NEURAL STEM CELL BIOLOGY AND NEUROGENESIS IN MOUSE MODELS OF AGING AND ALZHEIMER'S DISEASE

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1. Summary

The etiology of Alzheimer's disease (AD) remains a great challenge for neurological research. Extensive investigations for almost one hundred years have led to profound insights of the pathological and molecular mechanisms that affect the AD brain, and there are several hypotheses about what causes the characteristic AD related dementia. The focus has fallen increasingly on the deposition of ß-amyloid (Aß) in the cortex and it is believed, that the generation and deposition of Aß is the leading cause of the disruptions observed in the AD brain. Aß has been shown to provoke neuron death, decreased synaptic plasticity, aberrant sprouting of growing axons, chronic inflammation and hyper-phosphorylation of tau.

In recent years, research on adult neurogenesis in the mammalian brain has led to surprising findings: new neurons are added daily to specific regions of the brain and growing evidence suggests that these new neurons play a critical role for learning and memory, mood and, to a limited amount, repair of damaged cortical areas. All of these functionalities of neurogenesis are affected in AD patients and the question must be raised, if in the AD brain, neurogenesis is directly disturbed. Defects in neural stem cell biology might significantly contribute to AD dementia and the examination of the relationship of AD lesions and neural stem cell biology might provide new insights for the understanding and treatment of AD.

Only recently has it become possible to investigate neural stem cell biology in the AD brain. This is partly because only recent findings revealed the function of adult neural stem cells, but also because animal models for AD have only been available for few years. However, most AD mouse models, which are genetically engineered for Aß deposition, do not develop significant amyloid plaques until past their median lifespan. This limits their availability and the specificity to Aß is reduced due to accompanying age effects.

In a first study of this thesis, age related changes of neurogenesis were investigated by monitoring the progressive stages of hippocampal neurogenesis: proliferation, survival and differentiation, in four different age groups of wild type C57BL/6J mice. Net-neurogenesis was rapidly reduced in adult compared to young mice, but remained stable at a low level in aged and senescent mice. This effect could be attributed mostly to an age related decline of proliferation with a concomitant increase of survival rates in aged mice. These results suggest that neurogenesis in aged mice remains as functional as in adult mice, although the plasticity of the neurogenic system appears to be reduced compared to young mice. The finding that a

reduced caloric diet, a treatment known to reduce age related defects, did not have an effect on neurogenesis confirmed the finding that neurogenesis is not impaired in aged mice compared to adult mice.

In a second study neurogenesis was studied in APP23 mice, a transgenic AD mouse model with progressive amyloid plaque load. Adult Aß pre-depositing and aged Aß high-depositing mice were investigated. Surprisingly, aged APP23 mice showed an increased number of new neurons in the hippocampus compared to age matching controls. For a closer investigation of the interaction of neural stem cells and Aß, we crossed mice expressing GFP under a stem cell specific promoter with a new AD mouse model with cortical plaque deposition in early adulthood. Stem cells were reduced in numbers, strongly attracted to Aß and morphologically altered. In addition, the population of more differentiated immature neurons appeared to be morphologically unaffected by Aß. These findings show that Aß affects neural stem cell biology concomitant with an up-regulation of neurogenesis.

Several reports claim that stem cells from the periphery are able to cross the blood brain barrier and are able trans-differentiate to the neuronal lineage. It has also been shown, that the number of cells immigrating from the periphery increases in AD mouse models. Thus, in a third study we investigated if stem cells from the peripheral hematopoietic system could participate in the repair or replacement of the damaged neuronal tissue. APP23 mice were deprived of their immune system by gamma irradiation and later reconstituted with genetically marked hematopoietic stem cells. We found a large number of these cells invading the brains of aged APP23 mice, but cell fate analysis revealed that these cells matured to macrophages or T-cells, but none differentiated towards the neuronal lineage. We conclude that the hematopoietic system is involved in the immune response in the brain, but we found no evidence that it is involved the in repair of the damaged network or in the alterations of neural stem cell biology described above.

In conclusion, the results of the present thesis provide evidence of a defective behavior of neural stem cells in the amyloidogenic brain, but also unveil the limitations in the function and ability of neural stem cells in the aged brain.

2. Introduction

2.1. Stem Cell Biology

Stem cell research has opened one of the most fascinating chapters in the history of biology. Traditionally belonging to the field of developmental biology, stem cells have become of increasing interest for biomedical research in more recent years. Tissue engineering, therapeutic cloning, transgenic animals and gene therapy are among the most discussed applications.

2.1.1. Background

Stem cells are undifferentiated cells that can divide indefinitely. They can either divide symmetrically, producing two identical daughter cells, or asymmetrically producing one identical and one more differentiated daughter cell [1]. The least differentiated stem cell type is the omnipotent or totipotent stem cell. It is found in early mammalian embryos (4 – 8 cell stage) and can form any cell type or tissue including the entire fetus and the placenta [2]. The inner cell mass of the blastocyst contains pluripotent stem cells [3]. These embryonic stem (ES) cells can be maintained in an undifferentiated state in culture, can differentiate in virtually any kind of cell type, but are not capable of forming an entire embryo. ES cells can be differentiated into multipotent stem cells, which are restricted to their specific lineage. These are hematopoietic, mesenchymal, endodermal or neural stem cells. These lineages follow specific differentiation patterns, with increasingly specialized cells. For example, neuronal stem cells can differentiate into glial-restricted progenitors, motor neuron progenitors, neural crest stem cells and neuron restricted progenitors. By applying the appropriate clues in a defined order it is possible to direct an ES cell towards a specific cell type [4]. However, the controversy over using human embryos as a source of these cells have led to intensified research to find ES-like cells in the adult, and to reverse the differentiation process to the pluripotent level [5,6]. Although some groups claim to have gained stem cells from skin, bone marrow or hair-follicle, these cells are often restricted to a distinct lineage (for these examples: mesenchymal, hematopoietic or neuronal, respectively). Thus, the only source of ES-like cells from the adult remains somatic cell nuclear transfer (referred to as therapeutic cloning).

2.1.2. Adult stem cells

For five decades hematopoietic stem cells have been the only adult stem cells known and investigated. Recently it was discovered, that numerous adult tissues contain stem cells. Normally these cells are involved in the homeostatic self-renewal and regenerative processes, but are occasionally activated for repair activity (for review see [7]). The lumen of the intestine for example is replaced about once a week. Blood and skin is renewed constantly, hair and nails constantly grow. All these systems depend on small local populations of stem cells, which are highly regulated. If the specific program of proliferation, migration and differentiation fails, the respective tissue will either become dysfunctional or cancerous. The arrangement of these proliferative systems is surprisingly conservative for the different tissues where adult stem cells are found. Generally a population of stem cells is harbored in a defined niche. The stem cells proliferate slowly, maintain the size of population and produce another population of transient amplifying precursor cells. These proliferate at a higher rate, and migrate towards the final destination of the specific mature cell type. This results in a differentiation gradient from the stem cell along to the migratory precursor cell to the fully differentiated cell (Fig. 1).

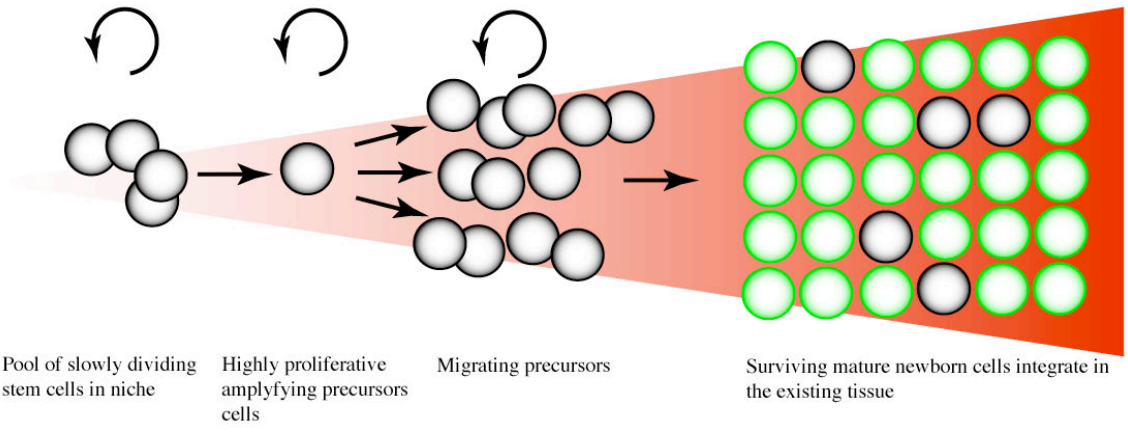

Fig. 1: *Schematic representation of adult stem cell differentiation. The differentiation process starts by asymmetric division of stem cells in a specific niche and continues during the migration towards the target tissue (green). The intensity of red background signifies the degree of differentiation.*

2.2. Adult Mammalian Neurogenesis

2.2.1. Neurogenesis in the adult brain

It has been a common understanding that in postnatal mammals no new neurons are added to the CNS and that any further changes can only be adopted through rewiring of the synaptic connections. In fact this dogma originates from early works at the end of the nineteenth century describing the developmental and adult brain of humans and other mammals [8-10]. These investigators found that the architecture of the brain appears to be fixed soon after birth. At the cellular level, neither mitotic nor developing neurons were observed. Although there were occasional reports on mitotic cells in the brain of adult mammals [11,12] there were no convincing methods to prove that these new cells would differentiate into neurons and be functionally integrated. Using autoradiography to track H^3 Thymidine, incorporated by proliferating cells during mitosis, Joseph Altman published a series of papers in the nineteen sixties showing evidence for adult neurogenesis in the adult rat and cat (for review see [13]). The scientific community did not recognize the significance of his results. Although Altman's experiments were repeated and combined with electron microscopy, and additional evidence for neurogenesis in songbirds was presented [14,15], the observation of neurogenesis in the adult brain did not get much attention. In the nineteen nineties new techniques emerged. Instead of tritiated Thymidine, Bromodeoxyuridine (BrdU) was used as a proliferation marker. BrdU can easily be labeled with immunohistochemical methods and investigated with brightfield and fluorescence microscopy. In addition specific antibodies against neuronal or glial markers were developed, providing easy methods to distinguish neurons from glia. With the help of these methods adult neurogenesis has been demonstrated to exist until senescence in numerous mammalian species including humans [16]. Finally, the neuronal behavior and integration into the network was confirmed by experiments testing long term potentiation (LTP), synapse formation and expression of immediate early genes after stimulation of the hippocampal network [17-20].

2.2.2. Neurogenic regions

In mammals three areas of ongoing neurogenesis have been identified: The subventricular zone (SVZ), the olfactory bulb (OB) and the granular cell layer (GCL) in the hippocampus (Fig. 2). Neural stem cells from other brain areas like the neocortex or the thalamus have been isolated *in vitro* by dissecting these areas and growing the precursors in a growth factor rich medium [21], but in vivo these cells appear to remain quiescent and have not been observed in an activated state. Three subtypes of cells have been identified in the SVZ [22]. 'Astrocyte-like' or 'Type B' stem cells cells are positive for glial fibrillary acid protein (GFAP) and most evidence suggests that this is the least differentiated neural stem cell. It has also been shown in embryonic tissue that radial glia cells can differentiate into neural precursor cells and one group was successful in re-differentiating neural precursor cells into radial glia [23]. Neural stem cells divide slowly in an asymmetric way, producing astrocyte-like cells and rapidly dividing precursor cells (type C). Type B cells and clusters of type C cells form channels within which the neuroblasts migrate along the sub ventricular zone. The migrating neuroblasts were named type A cells and differentiate from type C daughter cells. The SVZ provides the OB constantly with a stream of progenitor cells, through a path called the rostral migratory stream (Fig. 2).

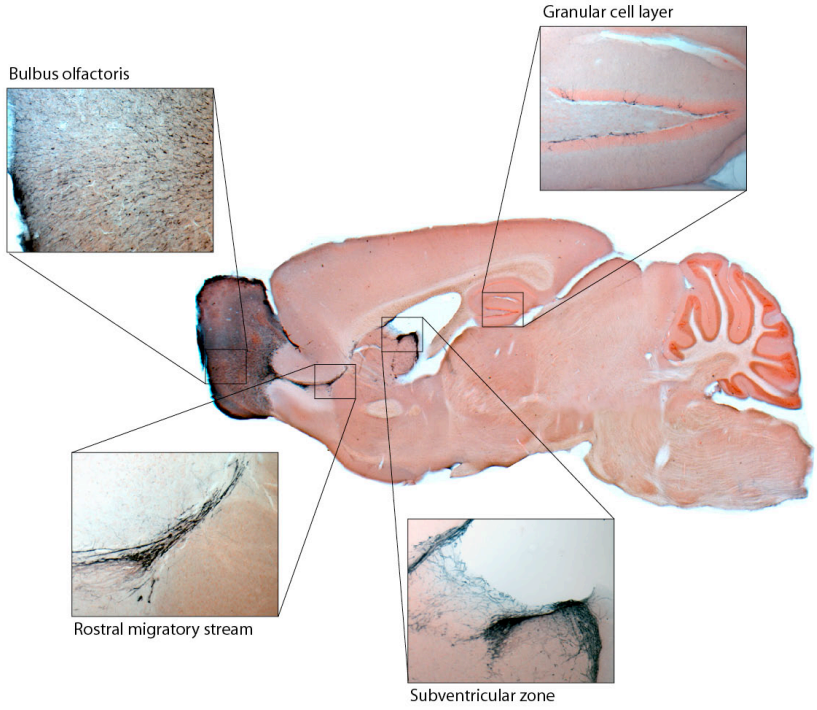

Fig. 2: *Sagittal section of a mouse brain showing the neurogenic regions by immunhistochemical staining of immature neurons (DCX).*

The migratory neuroblasts divide until they integrate as granular cells in the OB. Only very few mature new neurons originating from the SVZ invade the rest of the cortex. This was only found after distinct lesions and not in significant enough numbers to replace the cells lost in the incident [24,25]. In the dentate gyrus of the hippocampus there is a similar progression of differentiation, but within a different architecture (Fig. 3). Stem cells located in the subgranular layer produce cluster forming precursors. From there, neuroblasts migrate into the GCL were they extend dendrites into the molecular layer (ML) and send mossy fibers to the CA3 region (Fig. 4). Many more cells are generated than the number that ultimately survives. Following the principle 'use it or lose it' the survival depends on how sufficiently the new cells are activated by incoming neural signals. From the neural stem cell to the mature neuron the cells go through defined steps of division, differentiation, migration and maturation. Using specific markers it is possible to investigate the different phases of development separately (Fig. 3).

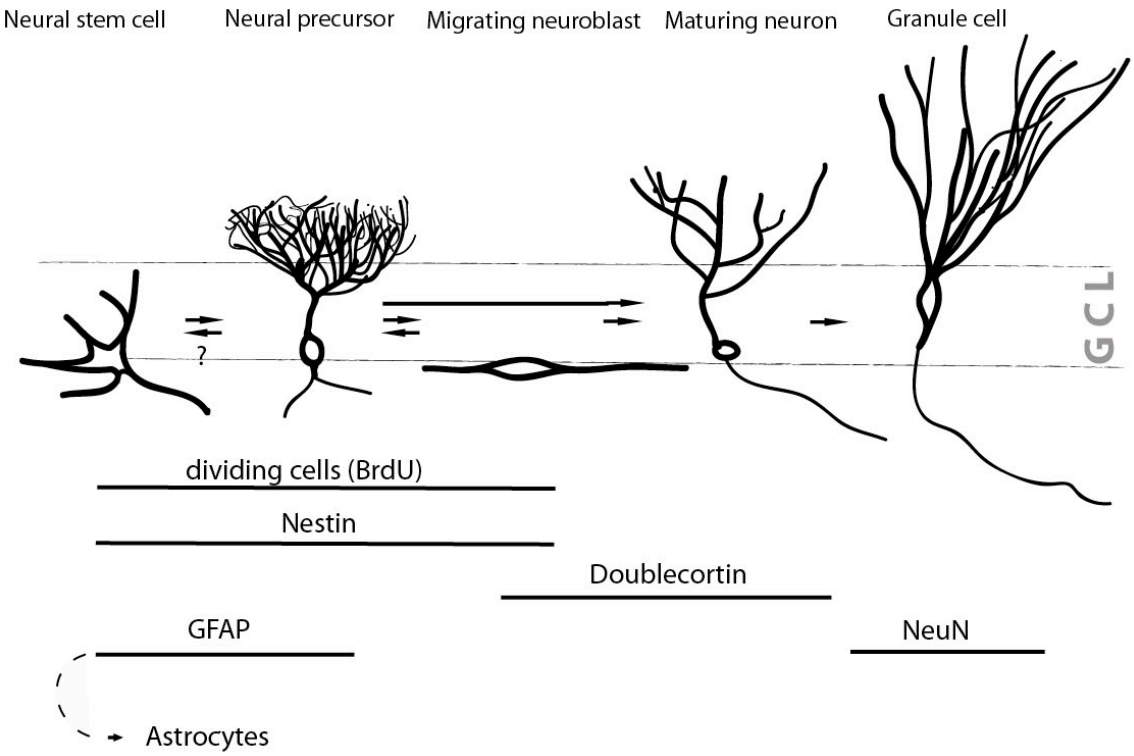

Fig. 3: *Differentiation of neural stem cells into granule cells in the GCL. It is still debated if precursor cells can dedifferentiate to stem cells. The different stages can be monitored by differential expression of specific proteins. Nestin and DCX is co-expressed for a very short time, and DCX and NeuN is not co-expressed.*

2.2.3. The neurogenic niche

Like all adult stem cells, neural stem cells are restricted to a particular niche. Interestingly, in the brain this is closely associated with the angiogenic niche [26]. Therefore, neural stem cells are always located close to the vasculature, and even react to the same stimuli as the endothelial stem cells do. This is even more astonishing, as neurogenic and endothelial stem cells are thought to arise from different lineages. However, a recent study reports that neural stem cells have been observed to differentiate into endothelial cells [27]. *In vitro* studies have shown that both endothelial cells and astrocytes can provide various factors to regulate neurogenesis in the vascular niche [28,29], and suppression of neurogenesis by irradiation was accompanied by a destruction of the niche [30].

2.2.4. Regulation of neurogenesis

Since the general recognition and acceptance of adult neurogenesis a large amount of studies have been conducted to investigate how neurogenesis is regulated. We know that Amphibians and Fish can regenerate neuronal tissue in the retina (for review see [31]) and findings from the investigations of retinal repair mechanisms have induced a multitude of experiments to study mammalian neurogenesis. The goal is to stimulate the neurogenic capabilities in the brain to induce an endogenous repair mechanism of the damaged mammalian CNS. These studies have revealed a complex regulative interaction of hormones, growth factors, cytokines and neurotransmitters conducted by environmental inputs like physical exercise, enriched environment or learning experiences.

Adrenal steroids may be one of the most important neurochemical regulators of neurogenesis. An increased plasma levels of corticosterone, as it appears as a reaction to applied stress, has negative effects on hippocampal neurogenesis [32-34]. Depleting the system from corticosterone by adrenalectomy reverses this effect. The influence of estrogen is more complex; ovariectomy decreases GCL precursor proliferation and if plasma levels are reconstituted, hippocampal neurogenesis is increased in the first hours. But if plasma levels stay high over prolonged time, an overall suppression of proliferation is observed [35,36]. Estrogen induced positive stimulation of proliferation is mediated by serotonin, downregulation of proliferation is mediated by adrenal steroids [36,37]. Estrogen as a positive regulator of neurogenesis is further modulated by interaction with IGF-1 [38].

IGF-1 itself is an important growth factor neurogenesis highly depends on. Plasma levels of IGF-1 are increased by exercise and this promotes major increases in GCL precursor proliferation [39]. This systemic IGF-1 is mostly synthesized in the liver and enters the brain through endothelial cells. It can be blocked by subcutaneous injection of an antibody against IGF-1, which blocks exercise induced increases of proliferation [40], or it can be simulated by peripheral injection of IGF-1 [41]. Like IGF-1, neurogenesis is stimulated by peripheral FGF-2 and VEGF, and similar experiments with subcutaneous injections of growth factors and antibodies were successfully performed [42,43]. However, even if growth factors from the periphery are blocked, a low level 'baseline neurogenesis' is observed in the GCL. This is due to local expression of these growth factors by astrocytes and neurons [43,44]. In addition, the regulation of baseline neurogenesis depends on other locally produced growth factors like EGF or BDNF and is further modulated by co-factors, such as Cystatin C and heparin, which facilitate growth factor binding to their receptors [45,46]. BDNF is a growth factor expressed in the hippocampus without further supply from the periphery. The up-regulation of BDNF expression is mediated through serotonin and noradrenalin, which also explains the high levels of BDNF and neurogenesis after antidepressant treatment [47,48].

