Adult hippocampal cell birth and death in relation to stress, aging and the vasculature
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Chapter 7

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Summary

Although neglected for years, it is now widely accepted that the central nervous system continually generates new neurons throughout adulthood, albeit in low numbers and only in restricted areas. The hippocampus, that plays a pivotal role in learning and memory, fear conditioning and neuroendocrine regulation, is one of the very few regions where adult neurogenesis has consistently been found. In the hippocampal dentate gyrus (DG), cell birth and cell death are closely associated, and a continuous cell turnover takes place, resulting in a highly heterogeneous cell population. This turnover is strongly influenced by various environmental as well as hormonal stimuli. Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis or stress exposure is believed to compromise hippocampal structure and function (e.g. learning and memory) and to be one of the risk factors contributing to the onset of depressive disorders. Both chronic stress and aging have been associated with HPA-hyperactivity in literature.

In this thesis, we therefore studied the effects of chronic stress and aging on the processes of neurogenesis and apoptosis. Furthermore, the cell cycle is involved in the regulation of proliferation as well as apoptosis. Various individual cell cycle markers together determine whether dividing cells arrest or progress through the cell cycle. These cell cycle proteins provide an attractive substrate for a better mechanistic understanding of how age- and stress-induced changes in neurogenesis are brought about. Since extracellular cues like corticosteroid exposure, can affect the cell cycle, we therefore addressed changes in protein expression of various important G1 cell cycle regulators in relation to chronic stress. Finally, a large amount of the newborn cells turn out to be intimately associated with blood vessels, while the processes of both neurogenesis and angiogenesis can be regulated by similar stimuli. Therefore, the presence of a microvascular niche is thought to be important in regulation of new neuronal birth. Since both chronic stress and aging have adverse effects on brain vasculature, and aging causes haemodynamic and vascular changes, we also examined changes in the extent of the ‘vascular-associated’ adult proliferation, as well as in angiogenic growth factors.

In Chapter 2, we studied lasting effects of stress on dynamic structural changes in the adult hippocampus and showed that both acute and chronic unpredictable stress decrease new cell proliferation in the adult rat dentate gyrus. Interestingly, apoptosis was increased after acute, but decreased after chronic stress. Moreover, stress induced reductions in hippocampal neurogenesis do not appear to be permanent or long lasting, as the impaired proliferation after chronic stress was already partly recovered 3 weeks.
after cessation of the stress, whereas the reduced proliferation after acute stress had even recovered already within 24 hrs.

In literature, hypercorticism has been frequently reported in relation to aging. Such increases were even expected to induce reductions in neurogenesis in old animals. In Chapter 3, we studied neurogenesis in relation to HPA axis activity in aging Wistar rats, but failed to demonstrate a correlation between decreased cell birth and increased corticosterone levels, or stress response parameters, indicating other factors must be involved. Despite this absence of HPA changes, both new cell birth and apoptosis slowed down profoundly with increasing age. Moreover, also migration and differentiation of the individual adult generated cells into a neuronal or glial phenotype, was strongly reduced already from 6 weeks of age onwards.

In subsequent chapters we further examined the possible mechanisms underlying stress- and age-related attenuation in proliferation.

Corticosteroids are released during stressful experiences and induce a wide variety of peripheral effects. In many vitro studies, corticosteroids also act as potent antimitotics that can affect the G1 phase of the cell cycle and even induce cell cycle arrest. We wondered whether steroids exert similar actions in vivo and in relation to hippocampal neurogenesis. We therefore studied in Chapter 4 changes in protein expression of three important G1 cell cycle regulators in the adult rat subgranular zone in relation to chronic unpredictable stress. Although the numbers of cyclin E- and cyclin D1-expressing cells did not change, chronic stress significantly increased the number of cells immunopositive for the cyclin-dependent kinase inhibitor p27Kip1, which is indicative of the involvement of a G1 arrest in the stress-induced decrease in hippocampal proliferation.

Since a considerable percentage of the proliferating cells are closely associated with the vasculature, we studied in Chapter 5 the involvement of the microvasculature in new cell proliferation in the dentate gyrus. We found 32% of the proliferating cells to be vascular-associated in 10 week-old rats. Interestingly, this proportion decreased considerably after chronic stress exposure, while a subsequent 3 week period of recovery restored the impaired proliferation that was not associated with the vasculature more effectively than the vascular-associated proliferation. Furthermore, vascular endothelial growth factor (VEGF) has been shown to have neurotrophic effects as well and may thus be related to proliferative changes. Immunocytochemistry revealed VEGF-expressing astrocytes in large numbers in the hilus, from where VEGF-positive end feet extend into the granule cell layer, ending in close apposition to the neuronal cell bodies. Chronic
stress decreased both the levels of VEGF and its receptor, i.e. Flk-1 protein, in the GCL, suggesting the involvement of a VEGF-mediated signaling pathway in the stress mediated inhibition of hippocampal neurogenesis.

Aging has been associated with profound haemodynamic changes and vascular alterations that may also affect the portion of vascular associated adult proliferation. In Chapter 6 we found that the vascular bed relatively increases during aging, as was evident from measurements of the surface area covered by the blood vessels. The proportion of cells proliferating near the vasculature rather decreased during aging, notably in a regionally dependent fashion. Furthermore, between two and six weeks of age, the proportion of actively cycling cells increased significantly. In the older animals however, mainly 'silent', single newborn cells were present, while also the number of cells positive for the cell cycle regulator p27Kip1 decreased, indicating a decline in the progenitor population as well.

In this thesis, we have shown that both chronic stress and aging have a profound effect on neurogenesis and apoptosis in the hippocampal dentate gyrus. It has been thought, that elevated corticosteroid levels contribute to these changes. In the aging animals, however, basal levels or stress response parameters did not correlate with the decreased cell birth. Chronic stress-induced changes in important regulators of the G1 phase of the cell cycle, influences cell division, apoptosis, as well as differentiation of the new cells. Our results on the chronic stress- and age-induced changes in the cell cycle, give better understanding of the impact of environmental factors on new neuronal birth. Furthermore, our data support the important involvement of the microvasculature in neurogenesis. Decreases in endothelial-derived neurotrophic factors, like VEGF, will contribute to the chronic stress- and age-related declines in neurogenesis. So, although elevated corticosteroid levels affect both cell cycle regulators as well as the vasculature, it is probably the interplay between intrinsic and extracellular factors that determine DG cell turnover. More knowledge of these and other regulating factors may contribute to 1) a better understanding of the onset and reversibility of hippocampal volume-related diseases, such as found in depression, and 2) the development of transplantation studies of healthy progenitor cells into an old or injured neuronal environment.
General Discussion

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1.4 Concluding Remarks / Future Directions
Ever since new cell birth was found to continue to occur in the adult brain, the regulation and function of neurogenesis and apoptosis in the adult dentate gyrus have received much attention. A lot of this interest focuses on how these two processes are regulated by environment, age and stress, and whether and how they contribute to structural and functional (e.g. cognitive) hippocampal changes as seen in e.g. ageing and depression.

The aim of this thesis (1.6) was to examine the effects of aging and chronic stress on neurogenesis and apoptosis in the adult hippocampal dentate gyrus, and to survey possible mechanisms by studying the role of cell cycle markers and microenvironmental factors like the vasculature.

1.1 Methodological Considerations

When discussing new cell birth in the adult brain, a few methodological considerations need to be addressed first to correctly interpret apparent discrepancies between different studies on adult neurogenesis (Prickaerts et al., 2004). We will clarify our choice of methodology to answer the research questions proposed in this thesis.

