ongoing adult proliferation and apoptosis (Biebl *et al.*, 2000; Levison *et al.*, 2000). Indeed, a continuous cell turnover occurs in the hippocampal dentate gyrus (Gould & McEwen, 1993). Programmed cell death or apoptosis is a highly regulated and short lasting process by which the organism eliminates unwanted cells without eliciting an inflammatory reaction. Generally neighboring, or macrophage-like, cells easily phagocytize the remainder of the apoptotic cells. Different specific morphological features characterize apoptosis: condensation of chromatin, cell shrinkage, membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and the formation of apoptotic bodies.

Several observations raise the possibility that molecular mechanisms controlling the cell cycle and cell death are related. In the first place, during both processes structural changes occur involving chromatin condensation, cell rounding, and cytoskeletal rearrangements. Furthermore, tumor suppressor genes and oncogenes can function as
regulators of cell death, e.g. induction of tumor suppressor gene p53 leads to cell cycle arrest or cell death (Ko & Prives, 1996). And finally, deregulation of cell cycle proteins like Cdns, cyclins and certain Cdk inhibitors, in proliferating cells can ultimately lead to cell death (Heald et al., 1993).

New cells need signals, survival and growth factors, from other cells or the environment to survive. Otherwise cells will activate their intracellular death program and die by apoptosis. For example during CNS development, an excess of new cells is produced that compete for limited amounts of survival factors secreted by target cells. Cell death in the adult dentate gyrus, under normal circumstances, also involves a large percentage of the newborn proliferating cells. For example Biebl et al (2000) showed that the majority of the apoptotic cells was found at the border between the hilus and granule cell layer, the neurogenic zone of the dentate gyrus. And further, the number of proliferating cells in this region declines markedly between 1 and 2 weeks after their birth, which appears to be caused by cell death rather than label dilution or migration (Cameron et al., 1993; Gould et al., 1999b; Dayer et al., 2003).

1.4 The Role of the Microenvironment in Neurogenesis

In the adult brain, only two regions (dentate gyrus and SVZ) produce new neurons. Although other brain regions also contain proliferating cells, in vivo these cells mainly become glial cells (Horner et al., 2000; Rakic, 2002). Under appropriate in vitro conditions, however, proliferating cells isolated from non-neurogenic regions can differentiate into both neurons and glia (Shihabuddin et al., 1997; Palmer et al., 1999; Kondo & Raff, 2000). Additionally, when neural stem / progenitor cells from neurogenic regions are transplanted into non-neurogenic regions, such as the cerebellum and spinal cord, they differentiate only into glia (Suhonen et al., 1996; Shihabuddin et al., 2000). These transplantation studies have provided evidence for the important role of environmental factors in the control of neural stem / progenitor cell fate choices. Furthermore, although the various developmental steps, which new cells undergo while integrating into an existing adult neuronal network, are probably highly comparable to those occurring during development, one major difference is that neurons generated in adulthood undergo these processes in a mature microenvironment. Various factors are known to contribute to this environment.
**Glia**

In the first place, hippocampal astrocytes were shown to be important in regulating neurogenesis by promoting proliferation and actually instructing new cells to adopt a neuronal fate (Toda *et al.*, 2000; Song *et al.*, 2002). Importantly, astrocytes from non-neurogenic areas do not have this ability, indicating that astrocyte populations have regional functional differences. In fact, cells that have structural and molecular characteristics of astrocytes were shown to be capable of producing new neurons, not only during development, but also in adulthood (Alvarez-Buylla *et al.*, 2002; Kriegstein & Gotz, 2003).

Radial glia are another relevant microenvironmental factor in this respect. Cell bodies of radial glia cells are located in the subgranular zone of the dentate gyrus and extend their processes into the granular cell and molecular layer (Alvarez-Buylla *et al.*, 1987; Schmidt-Kastner & Szymaś, 1990; Seki & Arai, 1999). These cells have been called residual radial glia, or remaining radial glia, because in the neocortex, most radial glial cells disappear once development is completed (Eckenhoff & Rakic, 1984; Rickmann *et al.*, 1987; Hartmann *et al.*, 1992). This is not so in the dentate gyrus, where small numbers of radial glial cells persist into adulthood. Confocal analysis showed that radial glial processes in the SGZ are in close contact with developing neurons, suggesting that these cells have a supporting role in adult granule cell development (Seki & Arai, 1999; Huttmann *et al.*, 2003).