Down regulation of neurogenesis has been reported from cytokines expressed as part of an inflammatory response. Induction of inflammation by LPS injection leads to an abrupt down regulation of neurogenesis. But this negative response to inflammation can be prevented with NSAIDs or with a blocking antibody against the cytokine IL-6 [49]. The suppressive effect of IL-6 on neurogenesis was also confirmed in a transgenic mouse engineered to over express IL-6 in the brain [50]. Another cytokine, CNTF promotes self renewal of stem cells and prevents the progeny from differentiating [51]. CNTF transmits its signal through gp130 and LIFR and promotes the expression of NOTCH, an inhibitor of neuronal differentiation [52,53].

The positive neurogenic influence of serotonin was already mentioned, but also the neurotransmitters dopamine and acetylcholine were found to directly stimulate neurogenesis [54,55]. It is therefore not surprising, that psychoactive drugs such as lithium and selective serotonin reuptake inhibitors as well as haloperidol (a dopamine antagonist) increase neurogenesis [56-58]. Deleterious drugs to neurogenesis are morphine, metamphetamine and alcohol [59-61].

The cortex is wired to the hippocampus through the entorhinal cortex (Fig. 4). This input connection has direct influence on neurogenesis; it has been shown that excitatory input from the entorhinal cortex through the NMDA receptor suppresses neurogenesis, while inhibition of this pathway through the blockage of NMDA receptors or the removal of input enhance it [62].

Finally, there are indications that neurogenesis can react to CNS insults. Brain injury through cerebral ischemia, seizure or neurotoxic lesion all lead to an increase of neurogenesis [63-65]. However, in all these experiments the damage was far greater than the response and the ability of neurogenesis to repair the damaged tissue seems very limited

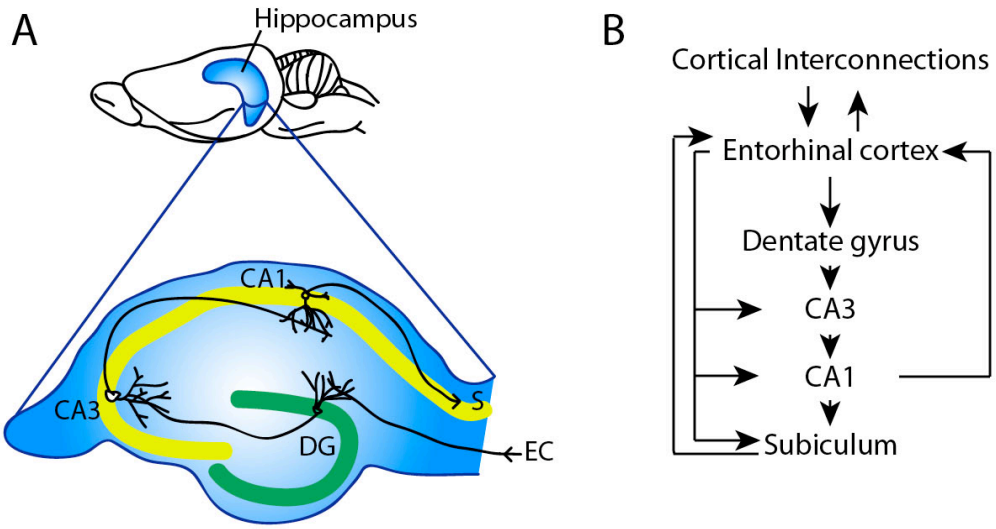

Fig. 4: *Organization of the hippocampal formation within the mouse brain. A: Schematic representation of the location of the hippocampus within the mouse brain. Enlarged is a coronal section through the hippocampus highlighting the basic circuitry B: Circuit diagram of the basic information flow in the hippocampus. EC: entorhinal cortex: DG: dentate gurus; S: subiculum; green: granular cell layer; yellow: CA3 and CA1.*

2.2.5. Neurogenesis and memory

The hippocampus is a brain structure critical for the formation and processing of memories. In humans it is involved in declarative memory, so called because of the involved synthesis of episodic representations and semantic knowledge. The hippocampus is especially important for the formation of memories in new situations that require an inference on the basis of linking distinct experiences in memory. Patients with a damaged hippocampus suffer from global anterograde amnesia, but normal cognitive function, immediate memory, remote childhood memories, and general world knowledge acquired early in life are not impaired [66-68]. In laboratory animals this is tested in hippocampus dependent spatial or temporal learning tasks [69,70]. The circuitry of the neuronal processing has been extensively studied and consists of a unidirectional message flow through the hippocampal subfields as well as direct inputs to all those subfields (Fig. 4). Studies on specific firing patterns of single neurons within this circuitry have revealed, that single neurons in the hippocampus are directly linked to spatial, and temporal memories, e.g. when a mouse walks through a maze, the same cell will fire whenever the mouse passes through a specific location. These cells where named place cells and the corresponding location the place field [71].

Recently published experiments suggest, that field representations within the entorhinal cortex are context-independent, while in the hippocampus they are context-dependent [72,73]. This means that the spatial organization of an environment is represented in the entorhinal cortex, but this is further processed in the hippocampal subfields in a temporal, social, emotional, etc context. These results seem to confirm previous findings that simple memories may be formed with a damaged hippocampus but inferential computing is impaired. In a classic transitive inference task animals learn a series of overlapping pair wise discrimination problems (A > B, B > C, C > D, D > E). Control animals can learn that B > D, animals with hippocampal damage are not able to do this transition [74]. Similarly, in a Morris water maze test animals with a damaged hippocampus are only slightly impaired to learn the location of the submerged escape platform when the starting point within the maze remains constant, but are unable to learn when the starting point is different from the previously learned one [75].

It is striking that neurogenesis occurs in the GCL of the dentate gyrus, a structure so central in the hippocampal memory system. However, only recently first links of memory and hippocampal neurogenesis could be established. Voluntary running and an enriched environment have both been shown to increase neurogenesis, this has been shown to be accompanied by improve performance in memory tasks [17,76,77]. When an anti-mitotic agent was

applied during environmental enrichment, memory did not improve, as neurogenesis did not increase [77]. Similarly, when VEGF, a growth factor through which an enriched environment increases neurogenesis is blocked, the beneficial effects of an enriched environment on neurogenesis and memory are lost [76]. Also blockage of neurogenesis can lead to substantial memory impairments, this has been shown by using a toxin for proliferating cells [78] and irradiation [79,80]. Learning abilities can be trained and therefore it is not surprising, that learning increases neurogenesis [81]. Plasticity seems to be especially high in newborn neurons in the GCL [82,83] and LTP in the dentate gyrus is increased after running [17]. Other studies on how neurogenesis is working in the system of memory formation suggests that, new neurons are important for memory clearance, and an impaired neurogenesis inhibits this clearance [84,85]. Computational models of the hippocampal learning structure predict, that increasing the number of replaceable neurons in the dentate gyrus would increase the capacity for learning new information and forgetting old information [86]. Although the involvement of adult neurogenesis in hippocampal learning and memory has only been studied for a brief period of time, the multitude of evidence suggests that it is indeed involved in this process, rather than being a residual of an evolutionary outdated repair ability.

2.2.6. Neurogenesis in the aging hippocampus

In the aging brain, neurogenesis remains functional, but the number of newly generated neurons declines gradually [87,88]. Is this the reason for age related impairments in learning capacity and if so what is the reason for this and why can't it been prevented? It was found that adult stem cells, in a variety of tissues, proliferate more slowly and become less productive in the aged. This may be because adult stem cells do have limits in their replication capabilities. As a result of the biochemistry of DNA replication 20-50 base pairs at the end of the chromosomes remain un-copied. To prevent damage to the genetic information, chromosomes have an extended DNA tail called the telomere. In ES cells the telomere is kept at a constant size by telomerase activity, but in adult stem cells telomerase activity is limited and they eventually reach senescence by telomere shortening [89]. A slower rate of proliferation might simply prevent these cells from becoming dysfunctional or cancerous. This hypothesis is underscored by a recent study finding that adult stem cells with reduced telomere length and decreased telomerase activity display a suppressed level of mitotic and migratory activity [90].

This lower level of neurogenesis starts in middle aged animals and is controlled by a reduced expression of the growth factors neurogenesis depends on. Indeed the down regulation of growth factors follows the same pattern as the down regulation of neurogenesis [87,91]. When factors like IGF, FGF or EGF are administered, neurogenesis can be elevated, but only to about 20% of the level seen in juveniles [46,92]. In fact, it has been postulated, that growth factor levels can not only influence neurogenesis and cognition, but also have direct influence on an animals lifespan [93].

2.3. Alzheimer's Disease

One hundred years ago, 1906, the German psychiatrist Alois Alzheimer (1864-1915) presented the case of a 54 years old woman who suffered from progressive dementia. He described the clinical and pathological symptoms for the first time, now known as Alzheimer's disease [94].

2.3.1. Clinical symptoms

AD is a progressive dementia with memory loss as the major clinical manifestation. Impairment of short-term memory is often prominent, but remote memory loss also appears to be affected over time. Other important features of AD are an altered behavior including paranoia, delusions, depression, impairments of attention, perception, reasoning and comportment, and a progressive decline in language function [95,96]. Normal aging does not necessarily imply that memory function remains intact [97]. However, the onset of AD related dementia is preceded by a transitional preclinical period of many years during which relatively isolated memory difficulties exceed those expected on the basis of normal aging without becoming severe enough to interfere with daily living activities [98,99]. The capacity for independent living wanes when mental functions and activities of daily living become progressively impaired [100]. However, motor functions remain initially intact but deteriorate in the final phase of the disease, leading to symptoms resembling motor disorders such as parkinsonism [101].

2.3.2. Pathophysiology

The main neuropathologic findings in the AD brain are amyloid plaques, neurofibrillary tangles and a degeneration and loss of synapses and neurons. Amyloid plaques are multicellular lesions containing extracellular deposits of amyloid-β protein (Aβ) consisting of amyloid fibrils (7-10 nm) intermixed with non-fibrillar forms of the Aβ peptide. The plaques are surrounded by dystrophic neurites, consisting of both axonal terminals and dendrites. Plaques often contain activated microglial cells situated near the amyloid core, reactive

astrocytes are found more at the periphery of the plaque [102,103]. Another form of Aß-deposition appears as diffuse or pre-amyloid plaques, where Aβ occurs in a non-fibrillar, less dense and amorphous form in the neuropil [104,105]. In brain regions with less significance to AD, like the cerebellum, the striatum and the thalamus, most Aβ deposits are diffuse with little or no glial and neuritic reactions [104,106]. Extracellular Aβ deposits are also found in the walls of cerebral and leptomeningeal blood vessels. This cerebral amyloid angiopathy (CAA) can be found in absence of AD. However, at the age of sixty and over CAA is detected in 30 % of the general population, compared to 80% in AD patients [107,108]. A growing amount of scientists support a theory called 'the amyloid cascade hypothesis', which states that the accumulation and deposition of Aβ in the brain is the primary influence driving AD pathogenesis and leading to neuronal dysfunction [109].

Neurofibrillary tangles are intra neuronal paired helical filaments composed of hyperphosphorylated isoforms of the microtubule-associated protein tau protein, which in normal cells is essential for axonal growth and development [110]. Neurofibrillary tangles generally occur in large numbers in the AD brain, particularly in neurons located in the entorhinal cortex, hippocampus, amygdala and frontal association cortex leading to cell death. The tau protein normally enhances the polymerization of tubulin into microtubules and stabilizes these organelles in neurons. But when it becomes excessively phosphorylated its affinity to microtubules is reduced [111]. Neurofibrillary tangles form in some cell bodies whose axons terminate in regions containing Aß [112]. It has been shown in mouse models that in these cases Aß leads to tangle formation, confirming the amyloid cascade hypothesis [113,114]. The AD brain is characterized by extensive neuronal and synapse degeneration found throughout a similar pattern as the neurofibrillary tangles [115-118]. In addition neuronal loss in the nuclei of major neurotransmitter pathways is prominent. The death of cholinergic neurons in the nucleus basalis of Meynert, serotonergic neurons in the median Raphe nuclei or adrenergic neurons in the locus coeruleus leads to a deficit in acetylcholine, serotonin or norepinephrine, respectively [119-121].

2.3.3. Epidemiology and genetics of Alzheimer's disease

Over 50% of the typical, late-onset cases of dementia are diagnosed as AD, and it is therefore the most common form of dementia in the elderly. The number of patients worldwide is estimated at approximately 20 million and is expected to keep growing as the current

population ages. At the age of 65 the risk of developing AD is estimated to be not more than 2%, but raises to 10-15% at the age of 80 [122-124]. Cognitive impairments start during the seventh decade in most individuals with AD, but may appear earlier, particularly in familial cases [125]. However, most AD cases are sporadic and occur with no clearly defined etiology. Familial AD (FAD) is autosomal dominant inherited, and except for the early age of onset (<60 years), clinically and pathologically indistinguishable from sporadic AD [126,127]. The proportion of Alzheimer cases that are genetically based is estimated between 5% and 15% [128,129].

The observation of an increased incidence of AD among older Down syndrome patients lead to a closer investigation of chromosome 21, and finally to the first genetic linkage discovery between a locus on chromosome 21q and FAD [130,131]. Screening of the sequenced APP gene for mutations led to the discovery of several missense mutations [132-134]. Most FAD cases do not have a mutation on APP, but a second locus on chromosome 14 was identified, and the gene was later isolated and named presenilin 1 (PS1) [135,136]. PS2 was found based on its homology to PS1 and mapped on chromosome 1 [137]. Other genetic risk factors have been identified that increase the risk of developing sporadic AD. These include the ε4 allele of the apolipoprotein E (APOE) gene, the proteinase inhibitor α2-macroglobulin, low density lipoprotein receptor-related protein (LRP), angiotensin converting enzyme (ACE) or insulin degrading enzyme (IDE) [138-142]. All of these risk factors of sporadic AD and the autosomal dominant mutations of FAD are involved in secretion, clearance or degradation of Aß protein, the primary component of β-amyloid plaques in the brain [143,144].

2.3.4. APP processing and Aβ

APP is a type-I integral transmembrane glycoprotein that is ubiquitously expressed, but the physiological function of APP in the brain remains unclear. It has been proposed to have functions in transmembrane signal transduction, calcium regulation, cell proliferation, cell-adhesion, neurite outgrowth and synaptogenesis [145-150]. This is reflected in APP knock out mice, which show cognitive deficits and reduced synaptic plasticity [151,152]. APP is processed by α-, β- and γ-secretase (Fig. 5). In the default, non amyloidogenic cleavage pathway, α-secretase cleaves within the Aβ region to produce the soluble α-APP (sAPP) and the 83 amino acid COOH-terminal fragment C83 [153,154]. Alternatively, processing with ß-secretase produces a 99 amino acid C-terminal (C99), which includes Aß [155] and a longer form of sAPP.

Both the C83 and C99 fragments remain anchored in the membrane where they are processed by γ-secretase to produce p3 from C83 or Aβ from C99 (Fig. 5). γ-Secretase cleavage occurs within the membrane, but is not sequence specific resulting in Aß peptides of varying length. The most common form is Aß40, but there is always a percentage of Aß42, which is less soluble and more likely to form toxic aggregates [156]. Extensive research has revealed that γ-secretase is a multi-protein high molecular weight complex and consists of presenilin-1 (PS1), presenilin-2 (PS2), nicastrin (Nct), anterior pharynx-defective phenotype (APH-2) and PS-enhancer (PEN-2) all of which are essential for its function [157-160].

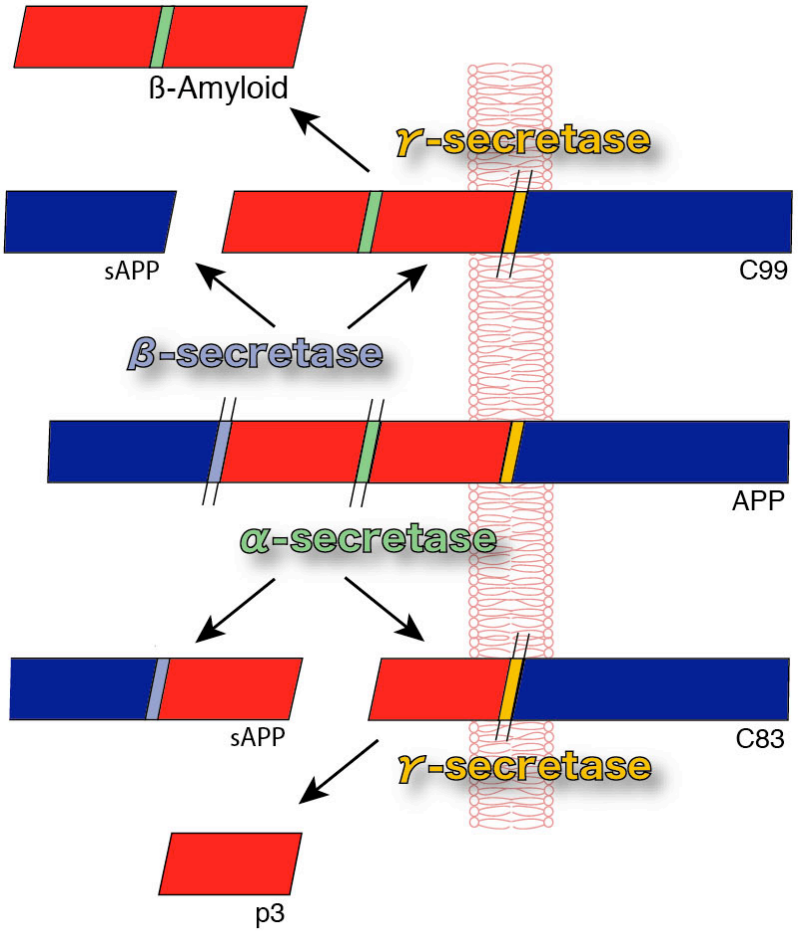

Fig. 5: *Proteolytic processing of APP by α-, β- and γ-secretase and the resulting protein fragments. Aβ can only be produced through the β-secretase pathway, while processing with α- secretase prevents Aβ formation. Mutations on APP that cause familial AD are localized within the Aβ domain near the cleavage sites of the secretases, here marked in green, grey and yellow.*

Missense mutations on the APP gene that have been linked to FAD are all located at the cleavage sites of α-, β- and γ-secretase. The different mutations have been named after the geographic area the FAD inheriting family lived in, e.g. Swedish, Dutch or London [132,134,161]. FAD mutations at the α- or β- secretase cleavage site favor ß-secretase cleavage, and therefore increase Aß production over p3 production [162]. Mutations at the γ-site promote the production of Aβ42 over Aß40 [163]. Both increased Aß production and a higher rate of Aß42 are responsible for increased deposition of Aß plaques.

2.3.5. Mouse models of Alzheimer's disease

The identification and sequencing of APP and presenilin opened the door for the engineering of transgenic mouse models to study pathogenic mechanisms of AD. The first successful mouse models over-expressed human APP with an FAD linked mutation in the brain. These mice exhibit Aß plaques, neuron loss, dystrophic neurites, inflammatory responses, learning impairments and deficits in synaptic transmission and/or long-term potentiation [164-169] (Fig. 6). The severity and onset time of AD like pathology is influenced by the level of transgene expression and the specific mutation. Other models combine the insertion of mutated APP with an insertion of mutated PS1, or insert APP with two mutation sites to have an even stronger phenotype [170,171]. None of these Aß depositing mouse models develop tau filaments. This has only been shown in mice expressing mutant human tau protein or by combining Aß deposition with a mutation in the tau gene [113,114,172].