BrdU Labeling

To study birth, survival and differentiation of newborn cells in an adult brain, many others, and we injected animals with the birth date marker Bromodeoxyuridine (BrdU). This S phase marker labels all cells that are in the process of replicating their DNA. This method not only allows to monitor and track newborn cells in the brain over time, α-BrdU immunocytochemistry can also be combined with immunofluorescent double labeling for phenotypic (neuronal or glial) markers. Although administration of BrdU is commonly used today to study adult neurogenesis, BrdU incorporation in DNA might in theory, affect cell viability, or e.g. detect ongoing DNA repair occurring at low levels in postmitotic neurons as well.

Cooper-Kuhn and Kuhn (2002) investigated these issues in 8 week-old Wistar rats by BrdU labeling at defined time intervals. Immunohistological analysis of the GCL revealed that BrdU-positive cells co-expressed markers for mitosis, progenitor, immature and mature neurons with increasing times after BrdU labeling. BrdU-positive cells did not colocalize with cells undergoing apoptosis, while irradiation, which stimulates DNA repair,
caused a decrease in BrdU labeling (Cooper-Kuhn & Kuhn, 2002). Apparently the dose of irradiation was rather high and induced apoptosis, preferentially in the newly generated cells. The authors concluded that incorporation of BrdU is a reliable method for detecting cells undergoing mitosis, and can be used to detect both neural stem and progenitor cells.

Other arguments in this respect are obtained from the work of Van Praag et al., (2002), who used Green Fluorescent Protein coupled retroviral labeling of dividing cells in adult mice. Indeed, a highly comparable anatomical distribution and frequency of the adult generated cells was found with this independent technique, confirming that BrdU indeed identifies newly generated and dividing cells and not just DNA repair (van Praag et al., 2002).

Still, there are a few other methodological aspects involving BrdU that should be considered. First, the estimated bioavailability of BrdU. This obviously will influence the numbers of cells in the brain that incorporate BrdU and depends on the dose and number of BrdU injections, but also on peripheral factors like blood brain barrier permeability, metabolic rate and liver function. BrdU was previously believed to be present peripherally for about 30 min before clearance by the liver was completed. However, Hayes and Nowakowski (2000) showed that BrdU is present for longer time and labeling might continue for up to 5-6 hours (Hayes & Nowakowski, 2000). Second, once BrdU is incorporated, labeled cells may stop proliferating and migrate out of the proliferative zone, but can also continue to proliferate again and again, thereby subsequently diluting BrdU label below detection level. It has been estimated from in vitro studies that at most, a fifth or sixth generation progeny can still be identified as immune positive. Although, in our studies also BrdU label dilution was visible, after our longest survival time of 4 weeks, immunopositive cells were still clearly identifiable. Contrary to tritiated thymidine (3H) incorporation, where actual grain number can be assessed stochastically in the nucleus of individual daughter cells, yielding truly quantitative data, BrdU immune signal does not allow such quantitative data, but has obvious other advantages, such as easy of use, and the possibility for phenotypic analysis.

Many studies, including our own, have shown that the number of BrdU-positive cells increases in the days after the first injection, peaking around 1-2 weeks later (Gould et al., 1999; Gould & Tanapat, 1999; Dayer et al., 2003). After this period, the number of dying cells exceeds the number of new cells being formed. The latter still contain sufficient amounts of label to be immunopositive. Thus, in conclusion, variation in literature regarding neurogenesis in absolute terms, depend to a large part on the BrdU dose, the use of single or multiple injections, the survival time points chosen, as well as environmental
factors, like blood-brain-barrier (BBB) permeability, liver activity. These will all affect the numbers of BrdU positive cells present in brain (Hayes & Nowakowski, 2000; Cameron & McKay, 2001) and may result in differential results even though e.g. fairly comparable designs were used (Gould et al., 1999; Greenough et al., 1999; van Praag et al., 1999). This needs to be considered and standardized as much as possible when comparing the results between different studies, and when planning studies on adult neurogenesis.

The animals in our studies that were studied for the effects of stress (Chapter 2), were injected once with a BrdU dose of 200 mg / kg. Contrary to the, at the time generally used dose of 50 mg/kg, the 200 mg/kg dose was shown to label all cells in S-phase, without inducing toxicity (Cameron and McKay, 2001). This implied that previous studies utilizing the lower dose, had all underestimated the true extent of adult neurogenesis. Indeed, from a rather rare event with only very low numbers of newborn cells that was difficult to consider a physiologically relevant or important phenomenon (Gould et al., 2001; Rakic, 2002), robust numbers were now found after 200 mg/kg injections, yielding estimates of around 9000 new cells born per day in young adult animals.

As will be discussed later, particularly in relation to stress, when e.g. BBB permeability might be affected, BrdU availability and incorporation in brain tissue also depends on the experimental conditions. Therefore, to confirm our BrdU findings and prevent misinterpretation, we additionally used immunocytochemistry for the endogenous, independent cell cycle marker Ki-67. The 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. Ki-67 was indeed shown to overlap nicely with the numbers of BrdU labeled cells in the DG after short survival times, and to respond to similar stimuli as does BrdU, such as irradiation (Kee et al., 2002). Moreover, Ki-67 is a classic and well-accepted proliferation marker in tumor biology (Gerdes et al., 1991).

We conclude that the BrdU injection protocols combined with the Ki-67 immunocytochemistry have been reliable tools to study the processes of adult proliferation and neurogenesis in sufficient detail, for the currently investigated questions.

**Stress Paradigms**

Concerning stress mediated effects on adult proliferation, various paradigms have been used previously. These differed in type (i.e. physical or psychological), species (i.e. rat vs. monkey), frequency (i.e. acute vs. repeated or chronic), severity (e.g. chronic mild stress vs. restraint) and predictability of the stressors involved. In general terms, most stress paradigms induce a reduction in adult proliferation rate in the short term, while for
chronic stress, differences may exist in the survival rate of the remaining adult generated cells, that appears to depend on whether or not the organism is capable of adapting to the stressor, when it is applied chronically. Furthermore, paradigms involving psychosocial stressors tend to have stronger effects on hippocampal proliferation (Gould et al., 1997; Czeh et al., 2002), whereas physical types of stressors often lead to adaptation.

For these reasons, we have chosen a well-established chronic stress paradigm of 3 weeks (Herman, 1995) that consists of an unpredictable combination of both psychosocial and physical stressors. Chronically stressed animals typically display specific symptoms like increased (basal) corticosterone levels, enhanced corticosterone or ACTH responses, an attenuated body weight gain, a decreased thymus and an increased adrenal weight, and a reduced surface area of the hippocampal CA3 region. Almost all these HPA-axis parameters were found in our animals, indicating they had indeed been exposed to chronic stress.

Another issue in this respect is whether certain forms of stress affect specific neuronal elements in different brain regions as well. In particular, increasing evidence supports a critical role for the amygdala and prefrontal cortex in fear, anxiety, and activation of the HPA axis (Davis et al., 1994; LeDoux, 1994). Anatomical studies indicate that limbic inputs onto the PVN and hypothalamic GABAergic neurons can be either excitatory from the hippocampus, and thereby enhancing GABAergic tone, or inhibitory from the amygdala, thereby reducing GABAergic tone (Herman et al., 1989; Jacobson & Sapolsky, 1991; Pitkanen & Amaral, 1994; Herman & Cullinan, 1997). This in turn implies that although enhanced hippocampal input would suppress the HPA axis, enhanced amygdaloid input could have an opposite effect on HPA activity. Vyas et al (2002) compared chronic unpredictable stress to chronic immobilization stress and found, indeed, contrasting patterns of dendritic remodeling in neurons of the amygdala and hippocampus (Vyas et al., 2002). Thus, studying the different effects of physical and psychosocial stressors, could give a better and more complete understanding of the specific aspects involved in the complex regulation of stress effects on new cell birth and death in the hippocampus.