**Vasculature**

A third factor that determines aspects of the local microenvironment is the vasculature that provides the surrounding brain tissue with glucose, oxygen and growth factors (Box 3). Interestingly, in the adult hippocampus, it has recently been shown that 37% of the cells generated in adulthood proliferate in close proximity to the blood vessel wall (Palmer *et al.*, 2000). Furthermore, neurogenesis and angiogenesis are closely related as they share many regulating factors (Jin *et al.*, 2002; Zhu *et al.*, 2003). This suggests that vasculature- or blood-derived factors can regulate neurogenesis. Indeed, in the adult songbird neostriatum, Louissaint *et al.* (2002) demonstrated that migration of neuroblasts first occurs along radial cells and then along blood capillaries, which release trophic factors to target and guide the new neurons.
Box 3: The vasculature

The formation of the vascular bed occurs via different well-controlled stages, i.e., vasculogenesis, angiogenesis and arteriogenesis (Yancopoulos et al., 2000; Carmeliet & Storkebaum, 2002). Vasculogenesis involves proliferation and differentiation of endothelial precursors within avascular tissue, after which the cells associate to form a primitive tubular network. During angiogenesis, this network further expands by sprouting or splitting of preexisting vessels. Finally, arteriogenesis takes place, which involves the enlargement of collateral arterioles to form larger arteries.

Figure 6: Formation of the vascular bed

(from Harrigan, 2003)

The processes neurogenesis and angiogenesis are closely related as they share many regulating factors (Jin et al., 2002; Zhu et al., 2003).

Angiogenesis

During angiogenesis, the sprouting phase consists of growth and stabilization of the new vessels. Growth of the preexisting vessels includes nitric oxide-mediated vasodilatation. This promotes the synthesis of the vascular endothelial growth factor (VEGF) family of angiogenic factors, which, subsequently, leads to VEGF-mediated increase in vascular permeability. Extravasation of plasma proteins supports endothelial cell proliferation, which causes endothelial cells and pericytes to migrate inwards simultaneously to form a lumen within the endothelial sprout. During stabilization, the basement membrane is reformed, and new vessels are invested with pericytes, a process that also inhibits endothelial cell proliferation (Folkman, 1995). The actual splitting of the vessels during angiogenesis involves the placement of cellular columns into the lumina of preexisting vessels. Growth and stabilization of these columns lead to partitioning and remodeling of the local vascular network.
Angiogenesis seems to be driven by the metabolic demands of, and soluble angiogenic factors produced by the expanding neuroectoderm (Harrigan, 2003). Vascular endothelium-specific growth factors include members of the vascular endothelial growth factor (VEGF), angiopoietin and ephrin family (Gale & Yancopoulos, 1999). In addition, many other growth factors that are not vascular endothelium-specific are involved in the formation of functional vessels, such as members of the platelet-derived growth factor or transforming growth factor-beta families (Carmeliet, 2000).

**VEGF**

VEGF is a highly conserved, secreted mitogen specific for endothelial cells subserving angiogenesis and permeability in development and injury (Ferrara, 2000; Ferrara et al., 2003). Five VEGF homologs, i.e., VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF), have been identified. VEGF can bind to two receptors, Flt-1 (VEGF-R1) and Flk-1 (KDR/VEGF-R2), which then activate their intracellular tyrosine kinase domains that subsequently influence several downstream signaling pathways (Larrivee & Karsan, 2000). VEGF has been observed in the periventricular matrix zone (Breier et al., 1995) while also receptors are expressed on invading endothelial cells (Millauer et al., 1993). In recent years, VEGF and its receptors have also been found on neurons, where they are likely to exert prominent roles in neurite outgrowth and neuroprotection as well, particularly after ischemia (Jin et al., 2000; Rosenstein & Krum, 2004).

**Blood Brain Barrier**

The blood brain barrier (BBB) is an important component involved in the integrity and permeability of the vasculature. It ensures homeostasis of the brain microenvironment, mostly through complex tight junctions between brain endothelial cells that prevent the passage of hydrophilic molecules from blood to brain and vice versa.

*Figure 7: Schematic representation of a cross-section of the CNS blood-brain barrier*
Tight junctions closely interconnect the endothelial cells. The basement membrane separates the pericyte, which is located at the abluminal side of the micro-vessel, from the endothelial cell and the astrocyte endfoot. Pericyte processes cover a substantial portion of the microvascular border including the endothelial tight junctions.

1.5 Regulation of Adult Neurogenesis

Cell birth in the adult hippocampal dentate gyrus, including the survival, migration and differentiation of the new cells, is influenced by many factors. Various growth factors, neurotrophins, hormones and neurotransmitters are known to regulate aspects of neurogenesis (Cameron et al., 1998; Gould & Gross, 2002). For example fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) are thought to be the primary mitogens to stimulate proliferation of progenitor cells (Cameron et al., 1998), while neurotrophins (NT-3) and brain-derived neurotrophic factor (BDNF) enhance neuronal differentiation (Henderson, 1996). Regulatory factors relevant to this thesis are discussed in more detail below.