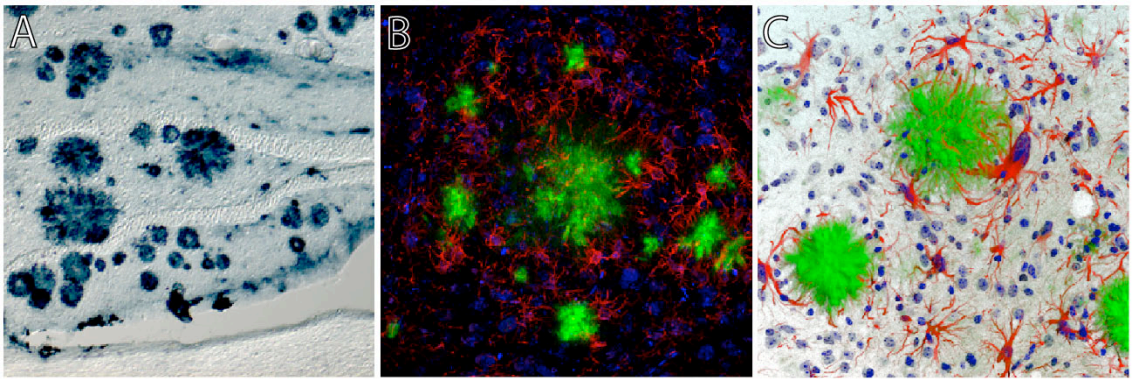

Fig. 6: *Amyloid plaques and glial reaction in transgenic APP23 mice. A: Immuno staining of Aß reveals large deposits in the hippocampus of a 24 month-old APP23 mouse. B: Projection of a stack of multiple confocal images demonstrating the distribution and morphology of microglia (red) around Aß plaques (green). Cell nuclei are stained blue. C: Three dimensional reconstruction of astrogliosis in APP23 mice; green: Aß; red: astroglia; blue: cell nuclei.*

2.4. Synthesis: Neurogenesis in the AD Brain

Given the complexity of the regulatory system of neurogenesis and the profound changes that are found in the AD brain, an altered behavior of neural stem and progenitor cells can be assumed. It is tempting to hypothesize that memory loss of AD patients is related to a disturbed neurogenesis in the hippocampus, and therefore a potential target for therapy. Even if neurogenesis is not directly impaired in AD, compensation for neuron loss in the hippocampus by controlled stimulation of endogenous neurogenesis might restore some of the lost hippocampal function. However, few studies about the influence of AD on neurogenesis have been published. Only one study investigated neurogenesis in AD patients [173]. The expression of markers of neurogenesis like DCX (a microtubuli binding protein in neuroblasts), PSA-NCAM (a plasma membrane glycoprotein expressed by neural progenitors) or NeuroD (a basic Helix-loop-helix protein expressed during neuronal differentiation) was measured in western blots from post mortem hippocampal tissue. Significant increases of these ‚neurogenic' proteins in AD brain led to the conclusion that neurogenesis is increased. However, other brain insults like ischemia or seizure, or brain damage through neurotoxic compounds tend to provoke an increase of neurogenesis or at least an increased proliferation rate of neural stem cells [63-65].

2.4.1. Influence of APP and Aß on neurogenesis

Neurogenesis has been investigated in AD mouse models, but the mouse models and methods differ and the results are controversial. In two different mouse lines expressing human APP with the Swedish mutation up to the age of 12 months, lower levels of precursor proliferation and survival compared to wild type littermates are reported, but only in one of these studies impairment in proliferation was reported at 3 months of age, therefore before Aß plaques are found [174,175]. In another mouse line expressing human APP with the Swedish and the Indiana mutation, proliferation is lower at 3 and 12 months [176]. Most of these mouse models over express APP, but often it is not known to what degree. It has been shown that sAPP can act as a growth factor by binding to neural precursor cells and blockage of sAPP can decrease neurogenesis in vivo [150]. In addition, APP seems to have an anti apoptotic effect on stressed

neurons. However, if APP over expression had an influence on neurogenesis one would expect an increased neurogenesis, especially in younger, Aß pre-depositing mice. In vitro experiments confronting precursor cultures with Aß are equally controversial: One study found Aß42 to be toxic to human neural precursor cells and inhibit migration [174], another study found that Aß42 provokes increased differentiation towards the neuronal cell fate, but had no effect on proliferation or apoptosis [177].

Neurogenesis depends on an angiogenic niche and is located in close proximity to the vasculature [26]. Disturbance of the niche may interfere with stem cell behavior. Aß deposition in the vasculature could induce such a disturbance, either through the loss of vascular cells [178], which provide growth factors, or through an accumulation of activated microglia, which influence neurogenesis through the expression of cytokines. Indeed, deficits in the brain vasculature of an AD mouse model increases with progressive age and Aß deposition [179]. A similar disturbance of the neurogenic/angiogenic niche by microglia activation and/or spatial disruption has been observed in irradiated mice [30,49]. There are only few mouse models for CAA available [178], and the impact on neurogenesis has not been investigated.

2.4.2. Angiogenesis in the AD brain

Neurogenesis in the vascular niche is regulated by the same growth factors angiogenesis depends on. Vascular injury, hypoxia and inflammation promote angiogenesis, which is stimulated by growth factors like VEGF and FGF-2, but also inflammatory factors like histamine. In AD patients increased CSF levels of VEGF were measured [180], which arguable might lead to the increased neurogenesis found in AD patients. In vitro, Aß stimulates angiogenesis via an FGF-2 dependent mechanism [181]. Interestingly, anti-histamines, lipid lowering statins, and some non-steroidal anti-inflammatory drugs, which all suppress angiogenic activity, reduce the risk of AD. Some authors argue that this injury induced angiogenesis results in increased secretion of Aß by these activated endothelial cells, and therefore plays an important role in Aß deposition [182]. Considering that neurogenesis too is altered by vascular injury and angiogenic reactions, its importance for AD might be wider than previously expected. The direct effect of anti-angiogenic drugs on neurogenesis has not been directly tested. However, the possibility that these drugs also lower neurogenesis has to be considered, and if neurogenesis in AD is abnormally high, a drug-induced down regulation might be part of the beneficial effect of these drugs on AD patients. Lipid lowering statins

reduce the risk of AD and were also found to inhibit angiogenesis [183]. Cdk-5, a kinase involved in cell cycle regulation is inhibited by statins and over expression of Cdk-5 has been shown to induce tau phosphorylation and cell death in neurons. In contrast it increases proliferation in undifferentiated precursor cells [184]. Thus angiostatin might block neurogenesis through this mechanism. Histamine promotes angiogenesis in a mechanism parallel to VEGF [185], but its influence on neurogenesis is not known. The inflammatory response involves factors for positive and negative regulation of neurogenesis and is discussed in the next paragraph.

Interestingly, vascular density in the brains of AD patients or AD mouse models was found to be either unaltered or lower than in age matching controls [179,186,187]. In the mouse model angiogenesis was found to be impaired despite an up regulation of angiogenic factors and before Aß deposition was detected [188]. Therefore, neurogenesis might be stimulated by a disturbed vascular biology/angiogenesis including the secretion of angiogenic proliferative.

2.4.3. Inflammatory factors and neurogenesis

AD is accompanied by a profound inflammatory response. Numerous inflammatory and pro-inflammatory factors such as complement, cytokines and acute phase reactants are up regulated in the vicinity of Aß and accumulate in the Aß plaque [189]. The neurogenic reaction to local inflammation has only been studied in few cases, and the abundance and complexity of actions of the released paracrine factors make it impossible to attribute the neuro-inflammatory response as exclusively benign or detrimental to neurogenesis. Pro-inflammatory cytokines are ligands of receptors on astrocytes and microglia, triggering the expression of a cascade of inflammatory cytokines in the CNS. TNF-α induces expression of IL-1 and IL-6, while IL-1 promotes growth factor and neurotrophin expression, IL-6 blocks IL-1 and TNF-α transcription [190]. IL-6 has also been studied in its effect on neurogenesis; in transgenic mice with chronic expression of IL-6 in astrocytes, neurogenesis is strongly reduced [50]. Systemic injection of LPS induces a neuroinflammatory response resulting in a decrease of neurogenesis, but administration of anti-inflammatory drugs or inhibition of microglia activation prevents LPS induced down regulation of neurogenesis [49,191]. An *in vitro* experiment co-culturing neural stem cells with activated microglia found the same result, and isolated exposure of neural stem cells to selected cytokines resulted in reduced neurogenesis by IL-6 and TNF-α, but not by INF-γ or IL-1ß [49]. Further evidence suggests that TNF-α acts

upstream of IL-6 and that IL-6 can activate NOTCH expression through the IL-6R/gp130 receptor complex [49]. Gp130 signaling and NOTCH expression has been shown to be important in maintaining neural stem cells in an undifferentiated state [53]. Alternatively, IL-6 expression concomitant to an inflammatory response is able to activate hormone production of the HPA-axis and increase corticosterone levels in the plasma [192], leading to higher corticosteroid levels in AD patients [193]. Anti-inflammatory drugs were found to lower the risk of AD [194,195]. Most of these drugs block the pathway leading to the expression of IL-6 and it could be argued, that a lower risk of AD is related to better functioning neurogenesis.

IL-1ß is another pro-inflammatory cytokine that is up-regulated in the AD brain [196]. It promotes the inflammatory reaction by increasing expression of IL-1, IL-6 and TNF-α and proliferation of astrocytes and microglia [197]. However, it is also involved in brain repair by stimulating IGF-1 and helps to initiate angiogenesis [198,199]. It might therefore protect neural stem cells from other detrimental effects of inflammation and even promote proliferation.

TNF-α is the most pivotal of the cytokines, it may either be neurotoxic or neurotrophic, but it is also central in promoting the inflammatory response [200]. It is highly expressed in the embryonic brain and *in vitro* it induces proliferation in primary astrocytes and glioma cells [201] or drives neuronal differentiation in neuroblastoma cells [202] and stimulates synthesis of NGF and BDNF [203]. However, high concentrations of TNF-α are toxic to neurons *in vitro* [204], it induces the release of NO and glutamate [204,205] and disturbs electrophysiological membrane properties [206]. NO has been identified to promote neuronal differentiation of neuronal precursor cells, and might be the mediator through which TNF-α interacts with neurogenesis [207]. The cytokines described here trigger the release of other cytokines from glia cells and neurons. Besides IL-6, Il-1 and TNF-α, IL-2, IL-3, IL-4, IL-8, IL-10, IL-12, IFN-α, IFN-ß, IFN-γ, TNF-ß and TGF-ß have been found in the AD brain [196]. Only few of the detailed interactions and regulative mechanisms of this inflammatory reaction and neurogenesis have been investigated. Despite first results that inflammation has an inhibiting effect on neurogenesis [49], more detailed experiments about the impact of Aß induced inflammation on neurogenesis are needed to understand the interactions of these brain processes.

2.4.4. Loss of neurotransmitter affects neurogenesis

AD is accompanied by a striking neuron loss within some major neurotransmitter systems. Most prominent is the decline of ACh neurons in the nucleus basalis of Meynert, but there are

also deficits of noradrenergic, serotonergic and dopaminergic neurons. All of these neurotransmitter systems have been related to a positive control of neurogenesis and their loss is linked to memory deficits [54,55,208]. Cholinesterase inhibitors are among the few drugs countering AD symptoms and known to ameliorate the AD related memory deficits. The loss of cholinergic neurons has been shown to have a negative effect on neural precursor proliferation, and the accompanying memory deficits may be related to the resulting decline of neurogenesis [55].

The loss of dopaminergic neurons is a prominent feature of Parkinson's disease, but it is also found in AD and is related to memory impairments [209]. Moreover, Dopamine is an important regulator of neurogenesis during ontogenesis and it is therefore no surprise that in the adult neurogenesis is dependent on dopamine as well [54].

Serotonin is not only involved in memory but also mostly known for an involvement in mood disorders such as depression. The loss of serotonergic input may lead to the mood disorders associated with AD [210]. Several links between depression and a low level of neurogenesis have been made, but it is unclear to date, which is the cause and which the effect. Neurogenesis and memory function is generally enhanced by selective serotonin uptake inhibitors [58]. And functional imaging and behavioral tests show impaired hippocampal function and decreased neurogenesis in depressed patients [211]. Taken together, the input of neurotransmitters is of great importance for adult neurogenesis, memory and mood. The loss of these neurons and/or disruption of the balance of neurotransmitter-release is likely to affect neurogenesis.

2.4.5. Influence of presenilin on stem cell biology

Although mutated presenilin (PS) is only accountable for a small percentage of AD cases, the behavior of non mutated PS might be altered by other factors involved in AD. PS is involved in the gamma secretase cleavage of APP processing [212]. Interestingly presenilin is involved in the processing of both APP and the notch receptor, producing an intracellular domain of the receptor that influences gene expression in the nucleus of the cell. In the ectodermal tissue of developing mesozoans binding and processing of Notch results in a suppression of the neuronal phenotype and is essential in mammals for the maintenance of the stem cell population [52]. Indeed, PS knock out mice are perinataly lethal and conditional ablation of PS in the adult forebrain or mutated PS results in impairments in neurogenesis and memory [213-215]. In contrast, over expression of PS results in increased neurogenesis [216].

2.4.6. Aß induces sprouting on growing axons

Close vicinity of axons to Aß plaques promotes axonal growth and the formation of distrophic boutons [217,218]. These abnormally sprouting axons form distrophic synaptic boutons around Aß deposits in the neuropil or vasculature, even if that particular cell does normally not innervate this area. Neurogenesis produces maturing neurons in the GCL that grow axons to the CA3 area and dendrites into the ML. Because the hippocampus is strongly burdened by Aß plaques in the AD brain, there is a strong possibility, that these growing processes are negatively influenced or misguided by the plaques close by. This would strongly impair the function of neurogenesis by inhibiting the correct connection to the existing network.

2.4.7. Hematopoietic stem cells in the AD brain

Hematopoietic stem cells from the bone marrow can be released to the blood stream and participate in angiogenesis [219]. Moreover if isolated and transplanted into blastocysts these cells are reported of being able to differentiate into mesenchymal, endodermal and neuroectodermal like cells [220]. Due to the chronic inflammation observed in AD, the blood brain barrier is weekend and the amount of peripheral cells entering the brain parenchyma is increased. Aß induced lesions and neuron loss raise the question if stem cells from the periphery are able to participate in neurogenic repair mechanisms by trans-differentiating to the neuronal lineage. Hematopoietic stem cells can be genetically marked and injected into mice deprived of their hematopoietic stem cell population by irradiation. The injected stem cells repopulate the bone marrow and reconstitute the blood system [221]. Invasion of the brain parenchyma by descendants of the hematopoietic lineage can be monitored effectively this way. Most of the cells passing the blood brain barrier are macrophages and seem to form an "exogenous" microglia population. However, there is some controversy if these cells can trans-differentiate to the neuronal lineage. Trans-differentiation of bone marrow derived cells into neurons, detected by co-expression with neuronal markers, has been observed in a variety of neuronal tissues; the neocortex, cerebellum, spinal chord and dorsal ganglia [222-224]. However, the ability for trans-differentiation of hematopoetic cells has been challenged by *in vivo* and *in vitro* experiments demonstrating that bone marrow derived cells spontaneously fuse with endogenous neurons [225,226]. Only one of the groups reporting neuronal trans-differentiation included controls that exclude cell fusion and concluded that a small number of

cells indeed turn into neurons [222]. It is also unknown, why hematopoietic cells fuse with neurons and what the function of such a fused cell is. However, if peripheral hematopoietic stem cells participate in neuronal repair mechanisms, this would have broad implications, not only for the ethiology and therapy of AD, but would also drastically alter our understanding of how the brain and the peripheral organs interact.

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3. Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice

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3.1. Abstract

Age-related changes in neurogenesis and its modulation by caloric restriction (CR) were studied in C57BL/6 mice. To this end, bromodeoxyuridine (BrdU) labeling was used to assess neuronal and glial precursor proliferation and survival in the granular cell layer (GCL) and the hilus of the dentate gyrus of 2-, 12-, 18-, and 24-month-old mice. For both regions, we found an age-dependent decrease in proliferation but not in survival of newborn cells. Interestingly, the reduction in proliferation occurred between 2 and 18 months of age with no additional decline between 18- and 24-month-old mice. Phenotyping of the newborn cells revealed a decrease in the neuron fraction in the GCL between 2 and 12 months of age but not thereafter. The majority of BrdU cells in the hilus colocalized with astrocytic but none with neuronal markers. CR from 3 to 11 months of age had no effect on neurogenesis in the GCL, but had a survival-promoting effect on newly generated glial cells in the hilus of the dentate gyrus. In conclusion, C57BL/6 mice reveal a substantial reduction in neurogenesis in the dentate gyrus until late adulthood with no further decline with aging. Long-term CR does not counteract this age-related decline in neurogenesis but promotes survival of hilar glial cells.

3.2. Introduction

The vast majority of cells in the CNS are generated during the embryonic and early postnatal period, but new neurons are continuously added in selected regions of the mammalian brain [1-5]. Neurogenesis has been described in the subgranular layer of the dentate gyrus [3,6] and the subventricular zone of the lateral ventricle [7-9]. Newborn cells in the subgranular layer migrate into the granule cell layer (GCL), where a portion differentiates into neurons, which then extend axons to the CA3 region [10-12]. Although the functional significance of this ongoing neurogenesis in the dentate gyrus remains to be fully established, evidence has been provided that newly produced neurons play an important role in learning and memory [13-15]. Because a decline of neurogenesis in the dentate gyrus has been reported with aging [5,16], a relationship between the age-related decline in neurogenesis and age-dependent cognitive impairments may be suggested [17].

In numerous rodent studies, caloric restriction (CR) has been shown to extend life span, postpone the onset of age-related changes including brain aging, and maintain physiological function at more youthful levels [18,19]. Thus, it is tempting to speculate that CR may also postpone the age-related decline in neurogenesis. Indeed, short-term CR has been reported to increase neurogenesis in the dentate gyrus of young mice and rats [20,21].

The aim of the present study was to investigate the effects of aging and CR on neurogenesis in the dentate gyrus of C57BL/6 mice. C57BL/6 mice were selected because this mouse strain has become the most common background for genetically engineered mouse models of age-related neurodegenerative diseases [22,23]. Moreover, we have previously assessed age-related changes in granule cell number in the dentate gyrus of this mouse strain [24] and thus have provided a basis for the interpretation of age-related changes in neurogenesis in the dentate gyrus of C57BL/6 mice.

3.3. Materials and Methods

3.3.1. Animals

To study age-related changes in neurogenesis, 2-month-old (n = 11), 12-month-old (n = 13), 18-month-old (n = 11), and 24-month-old mice (n = 5) male C57BL/6 mice were used. Their respective mean body weights were: 24.4, 31.5, 32.6, and 32.0 g (S.E.M. ± 0.7 to ± 1.3 g). To examine the effects of the diet manipulation on aging, 11-month-old C57BL/6 mice ad libitum (AL) fed (n = 13) and caloric restricted (n = 9) were used. Their respective mean body weights were: AL fed, 31.3 ± 0.7 g; CR, 23.8 ± 0.5 g. CR was initiated at 10% at 14 weeks of age, lowered to 25% at 15 weeks of age and was set at 40% from 16 weeks on [25]. All mice had been housed in standard cages in a colony maintained by the National Institute on Aging at Harlan Sprague–Dawley (Indianapolis, IN).

3.3.2. BrdU labeling and cellular phenotyping

For labeling of newly generated cells mice were given daily single injections of bromodeoxyuridine (BrdU; 50 μg/g body weight, i.p., Sigma) for five consecutive days. Half of the mice of each age group were killed 2 h after the last BrdU injection to study proliferation of dividing cells. The other half were killed 4 weeks later to study survival and differentiation of newly produced cells. In 24-month-old mice, only the 4-week time-point was examined. Mice were overdosed with pentobarbital and transcardially perfused with 4% paraformaldehyde in PBS. Brains were removed and postfixed in the same fixative overnight and then placed in 30% sucrose in PBS for 2 days. Brains were subsequently frozen in 2-methylbutane at –25°C and serially sectioned on a freezing-sliding microtome at 40μm. For detection of BrdU-positive cells, sections were pretreated as previously reported [5]. Briefly, sections were incubated in 50% formamide in 2 x SSC for 2 h at 65°C, followed by 10 min in 2 x SSC, 30 min in 2N HCl at 37°C and 10 min in 0.1M borate buffer. Sections were then incubated in 0.08% H_2O_2, followed by 0.3% Triton X-100, and blocked in 5% rabbit serum, all in TBS. Rat monoclonal antibody against BrdU (MAS250c; Accurate Ltd., Westbury, NY) was diluted 1:1000 in TBS with 2% serum and 0.3% Triton X-100. Sections were then incubated in biotinylated anti-mouse IgG followed by the avidin–biotin–peroxidase complex solution (Vector Laboratories). Sections were reacted with 3,3-diaminobenzidine (0.08%,

Sigma) and 0.03% hydrogen peroxide in PBS for 2 min, rinsed, dehydrated, cleared, and coverslipped. To study the cellular phenotype of BrdU-labeled cells, double and triple immunofluorescence stainings were performed with a combination of antibodies to BrdU (see above), NeuN (mouse monoclonal, 1:1000, Chemicon, Temecula, CA) and S100 (rabbit polyclonal, 1:2000, Swant, Bellinzona, Switzerland). The secondary antibodies were Alexa488 goat anti-mouse IgG, Alexa568 goat anti-rat IgG and Alexa633 goat anti-rabbit IgG (1:400, Molecular Probes). In case of high autofluorescence background, sections were incubated in 70% ethanol/0.3% Sudan Black B (Merck, Darmstadt, Germany) and coverslipped in mounting medium Vectashield (Vector Laboratories). Sections were analyzed with a Confocal Laser Scanning Microscope LSM 510, inverted Axiovert 100M (Zeiss).