**Aging**

When studying the effects of aging on neurogenesis in rodents (Chapter 5), some methodological issues need to be considered. Our animals were injected 3 times with a BrdU dose of 50 mg/kg, with intervals of 2 hours. The rationale for these multiple injections was born out of the expected low numbers of newborn cells in middle-aged and older animals, and, based on general experience of, the occasional failure to deliver BrdU.
using i.p. injections in (large) aged animals, that end up more frequently in fat tissue or the bladder. For the same reasons as above, we could beforehand not exclude that aging would affect metabolism or liver function in these old animals and hence leave BrdU availability unaffected. We therefore also included Ki-67 immunocytochemistry as an additional independent marker, that indeed revealed comparable (low) numbers of proliferating cells as after BrdU, in e.g. the oldest age group.

Another methodological issue on age is the criteria for what is considered an "aged" population of animals. This is important for the experimental design, as in literature clear differences from young animals have been described in middle aged groups, that again reversed, or normalized in old animals, or vice versa (Coleman et al., 1990). For these reasons, and to better understand the development of age-related changes, multiple time points, and also middle-aged animals, were included. As to criteria for aging, literature requires that a population of aged animals should follow a more or less rectangular survival curve, i.e. there should be a rapid decline of the number of surviving animals with advanced age. Also, when animals die of old age, they should do so of multiple pathologies, rather than from recurrent inbred strain specific pathology (Mos & Hollander, 1987). In our studies, the oldest animals were 24 months of age, which is well within the ranges of median life span data for Wistar rats (Mos & Hollander, 1987). All our animals were in good shape. In view of the high prevalence of pituitary or other hormone producing tumors in old animals of other inbred rat strains, excessive levels of secreted androgens or other steroids were expected. Yet, no indications were found for such tumors in our Wistar cohort, neither on postmortem inspection of the pituitary and hypothalamic area, nor from the corticosterone plasma values measured, that in fact did not fail to show increases in the old animals. Thus, our cohort studied in chapter 3 represented healthy, aged animals.

We have shown that the high numbers of adult generated cells in the 2 weeks old group declined already very early, i.e. from 6 weeks of age onwards with very low numbers in 12 and 24 months old rats. Specific stimulatory changes and challenges in the environment, such as present in "enriched" environmental housing conditions, are known to particularly, and profoundly stimulate hippocampal neurogenesis (Kempermann et al., 1997, 1998; Nilsson et al., 1999). Interestingly, this potential was shown to be retained even in middle aged rodents, as housing mice from the age of 10 to 20 months in an enriched environment, induced a five fold increase in hippocampal neurogenesis, parallel to significant improvements in learning, exploratory behaviour and locomotor activity (Kempermann et al., 2002). Although similar increases can be induced in the old rat (Kempermann et al., 1998), the animals we studied, like most laboratory animals,
obviously, did not not lead an "active" or challenging life, and were in that respect in fact studied under "environmentally impoverished" conditions. It should be noted therefore that, regarding neurogenesis, aging studied in the present laboratory setting is likely to be very different from animals living in the wild. One may wonder in this respect whether the housing condition and life style of aging rats reflects in any way the lifelong challenges and lasting learning experiences in human aging.

In addition to the points discussed above, the use of different strains, species, age and sex obviously also may contribute to different results found in literature. It is likely that together, they determine DG proliferation and survival rate.

1.2 Hippocampal Structural Dynamic Alterations after Stress and during Aging

Hyperactivity of the HPA-axis, or chronically elevated corticosteroid levels are believed to compromise hippocampal structure and function (e.g. cognition) and to be one of the risk factors contributing to (aspects of) the age-related decline in cognitive function, or the onset of depressive disorders (see for details below). The hippocampus is of particular interest in this context in view of its role in learning and memory, its involvement in various neurodegenerative disorders, its high density of mineralocorticoid (MR) and glucocorticoid receptors (GR) and its supposed role in feedback inhibition of the HPA axis. But do chronic stress and aging really change neurogenesis and if so, does this contribute to changes in hippocampal volume?

At the start of this project, acute stress was known to reduce cell birth in the hippocampal dentate gyrus. However, little was known on how chronic stress would affect both the processes of neurogenesis and apoptosis, and if so, whether this would indeed result in structural changes in the DG. Also, to obtain a better understanding of the lasting nature of the stress-related hippocampal volume changes, such as documented in depressed or Cushing patients, we further investigated whether chronic stress-induced changes could recover after a period of rest, lasting as long as the stress period. Our chronically stressed animals were indeed chronically stressed as they displayed all the symptoms of stress exposure such as increased basal corticosteroid levels, changed thymus/adrenal weights and CA3 atrophy.

Chronic stress decreased both cell birth and death in the dentate gyrus, whereas apoptosis increased in the DG after acute stress. One clear difference between the two groups is that, at the moment the acutely stressed animals were studied for cell death, their
numbers of proliferating cells were back to basal. By contrast, the chronically stressed rats still showed decreased numbers of Ki-67-positive cells.

Glucocorticoids can induce apoptosis in different brain regions under specific conditions. This occurs likely through a differential occupation of the mineralocorticoid (MR) and glucocorticoid receptors (GR) that may explain the opposing actions of corticosteroids on neuronal proliferation, survival, and death (Cameron & Gould, 1996; Hassan et al., 1996; Reagan & McEwen, 1997). While predominant MR occupation is associated with cell survival, additional GR occupation may endanger hippocampal neurons. GR occupation was shown before to stimulate apoptosis within the granular and hilus cell populations, an effect even more pronounced in aged rats (Hassan et al., 1996). MR activation, on the other hand, appears essential for maintenance of the granule cell layer (Sloviter et al., 1989; Woolley et al., 1991; Sousa et al., 1998) as is clear from conditions of steroid depletion like adrenalectomy, that induces massive apoptosis selectively in the DG (Sloviter et al., 1989; Hassan et al., 1996).

Of the many theories on brain aging, focusing on age-related changes in membrane composition, energy metabolism, free radical or calcium levels (reviewed by Sapolsky & Meaney, 1986; McEwen, 2000), GC hypersecretion is often seen as a key factor. Elevated GC levels may be neurotoxic and “endanger” hippocampal neurons by lowering the sensitivity threshold for hippocampal insults, toxins, or transmitters like the excitatory amino acids. GCs can induce structural neuronal remodeling by altering the extent of the dendritic tree of mainly pyramidal neurons, but also interfere with basal electrophysiological properties (Mesches et al., 1999; Alfárez et al., 2003; van Riel et al., 2003) that are thought to underlie cognitive functions both in rodents (Landfield et al., 1978; Sapolsky, 1992) and in humans (Lupien et al., 1994; Seeman et al., 1997; Lupien et al., 1998).