Enriched Environment and Exercise

Several environmental factors have been shown to modulate hippocampal neurogenesis. Housing mice in an enriched environment i.e., a laboratory-designed relatively complex environment, enhances the survival of newly generated cells in the hippocampus (Kempermann et al., 1997, 1998; Nilsson et al., 1999). That is, animals living in standard laboratory conditions probably lose more new cells than those living in relatively complex and challenging environments. One of the components of this enriched environment was a running wheel. When studied separately, it appeared that running alone already boosted cell survival (van Praag et al., 1999), demonstrating that voluntary exercise is an important neurogenesis stimulus. Furthermore, several learning tasks that depend on the hippocampus, such as place learning and trace eyelid conditioning increased survival of the newborn cells (Gould et al., 1999b).

Moreover, injury involving the hippocampus causes profound increases in hippocampal neurogenesis. This has now been shown for various trauma types, including ischemia, epilepsy but also for pharmacological or physical injury (Gould & Tanapat, 1997; Parent et al., 1997).
Stress and Glucocorticoids

Acute stressful experiences, which elevate corticosteroid levels and increase glutamate release in the hippocampus ((Moghaddam et al., 1994), decrease new cell proliferation rate. Psychosocial stressors, like exposure to predator odor in adult rats (Tanapat et al., 2001), the dominant-subordinate model in tree shrews (Gould et al., 1997; Fuchs et al., 2001) or resident-intruder stress paradigms in rat and monkey (Gould et al., 1998; Czeh et al., 2002), all inhibit adult proliferation in the dentate gyrus. These effects are generally more profound when applied in early life (Gould & Tanapat, 1999). Prenatal stress induced lifespan reductions in neurogenesis and cell death changes in the dentate gyrus (Lemaire et al., 2000), which, notably, correlated with behavioral performances of the offspring. Even during the hyporesponsive period at the first week of life, when rat pups exhibit a diminished response to stressors, exposure of male rat pups to odors of unfamiliar adult male rats decreased proliferation (Tanapat et al., 1998). Taken together, these studies demonstrate that activation of the HPA axis decreases proliferation in the hippocampal dentate gyrus.

The increased glucocorticoid levels during stressful experiences are thought to be main regulators of the decreased proliferation. The rate of neurogenesis, as well as death of granule cells, is under tight control of glucocorticoids. Adrenal steroid levels are e.g. inversely correlated with the rate of cell proliferation in the dentate gyrus (Sapolsky & Meaney, 1986). The hyporesponsive period, when adrenal steroid levels are low, coincides with maximal granule cell production in the dentate gyrus (Schlessinger et al., 1975). When, during this phase, corticosterone levels are experimentally elevated, the number of proliferating cells in this structure is diminished (Gould et al., 1991), similar to GC increase in the adult rat (Cameron & Gould, 1994). Furthermore, elevated corticosteroids levels in adulthood diminished cell death in the granule cell layer (Gould, 1994).

Conversely, removal of adrenal steroids by adrenalectomy (ADX) stimulated the production of new granule cells (Cameron & Gould, 1994). Moreover, also cell loss occurs that is particularly prominent 3 days after ADX and even more pronounced at 7 days (Gould et al., 1990). Both processes could be prevented by low doses corticosterone replacement. Subsequent studies revealed that aldosterone (MR agonist) replacement also reversed the effects of ADX, indicating that MR occupation is sufficient to protect against ADX induced cell loss (Woolley et al., 1991).

Effects of chronic stress were studied in the dominant-subordinate model in the tree shrew, showing a decreased production of new granule cells (Gould et al., 1997; Czeh et al., 2001), and a decline in the number of apoptotic cells in the hilar region of the dentate
General Introduction

gyrus (Lucassen et al., 2001a). Yet, the effects of chronic stress on the turnover of new granule cells in rat were hardly studied at the time this thesis-project was started.

Aging

While a growing number of studies support the idea that adult neurogenesis is involved in hippocampal function, neurogenic dysregulation is more often associated with hippocampal pathology. Subsequently, assumptions are made that the age-related decline in neurogenesis might contribute to age-related memory and learning deficits (Kempermann et al., 2002; Prickaerts et al., 2004). Some studies suggested, that the diminished production of granule cells during aging was due to elevated steroid levels (Kuhn et al., 1996; Gould & Tanapat, 1999), as frequently found in aged individuals. In agreement, removal of adrenal steroids during aging did stimulate new cell proliferation (Cameron & McKay, 1999), suggesting a possible causal relation between elevated corticosteroid levels and the age-related decline in neurogenesis. However, it has not been demonstrated whether those individuals within an aging cohort that have higher basal corticosteroid levels, also expose a lower proliferation rate than the others.