3.3.3. Stereological analysis

The total number of BrdU-positive cells in the GCL and hilus of the dentate gyrus was estimated using a variant of the optical fractionator technique [26]. Quantifications were performed on systematic random series of every sixth coronal section throughout the entire right dentate gyrus (12–16 fixed-frozen sections per animal). Cells were counted using a 100x, 1.3NA objective and an accurate three-dimensional stage movement coupled to a video-microscopy system (Systems Planning and Analysis Inc., Alexandria, VA). The mean section thickness was 17.9 ± 0.5μm. Cells touching the GCL were considered part of the GCL. To avoid any artifacts at the section edges, cells falling in a 3μm guard height were ignored. The sum of counted cells was multiplied by the reciprocal of the fraction of the brain region sampled, resulting in the total number of BrdU-positive cells/region. Results were analyzed using ANOVA with the help of the statistical software package SPSS 10.0.1. Data are presented as the mean and S.E.M. Statistical significance was accepted as $P < 0.05$.

3.4. Results

3.4.1. Aging and neurogenesis

To study the impact of aging on neurogenesis in the dentate gyrus, C57BL/6 mice of four age groups (2-, 12-, 18-, and 24-month-old) were analyzed 2 h and 4 weeks after the last BrdU injection. Two hours post-BrdU, cell proliferation was predominantly conferred to the subgranular layer (Fig. 1A, C, and E). The BrdU-positive nuclei were often clustered in the subgranular layer and exhibited variable shapes. In contrast, the majority of BrdU-labeled cells 4 weeks after BrdU treatment were located mostly inside the GCL and revealed large round nuclei (Fig. 1B, D, and F). Qualitative assessment indicated a robust increase in number of BrdU-labeled cells in the 2-month-old mice when compared to older mice. The hilus of both the 2-h post-BrdU and the 4-week post-BrdU groups revealed only few BrdU-positive cells (Fig. 1). Their distribution appeared random and an age-related decrease was also apparent.

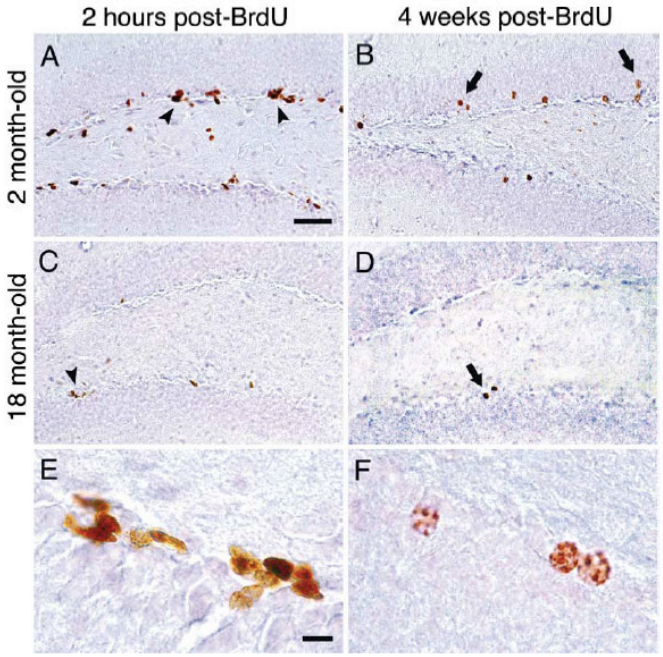

Fig. 1*. Age-dependent proliferation and survival of BrdU-labeled cells in the dentate gyrus. Mice received BrdU injections on 5 consecutive days and were analyzed 2 h (**A, C**, and **E**) and 4 weeks (**B, D,** and **F**) later. Two hours post-BrdU cells are predominantly aligned and clustered at the hilar-granule cell border (arrowheads in **A** and **C**). BrdU-positive cells exhibit a variety of shapes (**E**). Four-week post-BrdU cells have partly migrated into the GCL (arrows in **B** and **D**) and reveal large round nuclei with a chromatin structure similar to mature granular cells (**F**). Note the dramatic decrease in BrdU-positive cells in 18-month-old mice (**C** and **D**) compared to 2-month-old mice (**A** and **B**). Scale bars are 50 m (**A – D**) and 10 m (**E** and **F**).*

Using stereological quantification, we observed an age dependent decrease in the number of BrdU-labeled cells in the GCL (Fig. 2A) and the hilus (Fig. 2B) in both the 2 h and 4-week post-BrdU groups. Overall, the hilus contained substantially less BrdU-positive cells than the GCL. Two-way ANOVA for the two main factors age and post-BrdU time was performed separately for the GCL and the hilus. To stabilize the variance in the age groups and to achieve a normal distribution, a logarithmic transformation was applied. Results revealed highly significant main effects for age and post-BrdU time for both regions (Table 1). Subsequent Scheffé post hoc analysis revealed robust age-dependent decreases in BrdU-positive nuclei up to 18 months of age in both the GCL and hilus. Interestingly, however, no further significant decline in BrdU-positive cell number was detected between 18- and 24-month-old mice.

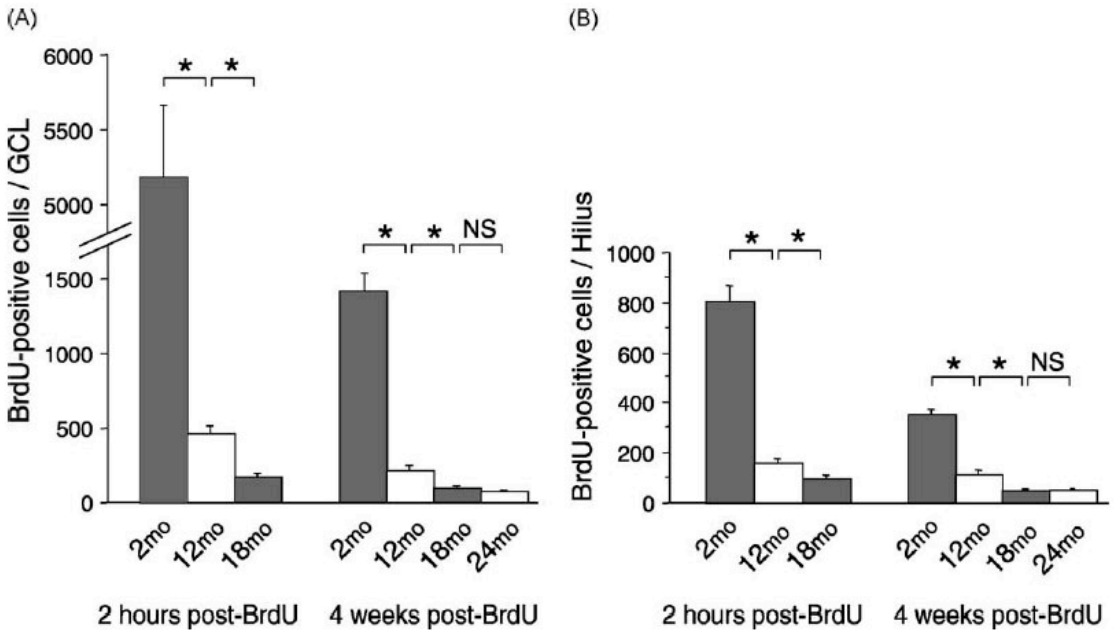

Fig. 2. *Stereological estimation of the total number of BrdU-positive cells in the dentate gyrus in aging C57BL/6 mice. The dentate gyrus was divided into the GCL (**A**) and the hilus (**B**), and these regions were analyzed separately. Results revealed that 2 h and 4 weeks post-BrdU, there was an age-related decrease in the number of newborn cells both in the GCL and in the hilus, *p < 0.01. Interestingly, however, in both regions, no further decline was seen between 18- and 24-month-old mice.*

To address the question of whether proliferation or survival of BrdU-labeled cells was decreased with aging, the number of BrdU-labeled cells 4 weeks post-BrdU was expressed as a ratio of the number 2 h post-BrdU. Results indicated an increase of the ratio with advancing age in both the GCL and hilus (27 and 44% at 2 months, 46 and 69% at 12 months, 55 and 53% at 18 months, respectively), which demonstrates that the age-related decline in BrdU-positive cells is solely attributable to an age-dependent decrease in proliferation but not survival of newborn cells.

Study	Region	Factor	*F*-value	d.f.	Residuals	*P*-value
Neurogenesis and aging	GCL	Post-BrdU time	29.2	1	36	<0.0001
		Age	85.2	3	36	<0.0001
		Post-BrdU time × age	0.99	2	36	0.38
	Hilus	Post-BrdU time	34.6	1	36	<0.0001
		Age	96.1	3	36	<0.0001
		Post-BrdU time × age	1.12	2	36	0.32
Neurogenesis and caloric restriction	GCL	Post-BrdU time	41.3	1	18	<0.0001
		AL/CR	0.06	1	18	0.81
		Post-BrdU time × AL/CR	0.15	1	18	0.70
	Hilus	Post-BrdU time	0.28	1	18	0.60
		AL/CR	3.50	1	18	0.08
		Post-BrdU time × AL/CR	5.85	1	18	0.03

Table 1 *Statistical parameters of two-way ANOVA analysis for number of BrdU-positive cells in the dentate gyrus. GCL: granular cell layer; d.f.: degrees of freedom.*

	2 months		12 months		18 months		24 months	
	GCL (103)	Hilus (39)	GCL (78)	Hilus (31)	GCL (34)	Hilus (16)	GCL (31)	Hilus (19)
NeuN (%)	68	0	39	0	33	0	30	0
S100β (%)	9	82	15	77	21	82	20	67

Table 2 *Phenotype of BrdU-positive cells in the GCL and in the hilus of 2-, 12-, 18-, and 24-month-old mice, 4 weeks after the last BrdU injection. BrdU-positive cells were labeled with markers for neurons (NeuN) and astrocytes (S100ß). Number of analyzed cells (in parenthesis) and percent of BrdU-colabeled cells are indicated.*

To phenotype the newly generated cells, immunofluorescence triplelabeling for BrdU, the neuronal marker NeuN, and the astrocytic marker S100ß was performed (Fig. 3). The dentate gyrus of three randomly chosen animals of each age group were analyzed with confocal microscopy. In the GCL, we found a considerable decrease in the fraction of BrdU/NeuN-colabeled cells between the 2- and 12-month-old mouse groups, with no further decline thereafter (Table 2). Interestingly, when the percentage of BrdU/NeuN-positve cells (Table 2) was multiplied by the survival ratios of BrdU-labeled cells of the corresponding age group (see above), neurons accounted for 18% of the surviving BrdU-positive cells in the GCL of all age groups. In contrast, an analogous analysis for BrdU/S100 –labeled cells showed that the ratio of cells committed to an astrocytic lineage tended to increase with age (2% at 2 months, 7% at 12 months, and 12% at 18 months). In the hilus, no BrdU-labeled neurons were found in any age group. The majority of newly produced cells in this region colocalized with the astrocytic marker S100ß (Table 2).

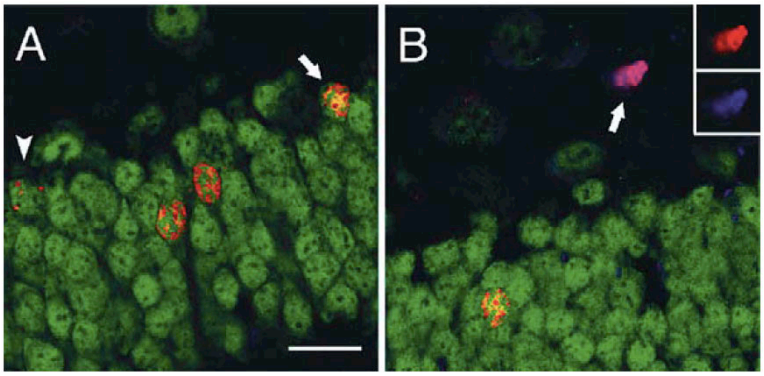

Fig. 3. *Cellular phenotyping of BrdU-positive cells. BrdU immunofluorescence (red) was combined with the neuronal marker NeuN (green) and the astrocytic marker S100ß (blue) and colocalization was assessed with confocal scanning microscopy. In 2-month-old mice, 4 weeks post-BrdU, the majority of the BrdU-labeled cells were positive for the neuronal marker NeuN (A). Note the variety of BrdU-labeling, ranging from dense stained neurons (arrow) to only partly labeled neurons (arrowhead), indicating a dilution effect of repeated stem cell divisions. In contrast to the GCL, BrdU-labeled cells in the hilus colocalized most frequently with the astrocytic marker S100ß in all age groups (arrowhead in* ***B****; shown is a 18-month-old mouse 4 weeks post-BrdU). The insert in* ***(B)*** *represents single confocal sections of BrdU and S100ß. Scale bar is 20 µm.*

3.4.2. Caloric restriction and neurogenesis

The effect of CR on neurogenesis in the mouse dentate gyrus was studied in 11-month-old mice. Qualitative analysis of the GCL revealed no effect of CR on the number of BrdU-labeled cells in either the 2-h post-BrdU or the 4-week post-BrdU group (Fig. 4). In contrast, 4 weeks post-BrdU, newborn cells in the hilus were markedly increased in CR mice compared to AL-fed mice (Fig. 4D versus 4B). Stereological quantification confirmed this qualitative assessment and revealed that CR affected only the survival of BrdU-labeled cells in the hilus but not in the GCL (Fig. 5). Consistently, two-way ANOVA revealed that the factor post-BrdU time was highly significant for both brain regions, but no significance was found for the factor AL/CR (Table 1). However, in the hilus, a significant interaction between AL/CR and post-BrdU time was found (Table 1). Scheffé post hoc analysis showed that CR mice had significantly more newborn hilar cells in the 4-week post-BrdU group ($P = 0.001$) but not in the 2-h post-BrdU group ($P = 0.78$) compared to AL mice. Cellular phenotyping of BrdU/NeuN-positive cells in the GCL revealed similar percentages of colocalization between the two groups (AL = 49%, CR = 37%, four randomly chosen animals of each group analyzed; 80 cells phenotyped per group). Again no BrdU/NeuN colabeling was found in the hilus (not shown), suggesting that the increased survival of newborn hilar cells is the result of an increased survival of glial cells in CR mice.

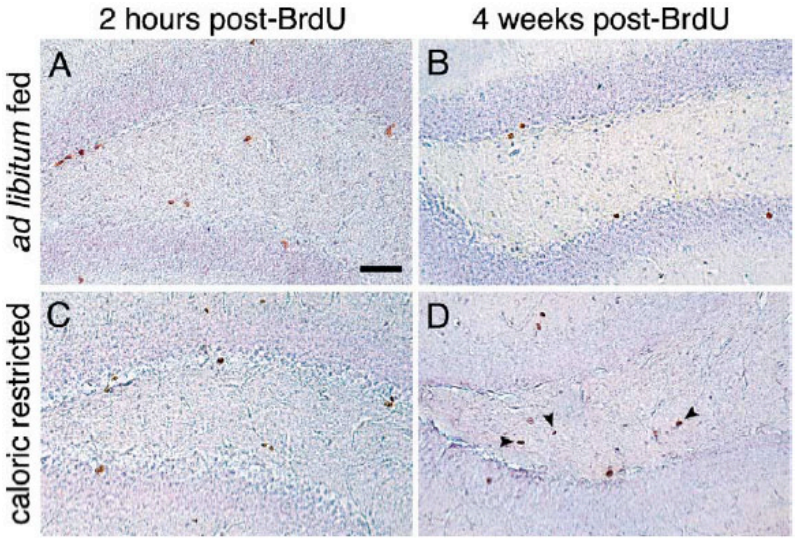

Fig. 4*. Proliferation and survival of BrdU-labeled cells in the dentate gyrus of ad libitum-fed (AL) and caloric-restricted (CR) mice. Eleven months old mice were killed 2 h (**A** and **C**) or 4 weeks (**B** and **D**) after the last BrdU injection. No qualitative difference was found when number of newborn cells in the GCL of CR mice (**C** and **D**) were compared to AL-fed mice (**A** and **B**). In contrast, the survival of BrdU-positive cells in the hilus was increased in the CR mice (arrowheads in **D**) when compared to AL-fed mice. Scale bar is 50 µm.*

3.5. Discussion

In the present study, we have investigated the impact of aging and CR on neurogenesis in the dentate gyrus of C57BL/6 mice. Proliferation of BrdU-positive cells was assessed 2 h post-BrdU, whereas a 4-week survival period was used to examine cell survival and differentiation.

3.5.1. Age-related changes in cell proliferation

Our stereological findings of a significant age-dependent decline in neurogenesis in the GCL parallel previous findings in mice [27] and rats [5,17,28,29]. The reduction of neurogenesis with aging was mainly caused by an age-related decrease in progenitor cell proliferation, whereas cell survival did not change with aging. In addition, the phenotypic differentiation of newborn cells into neurons decreased with age. Interestingly, all these changes occurred mainly between 2 and 12 months of age, with some additional decline until 18 months of age, but no further reduction in neurogenesis thereafter. In previous studies, the impact of aging on neurogenesis was examined in only two or three age groups, and thus the progression of the decline in neurogenesis could not be examined in detail [5,17,27,28]. Thus, the current findings would suggest greater functional significance of neurogenesis in younger mice with less impact on those approaching their median life span.

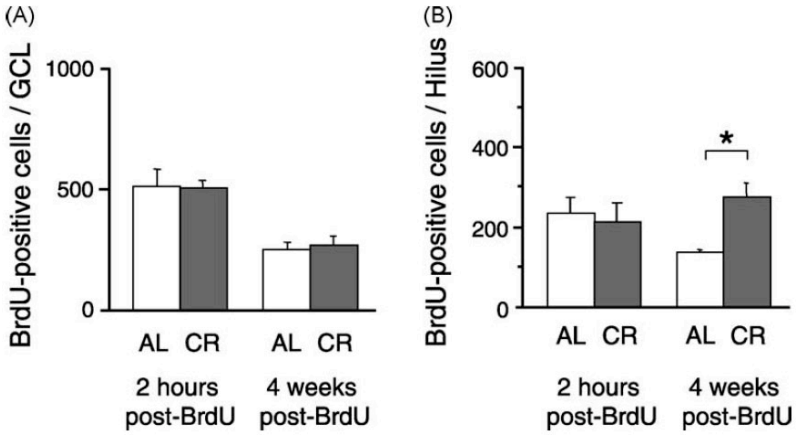

Fig. 5*. Stereological estimation of the total number of BrdU-positive cells in the dentate gyrus of caloric-restricted (CR) mice. CR had no effect on the total number of newborn cells in the GCL neither 2 h nor 4 weeks post-BrdU in comparison to ad libitum (AL) fed mice (**A**). Interestingly, in the hilus, where no BrdU/NeuN colabeled cells were detected, CR did not influence proliferation (2 h post-BrdU) but increased significantly survival (4 weeks post-BrdU) of newborn cells (**B**), $P < 0.05$.*

The decrease in progenitor cell proliferation with aging may be due to a decline in the pool of neuronal progenitors [30] or a lengthening of the cell cycle with aging [31]. The decrease in neurogenesis has also been explained by an age-related increase in stress-induced glucocorticoids [32,33]. Indeed, adrenalectomized rats show a markedly higher proliferation of neural precursors and no age-related decrease in neurogenesi [17]. Furthermore, physical activity, which has been shown to increase neurogenesis [13], declines in an age-dependent manner in rodents, including C57BL/6 mice [34,35]. Unfortunately, none of the studies examined whether such changes also decline until late adulthood but then remain stable in old age.

A relationship between the age-related decline in neurogenesis and age-dependent cognitive impairments has been suggested [17]. Thus, it could be speculated that the age-related reduction in neurogenesis and/or decreased neuronal turnover may underlie learning and memory deficits with aging in mice. However, we have previously reported that C57BL/6 mice are relatively resistant to age-related decline in spatial memory performance and show either no significant (C57BL/6J) or only very subtle (C57BL/6Nnia) cognitive changes with aging in hippocampus-dependent tasks [22,24]. Although we cannot exclude that age-related changes in neurogenesis contribute to cognitive changes in hippocampus-dependent tasks, the present results do not support this view. In future studies, it will be interesting to analyze age-related changes in neurogenesis in other inbred mouse strains that reveal more robust age-related cognitive changes such as the 129 mouse strain [22,24].

In the hilar region of the DG, neurogenesis was not apparent, and the majority of the BrdU-labeled cells revealed an astrocytic phenotype. This finding is consistent with previous studies, however, a smaller percentage of BrdU-positive cells with an astrocytic phenotype has been reported [36,37]. Interestingly, in the present study, we found a significant age-related decrease in the proliferation of such hilar astrocytes. Similar qualitative assessment in rats also revealed modest age-related decrease in astrocyte proliferation [5,28]. These observations suggest that aging decreases the astrocyte turnover in the DG because in previous studies we found no age-related change in total number of astrocytes in male C57BL/6J mice [38,39].