The ‘glucocorticoid cascade hypothesis’ of aging (Sapolsky & Meaney, 1986; Sapolsky, 1992; Landfield & Eldridge, 1994; McEwen et al., 1999) proposes an initial accumulation of glucocorticoid-induced damage to the hippocampus, that in turn, disinhibits the negative feedback on the HPA axis and thereby promotes a feedforward cascade of progressive elevations of adrenal steroid levels, inducing a further dysregulation of the HPA axis, that would affect brain as well as peripheral functions (Sapolsky & Meaney, 1986). As studied earlier in rats, the age-related changes in GC levels and other HPA parameters, are clearly strain dependent (De Kloet et al., 1998; Lucassen & De Kloet, 2001). In addition, it is still unclear whether this hypothesis applies also to the human.
situation (Swaab, 1998; Lucassen & De Kloet, 2001; Muller et al., 2001), or whether all individuals are equally susceptible to the GC mediated, supposedly deleterious effects.

At the start of this project, it was known that cell birth, i.e. proliferation rate, decreases during aging. We initially hypothesized that an age-related decline in new cell birth would correlate with an age-related increase in basal corticosteroid levels, and that also other parameters, like migration and differentiation of the individual newborn cell into an adult phenotype, or apoptosis, might be affected. Clearly, aging Wistar rats do not show hyperactivity of their HPA-axis, as measured by their basal corticosteroid levels and their stress responses to a mild stressor. Nevertheless, aging profoundly reduced both cell birth and death. Although both processes changed at different rates in absolute terms, the cell number and volume of the hippocampal dentate gyrus remained, however, constant during aging, which indicates that cell birth and death must be in balance.

Although steroid-mediated hippocampal cell loss was previously thought to occur during aging, the introduction of accurate stereological techniques has in fact provided very little support for major pyramidal cell loss in aged, cognitive impaired animals or even in stressed or GC-exposed rats (Rapp & Gallagher, 1996; Rasmussen et al., 1996; Vollmann-Honsdorf et al., 1997; Leverenz et al., 1999; Sousa et al., 2000). Nevertheless, hippocampal volume loss (albeit limited, 5-8%) does occur in these conditions, which could be transient and regionally dependent, e.g. affecting the DG. Several studies have indeed shown that GC excess or chronic stress induces dendritic atrophy in the CA3 pyramidal cell layer (Woolley et al., 1990; Watanabe et al., 1992; Magarinos et al., 1996), and that this retraction could be reversed after cessation of the stressor, or following antidepressant drug treatment.

Which subregion contributes most to the volume changes of the hippocampus as a whole, when detected by means of NMR imaging, is still unclear. Despite the reduction in CA3 surface area, chronic stress did not affect the structural parameters volume and cell number of the DG. This implies an adaptation in the DG turnover rate, as was confirmed by our analysis of neurogenesis and apoptosis that indicated both processes were indeed in balance. Apparently, 3 weeks of exposure to the present choice of unpredictable stressors, may not have been sufficiently long, or not sufficiently strong enough to induce volume changes in the DG. Such changes may become apparent only when animals are exposed to even longer periods of (more severe) stressors, as was shown by Pham et al (2003), who found small but significant reductions in the total number of granule cells (13%) and in granule cell layer volume (5%) following 6 weeks of restraint stress (Pham et al., 2003).
Summary - General Discussion

Although in our and other studies (Vollmann-Honsdorf et al., 1997; Leverenz et al., 1999; Heine et al., 2004b), DG volume remained thus relatively stable, a recent report identified functional, rather than structural, changes, particularly in the DG of aged monkeys as a hotspot in age-related cognitive decline.

At present it is also unknown which factors maintain the balance between cell birth and death and why one cell survives, while the other one dies. Although we did not address this question mechanistically, the numbers of proliferating and dying cells showed clear regional differences. In the dentate gyrus, birth of new cells continues throughout life, but at a considerably lower pace during aging. Remarkably, already between 2 week- and 6 week-old animals, a profound drop in the rates of proliferation, migration and differentiation speed occurred with most proliferating cells present in the SGZ. In the same age group, also in this region, most dying cells reside. These dying cells are thus likely to represent newborn cells. This view is strengthened by our observation, that less than half of the cells born at the age of 2 weeks did not survive the following 4 weeks (Chapter 3). Also others indicated that around 50% of the new cells die shortly after birth (Biebl et al., 2000), indicating that rather the newly generated instead of the mature ones, undergo apoptosis in the adult dentate gyrus (Biebl et al., 2000). This suggests a mechanism similar to that occurring during embryogenesis and CNS development, where a surplus of neurons is generated and consequently selected based on the establishment of functional connections and trophic support. Thus, these developmental ‘use it or lose it’ principles may also relate to the newly generated cells in an adult environment as well.

Finally, it remains unclear why the adult generated cells fail to survive, at what specific periods after their birth, or which factors can trigger survival of selected cells. Structural adaptation through hippocampal dependent learning has been implicated as one of the stimulating mechanisms involved in the survival decision of the newly generated cells (Gould et al., 1999). Aside from the local presence of obvious growth factors like the neurotrophins, including BDNF, FGF-2 and others, enriched environment could, in this respect, also be interpreted as an activating stimuli for the new cells in the hippocampus, as mainly survival rate instead of proliferation rate is affected, together with a shift in phenotype towards more hippocampal neurons (Kempermann et al., 1998; Brown et al., 2003; Ehnninger & Kempermann, 2003). Consistent with this, Young et al (1999) found that in mice living in an enriched environment, the number of apoptotic cells in the dentate gyrus was decreased (Young et al., 1999). Also after hippocampal insults like epilepsy or ischaemia, stimulation of neurogenesis through enriched environmental housing or running improved recovery and cognitive function parallel to reductions in apoptosis.
1.3 Potential Mechanisms

1.3.1 Cell Cycle Regulation of Cell Birth and Death

Having addressed the age- and stress-induced structural dynamic changes in the hippocampus, I will now discuss possible underlying mechanisms by focusing on the role of the cell cycle and the vasculature.

Cell Cycling changes during Aging

In Chapter 6, we analyzed the proliferative activity of the newly generated cells, cycling in clusters. By double labeling cells with the S-phase marker BrdU (24 hours survival time) and the endogenous proliferation marker Ki-67, present during the late G1 till the M phase, the temporal dynamics of newborn, proliferating cells can be studied. This provides information on possible changes in cell cycle length and on the speed at which newborn cells leave the cell cycle and differentiate.

We showed an age-related increase in the number of BrdU-positive cells (24 hours survival time) co-labeled with Ki-67 between 2 and 6 weeks of age. In the middle-aged and old animals, most BrdU-positive cells did not co-label with Ki-67. This indicates that these BrdU-positive cells were not actively engaged in cell cycle anymore and apparently progress through the cycle very slowly. Also in our other aging study (Heine et al., 2004a) (Chapter 3), we indeed found that not only proliferation, but also migration and differentiation of the newborn cells are strongly attenuated in middle aged and old animals. In contrast, proliferating cells in the young and young-adult rats, showed many actively cycling clusters, with 6 week-old animals even showing the highest amount of double labeled cells, which suggests that many proliferating cells continuously reenter the cell cycle at this age.