1.6 Outline of this Thesis

Hyperactivity of the HPA-axis is believed to impair hippocampal structure and function (e.g. cognition) and to be one of the risk factors contributing to the onset of depressive disorders. Furthermore, elevated levels of glucocorticoids, found during stressful experiences, are often associated with aging, and may contribute to (age-related) memory and learning deficits. Moreover, elderly individuals and depressed patients often show a decreased hippocampal volume, in relation to an increased HPA axis activity. Although increased glucocorticoid levels can affect adult DG proliferation, it was unknown at the start of this project how prolonged stress or aging exactly affect DG cell turnover.

In this thesis, we therefore examined the effects of chronic stress and aging, both associated with HPA-axis hyperactivity and morphological changes in the hippocampus, on neurogenesis and apoptosis, as well as on structural parameters in the hippocampal dentate gyrus (part I). We next focused on the role of cell cycle components and the vasculature in the observed changes (part II).
Chapter I

Research Questions

In the adult DG continuous cell turnover takes place. Although the effect of acute stress on proliferation in the rat DG has been well studied, relatively little is known about how chronic stress affects turnover of the DG. In Chapter 2, we investigated which structural changes are induced in the rat DG after 3 weeks of chronic unpredictable stress. Furthermore, because it is presently unclear whether reductions in proliferation after chronic stress are lasting, we also studied another group of animals that was allowed to recover for an additional 3 more weeks after chronic stress exposure. Similarly, for acute stress, changes in cell birth and death were examined as well, after 1 day of recovery.

Aging in rodents is often associated with hypercorticism. Elevated glucocorticoid levels are thought to be responsible for the age-related decline in proliferation. However, the influence of age on rates of various aspects of neurogenesis as well as apoptosis has not been studied in detail. Moreover, relatively little is known about the differentiation of the individual newborn cells as the organism ages. In Chapter 3 of this thesis, we examined the hypothesis that age of the animal influences (1) the balance between neurogenesis and apoptosis, and (2) the process of migration and neuronal maturation of individual newborn cells. To test this, we stereologically assessed birth rate, migration, and survival of the newborn cells, as well as cell death and structural maturation of the DG in young (2 weeks), young-adult (6 weeks), middle-aged (12 months), and old (24 months) rats. Finally, basal corticosterone levels, frequently elevated in aged rats, and the corticosterone response to a mild stressor (indicators of hypothalamus–pituitary–adrenal (HPA) axis activity and feedback function, respectively) were determined and correlated with age-dependent changes in dentate cell turnover.

In part II, we further examined the putative role of cell cycle factors and the vasculature on stress- and age-dependent changes in neurogenesis.

Regulation of mammalian cell proliferation by extracellular signals is thought to occur primarily during specific phases of the cell cycle. Many in vitro cell models have demonstrated that GCs can regulate \( G_1 \) cell cycle proteins, leading to an inhibition of proliferation and often cell cycle arrest. In Chapter 4 we tested the hypothesis that chronic stress inhibits progression of the cells through the \( G_1 \) phase by regulating expression of \( G_1 \) cell cycle proteins. This would cause less cells to enter the S phase and complete the cell cycle. We used quantitative immunocytochemistry and stereologically assessed the total
numbers of cyclin E, D, and p27Kip1-positive cells in the hippocampal SGZ.

Recent evidence has shown that adult proliferation takes place near the local microvasculature of the hippocampus, suggesting that the regulatory signals for adult proliferation are at least partially derived from the endothelium. Furthermore, angiogenesis and neurogenesis can be modulated by similar stimuli, including corticosteroids. In Chapter 5, we therefore questioned whether the effects of chronic stress on new cell proliferation are (at least in part) mediated through effects on the vasculature, and whether chronic stress affects an angiogenic-signaling pathway. To that end, we measured blood vessel density, the number of proliferating cells that is associated with the vasculature, and analyzed VEGF and Flk-1 receptor protein expression in the hippocampus of control and chronically stressed rats, and in rats that were allowed to recover after stress.

Since aging is known to cause adult haemodynamic changes, we further questioned if the vascular bed changes during aging and whether the vascular-associated proliferation changes in parallel. In Chapter 6, blood vessel density was measured, together with the number of vascular-associated proliferating cells in young, young-adult, middle-aged, and old Wistar rats. Additionally, confocal microscopy and two proliferation markers (Ki-67 and BrdU) were used to analyze the temporal dynamics of the clusters of proliferating cells, which cycle near the vasculature.

In summary, this thesis will address the following questions:

Does chronic stress affect birth, survival, migration and neuronal maturation of new cells, and cell death?
If so, how do these effects relate to changes induced by acute stress?
Are the stress-induced structural dynamic changes in the DG reversible after a recovery period?
How does aging affect birth, survival, migration and neuronal maturation of new cells, and cell death?
Is there a correlation between a decreased DG cell turnover and increased basal corticosterone levels (or changed corticosterone response) in old animals?
Is the G1 phase of the cell cycle changed after stress?
Is the microvasculature or angiogenic growth factor expression affected by stress?
How does aging affect vascular-associated proliferation?