3.5.2. Caloric restriction and neurogenesis

No difference in either proliferation, nor survival and differentiation of newly generated cells was observed in the GCL of CR compared to AL mice. Many studies have reported how CR in mice can attenuate several age-related physiological changes [18,40,41], thus, an effect of CR on

neurogenesis may have been expected. Indeed, in two previous studies, an increase in neurogenesis in adult CR rats and mice has been reported [20,21]. The different outcome in the current study may be due to differences in methodology. First, the feeding schedule differs among the studies, in that in the previous studies CR was imposed by providing the animals with food on alternate days. One speculative explanation would be that these animals are exploring their cage for food on the days maintained on diet and therefore their level of physical activity is increased compared to our mice. This in turn could increase their neurogenesis, as shown for mice with increased physical activity [13]. Second, the previous studies were performed with 6-month-old animals that have been food restricted for only 3 months, while in the present study 11-month-old mice that have been on DR for 8 months have been used. Third, the previous studies did either not differentiate between hilus and GCL [20] or did not phenotype BrdU-positive cells in AL-fed mice [21] making a direct comparison difficult.

Surprisingly, in contrast to the lack of CR on neurogenesis, we found an increased survival of glial cells in the hilus of CR mice compared to in AL-fed mice. This increase might occur because of a mild metabolic stress that undernourished mice are afflicted with [18,42]. In a transgenic mouse model of Alzheimer's disease, we have previously reported an increase in glial proliferation in vicinity of amyloid plaques that may be considered as another form of stress although it may simply be viewed as a response to injury [43,44].

In summary, our findings demonstrate that neurogenesis in the dentate gyrus of C57BL/6 mice decreases with advancing age and reaches a plateau around 18 months of age and remains stable thereafter. CR has no significant effect on the number of newly generated hippocampal neurons in adult mice but may increase the survival of glial precursors in the hilus.

3.6. References

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Submitted

4. Increased neurogenesis and alterations of neural stem cells in Alzheimer's disease mouse models

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4.1. Abstract

Alzheimer's disease (AD) patients have a depletion of hippocampal neurons with accompanying deficits in learning and memory, and mouse models forming ß-amyloid (Aß) deposits show hippocampal deficits as well. In neither case is it known whether alterations in function are accompanied by alterations in neural stem cell biology and/or hippocampal neurogenesis. Here, hippocampal neurogenesis was investigated in amyloid precursor protein (APP) transgenic mice that develop abundant Aß deposition with aging. Neurogenesis in adult APP23 mice prior to Aß deposition showed no clear difference relative to age-matched controls but aged amyloid-depositing mice showed significant increases in neural precursor proliferation and neurogenesis. To further investigate how Aß affects neural stem cell biology, nestin-positive neural stem cells were analyzed in APPPS1 mice, a transgenic mouse model with early and aggressive cerebral amyloidosis. Results revealed a decrease in quiescent astrocyte-like stem cells, but not transient amplifying progenitor cells. This was accompanied by an aberrant morphologic reaction of hippocampal neural stem cells towards Aß-deposits. Our results provide evidence for a disruption of neural stem cell biology in an amyloidogenic environment with a concomitant stimulation of neurogenesis.

4.2. Introduction

Neurogenesis in the mammalian brain continues through adulthood and into old age primarily in the subventricular zone (SVZ) of the lateral ventricles, the olfactory bulb and the granular cell layer (GCL) of the hippocampus [1-3]. GCL neurons are generated in high numbers in young animals and despite a significant decrease with progressive age, neurogenesis continues until senescence [4,5]. The function of neurogenesis in the hippocampus remains elusive, but growing evidence suggests its importantance for certain memory tasks that are also strongly affected in Alzheimer's disease (AD) [2,6].

The neuropathological characterization of AD involves a progressive deposition of ß-amyloid (Aß) protein in the brain parenchyma, often accompanied by Aß deposition in cerebral blood vessels with an accompanying vascular angiopathy [7,8]. It has been suggested that other hallmarks of AD, i.e. an increased inflammatory response, neurofibrillary tangles, dystrophic neurites, neuron loss or cognitive deficits are triggered by the accumulation of Aß and that this combined insult influences the cognitive outcome [9].

The hippocampus is one of the regions of the AD brain most heavily burdened with amyloid plaque and is also one of the few locations in the adult brain where neurogenesis continues throughout adult life. Several attributes of adult hippocampal neurogenesis suggest that amyloid deposition may influence neurogenesis. Proliferative amplification of neural progenitor cells occurs within the microvascular niche [10], an area prone to amyloidogenesis and affected in virtually all AD cases [11-15]. Numerous growth factors are known to be up-regulated in vicinity of amyloid plaques [16-18] and the same growth factors are also known to be potent modulators of neural stem cell activity [19-24]. Conversely, inflammation that accompanies amyloid deposition [25] may negatively impact neurogenesis given the potent down-regulation of neurogenesis observed in pro-inflammatory environments [26,27].

In the present study, two amyloid precursor protein (APP) transgenic mouse models were used to evaluate the potential impact of Aß deposition on neural stem cell biology. Our results provide evidence for a disruption of neural stem cell biology in the amyloidogenic brain and an accompanying increase in neurogenesis.

4.3. Materials and Methods

4.3.1. Animals

In a first experiment adult (5 month-old) and aged (25 month-old) female APP23 transgenic mice and corresponding non-transgenic control mice were used [28]. APP23 mice express KM670/671NL mutated human APP under a murine Thy-1 promoter element. Mice have been generated on a B6D2 background and in the following, have been backcrossed with B6 mice for more than ten generations. In a second experiment APPPS1 transgenic mice expressing both KM670/671NL mutated human APP and L166P mutated human PS1 under the same Thy-1 promoter element [29] were crossed with transgenic mice expressing enhanced green fluorescence protein (GFP) under a CNS-specific nestin promoter element [30]. APPPS1 mice have been generated on a pure B6 background, while Nestin-GFP transgenic mice have been generated on a B6D2 background and were backcrossed to B6 for at least three generations. Adult (8 month-old) female $GFP^+/APPPS1^+$ (GFP-APPPS1) and age-matched littermate $GFP^+/APPPS1^-$ (GFP-wt) control mice were used. All experimental procedures were in accordance with the local veterinary office regulations.

4.3.2. BrdU treatment

5-Bromo-2-deoxyuridine (BrdU) was obtained from Sigma (Taufkirchen, Germany). During the first seven days of the experiment animals received daily i.p. injections of 50 μg BrdU/g body weight at a concentration of 10 mg/ml in 0.9% NaCl. Mice were sacrificed three weeks after the last BrdU injection.

4.3.3. Tissue preparation

Mice were anesthetized with an overdose of 0.8% ketamine and 1% xylazine in 0.9% NaCl, transcardially perfused with PBS followed by ice-cold 4% paraformaldehyde (PFA) in 0.1M PBS. Brains were removed and postfixed at 4°C in 4% PFA, dehydrated in 30% sucrose, and frozen. Coronal serial 40μm sections were cut with a microtome and collected in cryoprotectant (30% glycerol, 45% ethylene glycol in PBS) and stored at –20°C until use.

4.3.4. Immunohistochemistry

Free-floating sections were processed for immunohistochemistry as described elsewhere [10,31]. Briefly, sections were washed in TBS and blocked with 3% goat or donkey serum (Vector Laboratories Inc., Burlingame, CA) in 0.3% Triton-X-100 (Fisher, Fair Lawn, NJ). The sections were incubated overnight with primary antibodies at 4°C in 2% serum and 0.3% Triton-X-100, washed three times with TBS and incubated for 3 hours with biotin-conjugated secondary antibodies. After repeated TBS washing, sections were stained by complexing with avidin-biotin and DAB or SG blue (Vectastain ABC elite kit; Vector Laboratories). Sections were mounted on pre-cleaned glass microscope slides (Superfrost® Plus; Langenbrinck, Teningen, Germany), dehydrated with an alcohol series, cleared in xylene and coverslipped in a xylene soluble mounting medium (Pertex®; medite GmbH, Burgdorf, Germany).

For immunofluorescence, a similar protocol was followed but with fluorophore coupled secondary antibodies. After the 3 hours incubation time with secondary antibodies, sections were repeatedly washed in TBS, mounted and coverslipped in polyvinyl alcohol with 2,5% DABCO (Sigma) or Vectashield® (Vector Laboratories) for GFP fluorescence.

Primary antibodies were used at the following concentrations: rat anti-BrdU (1:1000; Accurate, Westbury, NY), goat anti-doublecortin (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-NeuN (1:1000; Chemicon Int., Temecula, CA), guinea pig anti-GFAP (1:1000; Advanced ImmunoChemical, Inc., Long Beach, CA), rabbit anti-S100ß (1:2000; Swant, Bellinzona, Switzerland) and rabbit anti-Iba-1 (Wako Chemicals, Neuss, Germany). As secondary antibodies Biotin-SP, Cy5, Cy3, (Vector Laboratories) or Alexa 488, Alexa 568 (Molecular Probes, Inc., Eugene, OR) conjugated goat or donkey IgG at a dilution of 1:500 were used.

For BrdU antigen retrieval, sections were treated for 30 minutes with 2 M HCl at 37°C followed by repeated washing with TBS. For immunofluorescence co-labeling experiments with BrdU, sections were first stained for NeuN and GFAP, then fixed for 10 minutes in 4% PFA followed by antigen retrieval processing and staining for BrdU.

Histochemistry

Fibrillar Aß was visualized by staining free floating sections with 0.005% thioflavine S (Sigma) for 1 min. Alternatively, mounted sections were incubated in 0.5% Congo red (Sigma) in NaCl-saturated 80% ethanol.

Quantification of cell number and plaque load

For the stereological analysis of the total number of BrdU and DCX positive cells in the GCL of APP23 mice, every sixth section through the hippocampus was immunohistochemicaly stained and BrdU and DCX-positive cell numbers were estimated under light microscopy using the 'Optical Fractionator' probe of Microbrightfield Stereo Investigator software (MSI; MicroBrightField, Williston, VT). Cells were included in the analysis, if located inside or within one cell diameter of the GCL.

For phenotyping of BrdU-positive cells, triple immunofluorescence was used using anti-BrdU, anti-NeuN, and anti-GFAP antibodies. Cells were scored using a Zeiss 510 Meta confocal microscope (Jena, Germany). Careful optical sectioning in the z-plane was used to avoid scoring closely juxtaposed cells as double-positive [32]. In 5 month-old APP23 mice, one hundred BrdU positive cells per animal were examined, while in 25 month-old animals, where BrdU labeling was often very low [4], 40 to 100 cells per animal were analyzed. The total number of BrdU-positive cells that co-labeled with a specific lineage marker was estimated by multiplying the lineage positive percentage by the total number of BrdU positive cells in the GCL as stereologically estimated.

The number of stem cells in the GCL of GFP-APPPS1 and GFP-wt mice was estimated using a variant of the optical fractionater technique as previously described [4]. Every sixth section throughout the entire hippocampus was stained immunohistochemically for GFP and analyzed under the light microscope. The total number of cells was estimated by multiplying the counted cell number with the reciprocal of fraction of the sampled region. GFP-positive cells were classified as quiescent astrocyte-like or transient amplifying cells, if the orientation of the dendritic processes were either perpendicular or horizontal, respectively, and if the cell body was located in the subgranular zone [33].

Amyloid load was estimated on every sixth section through the entire dentate gyrus of the hippocampus using the 'Area Fraction Fractionator' probe of MSI software using a similar protocol as previously described [34]. The region was determined according to a mouse brain atlas [35].

4.3.5. Statistical analysis

Statistical analysis was performed using ANOVA (Statview 5.0 for Macintosh). Throughout the paper the mean ± standard error of the mean is indicated. Statistical significance was accepted as $p < 0.05$.

4.4. Results

4.4.1. Increased proliferation in aged APP23 mice

The incorporation of BrdU in the S-phase of mitotic cell division was used to monitor proliferative activity in the GCL. The effect of age (5 mo vs. 25 mo) and genotype (wild type vs. transgenic mice) on the number of newly generated cells in the dentate GCL of APP23 mice was estimated using unbiased stereological methods (Fig. 1). ANOVA revealed a significant effect of age [$F(1,20) = 11.16$; $p < 0.01$] and an age and genotype interaction [$F(1,20) = 4.89$; $p < 0.05$; n=6/group]. In young 5 mo-old mice there was no statistical difference between transgenic mice and wild type mice, although transgenic mice appeared to have less BrdU-positive cells. In aged 25 mo-old mice, a significantly higher number of BrdU positive cells was found in the GCL of transgenic compared to control mice [$t(10) = 4.95$; $p < 0.001$], reaching almost the level of adult 5-mo-old APP23 mice (Fig. 1).

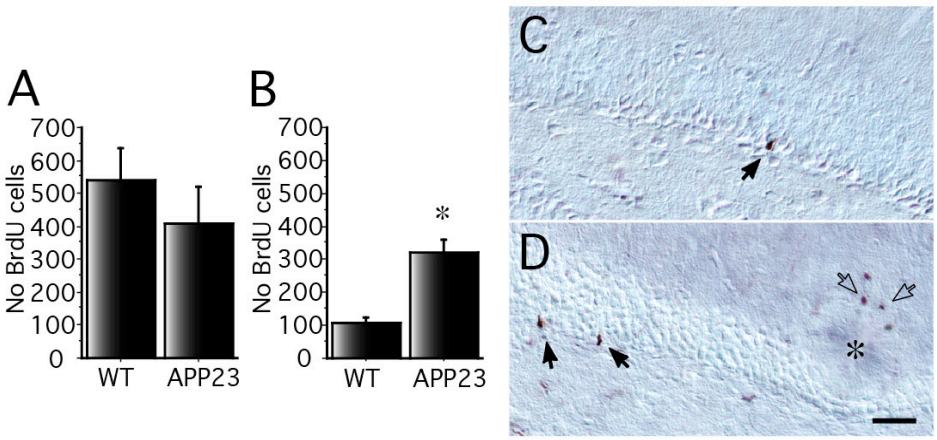

Fig. 1: *BrdU-positive cells in the GCL. Three weeks after BrdU injections cell proliferation in the GCL was evaluated in adult and aged APP23 mice and littermate control mice by stereological estimation of the number of BrdU positive cells in the GCL (n=6/group). (**A**) In 5 mo-old APP23 mice no difference was found (6 mice/group). (**B**) In aged 25 mo-old APP23 mice, the number of BrdU positive cells was significantly increased compared to wild type (WT) mice (6 mice/group; * $p < 0.001$). (**C, D**) BrdU positive cells (closed arrows) in aged 25 mo-old WT (C) and APP23 (D) mice were found close to the SGZ of the dentate gyrus. In aged APP23 mice proliferating microglia (open arrows) were often found in the periphery of amyloid plaques (asterisk), but this was rarely found within the GCL. Scale bar: 50µm.*

Stereological estimation of the amyloid load in the dentate gyrus of 25 mo-old mice was 14.0 ± 1.9% while no amyloid deposition was found in the 5 mo-old APP23 mice, demonstrating that the amyloidogenic environment of aged APP23 mice was accompanied by increased proliferative activity in the dentate gyrus.

4.4.2. Increased neurogenesis in aged APP23 mice

To determine whether this increased proliferation reflects neurogenesis, BrdU labeling was combined with confocal microscopy and fluorescent multiple labeling for the neuronal marker NeuN and the glial marker GFAP. In 5 mo-old APP23 mice no difference in the total number of BrdU and NeuN co-labeled cells was found (Fig. 2). There was also no difference in the fractions of BrdU positive cells co-labeling with NeuN in transgenic and wild type mice (71.4% ± 5.3% and 67.0% ± 5.9%, respectively; $p > 0.05$). In contrast, aged APP23 revealed a six fold higher number of total newborn neurons compared to wild type mice [$t(10) = 2.73$; $p < 0.05$] (Fig. 2). The fraction of BrdU cells expressing NeuN also appeared greater however did not reach significance in aged APP23 mice compared to wild type animals (10.9% ± 4.0% vs. 3.7% ± 0.8%; $p > 0.05$).

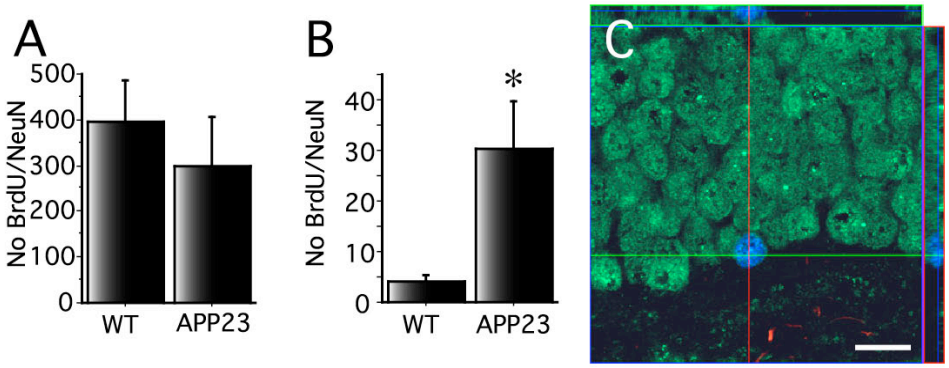

Fig. 2: *Neurogenesis in the GCL. Estimated numbers of BrdU-positive cells co-labeled with the neuronal marker NeuN in adult and aged wild type (WT) and APP23 mice (6 mice/group). (**A**) In 5 mo-old mice no difference was found in the number of newly generated neurons between WT and APP23 mice, while (**B**) in 25 mo-old APP23 mice a significantly higher number of BrdU/NeuN double positive cells was found compared to WT (* $p < 0.05$). (**C**) Capture of multiple confocal images in the Z-dimension allows the evaluation of the co-localization of different markers in all 3 dimensions. Green: NeuN; Blue: BrdU; Red: GFAP; Scale bar: 10µm.*

The percentage of BrdU positive cells co-labeled for GFAP was low in both 5 mo-old (0.9% ± 0.6% and 2.4% ± 1.2% in wild type and transgenic, respectively) and 25 mo-old animals (1.4% ± 0.9% and 4.3% ± 1.3% in wild type and transgenic, respectively) resulting in very few BrdU-labeled astrocytes in the GCL. In 5 mo-old APP23 animals no significant difference was observed in newborn glia between transgenic and wild type mice (6.5 ± 3.6 and 5.1 ± 3.3 newborn astrocytes per GCL respectively), while in 25 mo-old animals significantly more newborn glia cells were found in transgenic compared to wild type control mice (12.3 ± 3.5 vs. 1.3 ± 1.0, newborn astrocytes per GCL respectively; $p < 0.05$).

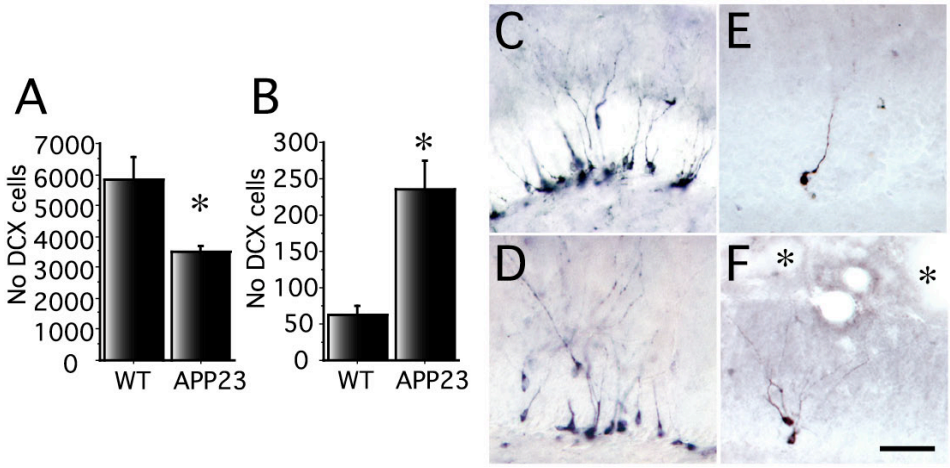

Fig. 3: *DCX-positive immature neurons in the GCL. Numbers of immature neurons in the GCL were estimated with unbiased stereological methods (**A**) In adult 5 mo-old wild type (WT) mice the pool of DCX positive cells was smaller compared to APP23 mice (* $p < 0.05$). (**B**) In aged 25 mo-old mice the population of immature neurons was significantly increased in APP23 mice (6 mice/group; * $p < 0.005$). This increase was most pronounced in the caudal pole of the dentate gyrus. (**C-F**) Representative micrographs of DCX positive cells in the GCL of 5 mo-old WT (**C**), 5 mo-old APP23 (**D**), 25 mo-old WT (**E**) and 25 mo-old APP23 mice (**F**). Astrisks indicate amyloid plaques. Scale bar: 50µm.*

4.4.3. Immature doublecortin-positive cells are also increased in aged APP23 mice

Because the incorporation of BrdU into dividing cells takes place after systemic administration of BrdU, it cannot be excluded that Aß induced changes in the vascular system influence the uptake of BrdU. Thus, we sought to confirm our results with an additional marker of neurogenesis. Neuroblasts committed to the neuronal lineage express doublecortin (DCX) during their last divisions but subsequently down-regulate DCX as NeuN is up-regulated during neuronal maturation [36,37]. DCX is therefore an excellent marker to monitor the abundance of immature neurons within the hippocampal dentate.