In a comparable study, Tropepe (1997) suggested an age-related increase in cell cycle length in proliferating cells in the forebrain subependyma of mice. Sequential labeling with BrdU and 3H-thymidine revealed, that in 23-25 month-old SW/COBS mice, ~50% of the proliferating progenitor cells had a longer cell cycle length than in the 2-4 month-old mice (Tropepe et al., 1997). Thus, not only the number of times newborn cells progress through the cell cycle, or its cycling activity, changes, also cell cycle length appears to be lengthened during aging.
Apoptosis

The processes of proliferation and apoptosis appear tightly coupled in the adult DG. Cell cycle regulators are involved in the control of both these processes (Meikrantz & Schlegel, 1995; King & Cidlowski, 1998). Progression through the cell cycle is monitored during specific cell cycle checkpoints at the G1/S phase boundary, in S phase, and during G2/M phases (Murray, 1993). If cells do not have the appropriate intracellular or extracellular environment, cell cycle progression is blocked at these checkpoints, which prevents them from progressing to the next phase. Cells either arrest to repair damage or wait for the right extracellular cues to resume. If the damage is too severe, cells can also choose to exit the cell cycle and go into apoptosis.

In vitro studies with thymocytes revealed that mature and proliferating cells likely utilize different GR activating signaling pathways leading to apoptosis (Cidlowski et al., 1996; Schaaf & Cidlowski, 2002). In both these pathways, glucocorticoids bind to the GR and induce changes in gene expression. In proliferating thymocytes, treatment with glucocorticoids involves changes in cell cycle components to activate apoptosis. However, in non-proliferating thymocytes, the final signaling pathway leading to apoptosis does not appear to involve changes in cell cycle proteins. Thus upstream signals induced by glucocorticoids can differ considerably depending on whether the cell is progressing through the cell cycle. The downstream components of both signaling pathways ultimately appear to activate apoptotic effector molecules that include the caspases and nucleases.

Similar to thymocytes, mature and proliferating cells in the hippocampus may utilize different GR activating signaling pathways leading to apoptosis. Moreover, the decreased apoptosis found in our study (Chapter 2) after chronic stress is solely due to the decreased apoptotic cell numbers in the SGZ, the main neurogenic region in the DG. The GCL showed increased apoptosis after chronic stress. We should note, though, that so far GR has not been identified in proliferating cells of the dentate gyrus (Cameron et al., 1993). However, GR expression is found on several human neuroblastoma cell lines, where the specific GR agonist dexamethasone (DEX) was able to decrease proliferation rate (Glick et al., 2000).

Even though older studies failed to identify GR on progenitor cells in the SGZ, it is still not known at what time in their development, adult generated cells in the DG start to express GR. This would be interesting to examine since GR expression is expected to make progenitor cells sensitive to direct steroid action, an action that might even overrule the well known role of the NMDA receptor in the stress-mediated suppression of
particularly the proliferation phase of adult neurogenesis (Gould *et al*., 1997). If the onset of MR and GR expression would develop over different but overlapping time windows, a differential receptor occupation during stress is expected, which could influence cell fate. Also, corticosteroid binding to steroid receptors on adult generated cells could possibly interfere with their fate determination i.e. to develop in a glial or neuronal phenotype (Tanapat *et al*., 1998). Double labeling studies for MR, GR and BrdU in the DG will have to be carried out before we can better appreciate the true effects of stress on the individual adult generated cells.

$G_j$ Phase

Regulation of mammalian cell proliferation by extracellular signals occurs primarily during the $G_j$ phase of the cell cycle (Pardee, 1989). In the $G_j$ phase of the cell cycle, cells decide to divide, differentiate or to go into apoptosis. A key cell cycle restriction point is located at the end of $G_j$ phase. If cells pass this point, they will almost invariably complete the cell cycle. This restriction point is regulated by many $G_j$ phase components, including Cdns, cyclins, Rb, E2F, p53 and Cdk inhibitors.

In Chapter 4, we studied amongst other marker proteins, expression of P27Kip1, a member of the CIP/KIP (cyclin-dependent kinase inhibitor) family, that can inhibit the action of cyclin/Cdk complexes (Sherr, 1994) thereby affecting cell cycle progression. Increased p27Kip1 expression is commonly interpreted as an induction of $G_j$ arrest. We showed that chronic stress increased the number of p27Kip1-positive cells, which indicates that neurogenesis is reduced after stress due to the fact that more cells are stuck in the $G_j$ phase.

It is so far unresolved via what mechanisms chronic stress induces cell cycle arrest. Some reports show that glucocorticoids can directly regulate p27Kip1 levels (Rogatsky *et al*., 1997; Zhuang & Burnstein, 1998; Rogatsky *et al*., 1999; Zhu *et al*., 2003), and that this signaling involves GR activation (Jiang *et al*., 2002). However, if GR is not present on progenitor cells, other pathways must be involved as well.

Various studies have shown a GR-induced cell cycle arrest, including in neuronal cells (Glick *et al*., 2000; Sengupta *et al*., 2000). Crochemore *et al* (2002) proposed a mechanism, in which GR-induced neural cell cycle arrest is associated with increases in nuclear translocation and transcriptional activity of p53. Potentiation of p53 may serve as a brake on cell proliferation and may prime cells for differentiation or death induced by other signals (Crochemore *et al*., 2002). Indeed, p53 seems to be a key player in the regulation of the proliferation and cell death (Miller *et al*., 2000). In agreement, Almeida *et
al (2000) showed that DEX-induced cell death was accompanied by increased p53 levels (Almeida et al., 2000). Adrenalectomy which increases both cell death (Sloviter et al., 1989) and neurogenesis (Gould & Tanapat, 1999) in the dentate gyrus, was also found to be accompanied by increased p53 gene expression (Schreiber et al., 1994).

In order to differentiate, cells need to leave the cell cycle in G₁ without passing the cell cycle restriction point. This implies that blocking the cell cycle in G₁ by overexpression of certain Cdk inhibitors should promote differentiation, whereas driving cells through G₁ should inhibit differentiation. This prediction is born out of experiments in the Xenopus retina, where overexpressing the Cdk inhibitor p27Xic1 blocks the cells in G₁ and potentiates the activity of various proneural genes. Driving cells through G₁, on the other hand, by overexpression of cyclin E1 rather reduced the activities of these proneural genes (Ohnuma et al., 2002). Accumulation of p27Kip1 promoting differentiation has been shown before in e.g. oligodendrocyte (Casaccia-Bonnefi, et al., 1997; Durand et al., 1997; Tikoo et al., 1998), retinal development (Cunningham et al., 2002) and differentiation of neuroblastoma cells (Matsuo & Thiele, 1998; Perez-Juste & Aranda, 1999; Borriello et al., 2000; Matsuo et al., 2001). In fact, nearly all components of the G₁ cell cycle regulation have been reported to influence neural determination, and most studies agree that the factors directing cell cycle arrest in G₁ somehow also activate determination pathways (Dyer & Cepko, 2000; Zezula et al., 2001; Carruthers et al., 2003; Vernon et al., 2003).

Similarly, we found increased p27Kip1 expression in parallel to a decreased proliferation after chronic stress. Numbers of differentiated neurons, however, remained unaltered in both the chronic stress and control group, where 50% of the newly generated cells expressed the neuronal marker NeuN after 3 weeks. Apparently, the remaining proliferating cell population has not lost its potential to differentiate and can catch up and expand rapidly, compensating the lower starting numbers to eventually even yield similar numbers of neurons. Although in theory, also neighboring (glia) cells may have been involved, the increased levels after stress suggest that also p27Kip1 may have been (indirectly) involved in promoting the expansion and differentiation of the suppressed progenitor population. This, however, waits to be established.

1.3.2 Vascular Involvement in the Neurogenic Niche

The importance of a specific microenvironment for neurogenesis is widely supported (Seki, 2003). Brain microvasculature plays a critical role in maintaining local brain perfusion to meet the dynamic metabolic needs for normal cerebral function (Kalaria,
1996; de la Torre, 1999) and possibly for the new cell proliferation in the dentate gyrus as well. Others have implicated before the importance of the vasculature, by showing that considerable numbers of adult generated cells proliferate in close association with the blood vessels in the rat hippocampus (Palmer et al., 2000) and subventricular zone (SVZ) (Capela & Temple, 2002) and in the songbird higher vocal center (Louissaint et al., 2002). Moreover, known robust stimuli for neurogenesis, like prolonged exercise, were shown to increase angiogenesis and cerebral blood volume as well (Swain et al., 2003), showing a close correlation between neurogenesis and the vasculature.

**Permeability and Integrity of Vasculature**

Many factors can regulate the integrity and permeability of the vasculature and thus affect its role in delivering metabolic fuels to the brain tissue. A few of them are mentioned here, before we discuss further down how these components are thought to be affected by aging and stress, and so could be involved in new neuronal birth.

A first example of an important component involved in the integrity and permeability of the vasculature, is the BBB and the molecular anatomical system of the tight junctions that characterize it. Due to its specific transport systems and tight barrier function, the endothelium selectively allows only selected compounds to pass the BBB. In this way, it exerts a protective barrier for the neuronal tissue, and helps to maintain a stable internal milieu. Astrocytic end feet surrounding the blood vessels are thought to play a dominant role in the development and maintenance of the BBB (Janzer, 1993).

Secondly, the basal lamina (BM) surrounds the endothelial cells and is often studied for its malformations under pathophysiological conditions (Kalaria, 1996; Farkas et al., 2000), such as ischemia (Mueliener et al., 2003) and also aging (Hicks et al., 1983).

Thirdly, pericytes, which are inserted in the BM and cover the vascular wall by their extended processes are considered to regulate capillary tone (Kelley et al., 1987), participate in immune responses (Thomas et al., 1999) and regulate vascular development by inhibiting endothelial cell proliferation and differentiation (Rucker et al., 2000).

Fourthly, the capillary endothelium isolates the neuropil from the peripheral circulation. Endothelial cells secrete factors that can have both mitogenic and anti-apoptotic effects on neuronal cells, such as BDNF (Leventhal et al., 1999; Linnarsson et al., 2000, Pencea et al., 2001; Zigova et al., 1998). Also the angiogenic factors VEGF and basic fibroblast growth factor (bFGF), can be produced by endothelial cells (Biro et al., 1994, Hoehn et al., 2002; Seghezzi et al., 1998) and are known to stimulate neurogenesis (Jin et al., 2002; Wagner et al., 1999).
**Age-related Effects on the Microvasculature**

We observed that between 6 weeks and 24 months of age the vascular bed in the hilus / SGZ increased significantly. This implies that even if the absolute number of proliferating cells stayed the same, chances that they were located in the vicinity of the vasculature increased. As it turned out, the proportion of proliferating cells near blood vessels actually decreased, contributing to a marked shift from vasculature-associated to non-associated cells.

In addition to the vasculature association of the proliferating cells that we studied, many more aspects of the vasculature may be changed with age or following stress.

The most consistent age-related change recorded in mammalian cerebral capillaries is thickening of the BM (Hicks et al., 1983; Honavar & Lantos, 1987; Kalaria, 1996). The deposition of fibrous material and collagen in the capillary wall, i.e. extensive fibrosis, has been reported in aging rats (De Jong et al., 1992; Knox et al., 1980) and humans (Kalaria, 1996). Such microvascular deposits have been considered to be associated with disturbed transport mechanisms over the endothelium (Mooradian et al., 1988).

Furthermore, aging in both animals and humans is associated with structural and functional alterations in the BBB (Shah & Mooradian, 1997). Thinning of the endothelium has been reported in aged rats (Hicks et al., 1983), primates (Burns et al., 1979) and humans (Mooradian et al., 1988), that might be caused by a general shrinkage of the cytoplasm or a loss of endothelial cells (Stewart et al., 1987). Permeability changes may also be related to a changed expression of tight junction associated structural proteins, such as occludin and zonula occludens-one (Hirase et al., 1997; Fruse et al., 1993). Also the cerebral occludin content reduces during aging, which may have an important impact on the functional and structural integrity of the BBB (Mooradian et al., 2003).

During aging, several active endothelial mechanisms such as choline transport over the BBB and glucose influx into the brain become affected (Mooradian et al., 1988; De Santi et al., 1995; Noda et al., 2002). Glucose is the main fuel for all brain cells. Therefore, both transportation of glucose and glycolytic metabolism of glucose in the endothelium of BBB are expected to affect brain function. Dysfunction of glucose transport and glycolytic metabolism in the endothelium of the BBB causes local hypoglycemia in the brain and has been proposed to initiate a process of dementia, atrophy of the brain and accumulation of amyloid in the brain (Silverman et al., 2001; Kalaria et al., 1997). Since pericytes surrounding the endothelium of the BBB of Alzheimer patients are prominently increased (Farkas et al., 2001), this has been interpreted as a key role in the dysregulation of microcirculation and
glucose metabolism in the BBB of aged subjects and AD patients.

In addition to its essential role in providing nutrition and oxygen to the brain, microvessels form a source of trophic factors that impact surrounding tissues (Delafontaine et al., 1995; Sonntag et al., 1998). IGF-I, NGF and BDNF e.g. are produced in the endothelium and, in the brain, are important in neurite (out)growth (Jones et al., 2003) as well as in the modulation of synaptic plasticity (Man et al., 2000; Thoenen et al., 2000). Interestingly, serum IGF-1 levels, which decline during aging (Busiguina et al., 2000; Carro et al., 2000) show a positive correlation with hippocampal neurogenesis (Anderson), and an inverse one with corticosterone, while treatment with IGF-1 even ameliorated the reduced neurogenesis in older rats (Lichtenwalner et al., 2001). A close correlation between serum IGF-I and cognition is further suggested by observations that serum levels of IGF-I are positively linked with cognitive performance in older subjects (Aleman et al., 1999). Of the various vascular related growth factors and their changes during aging, we conclude that IGF is a very promising one particularly when studied in relation to neurogenesis and cognitive functions.

Stress-related Changes in the Vasculature

In Chapter 5, we used Ki-67 and RECA immunocytochemistry and found that 32% of the new cells indeed proliferate in close proximity to blood vessels. Chronic stress exposure significantly decreased this percentage whereas three weeks of recovery restored the decreased proliferation not associated with the vasculature more effectively than the vascular-associated proliferation. Although we did not study longer recovery periods, this indicates that the stress effects are more profound and longer lasting in the vascular associated cells, and suggests an enhanced sensitivity (or vulnerability) of the population in this vascular microenvironment to stress.

Which factors determine this sensitivity, is still unclear. Already in the early phases of neurogenesis, clusters of newly born cells are observed within neurogenic regions, consisting of astrocytes, immature cells, as well as endothelium and extravascular basal lamina of blood vessels. Ventricular zone cells produce VEGF to attract growing vessels (Breier et al., 1992). Shen et al. (2004) even showed that endothelial cells are critical components in the neural stem cell niche, as they secrete soluble factors that maintain CNS stem cell self-renewal and their neurogenic potential (Shen et al., 2004). These authors furthermore showed that stem cells derived from the cerebral cortex of 10 to 11 day-old mouse embryos, proliferate slowly, when cultured under FGF2 rich conditions, considerable amounts of cells exiting the cell cycle, choosing to differentiate.
instead (Shen et al., 2004). However, when cells were co-cultured with endothelial cells, their proliferation rate doubled, resulting in the formation of large interconnected sheets of undifferentiated cells. Thus, the presence and connections to endothelial cells as well as endothelial factors such as FGF2 promote neural stem cell proliferation and appear even required for stem cell renewal.