ANOVA revealed significant effects of age [$F(1,20) = 143.6$; ($p < 0.001$)], genotype [$F(1,20) = 8.38$; $p < 0.01$] and of the interaction of age x genotype [$F(1,20) = 11.2$; $p < 0.01$]. Five mo-old APP23 mice showed significantly fewer DCX positive cells than age-matched controls [$t(10) = 3.13$; $p < 0.05$] (Fig. 3). In contrast, aged 25 mo-old APP23 mice showed a significant increase in DCX positive cells compared to age matched controls [$t(10) = 4.23$; $p < 0.01$] (Fig. 3), supporting the conclusion that an amyloidogenic environment stimulates neurogenesis and/or survival of newborn neurons.

4.4.4. Alterations of neural stem cells contacting Aß deposits

Next we intended to study whether this increase in neurogenesis is the result of an increased number of nestin-positive neural stem cells. Nestin is an intermediate filament protein expressed in neural stem and progenitor cells [10,38,39]. However, consistent with previous reports of a striking age-related decrease in stem cells in the SVZ [40] and poor detection of mouse nestin with commercially available antibodies we could not detect nestin-positive cells in significant numbers in the dentate gyrus of aged 25 mo-old mice. Thus, a recently generated APPPS1 mouse model was used that develops cerebral amyloidosis at a much younger age compared to APP23 mice (Radde et al., 2005). To further improve detection of stem cells we crossed such APPPS1 mice with mice expressing GFP under a nestin promoter [30]. GFP positive cells were located at the subgranular zone (SGZ) and often extended a small but very dense dendritic tree into the inner molecular layer (ML) (Fig. 4A).

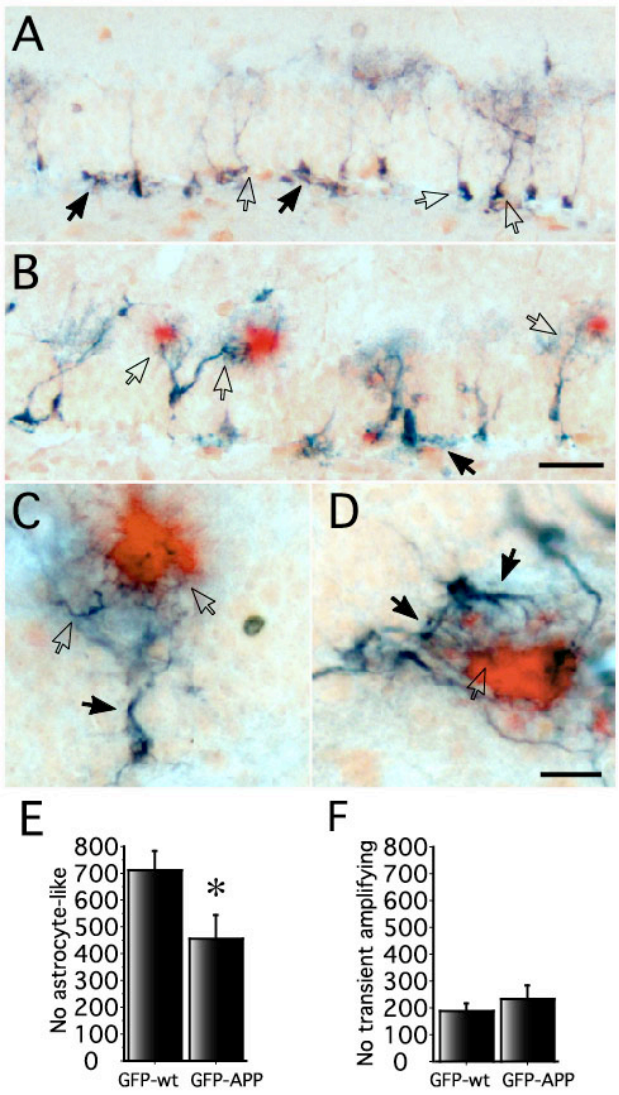

Fig. 4: *Neural stem cells in the GCL interact with amyloid plaques. (**A**) Adult nestin-positive stem cells in the GCL of aged 8 mo-old GFP-wt mice. Quiescent astrocyte-like stem cells (open arrows) were located along the GCL, with their cell body in the SGZ and dendrites in the molecular layer perpendicular to the GCL. In contrast, transient amplifying precursor cells (close arrows) in the SGZ displayed a horizontal orientation (**B**) In GFP-APPPS1 mice, amyloid deposits (congo red staining in red) strongly attracted the dendrites of quiescent astrocyte-like (open arrows) and transient amplifying cells (close arrow). Scale bar: 50µm. (**C**) High magnification of the typical morphological alterations of a quiescent astrocyte-like cell attracted to Aß (red). Note the thickened main branch of the dendrite tree (close arrow) and the thickened dendrites in close vicinity to the plaque (open arrows). (**D**) Transient amplifying cells were found to react to the presence of Aß similar to quiescent astrocyte-like cells with thickened processes (close arrows) and an aberrant dendritic tree sheathing the amyloid plaque with dystrophic bouton-like structures (open arrow). Scale bar: 10µm. (**E**) Stereological estimates of quiescent astrocyte-like stem cells revealed a significant reduction in 8 mo-old GFP-APPPS1 (n=8) compared to age-matched GFP-wt mice control mice (n=7) (*p < 0.05). (**F**) No difference occurred in the number of transient amplifying progenitor cells in GFP-APPPS1 compared to GFP-wt mice.*

These putative neuronal stem cells have been described as radial astrocytes [41] or as quiescent astrocyte-like "Type -1" cells [42,43] and are characterized by co-expression of nestin and GFAP but lack of S100ß or DCX expression. This phenotyping has also be confirmed in our mice using confocal microscopy and immunofluorescent multi-labeling (results not shown).

Remarkably, in 8 mo-old GFP-APPPS1 mice Aß deposits in the ML strongly attracted dendrites of these putative neural stem cells. This was accompanied by morphological alterations in these cells, including thickened processes and hypertrophic dendrites aberrantly contacting Aß plaques (Fig. 4). Estimates of total nestin-GFP positive quiescent astrocyte-like stem cells in amyloid-depositing 8 mo-old GFP-APPPS1 mice showed a significant reduction in number compared to GFP-wt control mice [$t(13) = 4.84$; $p < 0.05$; n=7-8/per group] (Fig. 4E).

As neural stem/progenitor cells begin to differentiate, GFAP expression is extinguished and cells adopt a migratory-like morphology with a major axis tangential to the GCL. These cells have been referred to as "Type-II" cells [42,43] or D cells [44] and represent a transient amplifying population of neural progenitor cells (Fig. 4). These GFAP-negative and nestin-positive cells were also found to extend aberrant processes to amyloid deposits (Fig. 4D), but their total number did not differ between GFP-APPPS1 and GFP-wt mice [$t(13) = 0.53$, $p>0.05$] (Fig. 4F).

Surprisingly, unlike nestin expressing stem cells, DCX-positive immature neurons appeared morphologically unaltered by Aß-deposits, extending dendrites close to and past amyloid plaques without showing any attraction or thickening (results not shown).

Together, these results provide evidence that neurogenesis and the population of immature neurons is increased in an amyloidogenic environment, while neural stem cells are decreased and strongly affected by the presence of Aß.

4.5. Discussion

In this study we investigated the behavior of neural stem cells and neurogenesis in the context of cerebral amyloidosis. Results revealed no differences in neurogenesis in pre-amyloid depositing 5 month-old APP23 mice but a significant increase in neurogenesis in aged 25 mo-old APP23 mice compared to age matched wild type mice.

In an effort to study whether the increase in neurogenesis is already reflected at the level of nestin-positive stem cells, we found that quiescent astrocyte-like stem cells were reduced in numbers in Aß-depositing APPPS1 mice compared to wild type mice, while the number of amplifying stem cells remained at a similar level. Transient amplifying cells are believed to be the more differentiated and more proliferative progeny of quiescent astrocyte-like cells [42-44], suggesting that chronic Aß deposition is accompanied by an increase in the ratio of transient amplifying / quiescent astrocyte-like cells, which in turn leads to increases in DCX positive cells and up-regulation of neurogenesis overall.

Previous results on the effect of Aß on neural progenitor proliferation in the hippocampus of transgenic mice revealed both increases and decreases of neurogenesis [45-47]. However, in these studies, transgenic mice were investigated at an early stage of amyloid deposition and thus, results may be related to both the over expression of APP and to accumulation of Aß [48]. Consistent with our results, investigation of the postmortem AD brain showed an increased expression of markers of proliferation and immature neurons in the hippocampus [49].

It is surprising that neurogenesis, which is thought to be important for hippocampus-related memory, is increased in both aged Aß-depositing mice and in AD patients. As in AD humans, previous behavioral experiments with APP23 and other AD mouse models have shown impairment in hippocampus-dependent memory tasks [50-53], suggesting that increased neurogenesis is not sufficient to overcome the more global neural dysfunction induced by amyloid deposition. Newborn neurons are known to be incorporated into functional neural networks and this has been shown to correlate with improved learning and memory [2,6,54]. However, the elevation in newborn neuron numbers in AD patients and mouse models is accompanied by deficits in learning and memory. Given our finding for grossly altered progenitor cell morphology and arborization, it seems possible that neurogenesis in an advanced amyloidogenic environment is accompanied by altered connectivity and perhaps aberrant function [55].

Amyloid-dependent up-regulation of neurogenesis is likely caused by a combination of several mechanisms. It is known that amyloid deposits stimulate the local accumulation of several growth factors that are potent regulators of neurogenesis; these include BDNF, VEGF, FGF, and CystatinC [16-18,56]. A neurogenic effect of Aß peptide on hippocampal neural stem cells has also been reported [57]. The elevation in neurogenesis may also represent an endogenous neural replacement response to cell death rather than a direct response to amyloidogenesis. Indeed, neurogenesis in the hippocampus is stimulated in response to a variety of insults such as cerebral ischemia, seizure or neurotoxic lesions [58-61]. In both AD patients and APP23 mice, neuronal cell loss in the hippocampus has been reported [62,63].

In conclusion, our present finding demonstrates a disruption of neural stem cell biology in an amyloidogenic environment with a concomitant stimulation of neurogenesis. This increase in neurogenesis may be compensatory and reflect a remarkable endogenous potential for neuronal replacement in an amyloidgenic environment. However, such a response still appears insufficient to overcome the more global neurodegeneration and associated cognitive deficits in AD and mouse models. It is also possible that neurogenesis in an amyloidogenic environment is aberrant and dysfunctional. Thus it remains to be determined if ablating neurogenesis in aged Aß-accumulating mice improves or harms hippocampal function which, in turn, will be of major relevance for the development of therapeutic strategies.

4.6. Supplementary Data

4.6.1. Neurogenesis in running mice

Although reports about the beneficial effects of exercise for AD patients are conflicting, studies with rodents provide growing evidence that exercise improves both cognitive performance and neurogenesis [5,64]. To determine the influence of running on neurogenesis in aged APP23 mice, 5 month-old and 25 month-old APP23 mice and littermate (N=6) were provided with running wheels and allowed to run voluntarily for 4 weeks. To determine if there were differences in voluntary use of the running wheel between APP23 and wild type mice, the night before sacrificing, running was monitored in a darkened room for two hours. Every five minutes (24 determinations over 2 hours), wheel-use was scored as + or -. We found that aged mice utilize the running wheel approximately one half as much as 5 month-old adult mice, but there were no significant differences between age-matched APP23 and wild type mice (96%, 100%, 40% and 52% for young APP23, young controls, old APP23 and old controls respectively).

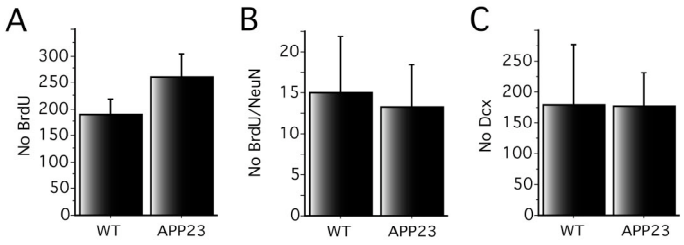

Fig. 5: *Exercise does not increase neurogenesis in aged APP23 mice. After four weeks with access to a running wheel the numbers of BrdU positive (**A**), BrdU/NeuN double positive (**B**) and DCX positive (**C**) cells in the GCL are at the same levels in 25 months old APP23 and age matching type control mice.*

Stereological evaluation of BrdU incorporation in 5 month-old animals showed that proliferation increased equally in both genotypes (data not shown). In aged animals, running also stimulated BrdU incorporation in wild type animals but had no significant effect in APP23 mice (Fig. 5A; $p > 0.05$). Similarly, comparing the numbers of BrdU/NeuN, and DCX positive cells in aged APP23 and control mice after 4 weeks of running revealed that running normalized the differences in net neurogenesis between APP23 and wild type mice (Fig.5 B-C). No differences in plaque load were observed between running and normally housed APP23 mice (14.0% ± 1,9% by tissue area in standard housed mice vs. 16.5% ± 1.5% in running mice). Together, the data suggests that plaque deposition is accompanied by an increased neurogenesis but that running has no further stimulatory effect on neurogenesis in these animals.

4.6.2. Accumulation of neurosphere building stem cells in the hippocampus of 24 months old APP23 mice

Because of the lack of good markers to label neural stem cells the methods to determine the number of neural stem cells in the hippocampus are limited. Incorporation of BrdU can only serve as an estimate of the numbers as the result is biased by availability of BrdU in the system of the animal or division rates of dividing cells. Isolated neural stem cells do not aggregate but grow clonally into floating spheres, called neurospheres [65]. In this in vitro essay, we estimated the number of stem cells by counting the neurospheres grown from the total stem cell population we extracted from the hippocampus.

Whole hippocampal lobes were dissected from 4 month and 24 month old APP23 and B6 control mice (N=3). Tissues were enzymatically digested with a mixture of papain, neutral protease and DNAse and then fractionated on a Percoll step gradient. Cells floating on a 65–35% interface were collected and plated into un-coated dishes in defined medium containing Neurobasal, B-27 supplement (Gibco, Carlsbad, California) and 20 ng/ml FGF-2 (Peprotech, Hill, New Jersey) and 20 ng/ml EGF (Sigma). After three days the number of spheres was counted at low magnification.

After three days in culture neural stem cells had grown into easily identifiable spheres (Fig. 6 A). To confirm the neuronal nature of the precursor cells, we induced differentiation and stained for neuronal and astrocytic markers (Fig. 6 B). The number of sphere growing stem cells confirmed the influence of Aß on neurogenesis that we portrayed in the results of the

experiments with BrdU and DCX; the number of spheres taken from young APP23 animals was reduced compared to controls, but we found a large excess of neurospheres from aged APP23 hippocampus relative to aged control mice (Fig. 6 C). These findings indicate that the effects of Aß on neurogenesis, are a result of the influence of Aß on the size of the stem cell population.

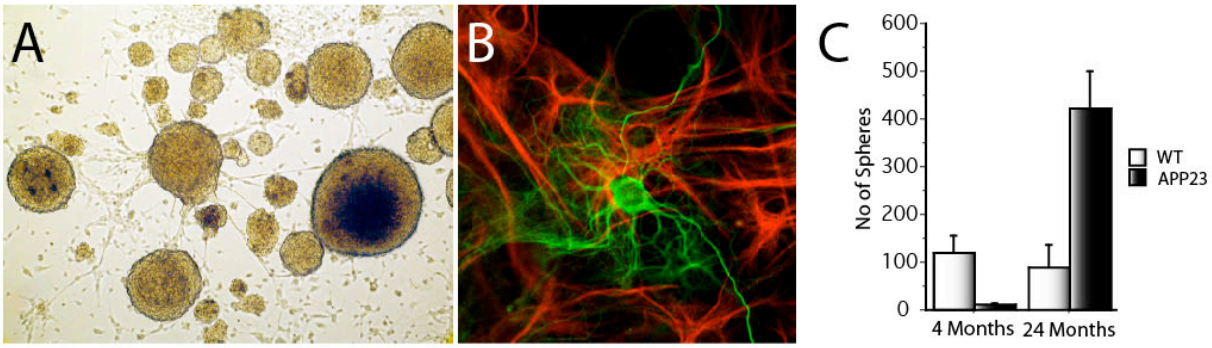

***Fig. 6**: Neurosphere assay from hippocampal progenitor cells. (**A**) Neural stem cells grow to Neurospheres in culture. (**B**) Cells outgrowing from neuropheres can differentiate into neuronal (ß-tubulin III, green) or Astrocytes (GFAP, red). (**C**) The number of neural stem cells does not wane in aged WT animals. In APP23 there is a reduction in predepositing mice, but increased numbers in aged mice with a large plaque load.*

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5. Invasion of hematopoietic cells into the brain of amyloid precursor protein transgenic mice

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5.1. Abstract

The significance of the peripheral immune system in Alzheimer's disease pathogenesis remains controversial. To study the CNS invasion of hematopoietic cells in the course of cerebral amyloidosis, we used a green fluorescence protein (GFP)-bone marrow chimeric amyloid precursor protein transgenic mouse model (APP23 mice). No difference in the number of GFP-positive invading cells was observed between young APP23 mice and non-transgenic control mice. In contrast, in aged, amyloid-depositing APP23 mice a significant increase in the number of ameboid-like GFP-positive cells was found compared to age-matched non-transgenic control mice. Interestingly, independent of the time after transplantation (3-10 months) only a subpopulation of amyloid deposits were surrounded by invading cells. This suggests that not all amyloid plaques are a target for invading cells or, alternatively, all amyloid plaques attract invading cells but only for a limited time, possibly at an early stage of plaque evolution. Immunological and ultrastructural phenotyping revealed that macrophages and T-cells accounted for a significant portion of these ameboid-like invading cells. Macrophages did not show evidence of amyloid phagocytosis at the electron microscopic level and no obvious signs for T-cell mediated inflammation or neurodegeneration was observed. The observation that hematopoietic cells invade the brain in response to cerebral amyloidosis may hold an unrecognized therapeutic potential.

5.2. Introduction

The pathological hallmarks of Alzheimer's disease (AD) include the presence of ß-amyloid plaques, neurofibrillary tangles and neuron loss [1]. These pathological changes are invariably accompanied by an associated inflammatory reaction involving microglia and astrocytes [2]. Accumulating data suggest, that microglia, as the brain resident macrophages, play a key role in mediating this inflammatory reaction [2-4]. Although excessive migration of peripheral cells into the CNS of AD patients has not been described, *in vitro* studies have demonstrated the ability of Aß to stimulate production of a number of cytokines and chemokines from human microglia [5-7]. Thus, it is likely that chemokines together with other inflammatory mediators are able to recruit peripheral immune cells into the AD brain, which, in turn, may contribute to AD pathogenesis.

The lack of a single defining marker to distinguish invading cells from resident and activated microglia makes the study of these potent immune regulatory cells difficult. Recently, the need for about ten different markers to unequivocally distinguish peripheral macrophages from resident microglia has been exemplified [8]. In response to these technical difficulties, bone marrow transplantation techniques have become an important tool to study the invasion of peripheral cells into the healthy or diseased brain [9,10]. Thus, to study the invasion of peripheral immune cells in response to cerebral amyloidosis, we performed bone marrow transplantation on different age groups of APP23 transgenic mice. APP23 mice develop amyloid plaques as well as cerebral amyloid angiopathy (CAA) with aging and show an inflammatory reaction to amyloid that is very similar to that seen in AD brain [11-13]. Our results in transgenic mice suggest an involvement of the peripheral immune system in cerebral amyloidogenesis.

5.3. Materials and Methods

5.3.1. Mice

Male APP23 transgenic mice and non-transgenic littermate controls were used [11]. APP23 transgenic mice have been backcrossed to C57BL/6J mice for more than ten generations. Hematopoietic donor cells were from C57BL/6J-TgN(ACTbEGFP)1Osb mice (Jackson Laboratories, Bar Harbor, MN [14]).

5.3.2. Transplantation

Host mice received two total body doses of cobalt radiation (500 cGray) within a three-hour interval to minimize gastrointestinal toxicity, followed by a single tail vein injection of approximately 5 million EGFP-positive donor cells in an injection volume of 200 μl.

Donor cells were prepared from timed pregnant embryonic day 17 old C57BL/6J-TgN(ACTbEGFP)1Osb mice. Embryos were removed from the uterus and screened for transgene expression using a fluorescent light. From the fetal livers of the EGFP-positive embryos a single cell suspension was prepared by passing the suspension through a descending line of smaller bore pipette-tips using semi-sterile conditions. Cells were washed with RPMI containing 5% FCS (GIBCO), and the density was adjusted to $25x10^3$ cells/μl in HBSS (GIBCO).

Transplanted mice were kept under sterile conditions for three weeks following the procedure before they were placed back in the regular SPF animal facilities. All experimental procedures were in accordance with the Swiss veterinary office regulation and performed under the approved protocol number 1864.

5.3.3. Tissue preparation and immunohistochemistry

Mice were overdosed with pentobarbital and transcardially perfused with ice cold PBS. Brains were removed and postfixed overnight in fresh 4% paraformaldehyde in PBS. After fixation the brains were placed in 30% sucrose in PBS for 2 days. Brains were subsequently frozen in 2-methylbutane and stored at –70°C until further use.

Coronal sections (25 μm) were cut on a freezing-sliding microtome into cryoprotectant solution (30% ethylenglycol, 20% glycerol, 50mM sodiumphosphate buffer, pH7.4). Sections were rinsed several times in PBS and incubated in 0.08% H_2O_2 in PBS, followed by an incubation in PBS containing 0.3% Triton X-100 and 5% serum. Primary antibody was diluted in PBS with 2% serum and 0.3% Triton X-100 all in PBS and incubated at 4°C overnight. Sections were then incubated with biotinylated secondary IgG followed by the avidin–biotin–peroxidase complex solution (all from Vector Laboratories; Burlingame, CA). Sections were incubated with Vector SG-blue (Vector Laboratories) in PBS for 2 min, rinsed, dehydrated, cleared, and coverslipped. The following antibodies were used: rabbit anti-GFP (Chemicon; Temecula, CA); rabbit anti-Aß (NT12; Gift of P. Paganetti, Basel, Switzerland; Sturchler-Pierrat et al., 1997); rat anti-F4/80 (BMA, Augst, Switzerland; Austyn and Gordon, 1981), rabbit anti-Iba-1 (gift of Dr. Y. Imai, Japan; Ito et al., 1998), hamster anti-mouse CD3 (Pharmingen; San Diego, CA), rat anti-B220 (Pharmingen), rat anti-mouse CD45 (Pharmingen), hamster anti-mouse CD31 (Pierce Biotechnology, Rockford, IL), mouse anti-NeuN (Chemicon); guinea pig anti-GFAP (Advanced Immunochemical Inc., Long Beach, CA); and rabbit anti-GFAP (Dako, Glostrup, Denmark). Amyloid plaques were visualized either by NT12 antibody or histologically by Congo red staining according to standard procedures.

For single and multiple immunofluoresent labeling a similar protocol was used. However, after incubation with the primary antibody, the sections were incubated for 3 hrs with the appropriate fluorophore-coupled secondary antibody. As secondary antibodies goat IgG coupled to AlexaFluor® 568, AlexaFluor® 633 (Molecular Probes; Eugene, OR) and Cy5 (Jackson Immunoresearch; West Grove, PA) were used. Sections were rinsed in PBS and coverslipped in polyvinylalcolhol mounting medium with DABCO (1,4-diazabicyclo {2,2,2} octane; Fluka, Buchs, Switzerland). Sections were analyzed with a Confocal Laser Scanning Microscope (LSM 510 Meta, Axiovert 100M; Zeiss, Jena, Germany).

5.3.4. Stereological assessment of GFP-positive cells

The total number of GFP-positive cells in the neocortex was estimated using the optical fractionator technique [15]. Neocortical borders were defined based on a mouse brain atlas [16]. Quantifications were performed on a systematic random series of every 12th GFP-peroxidase stained coronal section throughout the entire neocortex. The sum of counted cells was

multiplied by the reciprocal of the fraction of the brain region sampled, resulting in the total number of GFP-positive cells/neocortex. In a second step, the GFP-positive cells in the cortex were recounted and subdivided in five morphological subgroups of ameboid, round, rod, elongated and stellate cell [9].

5.3.5. Quantification of plaque load and vascular amyloid

A systematic random series of every 12th Aß-immunostained (NT12 antibody) section throughout the entire neocortex was selected per animal. Plaque load was estimated by calculating the area fraction occupied by parenchymal amyloid in two-dimensional counting frames on a single focal plane (20X objective, 0.6NA) [17]. Stereological analysis was performed with the aid of Stereo Investigator software (Microbrightfield; Williston, VT) and a motorized x–y–z stage coupled to a video-microscopy system (Systems Planning and Analysis Inc.; Alexandria, VA). On the same set of Aß-immunostained sections cerebral amyloid angiopathy (CAA) was quantified by estimation of CAA frequency, severity and CAA score according to a previously published method [12].

5.3.6. Association of GFP-positive cells with amyloid plaques and vascular amyloid

A systematic random series of every 12th section was stained for Congo red and immunostained for GFP. Plaques with one or more GFP-positive cells touching the congophilic center or their direct perimeter (halo) were counted.

5.3.7. Electron microscopy

Mice were deeply anaesthetized and transcardially perfused with a fixative containing 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and postfixed for 24 h in 4 % paraformaldehyde in 0.1 M PB. Frontal sections of the hippocampus (50 μm) were cut with a vibratome and washed in 0.1 M PB. After a blocking step (5 % BSA in 0.1 M PB), free-floating serial sections of each brain were incubated with an anti-GFP antibody (Clontech; Palo Alto, CA) at 4° C for 24 h. The sections were then incubated with a biotinylated secondary antibody (anti-rabbit IgG; Vector Labs) at room temperature for 90 min. After rinsing in 0.1 M PB, sections were exposed to an avidin-

biotin-peroxidase complex (ABC-Elite, Vector) for 2 h at room temperature. Following several washes, the sections were immersed in a 3,3' diaminobenzidine solution and washed again several times. They were then contrasted with osmium (0.5 % OsO4 in 0.1 M PB) for 20 min, dehydrated (70 % ethanol containing 1 % uranyl acetate) and embedded between liquid-release-coated slides and coverslips. Selected sections were re-embedded in Durcupan blocks (Fluka, Steinheim, Germany) for ultrathin sectioning. Sections collected on single-slot Formvar-coated copper grids were examined in a Zeiss electron microscope.

5.3.8. Quantitative assessment of GFP-positive macrophages and T cells

To estimate the percentage of macrophages in the population of GFP-positive ameboid cells, 4 sections from the systematically sampled set of sections were double-stained for Iba-1 and F4/80 and analyzed by confocal microscopy. The percentage of macrophages was estimated by analyzing 40 GFP-positive ameboid cells per animal that were labeled with at least one of the two macrophage markers. To identify T cells among the GFP-positive ameboid cells another set of 4 sections per animal was stained using a T cell specific antibody (CD3). The percentage of T cells was estimated by counting 30 GFP-positive ameboid cells per animal and calculating the proportion of CD3-positive cells.

For ultrastructural identification and phenotyping, 34 randomly selected plaque associated GFP-immunopositive cells in two animals were analyzed and phenotyped based on size, shape, lymphocyte-typical nuclear fold, and/or the presence macrophage-typical phagosomes.

5.3.9. Statistical analysis

Results were analyzed using Student's t-test with the help of StatView 5.0.1. Where appropriate Bonferroni correction for multiple data sets was used. Data are presented as the mean $\pm$ SD. Statistical significance was accepted as $p<0.05$.

5.4. Results

To examine the invasion of bone marrow-derived cells in response to cerebral amyloidosis, we reconstituted irradiated APP23 mice with syngenic hematopoietic cells expressing GFP. Experimental groups of non-transgenic and transgenic male mice were transplanted at different stages of amyloidosis with various times of survival thereafter (Fig. 1). Group 1 was transplanted at 6 months, an age prior to the deposition of cerebral amyloid, and was sacrificed 10 months later. In contrast, group 2 and 3 received their hematopoietic graft at an age with existing amyloidosis (15 and 20 months) and were analyzed 3 and 5 months later, respectively. As control, group 4 was transplanted and analyzed before mice developed amyloid deposits (Fig. 1).

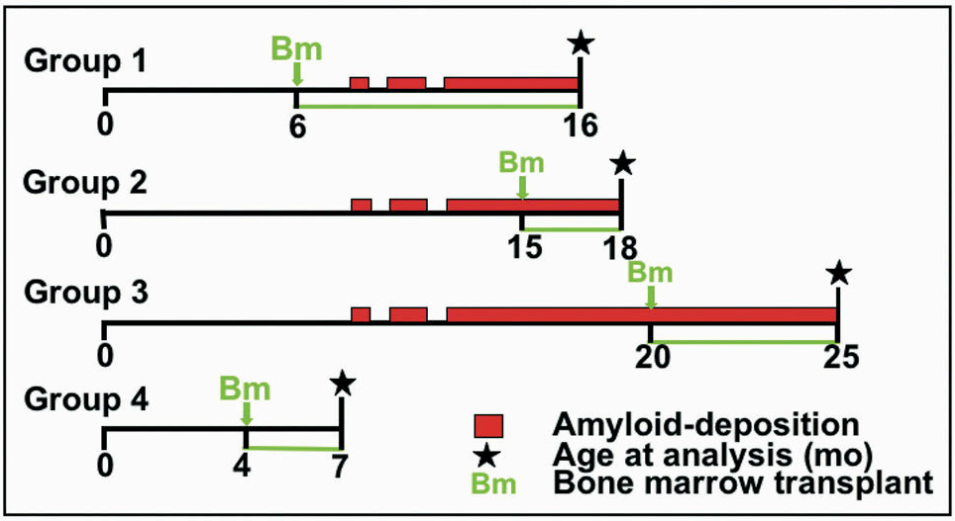

Figure 1: *Experimental groups studied. APP23 mice and littermate controls at various ages were lethally irradiated and transplanted with GFP-positive bone marrow (Bm). After different times following this procedure the invasion of peripheral bone marrow cells was analyzed in the neocortex. The following numbers of mice were used per group: Group 1: 3 controls; 8 APP23. Group 2: 4 controls; 4 APP23. Group 3: 6 controls; 3 APP23. Group 4: 5 controls; 5 APP23.*

5.4.1. Increased invasion of hematopoietic cells in amyloid-bearing APP23 mice

To determine the number of bone marrow-derived cells in the brain of APP23 mice, GFP-positive cells were visualized by immunohistochemistry with an antibody against GFP (Fig. 2). Analysis focused mainly on the neocortex because this region exhibited the most robust amyloid pathology.

In non-transgenic control mice, the majority of GFP-positive cells were located in perivascular spaces, in the meninges and in the ependymal lining of the ventricles and choroid plexus. Only few cells were seen in the parenchyma, and the vast majority of these cells were vessel associated (Fig. 2A). This observation is consistent with previous reports [9,18] and was true for all non-transgenic animals of the four experimental groups, i.e. independent of the age at which the mice received the bone marrow transplant and independent of the age at which the mice were analyzed (Fig. 1).

In contrast, in amyloid-depositing APP23 mice (group 1-3, Fig. 1) a significant portion of GFP-positive cells was found in the parenchyma in particular in neocortical areas with a high amyloid load (Fig. 2A). The total number of GFP-positive cells was strikingly increased compared to non-transgenic control mice. Stereological analysis revealed a 52% increase for group 1 (Fig. 2C) and 76% and 85% increases for group 2 and 3, respectively.

Morphological analysis of GFP-positive cells in non-transgenic and transgenic mice revealed five subgroups of GFP-positive cells consistent with morphological criteria previously suggested [9]: *Ameboid-like cells* (Fig. 2B) contained extensive cytoplasm, had often small projections and were predominately found in the brain parenchyma. They were rarely seen in the neocortex of non-transgenic control mice, but were abundant in amyloid-bearing APP23 mice. Quantitative analysis demonstrated a 7-fold increase of these cells in amyloid-bearing mice compared to non-transgenic controls (Fig. 2C). *Round cells* (Fig. 2B) were smaller, typically only 5-10μm in diameter, found mainly in vessels and did not enter the parenchyma. No difference was found between non-transgenic and amyloid-bearing APP23 mice (Fig. 2C). *Rod-like cells* (Fig. 2B) showed a distribution similar to the round cells. Again, no difference was found between non-transgenic and APP23 mice (Fig. 2C). *Elongated cells* (Fig. 2B) extended up to 100 μm along the vasculature and were always intimately associated with vessels of all calibers. They were the most abundant cells in non-transgenic control and in amyloid-bearing APP23 mice. However, no difference was noted between the groups (Fig. 2C). *Stellate cells* (Fig. 2B) were only found in the subventricular regions that lack a blood

brain barrier (e.g. arcuate nucleus, median eminence). No difference was noted between non-transgenic control and amyloid-bearing APP23 mice (Fig. 2C).

In APP23 transgenic mice sacrificed prior to the onset of cerebral amyloidosis (group 4), no significant increase of the total or any subgroup of GFP-positive cells was found (Fig. 2C). This observation suggests that the mere overexpression of APP and the presence of soluble human Aß did not affect the migration pattern of peripheral cells.

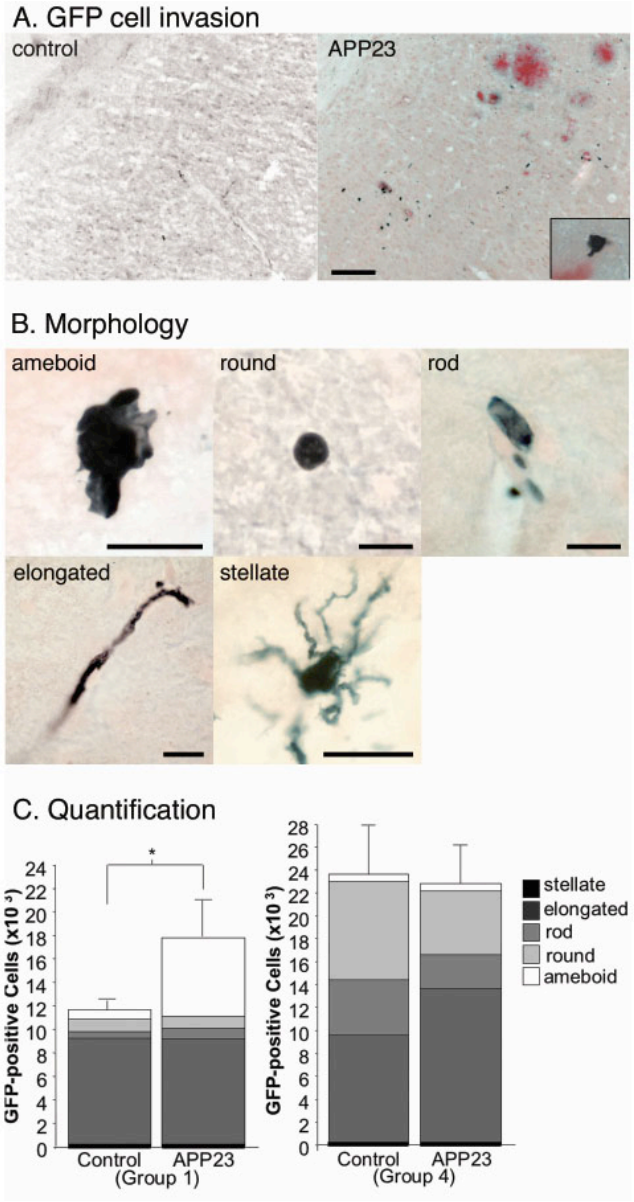

Figure 2: *Increased invasion of peripheral cells into the neocortex of amyloid-depositing APP23 mice. (**A**) Neocortex of a 16 mo-old control mouse and 16 mo-old APP23 mouse showing the distribution of GFP-positive cells (here immunostained with an antibody to GFP). Congophilic plaques are shown in red. Calibration bar is 100µm. Insert shows a GFP-positive cell closely associated with an amyloid plaque. (**B**) GFP-positive cells were morphologically classified in 5 distinct subgroups. Scale bars are 20 µm. (**C**) Quantification of total GFP-positive cells revealed a 52% increase (*p<0.05) in neocortex of APP23 mice compared to control mice (shown are results for Group 1, see Fig. 1). When GFP cells were sub-grouped, only ameboid cells were significantly increased (7-fold, p=0.002). Similar analysis for Group 2 and 3 also revealed significant increases in ameboid cells (p=0.006 and p=0.01, respectively) but not any other cell subtype. No differences in total number or any subgroup of GFP-positive cells were found in young, pre-depositing APP23 mice (Group 4, see Fig. 1).*

5.4.2. Association of ameboid cells with amyloid

To investigate the amyloid dependence of ameboid cell invasion, correlative analysis was done between plaque load and number of ameboid cells in neocortex of mice in group 1. Although results indicated a positive relationship, analysis did not reach statistical significance (Fig. 3A). A similar analysis for vascular amyloid (CAA), the extend of which is not dependent of plaque load [12], also failed to reveal a significant correlation, although a positive trend was again observed (Fig. 3B). The results obtained for group 2 and 3 showed the same trend (data not shown).

Histological analysis of the association of ameboid cells with amyloid plaques revealed that only a subpopulation of amyloid plaques was decorated with ameboid GFP-cells (Fig. 3C) while neighboring amyloid lacked completely any association with ameboid GFP-positive cells. The mere size of the plaque appeared not to be critical for an association with GFP-positive ameboid-like cells. To estimate the percentage of amyloid plaques decorated with ameboid GFP-positive cells, the number of congophilic plaques with associated GFP-positive cells was quantified. Results for group 1 revealed that 20±4% plaques were associated with invading ameboid cells. No other morphological difference was noted between plaques with or without such invading cells (Fig. 3C).

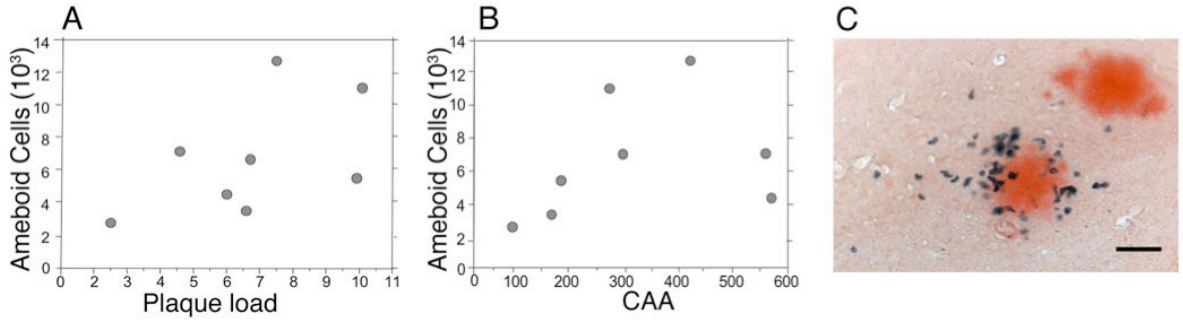

Figure 3: *Ameboid-like cells are associated with a subpopulation of amyloid deposits. (**A**) Neocortical plaque load of the APP23 mice of group 1 was stereologically estimated and correlated with the number of invading ameboid-like GFP-positive cells. Results indicate a positive relationship between plaque load and number of invading cells, although the correlation did not reach statistical significance. (**B**) Similarly, correlative analysis between CAA frequency and ameboid cell count revealed a positive trend. (**C**) Photomicrograph exemplifying the heterogeneous distribution of invading peripheral cell in relation to amyloid plaques. Some plaques were surrounded by a high number of invading cells, while neighboring plaques completely lacked invading peripheral cells. Amyloid is stained with Congo red, GFP-immunopositive cells are represented in black. Calibration bar is 75µm.*

To follow the hypothesis that newly developing amyloid deposits are able to attract peripheral cells in a time dependent fashion, the same analysis was done for groups 2 and 3, which varied in the time they held the transplant (3 and 5 months, respectively, as compared to 10 months in group 1). Results revealed that 27±2% and 18±4% of amyloid plaques in group 2 and 3, respectively, were surrounded by invading cells. This observation suggests that the association of peripheral cells with amyloid plaques is not increasing with time or with amyloid load.