Also for the chronically stressed animals, we measured the surface area covered by the blood vessels. Contrary to aging (Chapter 6), stress did not affect this parameter. However, the percentage of new cells proliferating near the vasculature decreased after chronic stress. So, although structural changes in the vascular bed were not responsible for the decreased vascular-associated proliferation, other vascular changes for instance in permeability or in expression of specific vascular-related signaling pathways cannot be excluded. To address this, we studied protein expression of VEGF and its receptor Flk-1. For both, decreased expression levels were found in the GCL of chronically stressed rats, suggesting an indirect pathway for the stress-mediated reduction in proliferation.

Recent studies have now shown that VEGF actions in nervous tissue are much more widespread than initially assumed. It is now apparent that VEGF can subserve multiple neurotrophic and neuroprotective roles in both the CNS and PNS. Different factors can regulate expression of VEGF and its receptors on both neurons as well as astrocytes (Reviewed by Rosenstein & Krum, 2004). Given that the neurotrophic effects of VEGF became apparent only in recent years, we therefore will highlight a few studies clarifying its involvement in new neuronal birth.

For example, Silverman et al (1999) demonstrated that VEGF application to organotypic fetal ventral mesencephalic explants has a significant effect on neurite outgrowth. In the same study, nascent mesencephalic neurons showed a strong and rapid upregulation of MAP-2, suggesting a role of VEGF in neuronal maturation as well. Also tyrosine hydroxylase-positive dopaminergic neurons showed increased survival compared to controls following VEGF application in vitro (Silverman et al., 1999). Very recent studies even revealed a direct effect of exogenous VEGF on neurite growth and MAP-2 expression in neocortical neurons in primary culture (Khaibullina et al., 2004; Rosenstein & Krum, 2004). In the PNS, Sondell et al (1999) demonstrated that VEGF treatment increased Flk-1-mediated axonal outgrowth and neuronal survival of dorsal root and superior cervical ganglia neurons. These studies further suggested that VEGF effects are mediated through Flk-1 without direct involvement of the Trk receptors (Sondell et al., 1999).

The Flk-1 receptor transduces most of VEGF-induced effects such as proliferation, chemotaxis, changes in protein expression and anti-apoptotic activity. Flk-1 activates the
MAPK signaling pathway and induces a mitogenic effect on brain endothelial cells (Mani et al., 2003), as well as the neurite outgrowth in cultured peripheral ganglia (Sondell et al., 1999) and CNS neurons (Khaibullina et al., 2004; Rosenstein & Krum, 2004). Furthermore, Flk-1 activates the PI-3 / Akt pathway, which is important in regulating cell proliferation and survival, and mediating an anti-apoptotic effect in endothelial cells (Thakker et al., 1999; Larrivée & Karsan, 2000), as well as transducing neuroprotective effects in an immortalized neuronal cell line (Jin et al., 2000).

Recently, it has been demonstrated that neural progenitor cells in the hippocampal SGZ and SVZ express Flk-1 (Jin et al., 2002; Maurer et al., 2003) suggesting that VEGF can exert a direct effect on the progenitor cells. Also more indirect effects have been proposed by Louissaint et al (2002), suggesting that in the vocal center nucleus of adult songbirds VEGF acts by upregulating BDNF, thereby stimulating the local microvasculature (Louissaint et al., 2002). Interestingly, also astrocytes and endothelial cells promote the production of new neurons in vitro via modulating BDNF (Leventhal et al., 1999; Song et al., 2002). Furthermore, Fabel et al (2003) showed that peripheral VEGF blockade abolishes running-induced neurogenesis, but does not affect baseline neurogenesis in non-running animals. This indicates that the local regulators that maintain baseline neurogenesis are not influenced by the physiological changes that occur during running and remain constant in both groups. It further demonstrates that peripheral VEGF is not a local regulator under basal conditions. In our study, VEGF expression levels in the astrocytes of the chronically stressed rats were not changed (although after three weeks of recovery, VEGF levels did increase). However, VEGF and Flk-1 expression levels in the GCL were decreased, which makes it more likely, that a direct local effect by VEGF was responsible for the reduced proliferation. Other properties of the vasculature may also be affected by chronic stress, although we did not study these.

BBB permeability has been shown to increase in response to stress (Sharma 1991; Fuchs and Flugge, 1998; Skultetyova et al, 1998). These BBB changes after stress are believed to be due to several factors, including stimulation of central catecholaminergic neurons, and noradrenaline release (Bradbury, 1979; Rapoport et al., 1980), increased local activation and release of serotonin (Sharma, 1991), changes in the circulating corticosteroid levels (Barr, 1985), and an increased cerebral blood flow and energy metabolism (Bryan, 1990). Stress-induced BBB damage further depends on the type and degree of stress as well as strain, species, and age of the animals (Fuchs & Flugge, 1998; Friedman, 1996; Telang, 1999; Keuker, 2003). For example, acute restraint stress increased BBB permeability in rats through CRF activation (Esposito et al., 2001), as well as the
activation of vasoactive mediators such as histamine, released from perivascular brain mast cells (Esposito et al., 2001). In addition, this group showed that brain microvessel endothelial cells contain CRF receptor proteins (Esposito et al., 2001), suggesting that CRF can directly affect the endothelial cells and so BBB permeability.

It remains puzzling though, that running increases glucocorticoid levels in humans (Heitkamp et al., 1998) and animals (Borer et al., 1992); and that both running and stress can increase BBB permeability, but nevertheless have contradicting effects on neuronal birth. Apparently, VEGF has the ability to increase BBB permeability, even though its levels are decreased by stress. Further research is needed to better understand these mechanisms.

In conclusion, the microvasculature is an important factor in the neurogenic niche. Changes in the vasculature or related local microenvironmental factors are likely to affect neurogenesis during aging and stress, and vice versa. However, the mechanisms and signaling pathways that involve the stress and age-related decreases in proliferation are poorly understood. Additional research should reveal whether the age-related decline concerns intrinsic changes in the stem / progenitor cell population or is, at least in part, induced by (local) environmental factors.

_Angiogenic Factors in Stress-induced G_i Cell Cycle Arrest_

In this thesis we have shown that chronic stress results in decreased proliferation, decreased VEGF and Flk-1 expression in the granule cell layer, as well as in an increase in p27Kip1 expressing cells in the SGZ. A remaining question is which biochemical pathway is involved in and how they induce a G_i cell cycle arrest?

Neurogenesis is regulated by several growth factors (Cameron et al., 1998), including EGF (Reynolds et al., 1992), FGF-2 (Wagner et al., 1999) and BDNF (Kirschbaum & Goldman, 1995). Each growth factor interacts with different receptor tyrosine kinases and activates different but overlapping sets of intracellular signal transduction pathways. VEGF interacts with two tyrosine kinase receptors, Flt-1 and Flk-1; however, only the Flk-1 has been expressed in the adult brain and is implicated in neurogenesis (Jin et al., 2002). Several pathways are involved in mediating the effects of VEGF, including phospholipase C (PLC), mitogen-activated protein kinase (MAPK), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and MEK extracellular signal-regulated kinase (ERK) (reviewed by Cross et al., 2003 and see Figure 1). The PI3K / Akt pathway also has an important role in VEGF-induced cell cycle progression in
endothelial cells (Thakker et al., 1999), and is necessary in mediating neurotrophic effects of several growth factors, including BDNF (Thakker et al., 1999).

Growth factor activated intracellular pathways regulate the expression of important regulators in the G\textsubscript{1} phase of the cell cycle (Lavoie et al., 1996; Weber et al., 1997; Gille & Downward, 1999; Jones & Kazlauskas, 2001; Zhu et al., 2003). A key response to growth factors in many cell types is the activation of Cdk4 by cyclin D (Sherr, 1994). Activation of cyclin / Cdk complexes are required for progression from the G\textsubscript{1} to S phase. Only in the presence of growth factors that specific genes involved in DNA synthesis are expressed. Zhu et al (2003) showed that the neuroproliferative effect of VEGF was associated with upregulation of cyclin D\textsubscript{1}, cyclin E and E2F transcription factors in vitro, all of which are necessary for the progression through the G\textsubscript{1} phase and the G\textsubscript{1}/S transition (Yoshikawa, 2000; Jones & Kazlauskas, 2001).

Extracellular cues, such as stress, can inhibit cell cycle progression by decreasing growth factor signaling, which subsequently would lead to increased cell cycle arrest and increased p27Kip1 expression levels in parallel. Still unresolved is how stress exerts its effects on the expression of growth factors, such as VEGF. Several in vitro studies have revealed that steroids like estrogen, dexamethasone and corticosterone, are all able to regulate VEGF and / or VEGF receptor mRNA expression (Cullinan-Bove & Koos, 1993; Klekamp et al., 1997; Nauck et al., 1998; Machein et al., 1999; Mueller et al., 2000; Sibug et al., 2002; Mallet et al., 2003; Clerch et al., 2004). These effects seem GR mediated, since e.g. inhibitory actions of dexamethasone could be reversed by GR antagonist application (Heiss et al., 1996; Gloddek et al., 1999). So, increased corticosteroid levels during stress could directly affect expression of VEGF. Clearly this would need to be investigated in our present experimental paradigms.

Since newborn cells fail to show GR expression (Cameron, 1993), alternative GR activation could occur via mature, neighboring cells or glial cells. Both mature cells in the GCL, as well as the astrocytes in the hilar region, express GR as well as VEGF. Neuronal progenitor cells have been shown to express Flk-1 \textit{in vivo} (Yang et al., 2003) and \textit{in vitro} (Fabel et al., 2003; Maurer et al., 2003), which would provide a signal transduction route for the relations between VEGF, stress, the vasculature and neurogenesis.

In conclusion, chronically stress-induced increases in corticosteroid levels could affect VEGF and / or Flk-1 expression, leading to, decreased growth pathway signaling. This would subsequently lead to enhanced G\textsubscript{1} cell cycle arrest, thus decreasing the number of Ki-67-positive and increasing the numbers of p27Kip1-positive cells (Figure 2).
1.6 Concluding Remarks / Future Directions

In this thesis we show that both environmental as well intrinsic properties are implicated in the regulation of DG turnover. Especially aging has a profound effect on new cell birth in which both intra- and extracellular factors play a pivotal role. To fully understand the age-related decrease in proliferation, and for instance the consequences for network function or its relation to hippocampal learning, both aspects need more research. As discussed, many microenvironmental factors are hampered during aging that could contribute to impaired neurogenesis. Obviously, the neurogenic niche should be a receptive environment to allow neurogenesis. More knowledge about the conditions creating this niche is needed. Particularly for the development of e.g. transplantation studies of healthy progenitor cells into the old, impaired brain. For example, next to growth factor application, additional supply of angiogenic factors could further improve donor cell survival. Moreover, like VEGF, also other ‘traditional’ angiogenic factors could have neurotrophic actions as well, that have so far been studied in little detail in relation to neurogenesis.

On the other sides, intrinsic mechanisms regulating the age-related decrease in proliferation are hardly known. Recently developed techniques could increase our knowledge about the identity of these new cells in the SGZ of the hippocampus and their changed expression profile as the organism ages. The introduction of the laser dissection microscope (LDM, see Addendum) in this respect gives the unique opportunity to isolate single or small groups of cells out of fixed material. Based on immunocytochemical labeling for specific proliferation and differentiation steps, as well for their location in the DG, cells could be isolated and grouped together. With the usage of RNA amplification techniques, the expression profile of these fixed cells could be studied in great detail. As shown before, RNA expression profiles isolated from fixed material are highly comparable to fresh cell material (Qin, Heine et al., 2003, see Addendum). Special interest would go to the temporal expression of receptors for growth factors, glutamate, glucocorticoids and endothelial-derived factors. Mapping the temporal onset of expression of these different factors would considerably increase our understanding of the susceptibility of the newborn surviving cells to environmental signals, like those we studied in this thesis. Other questions that can be addressed: Do the expression profiles of the aging newborn cells in young animals differ from adult-generated cells in old animals? How do these patterns change with aging of the individual cell in the different microenvironment of young and old brain? In addition, since the LDM also allows dissecting neuropil, glia or vascular
Figure 1: VEGF activated signalling pathway effecting the cell cycle

Figure 2: In the schematic representation, the neurogenic hippocampal SGZ, a control and chronic stress situation is shown. New proliferating cells, which are surrounded by blood vessels and innervated by protrusions of astrocytes, occupy the SGZ. Chronic stress increases the number of p27Kip1- and decreases the number of Ki-67-expressing cells, which suggests a G1 arrest situation:
regions, e.g. close to newly generated cells, one can further wonder, whether the new cells that proliferate in the vicinity of the vasculature have other profiles or phenotypic potentials; as such can they become endothelial cells as well? Finally, have the adult-generated cells in the hippocampus the same identity as those proliferating in the SVZ?

Chronic stress was shown to affect the microvasculature, the expression of angiogenic factors, as well as cell cycle regulators. It is, however, still unknown via what signaling pathway the increased corticosteroid levels regulate new cell birth. Which receptors (e.g. GR or NMDA) are involved? Studies should be carried out to determine the temporal expression pattern of the GR, MR and NMDA receptor (subunits) in relation to development of the adult generated cell. This will help to answer the question when exactly in their development, new surviving cells become sensitive to glucocorticoid. As mentioned earlier, experiments involving the combination of BrdU immunocytochemistry, LDM and RNA amplification, could resolve these questions.

As proposed, glucocorticoids could regulate expression of other receptors, like Flk-1, and so (indirectly) influence new neuronal birth as well. To confirm our hypothesis that chronic stress-induced increases in corticosteroid levels through changes in VEGF and/or Flk-1 expression, would lead to a decreased proliferation, the following experiments could be carried out. Applications of corticosteroids alone should regulate VEGF and Flk-1 expression in vivo. Furthermore, it should be addressed whether decreased Flk-1 expression leads to a diminished PI3K/Akt pathway activation, and subsequently to changes in the expression levels of G1 cell cycle regulators.

Clearly, more study is needed to reveal regulating factors and biochemical pathways involved in the chronic stress- and age-induced change in the turnover of dentate granule cells. More knowledge might contribute to a better understanding of the onset and reversibility of hippocampal volume-related diseases, such as found in depression.