5.4.3. Identification of cell type and function

Phenotyping of ameboid-shaped GFP-positive cells was done using confocal and electron microscopy (Fig. 4). To identify macrophages at the confocal level, a combination of two markers (F4/80, Iba-1) was used to ensure the detection of different activation states of these cells [8]. GFP-positive cells were counted as macrophages if they expressed at least one of the two markers (Fig. 4A-E). Quantitative analysis (5 randomly selected mice of group 1) demonstrated that 7% of GFP-positive ameboid cells were macrophages. A similar analysis using an antibody against the pan-T cell marker CD3 was done to identify T-cells among the GFP-positive ameboid cells (Fig. 4F-I). Results revealed an unexpectedly high percentage of 27% T-cells amongst the ameboid GFP-positive cell population in neocortex. Both, macrophages and T-cells, were randomly distributed throughout the neocortex and both revealed an association with a subpopulation of amyloid plaques. These amyloid plaques were always also surrounded by resident microglia, i.e. GFP negative and Iba1-positive cells. No B220-positive B cells could be detected (data not shown).

All GFP-positive cells found in the neocortex, independent of their morphological appearance, were positive for CD45 confirming their hematopoietic origin (data not shown). Consistent with this observation, confocal analysis using markers for endothelial cells (CD31), neurons (NeuN), and astrocytes (GFAP) did not reveal any GFP-positive cells that expressed one of these markers. These observations suggest that invading cells kept their hematopoietic identity and did not differentiate into another brain resident cell type. To confirm the identity of macrophages and T-cells at the ultrastructural level, electron microscopic analysis was done in two additional APP23 mice with a bone marrow transplant analogous to group 3.

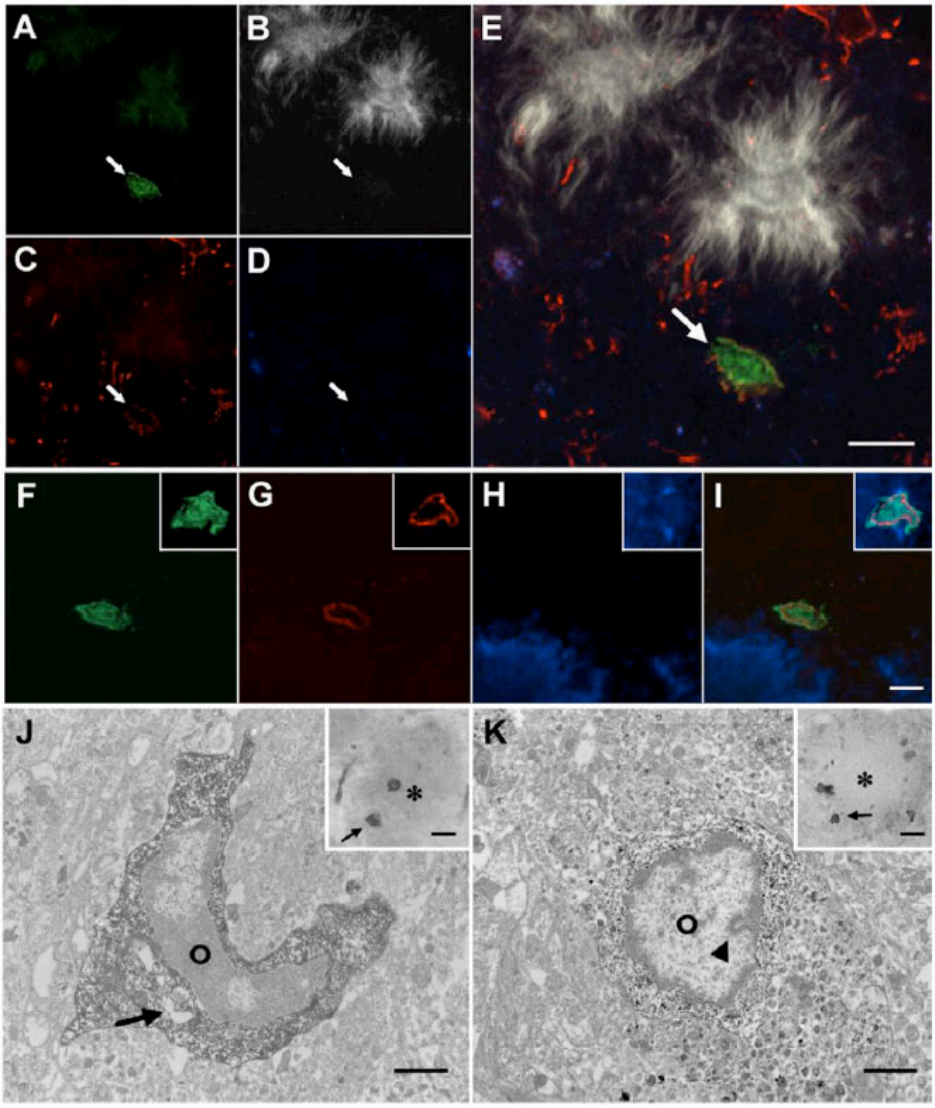

***Figure 4**: A subpopulation of the ameboid-like cells are macrophages and T-cells. **(A-I)** The fraction of macrophages and T-cells in the ameboid cell population was determined by investigating ameboid-shaped GFP-positive cells for co-labeling with a combination of different antibodies using confocal microscopy. To identify macrophages, ameboid-like cells that are positive for GFP and positive for either Iba-1 and/or F4/80 were identified. (**A**) GFP; (B) amyloid-staining using Thioflavin-S; (**C**) Iba-1; (**D**) F4/80; (**E**) merged images. Note the ameboid-like GFP-positive macrophage which is Iba-1-positive but apparently negative for F4/80 (arrow). To identify T-cells, ameboid-like GFP-positive cells that are positive for CD3 were identified. (**F**) GFP; (**G**) CD3; (**H**) amyloid staining using NT12; (**I**) merged images. Another GFP-positive and CD3-positive ameboid-like cell is shown in the insets (**F-I**). **(J,K)** Electron microscopy was used to identify GFP-positive cells that can be appreciated by the electron-dense immunoprecipitate in the cytoplasm. Shown in (**J**) is a macrophage that contains a phagosome (arrow) in its cytoplasm. The nucleus is indicated by a circle. A T-cell-like cell is shown in (**K**) with its typical nuclear fold (arrowhead). The insets (**J,K**) are lower magnifications (small arrows indicate the analysed cell; asterisks indicate the amyloid plaque). Scale bars: 10 µm (**E, I**); 2 µm (**J, K**); and 20 µm (insets in **J**, **K**).*

Amyloid-associated GFP-positive cells were randomly analyzed and the percentage of macrophages and T-cells determined. Cells that were 9-12 μm in diameter and contained a round nucleus and phagosomes were counted as macrophages (Fig. 4J). Cells that were 6-10 μm in diameter with a characteristic nuclear fold were identified as lymphocytes (Fig. 4K). Results revealed that 23% were macrophages and 31% lymphocytes. The higher percentage of macrophages at the ultrastructural level compared to the confocal analysis is at least partly the result of the different sampling methods. A random selection of ameboid cells in the neocortex was analyzed at the confocal level, while for electron microscopy preferentially plaque-associated cells were analyzed (but see also discussion). The high percentage of T-cells at the ultrastructural level confirmed the observation at the confocal level.

5.4.4. Lack of amyloid phagocytosis by peripheral macrophages

Our previous work suggested lack of amyloid phagocytosis in aged APP23 transgenic mice [13,19]. To examine the possibility that peripheral macrophages rather than microglia are capable of phagocytosis we examined the 8 previously identified GFP-labeled ameboid cells with macrophage-like ultrastructural features (Fig. 4J). However, analysis of GFP-positive macrophages did not reveal any indication of amyloid phagocytosis of these cells although they were in close vicinity of amyloid deposits (Fig. 4J). However, many macrophages with phagosomes containing unidentified, nonfibrillar material were observed.

5.4.5. No signs of T-cell mediated inflammation

T-cells were distributed throughout the parenchyma with some cells loosely associated with a subpopulation of amyloid plaques. However, there was no indication of a productive inflammation or even encephalitis, with associated tissue damage, edema, or vascular changes (data not shown). While the number of peripheral cells was clearly increased in the brain of APP23 mice, the distribution of cells was highly variable and there were no significant clusters of T-cells either around vessels (vasculitis) or around plaques or other structures indicative of a destructive process.

5.5. Discussion

Neuroinflammatory changes in human brain and transgenic mouse models are an integral part of the AD pathogenesis. While the significance of this neuroinflammation is a matter of lively debate (reviewed in [2], the contribution of the peripheral immune system to neuroinflammation and AD pathogenesis has not received similar attention. However, in the light of recent therapeutic Aß vaccination trials, the role of the peripheral immune system has been exemplified by the unexpected immunotherapy-induced occurrence of meningoencephalitis [20-22].

Our present results demonstrate that bone marrow-derived cells access the amyloid-laden brain in significantly higher numbers than compared to controls. The invading cells remain of hematopoietic phenotype and are heterogeneously scattered throughout the brain. About 20% of congophilic plaques were surrounded by GFP-positive ameboid-like peripheral cells, and this percentage did not change with increasing time after bone marrow transplantation. Even in mice that received the transplant before the onset of cerebral amyloidosis, the percentage of plaques that were surrounded by such peripheral ameboid-like cells remained approximately 20%. This observation indicates that only a subpopulation of amyloid plaques is a target for invading cells. Alternatively, all amyloid plaques might attract invading cells but only for a limited time, possibly at an early stage of plaque evolution.

Dying cells are the classical activator and target of phagocytic cells such as bone marrow derived invading macrophages [10,23]. It is therefore interesting to speculate**,** that plaques with GFP-positive cells represent a toxic stage, which attract macrophages to clear the cellular debris. However, the significance of such phagocytosis by peripheral macrophages and its relation to the proliferation of amyloid associated resident microglia is not clear [17,24]. Such an interpretation would however imply that only a subpopulation of plaques are toxic to their environment. Alternatively, plaques might be toxic only at an early stage of development and the presence of peripheral cells leads to a detoxification.

The finding that only a small percentage of the invading ameboid-like cells could be identified at the light microscopic level as macrophage is at first glance surprising. However, it is known that many of the macrophage markers, including Iba-1 and F4/80, are differentially regulated in the activation process and are downregulated when the cells enter

the CNS [25]. Thus, it is possible that the number of invading macrophages is underestimated by number supporting the observations at the ultrastructral level.

Peripheral macrophages are predominantly MHCII- and Fc gamma receptor (FcgR)-positive and represent the innate immune response. They are thought to be involved in amyloid phagocytosis [26]. However, although our results point to a tight association of such macrophages with amyloid bundles at light microscopic levels, electron microscopy did not reveal any signs of amyloid phagocytosis, i.e. complete engulfment of amyloid-fibrils by the macrophage cytoplasm. We have previously shown, that the same observation is true for activated resident microglia, which also lack the capacity for amyloid phagocytosis in APP23 transgenic mice [13,19].

The present finding of significant invasion of T-cells in response to cerebral amyloidosis was unexpected in light of previous findings. In an earlier study we did not detect any lymphocytes in APP23 transgenic mouse brain [27]. In the present study we have irradiated the mice, a process that is known to temporarily activate and open the blood brain barrier and increase the level of inflammation by activation of glial cells [28]. Thus, it is possible that this irradiation-induced increase in the level of chemoattractants and cytokines contributed to the invasion of T-cells. This would be consistent with the view that the lack of lymphocytes in most animal models of AD is due to the generally lower level of inflammation in the mice compared to the AD brain.

However, irradiation itself cannot be the sole cause of the increased lymphocytic infiltration in the present study since such an increased invasion was not found in control mice. Moreover, at the time points examined in this study there was no detectable blood brain barrier leakage (assessed by anti-IgG immunostaining). However, it is possible that the presence of vessel-associated amyloid prevents the “healing” of the radiation-induced vessel damage, allowing for an increased infiltration of leucocytes in the transgenic mice. This hypothesis could be addressed by irradiating the mice and protecting the brain by lead hats [29]. The discrepancy between the failure to see lymphocytes in our previous study and the invasion of T-cells in the present study, may also be that in the present study peripheral cells can easily be detected by their GFP-expression, thereby increasing sensitivity and specificity of detection.

In AD brain the presence of T-cells is well documented, but their role in the disease process is not clear [30,31]. In addition, most AD patients have peripheral T-cells which are self reactive against Aß. This is further evidence for the involvement of the peripheral immune system, in particular the lymphocytic compartment in the pathogenesis of AD [32]. However, when compared to the overt inflammatory response associated with an acute injury of the brain or to multiple sclerosis, the recruitment of peripheral cells in AD remains atypical. For example, there is no indication for a productive inflammation or even an encephalitis with associated tissue damage, edema, or vascular changes in AD [33]. Similarly, while the number of peripheral cells was clearly increased in APP23 mice, the distribution of cells was highly variable and there were rarely clusters of T-cells observed, neither around vessels (vasculitis) nor around plaques or any other structures, which would be indicative of a destructive process.

It is possible that such T-cells have a function in protecting the injured CNS [34]. Recent studies suggest indeed a regulatory interaction between T cells and microglia, where T-cells limit the destructive potential of the inflammatory reaction [35,36]. Thus, the role of T-cells in AD brain remains speculative; T-cells are present but do not show any association with tissue damage. Further studies into the role of T-cells in AD pathogenesis are needed and will allow to develop new and better immunization strategies, without the observed T cell-mediated menigoencephalitis as an unwanted side effect [20-22]. To this end, the herein described bone marrow reconstituted APP transgenic mouse model may serve as a model to study such immunotherapeutic strategies.

While the number of invading bone marrow-derived ameboid cells increased in response to cerebral amyloidosis, the elongated vasculature-associated cells did not increase in number and appeared not to be activated. These vasculature-associated invading cells most likely represent perivascular macrophages. The observation, that the population of perivascular macrophages remained unchanged was unexpected. It is thought that these cells participate in the control and regulation of homeostasis of the interstitial space [37], which is part of the perivascular clearance pathway of Aß [38]. In atherosclerosis and stenosis the number of new endothelial cells increases and helps to repair and restore endothelial function [39]. Therefore, we also evaluated the possibility, that these vessel associated-cells were endothelial cells. However, based on the lack of CD31 expression and the presence of CD45, no trans-differentiation to the endothelial cell type was noted.

In conclusion, this study demonstrates that the peripheral immune system is associated and likely has a role in AD pathogenesis. We show that an increased number of leucocytes enters the brain during amyloidosis, although this does not lead to an obvious functional and progressive immune response in the CNS. Therefore, it remains to be evaluated whether these cells are causal, contributory or sequels of the disease process. However, the fact that they invade and associate with the amyloid plaques together with their easy accessibility and genetic manipulation, makes them interesting as a therapeutic vehicle for targeted intervention [10,40] which recently has been successfully demonstrated for a lysosomal storage disease [10,40]. Harnessed with an amyloid degrading enzyme or an immune modulator, autologous bone marrow-derived cells may offer a potent, targeted therapeutic strategy of clearing amyloid deposition and/or reducing amyloid-associated neurodegeneration.

5.6. References

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6. Conclusions

The goal of these studies was to investigate the role of hippocampal neurogenesis in the aged and AD brain, to identify the disturbances of neural stem cell biology in the amyloidogenic environment and to assess strategies to ameliorate impaired neurogenesis.

We found a decreased neurogenesis in aged animals, with a major decrease from juvenile to adult mice, but only little decline in aged compared to adults. The finding that calorie restriction, a treatment that is consistently beneficent to age related deficits, does not increase the rate of neurogenesis confirmed that at least at the adult stage, there is only a minor age related defect of neurogenesis.

The level of neurogenesis has been shown to correlate with the performance in hippocampus dependent memory tasks [1-4]. In aged animals however, the relationship of memory and neurogenesis remains controversial [5,6], indicating that neurogenesis might only be relevant for memory if it is above a certain threshold and/or that neurogenesis becomes irrelevant for memory with progressive age. We found an increased neurogenesis in aged APP23 mice, which are known to be impaired in hippocampus dependent memory [7-9], providing further evidence that neurogenesis in aged mice is irrelevant to memory.

An experiment investigating neurogenesis in APP23 mice with access to a running wheel strengthened this hypothesis even more; voluntary running is known to increase neurogenesis and has also been shown to have a beneficial effect on memory [1,10] and more importantly on cognition of senile and AD patients [11-13] and AD mouse models [14,15]. Here, we found no increase beyond the pre-existing elevation in neurogenesis in exercising animals carrying heavy plaque loads. Again, this suggests that cognitive performance in animal models of AD does not dependent on neurogenesis. Recent studies with AD mouse models suggest, that Aß related memory deficits are caused by small assemblies of Aß that occur as a pre-stage of Aß plaques and affect the synapses [16-18]. Removal of these assemblies resulted in improved memory, without reducing plaque load [17,19], and we speculate that running induced memory enhancement in AD mouse models depends on a similar mechanism. We surmise that hippocampus dependent memory depends on neurogenesis in juvenile and early adult mice, but with progressive age relies more on synaptic plasticity of the existing cellular architecture.

Our observations in Aß depositing mice with genetically marked stem cells demonstrate, that stem cells residing in the GCL are highly disturbed and reduced in numbers. However, the

numbers of more differentiated, and highly proliferative neural precursor cells remained constant and are most likely responsible for the increased neurogenesis found in the AD mouse model. This result was confirmed by the finding that an excess number of neurospheres was produced by the extracted population of neural precursor cells from aged APP23 hippocampi. The functional reason of this increased neural stem cell proliferation could not be investigated, but it is possible that the up-regulation of neurogenesis reflects a compensational mechanism for AD related defects in the hippocampal network.

Potentially stem cells could be used as therapeutic agents in neurodegenerative diseases. Most elegantly, the endogenous stem cell population could be stimulated for the regeneration of the damaged tissue. Here, we found that although running can stimulate neurogenesis in adult and aged laboratory mice, it does not increase neurogenesis in the AD mouse model. Moreover, new neurons in aged animals are produced at such a low rate, that it seems unlikely, that neurogenesis can participate in the replacement of neurons in the degenerating brain. Our studies with hematopoietic stem cells invading the brain of APP23 mice revealed that peripheral cells do not replace damaged neurons. However, the invasion of peripheral cells into the brain was much greater than expected and the attraction of certain cells to Aß deposits makes them a potential carrier to deliver a neuroprotective drug or an Aß dissolving agent to the site of the Aß plaque.

In summary, we conclude that hippocampal neurogenesis has only limited importance for memory in aged mice. Consequently, we surmise that the various alterations of neural stem cell biology induced by Aß do not contribute to AD related memory impairments. In addition, we found that the endogenous hippocampal stem cell population could not be stimulated to a degree that would enable the repair of serious brain damage or neuronal degeneration. However, it is astonishing that neurogenesis persists until senescence. The role of adult neurogenesis remains elusive, but the Aß induced disturbances described here raise new questions about its function (or dysfunction in the AD brain) and provide a background for future investigations.

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Abbrevations

Aß	ß-amyloid
ACE	angiotensin converting enzyme
AD	Alzheimer's disease
AL	ad libitum
ANOVA	analysis of variance
APH-2	anterior pharynx-defective phenotype
APOE	apolipo protein E
APP	amyloid precursor protein
BDNF	brain derived growth factor
Bm	bone marrow
BrdU	bromodeoxyuridine
BSA	bovine serum albumine
CAA	cerebral amyloid angiopathy
CdK	cyclin dependent kinase
CNS	central nervous system
CNTF	cilliary neurtrophic factor
CR	caloric restriction
DAB	3,3'-diaminobenzidine
DCX	doublecortin
DG	dentate gurus
EC	entorhinal cortex
EGF	endothelial growth factor
ES cells	embryonic stem cells
FAD	familial Alzheimer's disease
FcgR	Fc gamma receptor
FGF	fibroblast growth factor
GCL	granular cell layer
GFAP	glial fibrillary acid protein
GFP	enhanced green fluorescent protein
Gp	glyco protein
HPA	hypothalamo-pituitary axis
IDE	insulin degrading enzyme
IgG	immune globuline G

IGF	insuline-like growth factor
IL	inter leukine
INF	interferon
LIFR	leucocyte inhibitory factor receptor
LPS	lipo poly saccharide
LRP	lipoprotein receptor related protein
LTP	long term potentiation
ML	molecular layer
Nct	nicastrin
NeuN	neuronal nuclei
NGF	nerve growth factor
NO	nitric oxide
NSAID	non steroidal anti inflammatory drug
OB	olfactory bulb
PB	phosphate buffer
PBS	phosphate buffered saline
PEN-2	presenilin-enhancer
PFA	paraformaldehyde
PS	presenilin
PSA-NCAM	polysialyated acid neural cell adhesion molecule
S	subiculum
sAPP	soluble amyloid precursor protein
SVZ	sub ventricular zone
TBS	tris buffered saline
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
WT	wild type

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